

**PURIFICATION OF CELLULASE AND XYLANASE
ENZYMES PRODUCED FROM POTENTIAL
MICROORGANISMS ISOLATED FROM COMPOST
FOR INDUSTRIAL APPLICATION**

Thesis

by

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*Submitted in partial fulfilment of the requirements
for the degree of*

**DOCTOR OF PHILOSOPHY
MICROBIOLOGY**



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CERTIFICATE - I

This is to certify that the thesis entitled, **“Purification of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost for industrial application”** submitted in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) is a record of bonafide research work carried out by **Mr. Sanjeev Kumar (F-2007-12-D)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations has been fully acknowledged.

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This is to certify that the thesis entitled, “**Purification of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost for industrial application**” submitted by **Mr. Sanjeev Kumar (F-2007-12-D)** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY** has been approved by the Student’s Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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With Immense servility, I would like to offer prime prayer at the lotus feet of "In the name of Almighty, The Most Gracious, and The Most Merciful. All praise belongs to Him, the lord (Who is the Creator, Sustainer and Guide) of the entire world". He created man from dust and awarded him with the soubriquet of "Master Creation". May peace, mercy and blessings of Almighty be upon the last Apostle.

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(Sanjeev Kumar)

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ABBREVIATIONS

%	:	Percent
&	:	and
°C	:	Degree centigrade
μ mole/min	:	Micro mole per minute
μg	:	Micro gram
μg/ml	:	Micro gram per ml
μl	:	Micro litre
μm	:	Micro meter
BSA	:	Bovine Serum Albumin
CBH	:	Cellobiohydrolase
CBM	:	Carbohydrate binding module
CCD	:	Central Composite Design
CFU	:	Colony forming units
cm	:	Centimeter
CMCase	:	Carboxymethylcellulose
DEAE	:	Diethylaminoethyl
DNA	:	Deoxyribonucleic acid
dNTPs	:	deoxyribonucleotide triphosphates
E.C.	:	Enzyme Commission
EG	:	Endoglucanase
Fig.	:	Figure
FPase	:	Filterpaperase
g	:	Gram
g/L	:	Gram per litre
GHF	:	Glycosyl hydrolase family
Gt	:	Gigatonne
h	:	hour or hours
Hz	:	Hertz
i.e.	:	That is
ITS	:	Internal Transcribed Spacer
IU	:	International units
kDa	:	Kilodalton
L	:	Litre
V _{max}	:	Maximum velocity
K _m	:	Michaleis constant
M	:	Molar

mA	:	mili ampere
mg	:	Miligram
mg/ml	:	Miligram per Millilitre
min	:	Minute
ml	:	Millilitre
mM	:	Milimolar
MW	:	Molecular weight
N	:	Normal
nm	:	Nano meter
O.D.	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis
PBD	:	Plackett Burman Design
PCR	:	Polymerase Chain Reaction
psi	:	Per square inch
RNase	:	Ribonuclease
rpm	:	Revolution per minute
rRNA	:	Ribosomal ribonucleic acid
RSM	:	Response Surface Methodology
SDS	:	Sodium dodecyl sulphate
SEM	:	Scanning Electron Microscopy
SmF	:	Submerged Fermentation
sp.	:	Species
SSF	:	Solid State Fermentation
U/gds	:	Unit per gram of dry substrate
U/mg	:	Unit per milligram
V	:	Volts
v/v	:	Volume per volume
v/w	:	Volume per weight
<i>viz.</i>	:	Vizually
vvm	:	Volume by volume per meter
W	:	Watt
w/w	:	Weight per weight
α	:	Alpha
β	:	Beta
ρ	:	Para

Chapter-1

INTRODUCTION

Lignocelluloses are the building blocks of all plants and are ubiquitous to most regions of our planet. Their chemical properties make it a substrate of enormous biotechnological value. The basic chemistry of cellulose, hemicellulose, and lignin has a profound effect on lignocellulose tertiary architect (Malherbe and Cloete, 2002). These intricate associations constitute physical and chemical barriers to lignocellulose utilization and biodegradation in natural and man-made environments. Overcoming these barriers is the key for unlocking the commercial potential of lignocelluloses. Understanding lignocellulose degradation of plant biomass under natural conditions ultimately forms the basis of any lignocellulose based application.

Plant cell walls are the most abundant renewable sources of fermentable sugars on earth (Himmel *et al.*, 1999; Saleem *et al.*, 2008) and are the major reservoirs of fixed carbon in nature. The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component. Plant biomass comprises on an average of 40% cellulose, 33% hemicellulose and 23% lignin by dry weight (Sa-Pereira *et al.*, 2003). Annually, 830 Gt of renewable plant biomass is formed during photosynthetic cycle (Rauscher *et al.*, 2006). This lignocellulosic waste generated in nature keeps on accumulating, thus causing serious environmental problems. Lignocellulosic biomass mainly comprising of forestry, agricultural and agro-industrial wastes can serve as an inexpensive energy source. This waste being rich in cellulose and hemicellulose can act as potential reservoir of value added products *viz.* enzymes, bioethanol and single cell proteins, etc. The non-use of these lignocellulosic materials constitutes a loss of potentially valuable sources (Mussatto and Teixeira, 2010). But lignocellulosic material being complex in nature is relatively inert for its hydrolysis releasing simple sugars that are the building blocks for their consequent bioconversion into industrially important substrates. Use of

hydrolytic enzymes of microbial origin *viz.* cellulases and xylanases is being proposed worldwide and has become most accepted mode of saccharification of lignocellulosic residue globally. Besides, cellulases and xylanases play pivotal role in many other fields and have become the focal biocatalysts due to their complex nature and wide spread industrial applications.

Over the years, due to their remarkable features, these enzymes have occupied the centre stage of all the biochemical and industrial processes. Their use has been preferred commercially because these can carry out myriads of biochemical reactions under ambient conditions, which make their use ecofriendly and offer the best alternative to polluting chemical technologies. The twentieth century saw an unprecedented expansion in the field of enzyme kinetics because new fields like microbiology and biotechnology have rapidly begun to gain ground, inturn promoting the use of enzymes at various industrial levels. Cellulases and xylanases are extracellular enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials. Efficient cellulose hydrolysis requires the cooperative action of endoglucanases (E.C. 3.2.1.4), which hydrolyze the cellulose polymer internally, exposing reducing and non-reducing ends, and exoglucanases or cellobiohydrolases (E.C. 3.2.1.91), which act on the reducing or non-reducing ends, releasing mainly cellobiose. The cellulose hydrolysis process is finalized through the action of β -glucosidase (E.C. 3.2.1.21), which cleaves cello-oligosaccharides into two molecules of glucose (Zhang *et al.*, 2011). Given the diversity of xylan structures, a complete hydrolysis of xylan involves the synergistic action of main chain degrading enzymes, including endo- β -1,4-xylanases (E.C. 3.2.1.8) and β -D-xylosidases (EC 3.2.1.37), and side group cleaving enzymes, including α -L-arabinofuranosidases (EC 3.2.1.55), α -glucuronidases (EC 3.2.1.139), acetyl xylan esterases (AXEs) (EC 3.1.1.72), and feruloyl esterases (EC 3.1.1.73).

Microbial cellulases and xylanases have the significant feature of being able to be produced in large quantities by established and optimized fermentation techniques. Enzyme production is closely controlled in microorganisms and

therefore, to improve its productivity these controls can be exploited and modified. To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favourable for the microorganism for over-production of the desired metabolite. An intensive investigation is therefore, required to establish the optimum conditions to scale up enzyme production under SmF/SSF in an individual fermentation process (Omojasola and Jilani, 2008).

Submerged fermentation is the cultivation of microorganisms containing soluble carbon sources and nutrients maintained under agitation. The use of submerged culture is advantageous because of the ease of sterilization and process control rendering it easier to design these systems. Depending on the strain and culture conditions, the enzyme can be constitutive or inducible, showing different production level and is a preferable method for enzyme production for bacterial fermentations (Vidhayalakashmi *et al.*, 2009). On the other hand, SSF is a highly attractive and alternative process needs to be exploited for generation of cellulase/xylanase enzymes with the use of cheaply available lignocellulosic residues and low input of process engineering. SSF has an edge over SmF due to low capital investment and operating cost, higher product yield, better product quality, cheaper product recovery and cheaper technology (Pandey *et al.*, 2003). Among different microorganisms evaluated so far, molds being best suited microorganisms to SSF for commercial cellulase and xylanase production.

Keeping in view the importance of cellulase and xylanase enzymes produced from potential isolates and their expanding use at commercial scale, the proposed study was carried out with the following objectives:

- ❖ To isolate and screen potential cellulase and xylanase producing strains.
- ❖ To sequence the genes of selected strains responsible for enzyme production
- ❖ To purify cellulase and xylanase for industrial application.

Chapter-2

REVIEW OF LITERATURE

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cell. All enzymes which have been purified are protein in nature, and may or may not possess a non-protein prosthetic group.

Enzymes occur in every living cell, hence are present in all microorganisms. Each single strain of organism produces a large number of enzymes which may be hydrolyzing, oxidizing or reducing and metabolic in nature. But the absolute and relative amounts of the various individual enzymes produced have been found to vary markedly between species and even between strains of the same species. Hence, it is customary to select strains for the commercial production of specific enzymes which have the capacity for producing highest amount of the particular enzymes desired. Commercial enzymes are produced from strains of molds, bacteria, and yeasts. Furthermore, optimizations of various environmental process parameters/genetic manipulation are highly suggestive methods escalate the production fold dramatically.

Lignocellulosic biomass contains three important classes of biopolymers viz. cellulose, hemicelluloses and lignin (Sharma and Bajaj 2005). In addition to the pivotal role in maintaining the structural integrity of plants, cellulose and hemicellulose serve as major source of nutrients for herbivores and as renewable substrate for the production of food, animal feed as well as textiles (Gilbert and Hazlewood, 1994). The cellulose and hemicelluloses are complex carbohydrates which are easily accessible to cellulolytic microorganisms and are degraded by them. Cellulose and hemicellulose are the renewable carbon source which are available in large quantity and can be a solution to the problem of energy,

chemicals and food (Romero *et al.*, 1999). Lignin is a three dimensional phenyl propanoids polymer linked by several different carbon to carbon and their linkages between monomeric phenylpropan phenylpropane units, most of which are not readily hydrolysable (Heck *et al.*, 2002).

2.1 STRUCTURE AND BIOCHEMISTRY OF CELLULOSE AND HEMICELLULOSE

2.1.1 Cellulose

2.1.1.1 Structure and Chemistry of Cellulose

Cellulose is a linear polymer of glucose linked by β -1,4-glycosidic bonds, having a simple primary and complex tertiary structures and its repeating unit is cellobiose (Bhat and Hazlewood, 2001) and these bonds can be hydrolysed by cellulolytic enzymes.

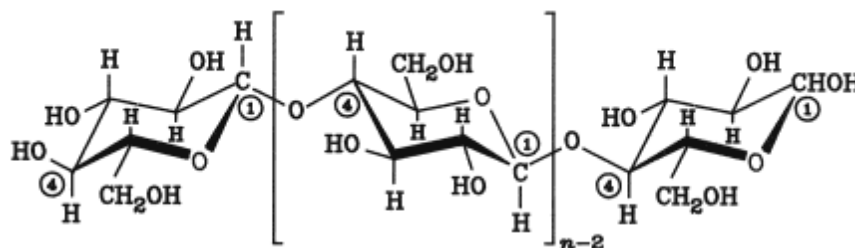


Fig. 1 Structure of cellulose (Harmsen *et al.*, 2010)

Many properties of cellulose depend on its degree of polymerization (DP), i.e. the number of glucose units that make up one polymer molecule. The DP of cellulose can extend to a value of 17000, even though more commonly a number of 800-10000 units are encountered. For instance, cellulose from wood pulp has a DP between 300 and 1700 (Kirk-Otmer, 2001).

The nature of bond between the glucose molecules (β -1,4 glucosidic) allows the polymer to be arranged in long straight chains. The latter arrangement of the molecule, together with the fact that the hydroxides are evenly distributed on both sides of the monomers, allows for the formation of hydrogen bonds between the molecules of cellulose. The hydrogen bonds in turn result in the

formation of a compound that is comprised of several parallel chains attached to each other (Faulon *et al.*, 1994).

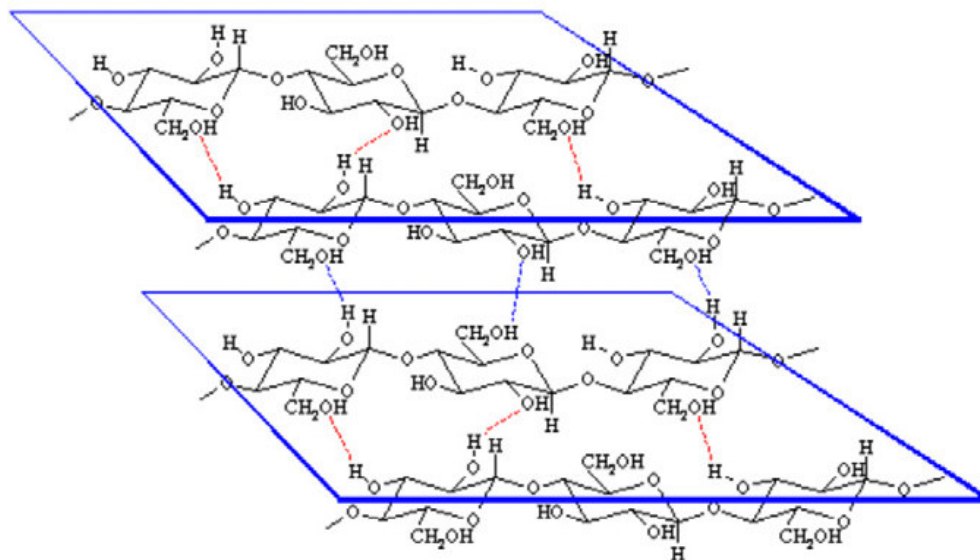


Fig 2. Demonstration of the hydrogen bonding that allows the parallel arrangement of the cellulose polymer chains (Harmsen *et al.*, 2010).

2.1.1.2 Biochemistry of Cellulose Degradation

Enzyme degradation of cellulose requires the synergistic activity of at least three enzymes (Julio *et al.*, 2002). Consequently, microorganisms that grow successfully on cellulose as a substrate are capable of producing a cellulase complex that consists at least three extra cellular enzyme activities:

- i. Endo-glucanase (EC 3.2.1.4, endo-1,4- β -glucanase)
- ii. Exo-glucanase (EC 3.2.1.91, 1,4- β -cellobiohydrolase)
- iii. β -glucosidase (EC 3.2.1.21)

The enzymatic hydrolysis of cellulose was found that is a promising method for its conversion to glucose.

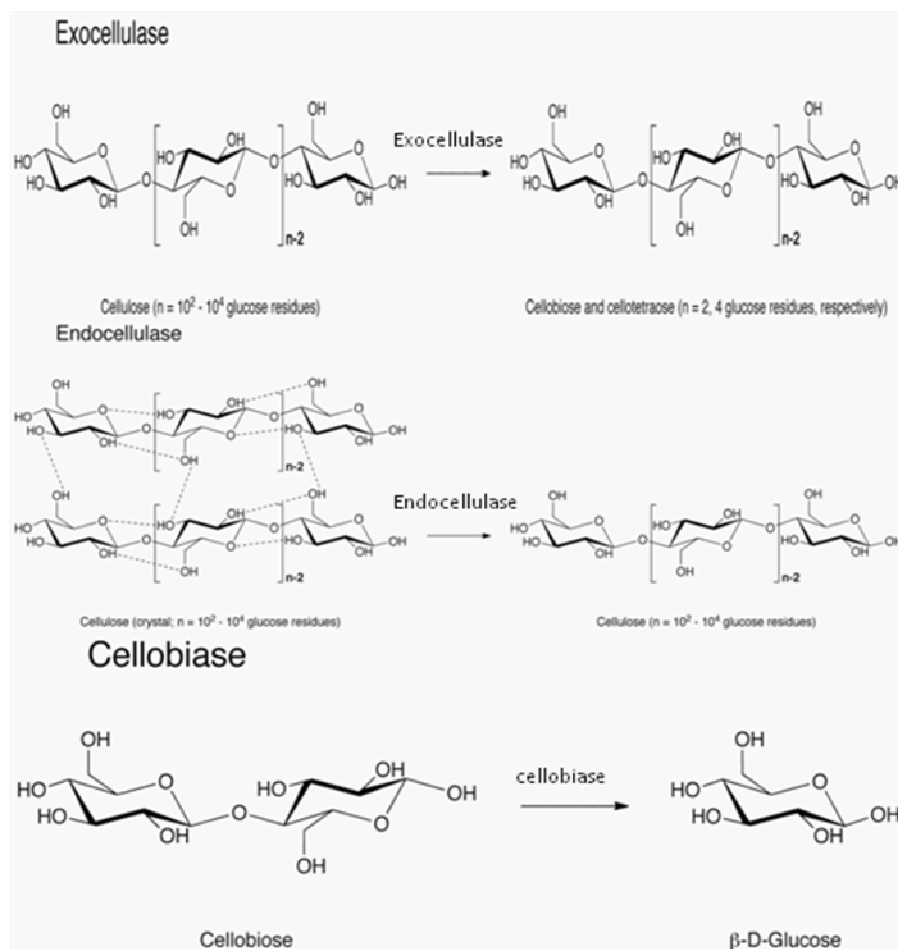


Fig. 3 Molecular structure of cellulose and site of action of endocellulase, exocellulase and cellobiase (www.wikipedia.com)

Chain length of cellulose can vary between 100 and 14000 glucose residues, which form microfibrils. The microfibrils consist of highly ordered crystalline regions interspersed by more disordered amorphous regions. The crystalline regions of cellulose are rigid and not easily accessible to endo-acting cellulases while the amorphous regions are easily attacked by dilute acid, endoglucanases or exoglucanases (Sinitzyn *et al.*, 2000). Cellulases (1,4-D glucan glucanohydrolase, EC 3.2.1.4) are multienzyme complexes, comprising three main components; endo-glucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β -glucosidase (EC3.2.1.21) which have been shown to act synergistically in the hydrolysis of celluloses (Fadel, 2000).

A first step is the degradation of polysaccharides chain by the action of extracellular endoglucanase and exoglucanases. Endoglucanases attack β -1, 4-

glucosidic bond of cellulose chain randomly and forming polymer chain ends and soluble oligosaccharides. Cellobiohydrolases cleave cellobiosyl residue from the end of celluloses chain and product formed is cellobiose which is further degraded by β -glucosidase. The last step is inhibitory to exo and endocellulases (Biosett *et al.*, 2001; Spiridonov and Wilsen 2001). All enzymes involved in the biodegradation of cellulose, cellobiohydrolases appears to be the important of key enzyme for the degradation of native crystalline celluloses (Kundanga and Mwenge, 2005)

Cellulolytic enzyme system from the filamentous fungi, especially *Trichoderma reesei*, contains two exoglucanase of cellobiohydrolases (CBH1 and CBH2), at least four endoglucanases (EG1, EG2, EG3 and EG5) and one β -glucosidase. These enzyme acts synergistically to catalyse the hydrolysis of cellulose (Kumar *et al.*, 2008). Exo-1, 4- β glucanases were essential components of fungal cellulose system. These enzyme degraded microcrystalline cellulose by liberating cellobiose from the non reducing ends of the β -1,4 glucan chains (Wood *et al.*, 2003) Exoglucanases were generally found to be inactive towards substituted cellulose derivative such as carboxymethylcellulose. In contrast, endo-1,4- β -glucanases usually highly active towards carboxymethylecellulose but unable to attack crystalline cellulose. For fungal cultures it has been proposed that cellobiohydrolases carry out the initial hydrolysis of native cellulose and the endoglucanases act on soluble cellodextrins (Beguin and Aubert 1994)

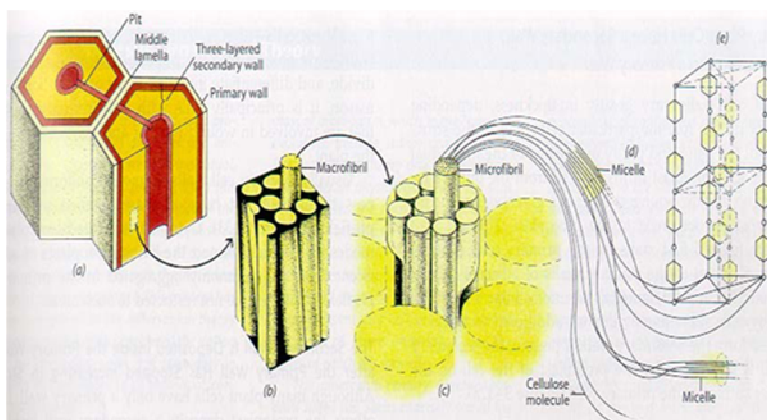


Fig. 4. Formation of micro and macrofibrils (fibres) of cellulose and their position in the wall Cellulose (Harmsen *et al.*, 2010)

2.1.2 Hemicellulose

2.1.2.1 Structure Chemistry of Hemicelluloses

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. Hemicelluloses are polysaccharides more heterogeneous than cellulose and are second most abundant organic in the plant cell wall. The major hemicelluloses polymer in cereals and hardwood is xylan, representing up to 30-35 per cent of the total dry mass (Joseleau *et al.* 1992)

Hemicelluloses are structurally unrelated to celluloses and are so named as these were incorrectly believed to be the precursor of celluloses (Singla and Chauhan, 1995). Among hemicelluloses, xylan is a heterogeneous polysaccharide in which β -1,4-linked D-xylopyranose residues are the main constituents depending on their origin, xylan may also contain variable amounts of arabinosyl and 4-O-methylglucuronic acid residues and acetyl groups. The most important enzyme in the xylan biodegradation is the endo-1, 4- β -xylanase (EC3.2.1.8) that releases xylopyranose units (Madlala *et al.*, 2001). It comprises up to 30 percent of cell wall material of annual plant, 15-30 per cent of hardwoods and 7-10 per cent of softwood (Jeya *et al.*, 2005).

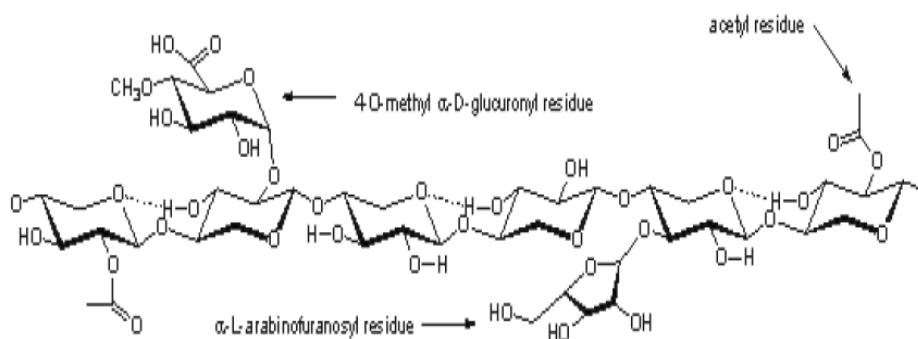


Fig 5. Structure of xylan (Harmsen *et al.*, 2010)

Xylan and glucomannan form the basic backbone polymer of hemicelluloses in wood. In hard wood there are twice as many 4-O methyl glucuronic acid side groups; on an average two out of ten xylan units are substituted with uronic acid. The ratio of arabinose side groups to xylose residues

in softwood xylan is 1:8. Xylan in hardwood and softwood differ in their degree of polymerization which in hardwood xylan is about 15-200 and in softwood about 7-130 (Fihlo *et al.*, 1994).

2.1.2.3 Biochemistry of Hemicelluloses

Xylan constitutes a group of complex structure polymers collectively referred to as “Hemicelluloses” (Silva *et al.*, 2005). Xylan being a heterogeneous polymer requires the concentrated action of complex enzyme system, degrading the backbone as well as side chains of xylans and converting it into constituent sugars (Amani *et al.*, 2007). Several hydrolytic enzymes are involved in complete breakdown of branched acetyl xylan which are given below (Dodd and Cann 2010).

1. Endo-1,4- β - xylanase (EC3.2.1.8)
2. β -D-xylosidase (EC 3.2.1.37)
3. α -L-arabinofuranosidase (AFs) (EC 3.2.1.55)
4. α -glucuronidase (EC 3.2.1.139)
5. Acetyl xylan esterase (EC 3.1.1.72)
6. Ferulic/coumaric acid esterase (EC 3.1.1.73)

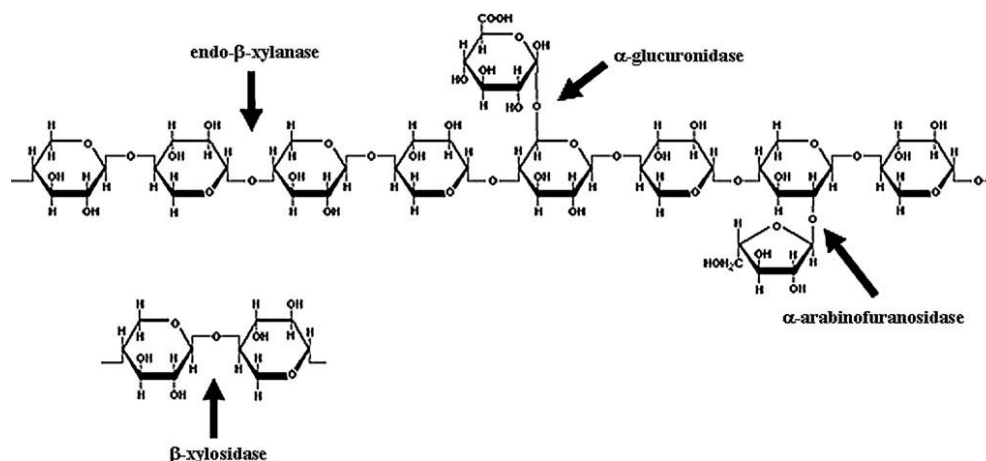


Fig 6. Polymeric chemical structure of hemicellulose and targets of hydrolytic enzymes involved in hemicellulosic polymer degradation (Kumar *et al.*, 2008)

The best known are endo-1,4- β - xylanase (xylanases), which attacks the polysaccharides backbone and β -xylosidase which hydrolyze xylo-oligosaccharide to D-xylose. Microorganisms often produce more than one type of xylanase. These differ in substrates binding site and consequently in the number of unsubstituted xylose units in series required in the polysaccharides backbone. Many of the microorganisms which degrade celluloses are also able to hydrolyze xylan, using the most part, distinct and different enzymes to attack the two substrates (Coughlan *et al.*, 1993). The complete hydrolysis of xylan requires number of enzymes with diverse specificity and mode of action. Endo-1,4- β -xylanase which hydrolyse the β -1,4-linked xylose backbone has been found to be the most important enzyme in hemicelluloses hydrolysis (Bhat and Hazlewood, 2001)

Xylanases are produced by a wide range of bacteria and fungi, including aerobes, anaerobes, mesophiles, thermophiles and extremophiles. Aerobic bacteria and fungi generally produce extracellular xylanases. β -1,4-endoxylanase (1,4-D-xylan xylohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in a decrease in the degree of polymerisation (DP) of the substrate. The attack of the substrates is random and the bonds to be hydrolysed depend on the nature of substrate. During hydrolysis of xylan, the main products formed are xylooligosaccharides (Wong *et al.*, 2000).

2.2 ISOLATION AND SCREENING OF CELLULOLYTIC AND HEMICELLULOLYTIC MICROORGANISMS

2.2.1 Isolation and Screening of Cellulolytic and Hemicellulolytic Bacteria

Plant wastes present high cellulose content, which is an ideal organic material for composting. Five strains of thermophiles from processed *Brassica* waste were isolated and the hydrolytic activity on various cellulosic biomass substrates and their temperature profiles were determined. 16S rRNA sequencing identified these strains as *Thermoactinomyces* and *Bacillus* sp. Maximal cellulase activity corresponded to 2.3 U mL⁻¹ of enzyme. The application of these strains on *Brassica rapa* residues demonstrates increased total nitrogen content. TA-3, a *Thermoactinomyces* sp. strain, performs best among all inoculants, increasing

the nitrogen content from 0.74 to 0.91%, and decreasing the carbon content from 15.4 to 12.2%, showing its high efficiency and bioactivity during compositing (Chang *et al.*, 2009).

Yung-Chung Lo *et al.* (2009) reported that nine cellulolytic bacterial strains were isolated from soil sample taken in southern Taiwan. Through 16S rRNA sequence matching; eight of those isolates belong to *Cellulomonas* sp., while the other one belongs to *Cellulosimicrobium cellulans*. The activity of cellulolytic enzymes (cellulases and xylanase) produced from those strains was mainly present extracellularly and the enzyme production was dependent on cellulosic substrates (xylan, rice husk and rice straw) used for growth. HPLC analysis confirmed the bacterial hydrolysis of these cellulosic substrates for soluble sugars production. The efficiency of fermentative hydrogen production from the enzymatically hydrolyzed rice husk was examined with seven hydrogen producing pure bacterial isolates. With an initial reducing sugar concentration of 0.36 g l^{-1} , only *Clostridium butyricum* CGS5 exhibited efficient hydrogen production from the rice husk hydrolysates with a cumulative hydrogen production and hydrogen yield of 88.1 ml l^{-1} and $19.15 \text{ mmol hydrogen (g reducing sugar)}^{-1}$ (or $17.24 \text{ mmol hydrogen (g cellulose)}^{-1}$), respectively.

Thermophilic cellulases producing bacteria have been isolated from an Egyptian hot spring by enrichment of the water and soil samples with cellulose for 3 weeks at 70°C were studied by Ibrahim and Diwany (2007). Three isolates termed EHP1, EHP2 and EHP3 have been isolated. The phylogenetic analysis of these strains using its 16S rDNA sequence data showed that strain EFP1 had highest homology (98.5%) with *Anoxybacillus flavithermus*, EFP2 showed 98.5 % similarity with *Geobacillus thermodenitrificans* and EHP3 showed 99.0 % similarities with *Geobacillus stearothermophilus*. Maximal cellulases production by *Anoxybacillus flavithermus* EHP2 was detected at the end of the stationary phase (36 h). The crude cellulase had activity toward avicell, CMC, cellobiose, and xylan, but there was no detectable activity on *p*-nitrophenyl- β -D-glucopyranoside. The rate of CMC degradation was higher than any other substrates used in this study. The optimum temperature and pH for the crude

enzyme activity was 75 °C and 7.5, respectively. Kumari *et al.* (2011) reported the screening and isolation of cellulolytic fungi from the soil samples collected from different areas of Hyderabad and identification of the isolates based on staining and molecular characterization and efforts were taken to optimize the cultural and environmental conditions for maximizing the yield of the enzyme. Identification of the fungal isolates was done based on biochemical and molecular characterization by sequencing the 18S rRNA coding gene.

Wedekind *et al.* (1988) observed that the fibrolytic microbiota of the human large intestine was examined to determine the numbers and types of cellulolytic and hemicellulolytic bacteria present. Fecal samples from each of five individuals contained bacteria capable of degrading the hydrated cellulose in spinach and in wheat straw pretreated with alkaline hydrogen peroxide (AHP-WS), whereas degradation of the relatively crystalline cellulose in Whatman no. 1 filter paper (PMC) was detected for only one of the five samples. The mean concentration of cellulolytic bacteria, estimated with AHP-WS as a substrate, was 1.2×10^8 /ml of feces. Pure cultures of bacteria isolated on AHP-WS were able to degrade PMC, indicating that interactions with other microbes were primarily responsible for previous low success rates in detecting fecal cellulolytic bacteria with PMC as a substrate. The cellulolytic bacteria included *Ruminococcus* spp., *Clostridium* sp., and two unidentified strains. The mean concentration of hemicellulolytic bacteria, estimated with larchwood xylan as a substrate, was 1.8×10^6 /ml of feces. The hemicellulose-degrading bacteria included *Butyrivibrio* sp., *Clostridium* sp., *Bacteroides* sp., and two unidentified strains, as well as four of the five cellulolytic strains. This work demonstrates that many humans harbor intestinal cellulolytic bacteria and that a hydrated cellulose source such as AHP-WS is necessary for their consistent detection and isolation.

Sangkharak *et al.* (2011) reported that five novel cellulase-producing bacteria were isolated and identified through 16S rRNA sequence as *Cellulomonas* sp. The activity of enzymes (mainly xylanase and endoglucanase) produced from isolated strains was almost present extracellularly and the production of enzyme was dependent on cellulosic substrate (xylan, rice straw

and wastepaper) used for growth. The optimal condition for cellulase production consisted of 4 of waste paper with controlled pH at 6 and cultivation temperature at 35°C. *Cellulomonas* sp. strain TSU-03 produced the highest activity of xylanase and endoglucanase at 1860.1 and 388.5 U mg⁻¹ protein, respectively. At 50°C, cellulase was highly stable and losing less than 20% of initial activity after 24 h of incubation. Cellulase production from strain TSU-03 can be an advantage as the activity of enzymes is the highest value ever reported from *Cellulomonas* sp. Therefore, waste paper hydrolysate was utilized as substrate for ethanol production using *S. cerevisiae* the highest ethanol production was 12.5 g L⁻¹ after 48 h of cultivation under separate hydrolysis and fermentation (SHF) process.

2.2.2 Isolation and Screening of Cellulolytic and Hemicellulolytic Fungi

Jahangeer *et al.* (2005) isolated of 115 fungal cultures from environmental sources including soil, air and infected plant, 78 (67.83%) were found to possess cellulose degrading ability. Cellulolytic fungi belonged *Aspergillus* sp., *Trichoderma* sp., *Fusarium* sp., *Alternaria* sp., *Penicillium* sp., and *Rhizopus* sp. Cellulase production by fungi was measured by using enzyme assays. Highest yield of enzyme was noted at 37°C while maximum activity in the range of pH 4 -4.8. Kinetics of enzyme production was also studied on selected *Aspergillus* isolates and mostly high enzyme production was observed after 7 days. Cellulase synthesis increased by 10 folds in the presence of cellulose while it repressed in the presence of glucose.

Chinedu *et al.* (2005) Wood-wastes from dump-sites at Okobaba Saw-mills on the western part of the Lagos lagoon were examined for cellulolytic microorganisms. Cellulolytic microfungi were isolated from the wastes using minimal salt agar medium containing 0.2% (w/v) crystalline cellulose, sugarcane pulp, corn cob or saw-dust as sole carbon/energy source. The colonies of cellulolytic microfungi which appeared on the plates increased in size and number as the incubation period (days) increased. Among the fungal isolates were two pathogenic *Aspergilli* (*A. flavus* and *A. fumigatus*), three different black *Aspergilli* (herein designated as *A. niger* I, *A. niger* II and *A. niger* III), *Botrytis cinerea*, *Fusarium* species and *Penicillium* species. Cell-free filtrates of 7 day

cultures of *A. flavus*, *A. niger* I, *A. niger* II, *B. cinerea* and *Penecillium* sp. grown on the minimal salt broth supplemented with crystalline cellulose as sole carbon/energy source showed very significant CM–cellulase activity. *Penecillium* species gave a very high value that was over 4 times the value for the closest organism, *Asperrgillus niger* II. There is a good prospect for cellulase production using the virgin strain of *Penecillium* species isolated from the wood-wastes.

Palaniswamy *et al.* (2008) reported that consortia of litter degrading fungal species were developed from different baiting substrates collected in and around Western ghat forest ecosystem, Coimbatore, Tamilnadu, India. Fifty-three litter degrading fungal species were isolated by nylon litter bag technique. The production of endo- β -1,4-xylanase (1,4-b- D-xylan xylanohydrolase, E.C. 3.2.1.8), b-D-xylosidase (1,4-b-xylan xylanohydrolase, E.C. 3.2.1.37) and protease was studied using oat spelt xylan as carbon source. Results showed that all fifty-three fungal species isolated from various litter samples produced fairly good xylanolytic enzyme activity. The xylanase and β -D-xylosidase activity ranges from 4.41 to 132.20 U and 48.72 to 1510.32 U, respectively. Growth was determined in terms of mycelial dry weight, which ranged between 0.209 and 1.047 mg/ml. The protease enzyme activity was from 19.7 to 60.8 U.

Soil samples of 200 g each were collected at random from 10 different locations at dump sites of Keffi town. Malt extract agar and potato dextrose agar were used for the isolation of mesophilic filamentous fungi at incubation temperature of 25°C by Makut and Godiya (2010). All the isolates were again inoculated on chemically defined cellulose agar medium to determine their cellulases producing abilities. The results revealed that the cellulolytic mesophilic fungi were present in the soil environment of Keffi metropolis and they included *Aspergillus niger*, *Aspergillus flavus*, *Trichoderma viride*, *Penicillium* sp. and *Fusarium solani*. The fungal isolates could be harnessed as inocula for use in solid waste management involved in the biodegradation of cellulose-containing materials. The results further demonstrate that the isolates could also be harnessed for the industrial production of cellulases.

Baharuddin *et al.* (2010) isolated cellulase-producing bacterium in thermophilic compost using a mixture of Empty Fruit Bunch (EFB) and Palm Oil Mill Effluent (POME) as composting materials. The isolation of microbes from compost heap was conducted at day 7 of composting process where the mixture of composting materials consisted of 45.8% cellulose, 17.1% hemicellulose and 28.3% lignin content. The temperature, pH and moisture content of the composting pile at day 7 treatment were 58.3, 8.1 and 65.5°C, respectively. The morphological analysis of the isolated microbes was conducted using Scanning Electron Microscope (SEM) and Gram stain method. The congo red test was conducted in order to detect 1% CMC agar degradation activities. Total genomic DNAs were extracted from approximately 1.0 g of mixed compost and amplified by using PCR primers. The PCR product was sequent to identify the nearest relatives of 16S rRNA genes. The localization of bacteria chromosomes was determined by Fluorescence *In Situ* Hybridization (FISH) analysis.

Soil fungi and bacteria were isolated from various locations visited throughout the expedition held at Bario Highlands. Nine fungal species were identified and among which four isolates demonstrated cellulolytic activities. Two of these isolates namely *Trichoderma* and *Aspergillus* were discovered to be highly cellulolytic compared to the rest (Kader *et al.*, 1999). Bakri *et al.* (2010) isolated fungi from soil. One hundred and thirty-six fungal isolates were screened for xylanase production. Two xylanase producing isolates, FSS117 and FSS129, were identified on the basis of analyses of 5.8S gene sequencing. The closest phylogenetic neighbors according to 5.8S gene sequence data for the two isolates were *Aspergillus tubingensis* and *Aspergillus terreus*, respectively. When birchwood xylan or corn cob hulls was used as a substrate for 5 days under submerged culture cultivation, xylanase production from *A. terreus* FSS129 was 113 and 174 IU ml⁻¹, respectively. The pH and temperature for optimum xylanase activity were 8 and 65°C.

Ivanen *et al.* (2009) reported that new substrates, 2-(2'-benzothiazolyl)-phenyl (BTP) cellooligosaccharides with degree of polymerization (d.p.) 2–4 (BTPG2–4) were synthesized for the screening of microbial cellulolytic activity

in plate assays. The substrates were very efficient that was shown for several cellulolytic bacteria, including yeast-like isolates from Kamchatka hot springs. Three tested bacterial strains and eighteen of 30 of the yeast isolates showed ability to degrade cellulose with cellobiohydrolase, β -glucosidase and endo-cellulase activities measured with standard substrates. The structures of 2-(2'-benzothiazolyl)-phenyl oligosaccharides were solved by NMR- and mass spectrometry. The usefulness of the 2-(2'-benzothiazolyl)-phenyl substrates were also shown during purification of the *B. polymyxa* cellulolytic complex, which consists of at least three types of the enzymes: cellobiohydrolase, endo- β -D-glucanase and β -glucosidase.

The soil samples were inoculated into culture plates prepared in sabouraud medium under sterilized conditions and incubated at 30°C for 4 to 7 days. The identified fungal species were incubated in self-designed cellulose medium for testing their cellulolytic ability. Forty-two species, including 2 nova species, representing sixteen genera showed growth and sporulation in the cellulose medium. Most of the isolated species were from genus *Aspergillus* and *Penicillium*. *Aspergillus niger* and *Mucor hiemalis* showed highest occurrence frequency (45% and 36% respectively), as these species were collected from about 80% of soil samples. Being agar free and cheaper, the new fungal medium designed showed results equivalent to Sabouraud medium (Mahmood *et al.*, 2006).

Some bacteria utilizing plum-seeds were isolated from the plum-grove soil samples by biodegradation assay. Among them, five bacteria had the activity of cellulase and xylanase. Four of the strains grew in the medium containing xylan instead of glucose. Partial sequencing of 16S rRNA and genome indicated that all the isolates belong to the *Pseudomonas* section but two bacteria are unknown. It is proposed that some *Pseudomonas* bacteria could be deployed as inoculant to attain the treatment of plum-seeds (Kurishita *et al.*, 2010).

Eida *et al.* (2011) studied the evaluation of cellulolytic and hemicellulolytic fungi isolated from sawdust compost (SDC) and coffee residue compost (CRC). To identify fungal isolates, the ITS region of fungal rRNA was amplified and sequenced. To evaluate enzyme production, isolates were

inoculated onto wheat bran agar plates, and enzymes were extracted and tested for cellulase, xylanase, β -glucanase, mannanase, and protease activities using different azurine cross-linked (AZCL) substrates. In total, 18 isolates from SDC and 29 isolates from CRC were identified and evaluated. Four genera (*Aspergillus*, *Galactomyces*, *Mucor*, and *Penicillium*) and five genera (*Aspergillus*, *Coniochaeta*, *Fusarium*, *Penicillium*, and *Trichoderma/Hypocrea*) were dominant in SDC and CRC, respectively. *Penicillium* sp., *Trichoderma* sp., and *Aspergillus* sp. displayed high cellulolytic and hemicellulolytic activities, while *Mucor* isolates exhibited the highest β -glucanase and mannanase activities. The enzyme analysis revealed that *Penicillium*, *Aspergillus* and *Mucor* isolates significantly contributed to the degradation of SDC, whereas *Penicillium*, *Aspergillus*, and *Trichoderma* isolates had a dominant role in the degradation of CRC. Notably, isolates SDCF5 (*P. crustosum*), CRCF6 (*P. verruculosum*), and CRCF2 and CRCF16 (*T. harzianum/H. lixii*) displayed high activity regarding cellulose and hemicellulose degradation, which indicates that these species could be beneficial for the improvement of biodegradation processes involving lignocellulosic materials.

Sridevi and Charya (2011) reported cellulase-free xylanase-producing fungi, screening and isolation of fungi was done using decaying wood, agricultural wastes and other lignocellulosic wastes as microbial source. Thirty fungal species were selected for further analysis based upon clearing zones formation on xylan enriched agar plates. Submerged fermentation was done with 30 fungal isolates to identify strain that could produce highest amount of cellulase-free xylanase at a pH of 5.5 and at $28 \pm 2^\circ\text{C}$ temperature. Under screening conditions, 90% of the strains produced xylanase (6.6 to 495 U/ml) and negligible amount of cellulase (0.6 U/ml) with growth. Growth was determined in terms of mycelial dry weight which ranged between 0.6 to 2.34 mg/ml. The amount of soluble protein was also determined which ranged between 102 to 300 $\mu\text{g/ml}$. The pH change after incubation was in between 5.0 to 8.0. Strain specific variability in xylanase production was confirmed in *Aspergillus* sp. and *Penicillium* sp. In addition, this study shows here that *Rhizopus* sp. can also produce xylanase under given conditions.

2.3 EFFECT OF PRETREATMENT ON LIGNOCELLULOSIC BIOMASS FOR ENZYME PRODUCTION

The inherent properties of native lignocellulosic materials make them resistant to enzymatic attack. The aim of pretreatment is to change these properties in order to prepare the materials for enzymatic degradation (Taherzadeh and Kirmani, 2008).

Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic structural and chemical composition to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars and to make cellulose more accessible to the enzymes (Chang and Holtzaple, 2000).

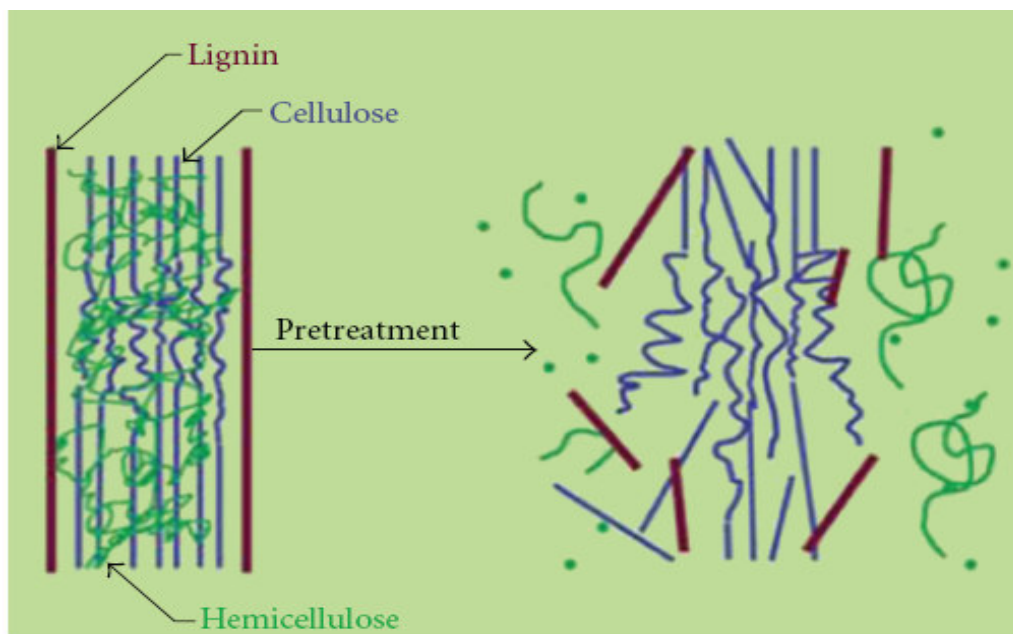


Fig 7. Schematic representation of the matrix of polymers in which cellulose exists. Pretreatment of biomass by different methods removes hemicellulose and lignin from this matrix before hydrolysis

Table 1. Pretreatment processes of lignocellulosic materials

Pretreatment method	Processes	Studied application	Possible changes in biomass	Notable remarks
	Milling:	Ethanol		
	- Ball milling			
	- Two-roll milling			
	- Hammer milling			- Most of the methods
	- Colloid milling			are highly energy-
	- Vibro energy milling		- Increase in	demanding
	Irradiation:	Ethanol and biogas	accessible surface area and pore size	- Most of them cannot remove the
	- Gamma-ray irradiation		- Decrease in	lignin
Physical pretreatments	- Electron-beam irradiation		cellulose crystallinity	- It is preferable not to use these methods
	- Microwave irradiation		- Decrease in degrees of	for industrial applications
	Others:	Ethanol and biogas	polymerization	-No chemicals are generally required for these methods
	- Hydrothermal			
	- High pressure steaming			
	- Expansion			
	- Extrusion			
	- Pyrolysis			
	Gas:	Ethanol	complete	the most promising
	Explosion:	Ethanol and biogas		
	- Steam explosion			
	- Ammonia fiber explosion (AFEX)			
	- CO ₂ explosion			
	- SO ₂ explosion			
	Alkali:	Ethanol		
	- Sodium hydroxide	and biogas		
	- Ammonia			
	- Ammonium Sulfite			
	Acid:	Ethanol	- Increase in	
	- Sulfuric acid	and biogas	accessible surface area	- These methods are among the most
	- Hydrochloric acid			effective and include
	- Phosphoric acid		- Partial or nearly	
Chemical and physicochemical pretreatments	Gas:	Ethanol and biogas	complete delignification	the most promising processes for industrial applications
	- Chlorine dioxide		- Decrease in cellulose crystallinity	- Usually rapid treatment rate
	- Nitrogen dioxide			
	- Sulfur dioxide			
	Oxidizing agents:	Ethanol and biogas	- Decrease in degrees of polymerization	- Typically need harsh conditions
	- Hydrogen peroxide		- Partial or complete	
	- Wet oxidation		hydrolysis of	- There are chemical requirements
	- Ozone		hemicelluloses	
	Solvent extraction of lignin:	Ethanol		
	- Ethanol-water extraction			
	- Benzene-water extraction			
	- Ethylene glycol extraction			
	- Butanol-water extraction			
	- Swelling agents			- Low energy requirement
Biological pretreatments	Fungi and actinomycetes	Ethanol and biogas	- Delignification - Reduction in degree of polymerization of cellulose - Partial hydrolysis of hemicellulose	- No chemical requirement - Mild environmental conditions - Very low treatment rate

(Taherzadeh and Karimi, 2008)

Several methods have been introduced for pretreatment of lignocellulosic materials prior to enzymatic hydrolysis or digestion. These methods are classified into “Physical pretreatment”, “Physicochemical pretreatment”, “Chemical pretreatment”, and “Biological pretreatment”.

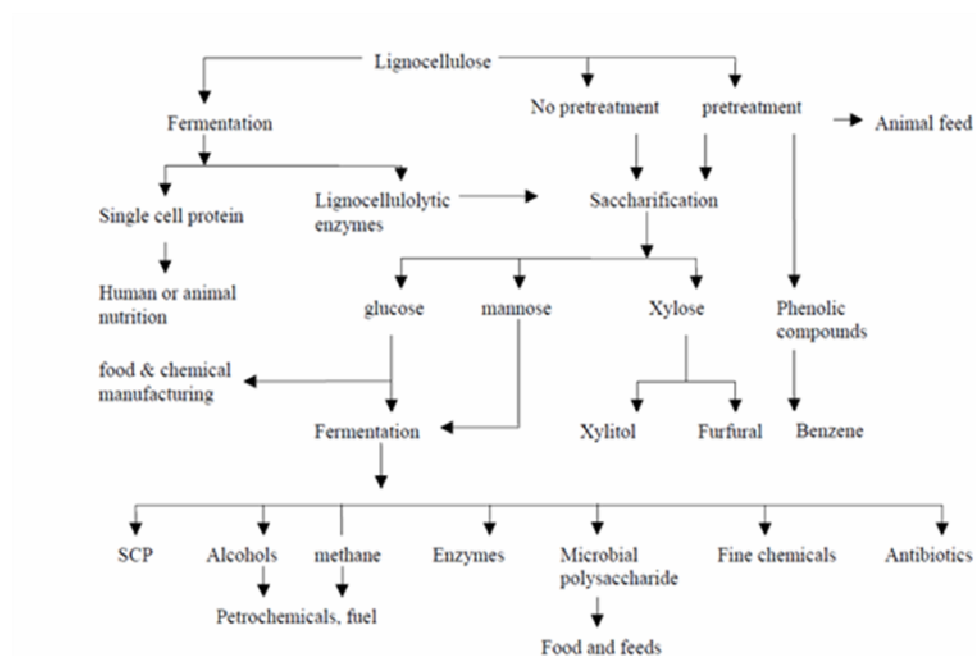


Fig. 8. Generalized process stages in lignocellulose bioconversion into value added bioproducts (Howard *et al.*, 2003)

2.3.1 Physical Pretreatment of Lignocellulosic Biomass

Physical pretreatment can increase the accessible surface area and size of pores, and decrease the crystallinity and degrees of polymerization of cellulose. Different types of physical processes such as milling (e.g. ball milling, two-roll milling, hammer milling, colloid milling, and vibro energy milling) and irradiation (e.g. by gamma rays, electron beam or microwaves) can be used to improve the enzymatic hydrolysis or biodegradability of lignocellulosic waste materials.

The agricultural residues, wheat bran and rice hulls, were used as substrates for cellulase production with *Trichoderma* sp. 32942 by solid-state fermentation. Microwave irradiation was employed to pretreat the substrates in order to increase the susceptibility. Although the highest cellulase yield was obtained by the substrates pretreated by 450W microwaves for 3 min,

pretreatment time and microwave power had no significant effect on cellulase production. The initial reducing sugar content (RSC) of substrates was decreased by microwave irradiation, but more reducing sugars were produced in later fermentation (Zhao *et al.*, 2010).

Shimokawa *et al.* (2007) reported the effect of gamma-ray irradiation on enzymatic hydrolysis of spent enokitake mushroom substrate containing corncob meal and rice bran as major components. Polysaccharides consisting of glucose and xylose residues were the major components of the spent substrate. The saccharification rate of the spent substrate doubled with irradiation at a dose of 500 kGy, and the irradiated sample formed finer particles than the untreated sample after the same period of grinding. The holocellulose was apparently depolymerized by irradiation, resulted in the fragility of the substrate. An appreciable amount of xylan-derived sugars were extracted with water from the irradiated sample. The enzymatic saccharification rate of the irradiated spent enokitake substrate increased to over 80% with cellulysin TP 25 from *Trichoderma viride*, which possesses endo- β -D-xylanase and β -D-xylosidase activities as well as cellulase activities.

Ma *et al.* (2009) investigated that Box–Behnken design and response surface methodology were employed to plan experiments and optimize the microwave pretreatment of rice straw. Experimental results show that microwave intensity (MI), irradiation time (IT) and substrate concentration (SC) were main factors governing the enzymatic saccharification of rice straw. The maximal efficiencies of cellulose, hemicellulose and total saccharification were respectively increased by 30.6%, 43.3% and 30.3% under the optimal conditions of MI 680 W, IT 24 min and SC 75 g/L. The chemical composition analysis of straw further confirmed that microwave pretreatment could disrupt the silicified waxy surface, break down the lignin–hemicellulose complex and partially remove silicon and lignin.

Zhu *et al.* (2010) reviewed the technical issues associated with cellulosic ethanol production from woody biomass: energy consumption for woody biomass

pretreatment, pretreatment energy efficiency, woody biomass pretreatment technologies, and quantification of woody biomass recalcitrance. Both total sugar yield and pretreatment energy efficiency, defined as the total sugar recovery divided by total energy consumption for pretreatment, should be used to evaluate the performance of a pretreatment process. A post-chemical pretreatment wood size-reduction approach was proposed to significantly reduce energy consumption and low liquid-to-wood ratio (L/W) to reduce thermal energy consumption for any thermochemical/ physical pretreatment in addition to reducing pretreatment temperature.

Ballesteros *et al.* (2002) evaluated the effect of particle size on steam-explosion pretreatment of herbaceous lignocellulosic biomass. Hemicellulose and cellulose recovery and effectiveness of enzymatic hydrolysis of the cellulosic residue is presented for steam-explosion pretreatment of an agriculture residue (*Brassica carinata*) using different particle sizes. The parameters tested were: particle size (2/5, 5/8 and 8/12 mm), temperature (190 and 210°C), and residence time (4 and 8 min). The compositional analysis of filtrate and water insoluble fibre after pretreatment and enzymatic digestibility. Larger steam-exploded particle (8/12 mm) results in higher cellulose and enzymatic digestibilities.

2.3.2 Chemical Pretreatment of Lignocellulosic Biomass

2.3.2.1 Alkali Pretreatment of Lignocellulosic Biomass

Chinedu *et al.* (2008) reported that *Aspergillus niger* (ANL301) and *Penicillium chrysogenum* (PCL 501) cultured in basal media with cellulose as sole carbon source yielded extracellular enzymes which partially hydrolyzed sawdust and sugarcane pulp into simple sugars. Pre-treatment of sawdust by ammonium hydroxide steeping increased the yield of simple sugars. The reducing sugars released from the pretreated sawdust by the crude enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL 501) were 3.58% and 7.02% of the total hydrolysable sugars respectively. This is in contrast to the 0.92% and 1.02% of the total hydrolysable sugars released respectively by the enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL 501) from the non-pretreated sawdust. Enzymatic hydrolysis of sugarcane pulp by the crude enzymes was not

significantly affected by ammonia pre-treatment. Reducing sugars released from non-pretreated sugarcane pulp by the crude enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL 501) were respectively 4.17% and 5.08% of the total hydrolysable sugars.

Cellulases produced by *Bacillus subtilis* were used for the saccharification of wheat straw, rice straw and bagasse. Pretreatment of these substrates with 2% NaOH was found to be more effective for increasing the saccharification. The saccharification rates of 33.0, 25.5 and 35.5% were obtained with 2% NaOH pretreated wheat straw, rice straw and bagasse, respectively. The saccharification of wheat straw was 33, 26 and 16.9% after 20h at 50°C when 4, 6 and 10% substrate was used, respectively. The per cent conversion reduced with increasing substrate concentration. Same trend in the hydrolysis rates of pretreated rice straw and bagasse were observed when used at increased concentrations. The per cent saccharification of these substrates decreased when the concentration was increased to 10%. It was observed that the per cent saccharification of these substrates increased with increasing enzyme concentration (Akhtar *et al.*, 2001).

Agricultural wastes contained a good amount of lignin, which protects the xylan to be attached by hydrolytic enzymes. To make components of lignocellulosic more accessible, substrates were autoclaved with 10 ml of different concentrations of alkali (0.1, 0.5 and 1.0N) for 30 min. After fermentation process filtrate was used for xylanase estimation (Goyal *et al.*, 2008).

To optimize the enzymatic conversion of widely available lignocellulosic biomass-wheat straw (WS), pretreatment facilitating the enzymatic saccharification process was first performed by alkaline peroxide, resulting in a substrate consisting of 60.17% cellulose, 29.53% hemicelluloses and 4.59% lignin. Using response surface methodology, the combined effects of enzyme loading, substrate concentration, surfactant concentration, and reaction time on hydrolysis yield from enzymatic saccharification of WS were further investigated. The results showed that both enzyme loading and substrate concentration had interactions with surfactant concentration. A quadratic

polynomial equation for predicting the hydrolysis yield was developed. The experimental results were in good agreement with predicted values. Therefore, the model could be successfully used to identify the effective combinations of the four factors for predicting hydrolysis yield (Qi *et al.*, 2009).

A new pretreatment technology using dilute ammonium hydroxide was evaluated for ethanol production on sorghum. Sorghum fibers, ammonia, and water at a ratio of 1:0.14:8 were heated to 160°C and held for 1 h under 140–160 psi pressure. Approximately, 44% lignin and 35% hemicellulose were removed during the process. Hydrolysis of untreated and dilute ammonia pretreated fibers was carried out at 10% dry solids at an enzyme concentration of 60 FPU Spezyme CP and 64 CBU Novozyme 188/g glucan. Cellulose digestibility was higher (84%) for ammonia pretreated sorghum as compared to untreated sorghum (38%). Fermentations with *Saccharomyces cerevisiae* D5A resulted in 24 g ethanol /100 g dry biomass for dilute ammonia pretreated sorghum and 9 g ethanol /100 g dry biomass for untreated sorghum (Salvi *et al.*, 2010).

Raghavendra and Geeta (2007) process was developed for hydrolyzing paddy straw, wheat straw and sugarcane bagasse into fermentable sugars. The powdered substrates were delignified with 3 per cent NaOH and subjected for microbiological and commercial cellulase enzyme pre-treatment. Among the fungal cultures *Trichoderma reesei* has yielded maximum reducing sugar of 22.30 mg/g in paddy straw, 25.56 mg/g in wheat straw and 28.56 mg/g in sugarcane bagasse. In case of commercial cellulase enzyme sugarcane bagasse has yielded maximum reducing sugar 136.86 mg/g after hydrolysis with 2.5 per cent enzyme concentration at 45°C for 24 h of incubation.

Han and Callihan (1974) investigated the effects of chemical, physical, and enzymatic treatments of rice straw and sugarcane bagasse on the microbial digestibility of cellulose have been investigated. Treatment with 4% NaOH for 15 min at 10°C increased the digestibility of cellulose from 29.4 to 73%. Treatment with 5.2% NH₃ could increase digestibility to 57.0%.

He *et al.* (2010) studied an integrated wet-milling and alkali pretreatment was applied to corn stover prior to enzymatic hydrolysis. The effects of NaOH

concentration in the pretreatment on crystalline structure, chemical composition, and reducing-sugar yield of corn stover were investigated, and the mechanism of increasing reducing-sugar yield by the pretreatment was discussed. The experimental results showed that the crystalline structure of corn stover was disrupted, and lignin was removed, while cellulose and hemicellulose were retained in corn stover by the pretreatment with 1% NaOH in 1 h. The reducing-sugar yield from the pretreated corn stovers increased from 20.2% to 46.7% when the NaOH concentration increased from 0% to 1%. The 1% NaOH pretreated corn stover had a holocellulose conversion of 55.1%.

2.3.2.2 Acid Pretreatment of Lignocellulosic Biomass

Saline crops were evaluated for their potential to be used as feedstock for fermentable sugar production via dilute acid pretreatment and enzymatic hydrolysis by Zheng *et al.* (2006). The saline crops included two woods, Athel (*Tamarix aphylla* L) and Eucalyptus (*Eucalyptus camaldulensis*), and two grasses, Jose Tall Wheatgrass (*Agropyron elongatum*) (JTW) and Creeping Wild Rye (*Leymus triticoides*) (CWR). Each of the biomass materials was first treated with dilute sulfuric acid under selected conditions (acid concentration, temperature and time) and then treated with enzymes (cellulases and β -glucosidase). The chemical composition (cellulose, hemicellulose and lignin contents) of each biomass material and the yields of total and different types of sugars after the acid and enzyme treatments were determined. The results showed that among the saline crops evaluated, the two grasses had the highest glucose yield (87% cellulose conversion) and fastest reaction rate during the enzyme.

Ethanol production from rice straw by simultaneous saccharification and fermentation (SSF) with *Mucor indicus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae* was investigated and compared with pure cellulose, Avicel, as a reference was studied by Karimi *et al.* (2006). The straw was pretreated with dilute-acid hydrolysis. The SSF experiments were carried out aerobically and anaerobically at 38°C, 50 g/l dry matter (DM) solid substrate concentration and 15 or 30 filter paper unit (FPU)/g DM of a commercial cellulase. The experiments were ended after 7 days, while an average of 2–3 days were usually enough to achieve the maximum ethanol yield. All the strains were able to

produce ethanol from the pretreated rice straw with an overall yield of 40–74% of the maximum theoretical SSF yield, based on the glucan available in the solid substrate. *R. oryzae* had the best ethanol yield as 74 from rice straw followed by *M. indicus* with an overall yield of 68% with 15 FPU/g DM of cellulase. Glycerol was the main byproduct of the SS by *M. indicus* and *S. cerevisiae* with yields 117 and 90 mg/g of equivalent glucose in the pretreated straw, respectively, while *R. oryzae* produced lactic acid as the major byproduct with yield 60 mg/g glucose equivalent in pretreated rice straw under anaerobic conditions.

Intra-particle diffusion of sulfuric acid into sugarcane bagasse, corn stover, rice straw and yellow poplar was investigated to determine the effective diffusivity of sulfuric acid within the porous biomass structure by Kim and Lee (2002). Diffusion experiments were conducted over 25–75°C for two different biomass sizes using dynamic diffusion test cells. Diffusivities of sulfuric acid in agricultural residues were significantly higher than those of hard wood. Diffusivity data for each biomass were fitted into the Arrhenius equation for extrapolation to higher temperatures. The diffusivity data were subsequently incorporated into a theoretical model to determine acid profile within the biomass matrix. The modeling results indicate that intra-particle diffusion of acid influences the rate of dilute-acid pretreatment if unground biomass feedstock is used under normal pretreatment conditions.

2.3.2.3 Chemical and Physicochemical Pretreatments of Lignocellulosic Biomass

Palmarola-Adrados *et al.* (2005) investigated the parameters optimized in the steam pretreatment step were residence time (5–15 min), temperature (190–215°C), and concentration of the acid catalyst (0 or 0.5% H₂SO₄). A microwave oven was employed for screening of the optimal conditions to obtain the highest sugar yield following combined pretreatment and enzymatic hydrolysis. The final optimization of the pretreatment prior to enzymatic hydrolysis was performed on a larger scale, in a steam pretreatment unit. Simultaneous saccharification and fermentation was carried out following steam pretreatment on 5 and 10% dry matter steam-pretreated slurries. Fermentability tests were performed to determine the effect of by-products (i.e. furfural and 5-hydroxymethyl furfural) in

the bioconversion of glucose to ethanol by baker's yeast. The maximum glucose yield, 88% of the theoretical, was obtained following steam pretreatment with 0.5% H₂SO₄ at 200°C for 10 min. Under these conditions, a sugar to ethanol conversion of 81% was attained in simultaneous saccharification and fermentation.

Wheat straw is a suitable raw material for ethanol production, since it has high cellulose content. In the bioconversion of lignocellulosic materials to ethanol, pretreatment of the material prior to enzymatic hydrolysis is essential to obtain high overall yields of sugar and ethanol by Zabihi *et al.* (2010). The pretreatment of wheat straw by steam explosion soaked with acetic acid or ethanol prior to the pretreatment was investigated by varying the temperature (180–225)°C and the retention time (3–60 min). The results showed that the pretreatment of wheat straw by steam explosion with soaked with acetic acid or ethanol was more effective than that by steam explosion alone. Additionally, the pretreatment of wheat straw by steam explosion at 210°C and 10 min, by steam/acetic acid at 220°C and 8 min and by steam/ethanol at 220°C and 5 min resulted in the best overall yield of reducing sugar which was found to be 177.3 g kg⁻¹ on dry solids (DS) basis, 244.1 g kg⁻¹ DS, and 264.3 g kg⁻¹ DS, respectively.

Thygesen *et al.* (2003) investigated the production of cellulose and hemicellulose-degrading enzymes by cultivation of *Aspergillus niger* ATCC 9029, *Botrytis cinerea* ATCC 28466, *Penicillium brasilianum* IBT 20888, *Schizophyllum commune* ATCC 38548, and *Trichoderma reesei* Rut-C30 was studied. Wet-oxidised wheat straw suspension supplemented with NH₄NO₃, MgSO₄, and KH₂PO₄ was used as cultivation medium aiming to obtain an enzyme mixture optimal for enzymatic hydrolysis of wet-oxidized wheat straw. The cultivations with *B. cinerea* and *P. brasilianum* gave the highest endoglucanase (EC 3.2.1.4) and $\square\beta$ -glucosidase (EC 3.2.1.21) activities, in contrast to the other fungi where lower activities were found. The culture filtrates were concentrated by ammonium sulphate precipitation. After enzyme concentration, the highest enzyme activities (1.34 FPU/ml) were found in the culture broth originating from *P. brasilianum*. Enzymatic hydrolysis of filter cake from wet-oxidized wheat straw for 48 h with an enzyme loading of 5 FPU/g

biomass resulted in glucose yields from cellulose of 58% (w/w) and 39% (w/w) using enzymes produced by *P. brasiliense* and a commercial enzyme mixture, respectively. At higher enzyme loading (25 FPU/g biomass) using either enzyme mixtures the glucose yield from cellulose was in the range of 77–79% (w/w).

The modified cellulose solvent-(concentrated phosphoric acid) and organic solvent- (95% ethanol) based lignocellulose fractionation (COSLIF) was applied to a naturally-dry bamboo sample. The biomass dissolution conditions were 50°C, 1 atm for 60 min. Glucan digestibility was 88.2% at an ultra-low cellulase loading of one filter paper unit per gram of glucan. The overall glucose and xylose yields were 86.0% and 82.6%, respectively. COSLIF efficiently destructed bamboo's fibril structure, resulting in a 33-fold increase in cellulose accessibility to cellulase (CAC) from 0.27 to 9.14 mg per gram of biomass. Cost analysis indicated that a 15-fold decrease in use of costly cellulase would be of importance to decrease overall costs of biomass saccharification when cellulase costs are higher than \$0.15 per gallon of cellulosic ethanol (Sathitsuksanoh *et al.*, 2010).

Atmospheric aqueous glycerol autocatalytic organosolv pretreatment (AAGAOP) to improve the enzymatic hydrolysis of lignocellulosic biomass. With the liquid–solid ratio of 20 g/l at 220°C for 3 h, the AAGAOP enabled wheat straw to remove 70% hemicelluloses and 65% lignin, with 98% cellulose retention. The pretreated fiber was achieved with 90% of the enzymatic hydrolysis yield after 48 h. At oven-drying, dehydration was likely to cause the horrifaction of fiber, which was responsible for the low enzymatic hydrolysis of dried fiber. With SEM observations, the AAGAOP disrupted wheat straw into thin and fine fibrils, with a small average size and more surface area Sun and Cheng (2002).

Different physical and chemical pretreatment with 2% solution of NaOH (300 ml), liquid NH₃, H₂SO₄ (3%), HNO₃ (3%), H₂O₂ (1%) and H₂O₂ (1%) + Mn⁺⁺ (100:1) were recommended to forest biomass waste at room temperature for better production of hydrolytic enzymes (Sharma *et al.*, 2006).

Riffat *et al.* (2005) isolated the two strains of *Streptomyces* sp. which were identified as *S. albus* and *S. chromofuscus*. Their maximum xylanase activity was 13.25, 19.31 and 32.53, 43.01 on untreated rice straw pulp and pulp treated with TiO₂ in both *Streptomyces* sp. respectively. The enzyme activity increased when both isolates were grown on yeast extract. Optimum production of xylanase was recorded after 5 days of fermentation. Xylanase produced with *S. chromofuscus* showed higher bleaching activity than that from *S. albus*. The enzyme enhanced the liberation of reducing sugar, which improved pulp bleach ability.

The application of Fenton's reaction to enhance the fermentability of prehydrolysates obtained from steam explosion pretreatment of poplar biomass was studied. Reaction conditions of temperature and H₂O₂ and Fe(II) concentration were studied by Oliva *et al.* (2005). The fermentability of prehydrolysate treated by Fenton's reaction was tested by using different inoculum sizes of thermotolerant strain *Kluyveromyces marxianus* CECT 10875. The highest percentages of toxic compound degradation (ranging from 71 to 93% removal) were obtained at the highest H₂O₂ concentration tested (50 mM). However, a negative effect on fermentability was observed at this H₂O₂ concentration at the lower inoculum loading. An increase in inoculum size to 0.6 g/L resulted in an enhanced ethanol fermentation yield of 95% relative to control

Haq *et al.* (2006) reported a thermophilic strain of *Humicola insolens* TAS-13 was locally isolated and was tested for cellulases production under solid-state fermentation conditions using sugar cane bagasse as substrate. The cultural conditions for the *H. insolens* were also optimized for the higher rate of cellulase secretion. In order to enhance the production rate of heterogenous cellulosic proteins, bagasse was pretreated with NaOH, H₂SO₄, H₂O₂ and H₂O₂+1.5%NaOH. The pretreatment of bagasse with 2.0% H₂O₂ along with 1.5% NaOH enhanced the biosynthesis of cellulases by *H. insolens*. Production rate was also optimized with different parameters like thickness of fermentation medium, initial pH, incubation time and temperature. The thickness of the fermentation medium of 0.8 cm (10 g) with pH range of 5.5 was found to be

better for enhanced production at 50°C. The yield of the enzyme was reached maximum with CMCase (18.98 U/g/min), FPase (13.63 U/g/min), β -glucosidase (19.54 U/g/min) 72 h after inoculation.

The focus of this study was to alter the xylan content of corn stover and poplar using SO₂-catalyzed steam pretreatment to determine the effect on subsequent hydrolysis by commercial cellulase preparations supplemented with or without xylanases by Chandra and Addler (2009). Steam pretreated solids with xylan contents ranging from 1 to 19% (w/w) were produced. Higher xylan contents and improved hemicellulose recoveries were obtained with solids pretreated at lower severities or without SO₂-addition prior to pretreatment. The pretreated solids with low xylan content (>4% (w/w)) were characterized by fast and complete cellulose to glucose conversion when utilizing cellulases.

2.3.3 Biological Pretreatment

Microorganisms can also be used to treat the lignocelluloses and enhance enzymatic hydrolysis. The applied microorganisms usually degrade lignin and hemicellulose but very little part of cellulose, since cellulose is more resistance than the other parts of lignocelluloses to the biological attack. Several fungi, e.g. brown, white and soft rot fungi, have been used for this purpose. White-rot fungi are among the most effective microorganisms for biological pretreatment of lignocelluloses (Sun and Cheng, 2002).

Patel *et al.* (2007) reported that bioconversion offers a cheap and safe method of not only disposing the agricultural residues, but also it has the potential to convert lignocellulosic wastes into usable forms such as reducing sugars that could be used for ethanol production. This paper reports a preliminary study on the microbial pretreatment and fermentation of the agricultural residues like wheat straw, rice straw, rice husk and bagasse. A combination of six different fungi obtained from screening was used for pretreatment and *Saccharomyces cerevisiae* (NCIM 3095) was used for carrying out fermentation. In case of Wheat straw and rice straw, pretreatment with *Aspergillus niger* and *Aspergillus awamori* and fermentation yielded highest amount of ethanol(2.5 g/L

and 2.2 g/L respectively), for rice husk and bagasse it was with *Aspergillus awamori* and *Pleurotus sajor-caju* (8.5 g/L and 9.8 g/L, respectively).

Cellulase production was carried out by solid state bioconversion (SSB) method using rice straw, a lignocellulosic material and agricultural waste, as the substrate of three *Trichoderma* spp. and *Phanerochaete chrysosporium* in lab-scale experiments by Khan *et al.* (2007). *Phanerochaete chrysosporium* was found to be the best among these species of fungi, which produced the highest cellulase enzyme of 1.43 IU/mL of filter paper activity (FPase) and 2.40 IU/mL of carboxymethylcellulase activity (CMCase). The “glucosamine” and “reducing sugar” parameters were observed to evaluate the growth and substrate utilization in the experiments. In the case of *Phanerochaete Chrysosporium*, the highest glucosamine concentration was 1.60 g/L and a high concentration of the release of reducing sugar was measured as 2.58 g/L obtained on the 4th day of fermentation. The pH values were also recorded. The range of the pH was about 5.15 to 5.56 in the case of *Phanerochaete Chrysosporium*.

Hydrolysis of the corn wet-milling co-product of corn fiber to simple sugars by the brown rot fungus *Gloeophyllum trabeum* was studied in suspended-culture and solid-state fermentations by Rasmussen *et al.* (2010). Suspended-culture experiments were not effective in producing harvestable sugars from the corn fiber. The fungus consumed sugars released by fungal extracellular enzymes. Solid-state fermentation demonstrated up to 40% fiber degradation within 9 days. Enzyme activity assays on solid state fermentation filtrates confirmed the involvement of starch and cellulose degrading enzymes. To reduce fungal consumption of sugars and to accelerate enzyme activity, 2 and 3d solid-state fermentation biomasses (fiber and fungus) were submerged in buffer and incubated at 37°C without shaking. This anaerobic incubation converted up to almost 11% of the corn fiber into harvestable reducing sugars. Sugars released by *G. trabeum* were fermented to a maximum yield of 3.3 g ethanol/100 g fiber.

The white rot fungus *Irpex lacteus* CD2 was incubated on corn stover under solid-state fermentation conditions for different durations, from 5 days up to 120 days. Lignocellulose component loss, enzyme production and Fe³⁺

reducing activity were studied. The average weight loss ranged from 1.7% to 60.5% during the period of 5–120 days. In contrast to lignin, hemicellulose and cellulose were degraded during the initial time period. After 15 days, 63.0% of hemicellulose was degraded. Cellulose was degraded the most during the first 10 days, and 17.2% was degraded after 10 days. Lignin was significantly degraded and modified, with acid insoluble lignin loss being nearly 80% after 60 days. Filter paper activity, which corresponds to total cellulase activity, peaked at day 5 and remained at a high level from 40 to 60 days. High xylanase activity appeared after 30 days. No ligninases activity was detected during the incipient stage of lignin removal and only low lignin peroxidase activity was detected after 25 days (Xu *et al.*, 2009).

Lee *et al.* (2007) investigated the effects of biological pretreatment on the Japanese red pine *Pinus densiflora*, was evaluated after exposure to three white rot fungi *Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis*. Change in chemical composition, structural modification, and their susceptibility to enzymatic saccharification in the degraded wood were analyzed. Of the three white rot fungi tested, *S. hirsutum* selectively degraded the lignin of this softwood rather than the holocellulose component. After eight weeks of pretreatment with *S. hirsutum*, total weight loss was 10.7%, while lignin loss was the highest at 14.52% among the tested samples. However, holocellulose loss was lower at 7.81% compared to those of *C. lacerata* and *P. brumalis*. Extracellular enzymes from *S. hirsutum* showed higher activity of ligninase and lower activity of cellulase than those from other white rot fungi. Thus, total weight loss and changes in chemical composition of the Japanese red pine was well correlated with the enzyme activities related with lignin- and cellulose degradation in these fungi. Based on the data obtained from analysis of physical characterization of degraded wood by X-ray Diffractometry (XRD) and pore size distribution, *S. hirsutum* was considered as an effective potential fungus for biological pretreatment. In particular, the increase of available pore size of over 120 nm in pretreated wood powder with *S. hirsutum* made enzymes accessible for further enzymatic saccharification. When Japanese red pine chips treated with *S. hirsutum* were enzymatically saccharified using commercial enzymes (Cellulclast

1.5 L and Novozyme 188), sugar yield was greatly increased (21.01%) compared to non-pretreated control samples, indicating that white rot fungus *S. hirsutum* provides an effective process in increasing sugar yield from woody biomass.

Ray *et al.* (2010) reported that the current barrier to the large-scale production of lignocellulosic biofuels is the cost associated with the energy and chemical inputs required for feedstock pretreatment and hydrolysis. The use of controlled partial biological degradation to replace elements of the current pretreatment technologies would offer tangible energy and cost benefits to the whole biofuel process. It has been known for some time from studies of wood decay that, in the early stages of growth in wood, brown rot fungi utilise a mechanism that causes rapid and extensive depolymerisation of the carbohydrate polymers of the wood cell wall. The brown rot hyphae act as delivery vectors to the plant cell wall for what is thought to be a combination of a localised acid pretreatment and a hydroxyl radical based depolymerisation of the cell wall carbohydrate polymers. It is this quality that we have exploited in the present work to enhance the saccharification potential of softwood forest residues for biofuel production. Here we show that after restricted exposure of pine sapwood to brown rot fungi, glucose yields following enzymatic saccharification are significantly increased. Our results demonstrate the potential of using brown rot fungi as a biological pretreatment for biofuel production.

Agaricus arvensis, a newly isolated basidiomycete fungus, was found to secrete efficient cellulases. The strain produced the highest endoglucanase (EG), cellobiohydrolase (CBH) and β -glucosidase (BGL) activities of 0.3, 3.2 and 8 U/mg-protein, respectively, with rice straw as the carbon source. Saccharification of the woody biomass with *A. arvensis* cellulase as the enzyme source released a high level of fermentable sugars. Enzymatic hydrolysis of the poplar biomass was optimized using the response surface methodology in order to study the influence of the variables (pH, temperature, cellulases concentration and substrate concentration). The enzyme and substrate concentrations were identified as the limiting factors for the saccharification of poplar wood biomass. A total reducing sugar level of 29 g/L (293 mg/g of substrate) was obtained at an enzyme

concentration of 65 FPU/g-substrate after optimization of the hydrolysis parameters. The model validation showed a good agreement between the experimental results and the predicted responses. *A. arvensis* could be a good candidate for the production of reducing sugars from a cellulosic biomass (Jeya *et al.*, 2010).

2.4. PRODUCTION OF CELLULASE AND XYLANASE FROM DIFFERENT MICROORGANISMS AND BIODEGRADATION OF LIGNOCELLULOSIC BIOMASS

Abo-State *et al.* (2010) reported that cellulase producer were isolated from agriculture waste and identified as *Aspergillus terreus* MAM-F23 and *Aspergillus flavus* MAM-F35. The results under solid state fermentation of wheat straw revealed that, the optimum pH values for the three cellulases (CMCase, FPase and Avicelase) and soluble protein production by *T. viride* as standard strain, *A. terreus* and *A. flavus* were ranging from 3.5 to 5.0 and the best inoculum size (level) was 0.5 ml spore suspension ($\sim 2 \times 10^7$ spores/ml). Tap water was the best moistening agent in producing CMCase, FPase and Avicelase by *A. terreus* MAM-F23, but phosphate buffer was the best moistening agent for CMCase and Avicelase by *T. viride* and CMCase and FPase by *A. flavus* MAM-F35. The trend for cellulases production as the incubation period increased was almost the same in the three tested strains (*T. viride*, *A. terreus* and *A. flavus*). The maximum CMCase had been reached after 6 days incubation period and soluble protein increased as incubation period increased. On the other hand, Avicelase recorded the highest productivity after 12 h incubation and decreased gradually as incubation period increased.

Trichoderma reesei LM-UC4, the parent strain, and its hypercellulolytic mutant LM-UC4E1 were co-cultured with *Aspergillus niger* ATCC 10864 in solid substrate fermentation on alkali-treated sugar cane bagasse for cellulolytic enzyme production. Bagasse was supplemented with either soymeal or with ammonium sulfate and urea, and fermented at 80% moisture content and 30°C. Mixed culturing produced better results with the inorganic supplement. The mutant strain was more responsive to mixed culturing than the parent strain when

A. niger was the cooperating partner. In a mixed culture of the mutant with inorganic N-source, 10% more biomass, but 63% more cellulase, 85% more endoglucanase and 147% more β -glucosidase was produced than in single culture. Since co-culturing helped enzyme production more than growth, it appeared that synergistic interactions not directly related to growth were responsible for increased enzyme production (Gutierrez-Correa *et al.*, 1999).

Liu and Yang (2007) investigated the cellulase production was carried out in solid-state fermentation using the waste from the vinegar industry as the substrate for *Trichoderma koningii* AS3.4262. This waste is porous and easy to degrade by cellulolytic fungi. The effects of water content, initial pH value in solid substrate and culture temperature on cellulase synthesis were observed for optimal production in flask fermentors. An orthogonal layout was employed in the statistical process and better cellulase activity was obtained in the fermentation batch. The optimal filter paper cellulase (FPase) activity of 6.90 IU/g of substrate dry matter (SDM), and carboxymethyl cellulase (CMCase) activity of 23.76 IU/g SDM were obtained after 84 h of incubation with media containing vinegar waste, with optimal moisture content of 50 %, pH=5.0, incubation temperature of 30 °C, and additional nutrients of inorganic salts in a certain amount. To produce cellulase on a larger scale, a deep trough fermentor with forced aeration was used, so that FPase activity of 5.87 IU/g SDM and CMCase activity of 12.98 IU/g SDM were reached after 84 h of solid-state fermentation. Results indicate the excellent scope of utilizing vinegar waste as solid substrate for commercial production of cellulase employing fungi

Xylanase production by wild-type *Aspergillus niger* ANL301, newly isolated from wood-waste, was monitored at 24 h intervals for a period 168 h in media containing different carbon sources by Okafor *et al.* (2007). The carbon sources were oat-spelt xylan (Fluka) and three agro-wastes (sawdust, sugarcane pulp and wheat bran). Highest xylanase activity of 6.47 units/mL was obtained at 96 h in media containing wheat bran as sole carbon source. Maximum activity value for the media containing sugarcane pulp was 0.95 units/mL obtained also at 96 h. Sawdust and oat spelt xylan gave the peak enzyme activities of 0.65 and

0.80 units/mL respectively at 120 h. High protein yield was obtained in media containing the agro-wastes, with wheat bran giving the highest value of 1.14 mg/mL at 96 h. The maximum specific xylanase activities were 3.86, 3.37, 5.69, and 9.36 units/ mg protein for sawdust, sugarcane pulp, wheat bran and oat spelt xylan, respectively.

Knob and Carmon (2008) reported the production of xylanase by the fungus *Penicillium sclerotiorum* under submerged cultivation. Oat spelts xylan and wheat bran were the best inducers of xylanase activity. Optimal xylanase production was obtained in liquid Vogel medium, pH 6.5, at 30°C, under stationary condition during 5 days. The temperature for optimum activity was 50°C and optimum pH 4.5. The enzyme was stable at 40°C, with a half-life of 72 min. and when it was incubated at 45 and 50°C, the half-life was 8 min and shorter than 4 min, respectively. High pH stability was verified from pH 3.5 to 7.5.

The growth and bioconversion potential of selected strains growing on cassava waste substrate during solid state fermentation were assessed by Pothiraj and Eyini (2007). *Rhizopus stolonifer* showed the highest and the fastest utilization of starch and cellulose in the cassava waste substrate. It showed 70% starch utilization and 81% cellulose utilization within eight days. The release of reducing sugars indicating the substrate saccharification or degradation potential of the organisms reached the highest value of 406.5 mg/ g by *R. stolonifer* on cassava waste during the eighth day of fermentation. The protein content was gradually increased (89.4 mg/g) on the eighth day of fermentation in cassava waste by *R. stolonifer*. The cellulase and amylase activity is higher in *R. stolonifer* than *A. niger* and *P. chrysosporium*. The molecular mass of purified amylase and cellulase seemed to be 75 KDa, 85 KDa respectively.

The effect of some natural wastes (orange pomace, orange peel, lemon pomace, lemon peel, apple pomace, pear peel, banana peel, melon peel and hazelnut shell) on the production of xylanase from *Trichoderma harzianum* 1073 D3 has been studied and maximum activity has been observed on melon peel

(26.5 U/mg of protein) followed by apple pomace and hazelnut shell. Also, molasses could be used as an additional carbon source as it decreased the production time approximately by 50 %. Finally, potential alternatives of organic nitrogen source (cotton leaf and soybean residue wastes) were analyzed and it was concluded that peptone could be replaced with these residues (Seyis and Aksoz, 2005).

The production of xylanase by a local isolate *Trichoderma* spp. FETL C32 via solid state fermentation system using sugar cane baggase: palm kernel cake as substrates was investigated by Pang *et al.* (2006). The optimized solid state fermentation (SSF) system consists of 5 g of sugar cane baggase: palm, kernel cake of ratio 90:10 (% w/w), moisture content of 75% (v/w), pH of moistening agent of pH 7.0, at 30°C and inoculum size of 1×10^8 spores/ml. The SSF system was also supplemented with 4% (w/w) dextrin and 6% (w/w) of tryptone as additional carbon and nitrogen sources, respectively. Cellulose at the concentration of 0.2% (w/w) was found to be a significant inducer for xylanase production. Using the optimized SSF system, a maximum xylanase productivity of 75.0 U per mg glucosamine after 4 days of fermentation time at 30°C was obtained. The modifications of the SSF system resulted an increase in xylanase productivity by 180% and growth by 40% compared to the basal SSF system.

The feasibility of using apple pomace for cellulase production by *Trichoderma* sp. under solid state fermentation was evaluated by Sun *et al.* (2010). Results indicated that initial moisture level of the medium, incubation temperature and inoculum size influenced the cellulase production greatly. The optimum initial moisture level, incubation temperature and inoculum size were 70%, 32 and 2×10^8 spores/flask, respectively. The supplement of lactose and corn-steep solid to the apple pomace favored the enzyme formation markedly.

Aslam *et al.* (2010) studied the production of easily fermentable low-cost sugars demands for an economic and less expensive method of enzymatic hydrolysis of cellulosic biomass. It is highly desirable to compare various fermentation media with different carbon sources for cellulase production. *T.*

harzianum was grown on different carbon sources and monitored for cellulase production. Glucose-grown cultures of *T. harzianum* showed high amount of mycelial growth but no yield of cellulase enzyme. Cellulase expression was also studied herein by comparing the cellulase activities using soluble and insoluble cellulosic carbon sources in the growth media in order to obtain less expensive fermentation media.

Sawdust and bagasse were used as lignocellulosic substrates for the production of cellulase enzyme using *Aspergillus niger* after ballmilling and pretreatment with caustic soda by Guruchandran and Sasikumar (2010). From the fermentation studies, Sawdust gave the best result with an enzyme activity value of 0.0846 IU/ml while bagasse gave 0.0682 IU/ml. The two lignocellulosics gave their maximum enzyme activities at about the 12th hour of cultivation, suggesting that the 12th hour is the optimum time when the enzyme may be harvested.

Jecu (2000) reported the cellulase production from lignocellulosic materials under solid state fermentation (SSF). The effects of fermentation conditions, such as moisture content, initial pH, temperature, and composition of mixed substrate (wheat straw and wheat bran) on endoglucanase production by *Aspergillus niger* 38 were optimized. With a moisture content of 74%, a pH range of 4.5–5.5 on mixed substrate containing wheat straw: wheat bran of 9:1, 14.80 international units (IU) endoglucanase activity/ml were obtained in 96 h.

Grigorevski-Lim *et al.* (2009) evaluated the production of cellulolytic enzymes by an *Aspergillus fumigatus* strain, isolated from sugar cane bagasse, according to its ability to grow on microcrystalline cellulose as the sole carbon source. The effect of the carbon source (brewer's spent grain, sugarcane bagasse, and wheat bran) and of the nitrogen source (corn steep liquor and sodium nitrate) on cellulase production was studied using submerged and solid state cultivations at 30°C. The highest levels of endoglucanase (CMCase) corresponded to 365 U L⁻¹ and was obtained using sugarcane bagasse (1%) and corn steep liquor (1.2%) in submerged fermentation within 6 days of cultivation. This supernatant was used to run a sodium dodecyl sulfate polyacrylamide gel electrophoresis that showed six bands with endoglucanase activity. CMCase activity was higher at

65°C and pH 2.0, indicating that this microorganism produces a thermophilic and acid endoglucanase. Solid state cultivation favored FPase production that reached 47 U g⁻¹ of dry substrate (wheat bran and sugarcane bagasse) within 3 days.

Rodrigo *et al.* (2010) evaluated cellulases, xylanases, and β -glucosidases produced by two fungi, the thermotolerant *Acrophialophora nainiana* and *Ceratocystis paradoxa*. Cells were grown in submerged culture on three carbon sources: lactose, wheat bran, or steam-pretreated sugarcane bagasse, a commonly used cattle feed in Brazil. Xylanase and endo-1,4- β -glucanase (CMCase) highest production were found in *A. nainiana* growing on lactose and reached levels of 2,200 and 2,016 IU/L, respectively. *C. paradoxa* showed highest activity for xylanase when grown on wheat bran and for β -glucosidase when grown on steam treated bagasse, at levels of 12,728 and 1,068 IU/mL, respectively.

da Silva *et al.* (2005) investigated the production of CMCase and xylanase by *Thermoascus aurantiacus* in solid state fermentation (SSF) using different agricultural residues (wheat bran, sugarcane bagasse, orange bagasse, corncob, green grass, dried grass, sawdust and corn straw) as substrates without enrichment of the medium and characterize the crude enzymes. All the enzymes were stable at room temperature by 24h over a broad pH range (3.0-9.0) and also were stable at 60°C for 1h. The optimum pH and temperature for xylanase and CMCase were 5.0-5.5 and 5.0 and 75°C, respectively. The microorganism grew quickly in stationary, simple and low cost medium.

Production of cellulases from *Aspergillus heteromorphus*, submerged fermentation was performed using wheat straw as substrate. Optimization of saccharification conditions like pH, temperature and time were studied. Highest reducing sugar was released on 5th day at 5 pH, 30°C temperature. When *A. heteromorphous* was grown on wheat straw in submerged fermentation after 5 days incubation at 30°C, 3.2 IU/ml and 83 IU/ml, filter paper activity and CMCase activity, respectively (Singh *et al.*, 2009).

Fatma and Fadel (2010) reported that the high cellulase activity was obtained when the fungus was cultivated on substrate with about 75 % (v/w) moisture, pH 4.8 for 5 days incubation at 28±2°C, as it gave 16.2 IU/g substrate.

The obtained cellulase of 1.2 IU/ml culture filtrate was applied for saccharification (5% w/v) of alkali treated rice straw, in 0.1M citrate buffer pH 4.8 in shaker water bath of 100 rpm. Sugary solution of 1.07 % glucose was achieved after 16 hrs. The sugary solution was concentrated to give 10% (w/v) glucose. Ethanolic fermentation was conducted by *Saccharomyces cerevisiae* SHF-5 under static condition giving 5.1% (v/v) ethanol after 24 hrs. The fermented mash contained 3.6 g/L yeast cell can be utilized as fooder yeast used for animal feeding.

2.5 OPTIMIZATION OF CELLULASE AND XYLANASE FROM DIFFERENT MICROORGANISMS UNDER SmF AND SSF

2.5.1 Optimization of Cellulase and Xylanase from Bacteria under SmF and SSF

Bakri *et al.* (2008) studied the xylanase production by a new *Cochliobolus sativus* Cs5 strain was improved under submerged fermentation. The xylanase was induced by xylan and repressed by glucose, sucrose, maltose, xylose, starch and cellulose. Highest enzyme production (98.25 IU/mL) was recorded when wheat straw (4 % by mass per volume) was used as a carbon source after 120 h of incubation. NaNO₃ increased xylanase production 5.4-fold as compared to the control. Optimum initial pH was found to be 4.5 to 5. The *C. sativus* Cs5 strain grown under submerged culture in a simple medium proved to be a promising microorganism for xylanase production.

Abou-Taleb *et al.* (2009) reported the effect of some nutritional and environmental factors on growth and cellulase production by *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23 was investigated. Results indicated that 1% carboxymethylcellulose (CMC) and 0.7% yeast extract were most effective as the carbon and nitrogen sources respectively. Initial pH 7 and 3% inoculum size found to be optimal for growth and cellulase production. Incubation temperature at 30 and 45°C achieved the highest activity of cellulase for *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23 respectively, and the suitable shaking rate was 150 and 200 rpm.

Sharma *et al.* (2007) studied the optimization of cultural variables resulted in a marked enhancement in the secretion of cellulase-free and alkali-

thermostable xylanase (EC 3.2.1.8) by an extreme thermophile *Geobacillus thermoleovorans*. The enzyme secretion was enhanced when the medium was supplemented with xylan (0.15%) and Tween-80 (0.1% v/v). In wheat bran tryptone medium, the peak in enzyme production was attained within 42h in a fermenter as compared to 72h in shake flasks. Optimization of the culture condition resulted in a 7.72-fold enhancement in enzyme production. The cellulase-free xylanase was optimally active at pH 8.5 and 80°C, and it was found to be useful in the pre-bleaching process of paper pulps.

Alkalophilic *Bacillus subtilis* ASH produced high levels of xylanase using easily available inexpensive agricultural waste residues such as wheat bran, wheat straw, rice husk, sawdust, gram bran, groundnut and maize bran in solid-state fermentation (SSF). Among these, wheat bran was found to be best substrate. Xylanase production was highest after 72h of incubation at 37°C and at a substrate to moisture ratio of 1:2 (w/v). The inoculum level of 15% resulted in maximum production of xylanase. The enzyme production was stimulated by the addition of nutrients such as yeast extract, peptone and beef extract. In contrast, addition of glucose and xylose repressed the production of xylanase. The extent of repression by glucose (10%, w/v) was 81% and it was concentration-dependent. Supplementation of the medium with 4% xylose caused 59% repression. Under optimized conditions, xylanase production in SSF (8,964 U of xylanase/g dry wheat bran) was about twofold greater than in submerged fermentation (Sanghi *et al.*, 2008).

Haq *et al.* (2004) studied the effects of time course; incubation temperature; medium pH and volume of fermentation medium on the production of xylanase were studied. The maximum xylanase production (250 U/mL) was observed at an incubation temperature of 30°C after 48 h of incubation period. The optimum initial pH of the medium was 5.0 because maximum production of xylanase (260 U/mL) was observed at this pH. The production of enzyme was found to be maximum (265 U/ml) when the volume of fermentation medium was kept as 25 mL/250 mL conical flasks.

Kapoor *et al.* (2007) studied the cultural conditions for xylanase production by an alkalophilic *Bacillus pumilus* strain MK001 were optimized

under submerged fermentation. An initial medium pH 9.0, agitation 200 rpm, inoculum size 1.25% (v/v) and inoculum age 2 h were found to be optimal for maximum enzyme production. The bacterium secretes high levels of xylanase on agricultural residues (wheat bran 1220.0 IU ml⁻¹; wheat straw 900.0 IU ml⁻¹) as well as on synthetic xylans (birch wood xylan 1190 IU ml⁻¹; oat spelt xylan 1150.0 IU ml⁻¹). Among different inorganic/organic nitrogen sources tested, a combination of yeast extract and peptone (0.66% N₂ equivalent) produced maximum (1288.0 IU ml⁻¹) xylanase titers. Among various amino acids, vitamins and surfactants, dl-β-phenylalanine, niacin, and polyethylene glycol (PEG)-3330 maximally enhanced xylanase production by 136.0% (2880.0 IU ml⁻¹), 79.0% (2190.0 IU ml⁻¹), and 107% (2536.0 IU ml⁻¹) respectively. Batch fermentation in 5-l laboratory fermentor allowed reduction (8.0 h) in incubation period for optimal (2886.0 IU ml⁻¹) xylanase production. Whole-cell immobilization of *B. pumilus* strain MK001 on inert supports (polyurethane foam, PUF and scotch brite, SB) was found to be superior in maximizing xylanase production (up to 4000.0 IU ml⁻¹) as compared to natural supports (cotton and silk) (up to 3854.0 IU ml⁻¹). Saccharification of chemically pretreated *Prosopis juliflora* by enzymatic cocktail (xylanase, cellulase and cellobiase) resulted in release of reducing sugars (15.5 g g⁻¹; saccharification efficiency of 20.0%). Fermentation of saccharified hydrolysates by *Pichia stipitis* resulted in an ethanol yield of 0.36 g g⁻¹.

The medium components for the production of extracellular cellulases by *Melanocarpus* sp. MTCC 3922 were optimized using solid-state fermentation. *Melanocarpus* sp. cultured in optimized medium containing 1.5% urea, and 0.12% KH₂PO₄ along with a trace element solution and surfactant (Tween 20), produced endoglucanase (142.4 U/g of substrate), avicel-adsorbable endoglucanase (27.0 U/g of substrate), Avicelase (0.65 U/g of substrate), FPase (39.9 U/g of substrate) and β-glucosidase (109.0 U/g of substrate) activities. The presence of sulphate ions in traces stimulated endoglucanase yields. The IEF fractionation of the crude proteins from *Melanocarpus* sp. showed the expression of 3, 1 and 11 isoforms of endoglucanase, β-glucosidase and xylanase, respectively (Jatinder *et al.*, 2006).

Gupta and Kar (2008) reported that different agro-residues were evaluated as substrates in solid state fermentation for xylanase production by a thermotolerant *Bacillus* isolate. Various fermentation parameters were optimized for enhanced endoxylanase production under solid state fermentation (SSF). Maximum enzyme production of 74.96 ± 5.2 U/gds took place at 45°C with corn cob and mineral salt solution (MSS) after 72 h, at pH 6.0 and particle size of 500 µm. A ratio of substrate: moistening agent of 1:2.5 was found to be optimum for corn cob, a solid substrate rarely utilized for bacterial xylanase in SSF. Continuous xylanase production by recycling immobilized cells could be achieved till 10 cycles with maximum enhancement of 156.05% and 219.21% after 5 and 4 cycles in static and submerged states respectively. Scale up in large trays under SSF yielded 157.12 ± 8.7 U/gds in static state and 111.47 ± 8.1 U/gds at 110 rpm.

Sepahy *et al.* (2011) reported that a xylanase producer *Bacillus mojavensis* strain, called AG137, isolated from cotton farm (Kashan-Iran). The optimal xylanase activity reached at 55°C and pH 9.0. Enzyme yield was studied using a medium with different agricultural wastes as inducers. Xylanase production of about 249.308 IU/mL was achieved at pH 8 and 37°C, within 48 h submerged fermentation in enzyme production medium supplemented with 2% (w/v) oat bran as an optimum carbon source. A mixture of 1% (w/v) yeast extract and 1% (w/v) tryptone as optimum nitrogen sources, agitation speed 200 rpm, and inoculum size 2% (v/v) were the optimums for maximum production. Accordingly, xylanase yield from 194.68 IU/mL under non-optimized fermentation condition enhanced to 302.466 IU/mL in optimized condition. Screened xylanase is thermostable, presenting 70% stability at 60°C during 30 min. Further enzyme incubation in higher temperature caused a decrease in the residual enzyme activity, yet it retained 68%-50% of its activity after 1 hour from 45°C to 55°C. Besides, it is stable in pH 9 and 10, maintaining over 70% of its activity for 2h. The enzyme also could preserve 71% and 63% of its initial activity after 3 h of pre-incubation in the same alkaline condition.

Gupta *et al.* (2001) studied the agro-residues such as wheat bran, sugarcane bagasse, corncobs and poplar wood when used as sole carbon source,

improved the xylanase yield by five-fold as compared to xylose and xylan. *Staphylococcus* sp. SG-13 also produced equally good amounts of xylanase when grown simply in deionized water (pH 8.0) supplemented with agro-residues as sole carbon source. In the biphasic growth system (lower layer containing agricultural residue set in agar medium with liquid medium above it), the prime substrate, wheat bran (1 w/v), resulted in maximum xylanase production of 4525 U/L (pH 7.5) and 4540 U/L (pH 9.2) at an agar: broth ratio of 4.0 after 48 h of incubation at 37°C under static conditions.

2.5.2 Optimization of Cellulase and Xylanase from Fungi under SmF and SSF

A local isolate of *Aspergillus niger* was cultivated under optimal growth conditions on wheat bran in solid state fermentation by Chandra *et al.* (2009). β -endoglucanase from fermented bran was separately extracted with different solvents to test recovery of enzyme. Among solvents tested, distilled water served the best leachate. Conditions were further optimized with this leachate. Two washes of fermented bran with the leachate for 30 min each under shaking conditions in a ratio of 1 g of wheat bran: 4 ml of distilled water together yielded maximum recovery of 16.7 U/g of wheat bran.

Nair *et al.* (2007) reported seventy fungal strains were isolated from soils collected from different parts of southern Kerala, India. The strains were screened for xylanase production using Czapek's agar medium. On the basis of clearing zones formed, 34 fungal strains were selected and identified. Solid state and submerged fermentation were done to identify strains that could produce maximum amount of xylanase, as well as to identify those strains that could produce cellulase-free xylanase under these conditions. All strains produced cellulase along with xylanase in solid state fermentation, while 70% of the strains produced cellulase-free xylanase during submerged fermentation.

Hemicellulose consists of non-cellulosic polysaccharides, with xylans and mannans as their main examples. In nature, xylan can be first degraded to xylooligosaccharides and finally to xylose by certain microorganisms. White-rot fungi basidiomycetes *Pleurotus* sp. BCCB068 and *Pleurotus tailandia* were used to degrade oat-spelts xylan under submerged fermentation for a period of 40

days. The study obtained activities of endo-1, 4- β -xylanase and β -xylosidase and determination of xylan products by degradation. The fungi reached significant levels of xylan degradation by *Pleurotus* sp. BCCB068 (75.1%) and *P. tailandia* (73.4%), following formations of xylooligosaccharides and sugar monomers. These *Pleurotus* strains proved to be a feasible alternative for biotechnological processes related to degradation of hemicellulose sources (de Menezes *et al.*, 2010).

An optimization of process parameters is a necessary step to get higher yield of product. Several kinetic parameters like pretreatment, extraction solvent, substrate concentration, initial moisture content, pH, incubation temperature and inoculum size were optimized for enhanced production of third most demanded industrially important cellulase. The maximum cellulase enzyme activity $398.10 \pm 2.43 \mu\text{M/mL/min}$ was achieved when proximally analyzed lignocellulosic substrate wheat straw incubated at 2% HCl as pretreatment tool along with distilled water as extraction solvent, 3% substrate concentration 40% moisture content with optimum pH 5.5 at 45°C incubation temperature and 10% inoculum size (Ahmed *et al.*, 2010).

Rhizopus oryzae PR7 MTCC 9642, a producer of endoglucanase was found to produce extra cellular exoglucanase or avicelase when grown on avicel or micro crystalline cellulose. In order to curtail the cost of production the strain was grown in media supplemented with various cellulosic wastes, of which dried flower showed the best result followed by the sweet lime peel at optimum pH 8.0 and 5.0 respectively at 37°C. Peptone was found to be the best nitrogen source for exoglucanase production whereas amongst metal ions Mn^{2+} and Fe^{2+} could bring a 1.23 fold increase in enzyme production in sweet lime peel supplemented culture. Under optimized condition, highest exoglucanase production was achieved at 96 h of growth. The enzyme showed optimum activity at pH 5.0 and 40°C and stability at pH range of 5-9 and about 90% activity was retained even after an exposure of 10 minutes at 80°C. The enzyme activity was enhanced in presence of Mn^{2+} and Fe^{2+} . The enzyme was found to saccharify avicel and the wastes into cellobiose (Mukherjee *et al.*, 2011).

Optimization of nutritive media composition for xylanase production by *Aspergillus awamori* RUS-8 was carried out. Full adequate regression model was obtained by Central Composition Plan. Maximum xylanase activity was achieved in optimized nutritive media on a following composition (g/L): wheat bran-20.02; corn cobs-30.85; corn steep liquor-20.38; NaNO₃-2.20; KH₂PO₄-1; KCl-0.5; MgSO₄-0.5 and FeSO₄-0.01 (Velkova *et al.*, 2007).

Thomas and Ambikapathy (2011) optimized cellulase production with *Hormodendrum cladosporioides* fungus as a potential producer of cellulase. Different cultural conditions were examined such as pH, temperature and incubation period to assess their effect in optimizing enzyme production. Alkaline pretreated (2N NaOH) leaf litters of *Artocarpus heterophylla*, *Mangifera indica* and *Tectona grandis* showed 1.371, 1.832 and 1.183 IU/mL cellulase activity, respectively. Optimum pH for cellulase production was 5, temperature 30°C and the incubation period 5th day. Submerged fermentation at 120 rpm at 30°C gave higher yields of cellulase. Among the five nitrogen sources used casein with 1.25% concentration showed 1.824 IU/mL cellulase activity followed by urea which showed 1.75 IU/mL and whey with 1.654 IU/mL in the same concentration.

Ali *et al.* (2011) reported that sugar cane waste was used as substrates for the production of cellulase enzyme using *Aspergillus niger* and *Trichoderma viride*. From the fermentation studies, sugar cane waste gave the best result with an enzyme activity value of (using *A. niger* and *T. viride*) 2.7 and 2.6 IU/ml respectively. It gave the maximum enzyme activity at about the 10 days of cultivation, suggesting that is the optimum time when the enzyme may be harvested. The fermentation experiments were performed in solid state fermentation (SSF). Incubation time and initial pH of fermentation medium was optimized with simultaneous mixed culture. It was revealed that the sugar cane waste at pH 4.5 and 40°C *Aspergillus niger* (37°C for *T. viride*) was the best source of carbon for the enhanced production of cellulase in the compatible culture experiments.

Gautam *et al.* (2010) isolated *Trichoderma viride* from municipal solid waste and optimized the physicochemical and nutritional parameters for cellulase

production. Municipal solid waste residue and yeast extract were found to be the best combination of carbon and nitrogen source for the production of cellulase by *Trichoderma* sp. Optimal concentration of municipal solid waste residue and yeast extract for the production of cellulase were 45% (w/v) and 1.0% (w/v), respectively. Optimum temperature and pH of the medium for the cellulase production by *Trichoderma viride* was 45°C and 6.5.

Alkaline cellulases were screened from the fifty four fungal isolates obtained from mangrove leaves and mangrove wood litters as endophytes and wood litter fungi respectively by Ravindran *et al.* (2010). Only three fungal isolates tested positive for alkaline cellulases under plate assay, among which *Chaetomium* sp. (NIOCC 36) was found to grow in a wide range of pH (between 4 to 12). This alkaline tolerant fungi was further tested for the production of alkaline cellulases (β -endoglucanase, β -exoglucanase, β -glucosidase), using agricultural and industrial wastes as substrates in the submerged (SMF) and solid state fermentation (SSF) processes. Cotton seed under SSF condition showed maximum enzyme production at high alkaline pH. The results revealed that cellulase enzymes produced under alkaline cotton seed SSF condition had high stability and activity than the non alkaline conditions used at pH 5, 7, 12 and at 50°C.

2.6 OPTIMIZATION OF CELLULASE AND XYLANASE USING RESPONSE SURFACE METHODOLOGY (RSM)

Statistically based experimental design was applied for the optimization of endoglucanase production in liquid state fermentation by *Rhizopus oryzae* MTCC 9642 using water hyacinth as sole carbon source. Effect of three critical culture parameters substrate concentration, cultivation temperature and pH on enzyme production, examined by Response Surface Methodology using Central Composite Design (CCD) indicated that although a substrate concentration of 1.25% was essential to maximize the production of the enzyme by the strain, but the combination of pH 6 and 40°C temperature were required for highest production of endoglucanase. Under the optimized cultivation condition the strain synthesized 450 IU/ml for endoglucanase utilizing water hyacinth in the media. A

verification experiment was accomplished and revealed approximately 95% model validity (Karmakar and Ray, 2011).

Hao *et al.* (2006) reported that the mutant strain *Trichoderma reesei* WX-112 with high cellulase activity was isolated by a newly invented plate. The mutant's ability to produce cellulase increased 1.95 times after the treatment with UV and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Also, the medium composition was optimized using response surface methodology (RSM). A fractional factorial design (2^2) was applied to elucidate the medium components that significantly affect cellulase production. The concentration of Avicel and soybean cake flour in the medium was significant factors. The steepest ascent method was used to locate the optimal domain and a central composite design was used to estimate the quadratic response surface from which the factor levels for maximum production of cellulase were determined. The composition of fermentation medium optimized with response surface methodology was (in g/L): wheat bran 30, Avicel 36.4, soybean cake flour 24.7, KH_2PO_4 4 and corn steep flour 5. Compared to the original medium, the cellulase activity increased from 7.2 to 10.6 IU/mL.

The enhancement of the cellulase activity of *Aspergillus nidulans* by combinational optimization technique was investigated by Jabasingh and Nachiyar (2010). The strain isolated from decayed, dry leaf of *Ficus caricus* was compared for the first time for its ability to produce cellulolytic enzyme in submerged fermentation (SmF). The medium ingredients enhancing the cellulase production were optimized by combinational statistical approach by one factor at a time methodology (OFAT), Plackett Burmann methodology (PB) and response surface methodology (RSM). A four-factor-five-level central composite design (CCD) was employed to determine the maximum activity of cellulase at optimum levels of carboxy methyl cellulose (CMC), ammonium nitrate and potassium dihydrogen phosphate at varying pH values. The optimum fermentation parameters were found to be 1.2 g/l CMC, 0.9 mg/l ammonium nitrate and 0.75 mg/l potassium dihydrogen phosphate at pH 6. The optimization of medium by combinational statistical approach led to the fine tuning of the cellulase

production thereby enhancing the cellulase activity from 4.91 U/ml to 39.56 U/ml.

Xu *et al.* (2008) studied the different nitrogen source such as NaNO_3 , yeast extract, urea, Na_2CO_3 , MgSO_4 , peptone and $(\text{NH}_4)_2\text{SO}_4$ were screened as the significant factors positively affecting the xylanase production by Plackett-Burman design. Evaluating the nitrogen sources effect, urea was proved to be the most effective and economic nitrogen source for xylanase production and used for further optimization. The CCD and response surface methodology (RSM) were applied to determine the optimal concentration of each significant variable, which included urea, Na_2CO_3 and MgSO_4 . Subsequently a second-order polynomial was determined by multiple regression analysis. The optimum values of the critical components for maximum xylanase production were obtained as follows: x_1 (urea) = 0.163 (41.63 g/L), x_2 (Na_2CO_3) = -1.68 (2.64 g/L), x_3 (MgSO_4) = 1.338 (10.68 g/L) and the predicted xylanase value was 14374.6 U/g dry substrate. Using the optimized condition, xylanase production by *Aspergillus niger* XY-1 after 48h fermentation reached 14637 U/g dry substrate with wheat bran in the shake flask.

Fermentation conditions were statistically optimized for producing extracellular xylanase by *Aspergillus niger* SL-05 using apple pomace and cotton seed meal. The primary study shows that culture medium with a 1:1 ratio of apple pomace and cotton seed meal (carbon and nitrogen sources) yielded maximal xylanase activity. Three significant factors influencing xylanase production were identified as urea, KH_2PO_4 , and initial moisture content using Plackett–Burman design study. The effects of these three factors were further investigated using a design of rotation–regression–orthogonal combination. The optimized conditions by response surface analysis were 2.5% Urea, 0.09% KH_2PO_4 , and 62% initial moisture content. The analysis of variance indicated that the established model was significant ($P < 0.05$), “while” or “and” the lack of fit was not significant. Under the optimized conditions, the model predicted 4,998 IU/g dry content, whereas validation experiments produced an enzymatic activity of xylanase at 5,662 IU/g dry content after 60 h fermentation (Liu *et al.*, 2008).

Maciel *et al.* (2008) studied that xylanase was produced by *Aspergillus niger* LPB 326 cultivated on lignocellulosic substrate composed by sugarcane bagasse and soybean meal in solid-state fermentation. The effects of various variables were observed and optimized by applying statistical experimental designs. The best xylanase activity was obtained in a medium containing 10g of sugarcane bagasse and soybean meal in the ratio of 65 and 35%, respectively, moistened to 85% of initial water content with a nutrient salt solution composed of (in g/L): CuSO₄ 0.4, KH₂PO₄ 1.5 and CoSO₄ 0.0012, and incubated for four days at 30°C. Under these optimized conditions, a xylanase activity of 3099 IU/g of dry matter was obtained.

Medium optimization was carried out using De Moe's fractional factorial design with seven components. Maximum production of xylanase 3249±9 U/g) was obtained in SSF with an optimized medium containing (g/l): NaNO₃, 20; K₂HPO₄, 20; MgSO₄, 10; FeSO₄, 0.001; KCl, 1; peptone, 10 and yeast extract, 10. Four components namely NaNO₃, MgSO₄, peptone and K₂HPO₄ significantly increased the xylanase production by *A. versicolor* MKU3 (Jeya *et al.*, 2005).

Amani *et al.* (2007) reported that the two simultaneous statistical experiments were applied in order to optimize medium conditions for xylanase production by *B. pumilus*. The first experiment was a 2ⁿ factorial design, which revealed the significance of glucose and tryptone components. The significant factors were further optimized using the steepest ascent method. Optimized medium (in g/l, tryptone, 4; yeast extract, 6; glucose, 10.4; and NaCl, 6) showed a 1.5 fold increase in xylanase activity. Optimized conditions were used with cells immobilized by using agar and alginate gel materials. Immobilized cells in 2% alginate showed a 2.6-fold increase in xylanase specific activity compared to free cells.

The effect of substrate concentration, pH and cultivation time on the production of endoxylanase from *Aspergillus awamori* ZH-26 were optimized by statistical analysis using response surface methodology (RSM) by Li *et al.* (2006). Endoxylanase production was optimized by central composite rotary

design. Statistical analysis of the results showed that the linear and quadratic terms of these three variables had significant effects. But no interactions between the three variables were found to contribute to the response at a significant level. The optimal conditions were: wheat bran 49.3 g/L, pH 4.14 and cultivation time 103.7 h. Under these conditions, the model predicted an endoxylanase activity of 28.25 U/mL. Verification of the optimization showed that endoxylanase production of 29.65 U/mL was observed under optimal conditions.

Khucharoenphaisan *et al.* (2008) studied a factorial design was performed to find the best conditions of pH and temperature for xylanase activity and to maintain its activity for prolonged periods of time of pure xylanase produced by newly isolated *Thermomyces lanuginosus* THKU-49. The central composite design (CCD) used for the analysis of treatment combinations showed that a regression models of optimization of xylanase activity and xylanase stability were good agreement to experimental results with $R^2 = 0.98$ and 0.99 , respectively. The maximum activity of xylanase was obtained at 66°C and pH 6.3. The temperature and pH for maximum enzyme stability was 70°C and pH 7.3, respectively. Under this condition xylanase having half-life of 825 min indicated the highest thermostable xylanase.

A statistical optimization was studied to design a media composition to produce optimum cellulolytic enzyme where palm oil mill effluent (POME) as a basal medium and filamentous fungus, *Trichoderma reesei* RUT-C30 were used in the liquid state bioconversion(LSB). 2% (w/v) total suspended solid, TSS, of the POME supplemented with 1% (w/v) cellulose, 0.5 % (w/v) peptone and 0.02% (v/v) Tween 80 was estimated to produce the optimum CMCase activity of 18.53 U/ml through the statistical analysis followed by the faced centered central composite design (FCCCD). The probability values of cellulose (<0.0011) and peptone (0.0021) indicated the significant effect on the production of cellulase with the determination coefficient (R^2) of 0.995 (Rashid *et al.*, 2009).

2.7 PURIFICATION AND CHARACTERIZATION OF CELLULASE AND XYLANASE ENZYME

2.7.1 Purification and Characterization of Cellulase and Xylanase Enzyme from Bacteria

2.7.1.1 Purification and Characterization of Cellulase Enzyme from Bacteria

An alkaline thermoactive cellulase from thermophilic actinomycete was purified obtaining 25.18-fold with a 23% yield and a specific activity of 24.83 U mg⁻¹ protein. The molecular mass of cellulase was determined as 90KDa. The enzyme was optimally active at pH 8 and 60°C and was stable from pH 6 to 9 with more than 80% activity remaining after incubation at room temperature for 12 hour. The kinetic parameters was also determined which showed the thermostability, pH stability of the enzyme and make it potentially useful in laundry detergents and other application (Aboul-Enein *et al.*, 2010).

Yin *et al.* (2010) investigated that broth incubated with *Bacillus subtilis* YJ1 at 37°C for 36 h was collected and removed bacterial cells by passing through a 0.45 µm membrane. A cellulase was purified to electrophoretical homogeneity by ammonium sulfate precipitation, Macro-Prep ion exchange and Bio-Gel P-100 chromatography. The recovery and purification fold were 9.7% and 289, respectively, after Bio-Gel P-100 chromatograph. The purified cellulase, with a molecular mass (M) of 32.5 kDa, had an optimal pH and temperature at 6.0 and 50-60°C, respectively. It was stable at pH 6.0-7.5 and <50°C. The purified cellulase was activated by Mn²⁺ and Co²⁺, but inhibited by SDS, *p*-CMB, DTT, Hg⁺, Cd²⁺, Fe²⁺ and Fe³⁺. According to substrate specificity, the purified cellulase has high specificity to CMC and was considered to be an endo-1, 4-glucanase.

Cellulose splitting enzyme, cellulase (extracellular) extracted from *A. oryzae* ITCC-4857.01 was purified by ion-exchange chromatography using DEAE-cellulose followed by Gel filtration was studied by Begum *et al.* (2009). The purification achieved was 53 fold from the crude extract with a yield of 49%. The purified enzyme was homogenous as judged by disc gel electrophoresis. The molecular weight as determined by gel filtration and SDS-polyacrylamide gel electrophoresis was 41,500 and 41,000 respectively. The purified extracellular

cellulase contained only one subunit. The purified extracellular cellulase in aqueous solution gave absorption maximum at 290 nm and minimum at 260 nm. The enzyme is a glycoprotein as nature and contained 0.72% neutral sugar. The apparent K_m value of the enzyme against cellulose was 0.67%. The affinity of the enzyme with different substrates showed as the highest relative activities on CMC followed by avicel, salicin and filter paper.

A halostable cellulase with a molecular mass of 29 kDa was purified from culture supernatants of the halophilic bacterium *Salinivibrio* sp. NTU-05 by way of the Fast Protein Liquid Chromatography method and the biochemical properties of the halostable cellulase was studied by Wang *et al.* (2009). The enzyme was active over a range of 0-25% sodium chloride examined in culture broth. The optimum cellulase activity was observed at 5% sodium chloride. Results from the salinity stability test indicated 24% of enzyme activity was retained at 25% sodium chloride for 4 h. The enzyme was also shown to be slightly thermostable with 40% residual activity under 60 °C for 4 h. The enzyme has a K_m of 3.03 mg/ml and a V_{max} of 142.86 mol/min/mg when tested using carboxymethyl-cellulose (CMC). The enzyme activity increased in the presence of K^+ , Mg^{2+} , Na^+ ions and decreased when Hg^{2+} ions were present.

Lee *et al.* (2008) reported a microorganism hydrolyzing rice hull was isolated from soil and identified as *Bacillus amyloliquefaciens* by analysis of 16S rDNA and partial sequences of the *gyrA* gene, and named as *B. amyloliquefaciens* DL-3. With the analysis of SDS-PAGE, the molecular weight of the purified cellulase was estimated to be 54 kDa. The purified cellulase hydrolyzed avicel, carboxymethylcellulose (CMC), cellobiose, β -glucan and xylan, but not *p*-Nitrophenyl- β -D-glucopyranoside (PNPG). Optimum temperature and pH for the CMCase activity of the purified cellulase were found to be 50 °C and pH 7.0, respectively. The CMCase activity was inhibited by some metal ions, *N*-bromosuccinimide and EDTA in the order of $Hg^{2+} > EDTA > Mn^{2+} > N$ bromosuccinimide $> Ni^{2+} > Pb^{2+} > Sr^{2+} > Co^{2+} > K^+$. The open reading frame of the cellulase from *B. amyloliquefaciens* DL-3 was found to encode a protein of 499 amino acids. The deduced amino acid sequence of the cellulase from *B. amyloliquefaciens* DL-3 showed high identity to cellulases from

other *Bacillus* species, a modular structure containing a catalytic domain of the glycoside hydrolase family 5 (GH5), and a cellulose-binding module type 3 (CBM3).

An alkaline cellulase from *Bacillus* sp. HSH-810 was purified 8.7-fold with a 30% yield and a specific activity of 71 U/mg protein. Kim *et al.* (2005) showed that it was optimally active at pH 10 and 50°C and was stable from pH 6 to 10 with more than 60% activity remaining after heating at 60°C for 60 min. The molecular mass of cellulase was 80kDa. It was inhibited by 50% by Fe³⁺ (1 mM) and Mn²⁺ (0.1 mM) but was relatively insensitive to Hg²⁺ and Pb²⁺ at 1mM.

Kotchoni *et al.* (2006) reported the isolation of a chemical-mediated mutant of *Bacillus pumilus* termed as BpCRI 6 for catabolite Repression Insensitive no. 6, which was tested for its ability to produce cellulase enzyme. The dimeric purified enzyme has a molecular mass of 80 and 170 kDa by SDS-PAGE and size exclusion chromatography, respectively and has been subsequently characterized in the present study. The enzyme was highly active on carboxymethyl cellulose (CMC) but much reduced or no activity on avicel, cellobiose and 4-methylumbelliferyl-β-D-cellobiose (MUC) and was suggested to be a CMCase/endoglucanase. The enzyme expression pattern in BpCRI 6 showed apparently stable biochemical characteristics over a long period when stored at -20°C and was ten times higher than in the wild type in presence of high concentration of glucose (40 mM).

Bacillus subtilis was cultured in solid-state fermentation (SSF) of Banana stalk to produce exoglucanase. The fermented biomass was harvested after 72h of SSF at pH 7 and temperature 35°C. It was filtered and centrifuged at 10,000 rpm at -10°C and supernatant was collected as crude enzyme extract. Maximum activity of exoglucanase (3.48 IU/mL/min) was obtained from the medium fermented with 70% moisture content, 5 mL inoculum, 0.1% peptone, 0.4% yeast extract and 0.2% Tween 80 at pH 7 and temperature 35°C. SSF was found to be more productive than liquid state fermentation (LSF) in terms of exoglucanase yields. The partial purification of exoglucanase was carried out through (NH₄)₂SO₄ precipitation and maximum purification was achieved with 20% (NH₄)₂SO₄ saturation (Shafique *et al.*, 2004).

2.7.1.2 Purification and Characterization of Xylanase Enzyme from Bacteria

Bacillus sp. 99 was isolated from sugarcane molasses is thermoalkalophilic by Kumar *et al.* (2011). It grows in wide range of different temperature and pH. It produced extracellular cellulose-free xylanase produced was purified and characterized. The Optimum temperature and pH of xylanase were 45°C and 10.0, respectively. Molecular weight of xylanase is 16-kDa was purified 64 fold. Xylanase gene of size 1.2 kb was amplified by PCR.

Widjaja *et al.* (2009) studied to optimize the activity and productivity of xylanase from xylanase producing strains of fungi *Aspergillus niger* and *Trichoderma reesei* was conducted as well as its purification strategy. Solid state fermentation (SSF) in which the ratio of solid wheat bran to water was 1:7 (12.5% w/v solid) gave higher enzyme activity than submerged fermentation (1% w/v solid). In all incubation conditions, although cellulase activity was also found in the crude cell extract, its value was much lower than xylanase activity. pH was found to play a very important role in enzyme purification using ion exchange chromatography. CM-Toyopearl, a weak cation exchanger, gave nearly a 100 % adsorption at pH range of 4 to 5, with maximum adsorption at pH 4.5. Using this chromatography column, a 10 times increase of the specific activity of xylanase and a total recovery of 81% were obtained. By SDS-PAGE, the molecular weight was determined to be 27 kDa.

Sanghvi *et al.* (2010) isolated strain of *Trichoderma harzianum* was studied for production of xylanase (EC 3.2.1.8) using lignocellulosic substrates for solid-state fermentation. Among the different substrates used, wheat straw produced the highest yields (146 IU/ml). The influence of temperature, pH, moistening agents, moisture level, carbon sources, nitrogen sources, pretreatments and metal ions were evaluated with respect to xylanase production. Highest xylanase production was obtained using wheat straw after 12 days of incubation. Different substrate combination ratios and the effect of particle size were also checked against xylanase production. Maximum xylanase production was observed in a wheat straw/rice straw ratio of 1:1, with a particle size of 0.45-0.5 mm. Media supplementation with xylose as a carbon source in a ratio 1:5 (carbon source/substrate) gave maximum activity (157 IU/ml). Nitrogen

supplementations from yeast extract produced xylanase activity of 141 IU/ ml. Optimum xylanase production was observed at 30°C and pH 5.0. Xylanase production was enhanced in the presence of Ca^{2+} and Zn^{2+} ions. Ammonium sulfate fractionation (20-80% saturation) of partially purified xylanase yielded 76.5% of the enzyme with a 3.53-fold purification. The molecular weight of xylanase was found to be ca. 29,000 Da by SDS–PAGE.

The alkalophilic bacteria *Bacillus licheniformis* 77-2 produces significant quantities of thermostable cellulase-free xylanases. The crude xylanase was purified to apparent homogeneity by gel filtration (G-75) and ionic exchange chromatography (carboxymethyl sephadex, Q sepharose, and Mono Q), resulting in the isolation of two xylanases. The molecular masses of the enzymes were estimated to be 17 kDa (X-I) and 40 kDa (X-II), as determined by SDS-PAGE. The K_m and V_{max} values were 1.8 mg/mL and 7.05 U/mg protein (X-I), and 1.05 mg/mL and 9.1 U/mg protein (X-II). The xylanases demonstrated optimum activity at pH 7.0 and 8.0–10.0 for xylanase X-I and X-II, respectively, and, retained more than 75% of hydrolytic activity up to pH 11.0. The purified enzymes were most active at 70 and 75°C for X-I and X-II, respectively, and, retained more than 90% of hydrolytic activity after 1 h of heating at 50°C and 60°C for X-I and X-II, respectively (Damiano *et al.*, 2006).

Sanghi *et al.* (2010) reported the one-step purification and characterization of an extracellular cellulase-free xylanase from a newly isolated alkalophilic and moderately thermophilic strain of *Bacillus subtilis* ASH. Xylanase was purified to homogeneity by 10.5-fold with ~43% recovery using ion-exchange chromatography through CM-Sephadex C-50. The purified enzyme revealed a single band on SDS-PAGE gel with a molecular mass of 23 kDa. It showed an optimum pH at 7.0 and was stable over the pH range 6.0-9.0. The optimum temperature for enzyme activity was 55°C. The purified xylanase did not lose any activity up to 45°C, however, it retained 80% and 51% of its activity after pre-incubation at 55°C and 60°C, respectively. The enzyme obeyed Michaelis-Menton kinetics towards birch wood xylan with apparent K_m 3.33 mg/ml and V_{max} 100 IU/ml. The enzyme was strongly inhibited by Hg^{2+} and

Cu^{2+} while enhanced by Co^{2+} and Mn^{2+} . The purified enzyme could be stored at 4°C for six weeks without any loss of catalytic activity.

Highest thermostability at 60°C, retaining approximately 97% of its relative activity following incubation for 4h. In the presence of 5 mM solution of CaCl_2 , the relative xylanase activity increased by 35.9%; however, it decreased significantly in the presence of 10 mM solution of Cu^{2+} . Among the xylan-based substrates tested, purified *L. sulphureus* xylanase showed the highest activity on beechwood by (Lee *et al.*, 2009).

Heck *et al.* (2006) Xylanase from Amazon isolate *Bacillus circulans* BL53 grown on solid-state cultivation was purified to apparent homogeneity by ammonium sulphate fractioning, cation-exchange and gel filtration chromatography. A purification factor of 428-fold was achieved, with the purified enzyme presenting a specific activity of about 37Umg⁻¹ protein. The xylanase molecular weight was calculated as 38 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and its isoelectric point was determined as 8.8. Determination of pH and temperature for the maximum activity was obtained using a 2² factorial design over an extensive range of pH (5.0-8.0) and temperatures (40-80°C). The enzyme follows Michaelis-Menten kinetics with K_m and V_{max} values of 9.9 mg xylan mL⁻¹ and 25.25μmol min⁻¹, respectively. The purified enzyme hydrolyzes soybean hull, soybean fiber, rice straw, grape skin and sugar cane bagasse. Its activity was stimulated by Co^{2+} , Mn^{2+} and protein disulphide reducing reagents, but strongly inhibited by Hg^{2+} ions and SDS.

The isolate *Bacillus pumilus* produced xylanase demonstrating maximal activity at 35°C and at pH 7.0. Crude extract fractionated by ammonium sulphate precipitation had a specific activity of 0.69 μM min⁻¹ mg⁻¹. Enzyme kinetics and properties were studied by DNS assay method. Xylanase had K_m of 4.0 mg ml⁻¹ and V_{max} of 0.068×10^{-4} mM min⁻¹ mg⁻¹. Molecular weight of xylanase was 19 kDa as determined on SDS PAGE. Enzyme was partially purified to a fold of 3.79 and a yield of 66% suggesting the method with fine-tuning could be suitable for scale-up process (Monisha *et al.*, 2009).

Sa-Pereira *et al.* (2002) reported a *Bacillus* sp. CCMI 966, characterized as *Bacillus subtilis*, has a duplication time of about 24 min. It produces at least two extracellular xylanases, Xyl I and Xyl II. The extracellular xylanase activity seems to be strongly correlated with the biomass growth profile. The Xyl II isoenzyme was purified by ammonium sulphate precipitation and anionic exchange chromatography, with a purification factor of 8.3. The molecular weight of the isoenzyme was estimated by SDS-PAGE revealing that Xyl II is a multimeric enzyme with a catalytic subunit of about 20 kDa. Under non-denaturing conditions, a molecular weight of about 340 kDa was obtained by native PAGE gel and of 20 kDa by gel filtration chromatography. The enzyme showed an optimum pH and temperature of 6.0 at 60°C. Xyl II was stable at 40°C for 180 min at pH 6.0. The specificity of Xyl II for different substrates was evaluated. Xyl II presents a higher affinity towards OSX, with a K_m of 1.56 g l⁻¹ and showed the ability to hydrolyse laminarin, with a K_m of 1.02 g l⁻¹. Xylotetraose is the main product of xylan degradation.

A highly thermostable alkaline xylanase was purified to homogeneity from culture supernatant of *Bacillus* sp. JB 99 using DEAE-Sepharose and Sephadex G-100 gel filtration with 25.7-fold increase in activity and 43.5% recovery. The molecular weight of the purified xylanase was found to be 20 kDa by SDS-PAGE and zymogram analysis. The enzyme was optimally active at 70°C, pH 8.0 and stable over pH range of 6.0–10.0. The relative activity at 9.0 and 10.0 were 90% and 85% of that of pH 8.0, respectively. The enzyme showed high thermal stability at 60°C with 95% of its activity after 5 h. The K_m and V_{max} of enzyme for oat spelt xylan were 4.8 mg/ml and 218.6 $\mu\text{M min}^{-1} \text{mg}^{-1}$, respectively. Analysis of N-terminal amino acid sequence revealed that the xylanase belongs to glycosyl hydrolase family 11 from thermoalkalophilic *Bacillus* sp. with basic pI. Substrate specificity showed a high activity on xylan-containing substrate and cellulase-free nature (Shrinivas *et al.*, 2010).

The results showed that crude extracellular, cellulase-free xylanase of an alkaliphilic *Bacillus* sp. strain K-8 could be purified in a single step by affinity adsorption–desorption on a corn husk column using a high flow rate, under the

conditions 25 mM acetate buffer, pH 4.0, 48°C, which prevented the hydrolysis of xylan by xylanase. After adsorption, the xylanase was eluted from the enzyme–corn husk complex with 500 mM Urea. The enzyme was purified 5.3-fold to homogeneity from culture supernatant. The molecular weight of the purified enzyme was 24 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The specific activity and recovery yield after purification were 25.4 U/mg protein and 42.3%, respectively (Tachaapaikoon *et al.*, 2006).

2.7.2 PURIFICATION AND CHARACTERIZATION OF CELLULASE AND XYLANASE ENZYME FROM FUNGI

2.7.2.1 Purification and Characterization of Cellulase Enzyme from Fungi

Singh *et al.* (2011) reported an extracellular endoglucanase (EG) from culture filtrates of *Aspergillus awamori* strain F 18 was 12 fold purified by ammonium sulphate precipitation followed by DEAE-anion exchange chromatography. The purified enzyme had an optimum pH of 4.0, temperature tolerance of 60°C and exhibited a K_m value of 17.24 mg/ml and V_{max} of 28.8 µg glucose/min/IU. This novel enzyme was thermostable retaining 65% of activity after incubation at 60°C for 2 h. SDS-polyacrylamide gel electrophoresis revealed the monomeric enzyme to have a molecular weight of about 43 kDa.

Lin *et al.* (2010) used native gradient-polyacrylamide gel electrophoresis and electroelution (NGGEE) to purify enzymatic proteins from *Trichoderma koningii* AS3.2774. With this method, we purified eight enzymatic proteins and classified them to the cellulase system by comparing secretions of *T. koningii* in inductive medium and in repressive medium. It resulted in 24-fold β-glucosidase (BG) purification with a recovery rate of 5.5%, and a specific activity of 994.6 IU mg⁻¹ protein. The final yield of β-glucosidase reached 8 µg under purifying procedure of NGGEE. β-glucosidase had one subunit with a molecular mass of 69.1 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The hydrolytic activity of the β-glucosidase had an optimal pH of 5.0, an optimal temperature of 50°C, an isoelectric point of 5.68 and a K_m for p-nitrophenyl-β-D-glucopyranoside of 2.67mM. Taken together, we show that

NGGEE is a reliable method through which μg grade of active proteins can be purified.

Beguml and Absar (2009) reported purification and characterization of intracellular cellulase produced by *A. oryzae* ITCC-4857.01 are reported. The enzyme was purified by ion-exchange chromatography using DEAE-cellulose followed by Gel filtration. The purification achieved was 41 fold from the crude extract with yield of 27%. The purified enzyme showed single band on poly acrylamide gel. The molecular weight as determined by SDS-PAGE and gel filtration was 38 KDa and 38.6 KDa respectively and contained only one subunit. The enzyme is glycoprotein as nature and contained 0.67% neutral sugar. The apparent K_m value of the enzyme against cellulose was 0.83%. The enzyme showed the highest relative activities on CMC followed by avicel, salicin and filter paper. The optimum pH of activity was 5.5 and very slight activity was observed at or above pH 7.5 as well as below pH 3.5. The optimum temperature of the activity was 45°C and the highest activity was exhibited in 35 to 45°C. The enzyme lost their activities almost completely (95~100%) at 80°C or above and as well as below 25°C.

Han and Chen (2008) reported purification and characterization of β -glucosidase from corn stover was performed and the enzyme was tried in SSF to evaluate the suitability of plant glycosyl hydrolases in lignocellulose conversion. A β -glucosidase with Mw of 62.4 kDa was purified to homogeneity from post-harvest corn stover. The following physicochemical and kinetic parameters of the β -glucosidase were studied respectively: optimum temperature, thermal stability, optimum pH, pH stability, K_m , V_{max} , V_i , cellobiose inhibition, tryptic peptide mass spectrometry and effect of metal ions and other reagents on the activity. The β -glucosidase activity on salicin was optimal at pH 4.8 and 37°C. The unique property of optimum temperature makes the β -glucosidase potentially useful in SSF. In SSF of steam explosion pretreated corn stover, the supplementation of the purified β -glucosidase was more effective than *Aspergillus niger* β -glucosidase.

Karnchanatat *et al.* (2008) studied a thermostable endoglucanase was purified to homogeneity from culture supernatants of the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb:Fr.) Rehm grown on 1.0% (w/v) carboxymethyl-cellulose using ammonium sulfate precipitation, ion-exchange, hydrophobic interaction, and gel filtration chromatography. The molecular weight of the enzyme was 46.4 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point of the enzyme was at pH 4.9. The temperature for maximum activity was 70°C, with 85% of its maximum activity retained after 150 min of incubation at 50°C, but was rapidly inactivated at 70°C. The pH optimum of the enzyme activity was 6.0, and it was stable over a pH range of 4.0–7.0 at 50°C. The enzyme was significantly inhibited by Hg^{2+} , Cu^{2+} , and Fe^{2+} , and stimulated by Ca^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} , glycerol, DMSO, DTT, and EDTA. The enzyme also hydrolyzed filter paper, and Avicel® PH-101 at rates of 25.8%, and 7.3%, respectively when compared with carboxymethyl-cellulose. The enzyme did not hydrolyze soluble starch, oat spelt xylan, birch wood xylan, or locust bean gum. The enzyme catalyzed the hydrolysis of carboxymethyl-cellulose with a K_m of 1.74 mg/ml and a V_{max} of 0.63 U/min/mg protein. This enzyme was competitively inhibited by glucose and cellobiose with K_m values of 0.67 and 0.45 M, respectively.

The accumulation dynamics of cellulolytic enzymes in culture media of the basidiomycete fungi *Panus tigrinus*, *Pleurotus ostreatus*, *Fomes fomentarius*, and the micromycete *Aspergillus terreus* were studied during a long incubation period. *A. terreus* was the most active producer of cellulolytic enzymes among the studied fungi. Two protein fractions with cellulase activity were isolated using gel filtration and ion-exchange chromatography. PAAG electrophoresis showed that fraction-I consisted of four components; fraction-II, an electrophoretically homogeneous protein (Mirzaakhmedov *et al.*, 2007).

Amouri and Gargouri (2006) reported an improved mutant was isolated from the cellulolytic fungus *Stachybotrys* sp. after nitrous acid mutagenesis. It was fed-batch cultivated on cellulose and its extracellular cellulases (mainly the endoglucanases and β -glucosidases) were analyzed. One β -glucosidase was

purified to homogeneity after two steps, Mono Q and gel filtration and shown to be a dimeric protein. The molecular weight of each monomer is 85 kDa. Besides its aryl β -glucosidase activity towards salicin, methyl-umbelliphenyl- β -glucoside (MUG) and *p*-nitrophenyl- β -d-glucoside (*p*NPG), it showed a true β -glucosidase activity since it splits cellobiose into two glucose monomers. The $V_{\max}$ and the K_m kinetics parameters with *p*NPG as substrate were 78 U/mg and 0.27 mM, respectively. The enzyme shows more affinity to *p*NPG than cellobiose and salicin whose apparent values of K_m were, respectively, 2.22 and 37.14 mM. This enzyme exhibits its optimal activity at pH 5 and at 50°C.

Rhizopus stolonifer was cultivated in wheat bran to produce a cellulase-free alkaline xylanase. The purified enzyme obtained after molecular exclusion chromatography in Sephacryl S-200 HR showed optimum temperature as 45°C and hydrolysis pH optima as pH 6.0 and 9.0. Xylanase presented higher $V_{\max}$ at pH 9.0 (0.87 mmol/mg protein) than at pH 6.0 and minor K_m at pH 6.0 (7.42 mg/mL) than at pH 9.0. (Goulart *et al.*, 2005).

Chaabouni *et al.* (2005) reported two endoglucanases (EGs), EG A and EG B, were purified to homogeneity from *Penicillium occitanis* mutant Pol 6 culture medium. The molecular weights of EG A and EG B were 31,000 and 28,000 kDa, respectively. The pI was about 3 for EG A and 7.5 for EG B. Optimal activity was obtained at pH 3.5 for both endoglucanases. Optimal temperature for enzyme activity was 60°C for EG A and 50°C for EG B. EG A was thermostable at 60°C and remained active after 1h at 70°C. EGs hydrolyzed carboxymethylcellulose, phosphoric acid swollen cellulose, and β -glucan efficiently, whereas microcrystalline cellulose (Avicel) and laminarin were poorly hydrolyzed. Only EG B showed xylanase activity. Furthermore, these EGs were insensitive to the action of glucose and cellobiose but were inhibited by the divalent cations Hg^{2+} , Co^{2+} , and Mn^{2+} .

A β -glucosidase was purified from the cell extracts of *Rhizopus oryzae* MIBA348 to homogeneity by successive anion exchange and size-exclusion chromatography and characterized. The enzyme has a molecular weight of

105,000. The optimum pH and the optimum temperature was 5.0 and 50°C, respectively. The enzyme was active on *p*-nitrophenyl- β -D-glucopyranoside, cellobiose and salicine. It hydrolyzed gentiobiose and amygdaline, but was inactive on Avicel, carboxymethylcellulose, maltose and *p*-nitrophenyl- α -D-glucopyranoside (Takii *et al.*, 2005).

An intracellular β -glycoside hydrolase with β -glucosidase and β -galactosidase activity, designated β -glucosidase BGL1 was isolated to apparent homogeneity from the thermophilic ascomycete *Talaromyces thermophilus* CBS 236.58. The monomeric enzyme has a molecular mass of 50 kDa (SDS-PAGE) and an isoelectric point of 4.5–4.6. The enzyme is active with both glucosides such as cellobiose and galactosides including lactose; based on the catalytic efficiencies determined glucosides are the preferred substrates. β -Galactosidase activity of BGL1 is activated by various mono and divalent cations including Na⁺, K⁺ and Mg²⁺, and it is moderately inhibited by its reaction products glucose and galactose. Its pH optimum for the hydrolysis of galactosides is in the range of 5.5–6.0, and its optimum temperature was found to be 50°C (15 min assay). In addition to its hydrolytic activity, BGL1 shows a significant transferase activity which results in the formation of galactooligosaccharides. These have recently attracted interest because of possible applications in food industry. The highest yield of oligosaccharides was approximately 20% when using 38 g L⁻¹ lactose as the starting material (Nakkharat and Haltrich, 2006).

2.7.2.2 Purification and Characterization of Xylanase Enzyme from Fungi

Zhou *et al.* (2011) reported a psychrotrophic fungus identified as *Trichoderma* sp. SC9 produced 36.7 U/ml of xylanase when grown on a medium containing corncob xylan at 20°C for 6 days. The xylanase was purified 37-fold with a recovery yield of 8.2%. The purified xylanase appeared as a single protein band on SDS-PAGE with a molecular mass of approximately 20.5 kDa. The enzyme had an optimal pH of 6.0, and was stable over pH 3.5–9.0. The optimal temperature of the xylanase was 42.5°C and it was stable up to 35°C at pH 6.0 for 30 min. The xylanase was thermolabile with a half-life of 23.9 min at 45°C. The

apparent K_m values of the xylanase for birchwood, beechwood, and oat-spelt xylans were found to be 3, 2.1, and 16 mg/ml respectively.

A xylanase was purified from symbiotic fungus, *Termitomyces* sp. by chromatography on columns of DEAE-Sepharose, CM-Sepharose, gel filtration and Phenyl-Sepharose. The preparation was shown to be homogenous by polyacrylamide gel electrophoresis. The purified enzyme displayed two protein bands on SDS-polyacrylamide gel electrophoresis and its molecular mass was estimated to 80-87 kDa. The xylanase exhibited maximum activity at 65-70°C and at pH 5.6, but it retained more than 80% of its activity in the pH range 5.0-6.0. The enzyme was stable for a long time-period up to 50°C and for 1 h at 60°C. Although the xylanase had a lower carboxymethylcellulase activity, it lacked activity towards substituted xylan, xylobiose, inulin, starch, polygalacturonic acid or pNP-glycosides. Kinetic parameters indicated higher efficiency in the hydrolysis of beechwood xylan and birchwood xylan. The xylanase activity was stimulated by K^+ , Mn^{2+} and dithiol-reducing agents and was sensitive to Cu^{2+} , Fe^{2+} , Zn^{2+} and detergent agents. The enzymatic activity was observed in presence of urea up to a 1% (w/v) concentration. The enzyme could also be used in the presence of organic solvents such as acetone or dioxane (5%, v/v) without loss of activity (Faulet *et al.*, 2006).

Chidi *et al.* (2008) investigated *Aspergillus terreus*, UL 4209 strain, isolated from the soil in South Africa was used to produce an extracellular cellulase-free xylanase in shake flask cultures containing oat spelt and/or birchwood xylans. Maximum xylanase activity (35 U/ml) was observed after 96 h at 35°C and pH 6 in 1% oat spelt xylan. The xylanase was purified to homogeneity by gel filtration on Sephacryl S-200. This enzyme was found to be a single subunit protein of 22 kDa showing optimal activity at 35°C and pH 6. The enzyme retained 95% activity at 35-40°C after 4h incubation at pH 6 and at 50°C the half-life was 5.8 h. The apparent K_m and V_{max} values were 3.57 mg/ml and 55.5 μ mol/min per mg protein, respectively. MALDITOF and LC mass spectroscopy gave 8 peptide ions whose sequence alignments showed that the

xylanase produced by this strain has homology with those of other *Aspergillus* strains such as *A. terreus* and *A. versicolor*.

Teixeira *et al.* (2010) studied production, purification and properties of a thermostable β -xylanase produced by an *Aspergillus awamori* 2B.361 U2/1 submerged culture using wheat bran as carbon source. Fractionation of the culture filtrate by membrane ultrafiltration followed by Sephacryl S-200 and Q-Sepharose chromatography allowed for the isolation of a homogeneous xylanase (PXII-1), which was 32.87 kDa according to MS analysis. The enzyme-specific activity towards soluble oat spelt xylan, which was found to be 490 IU/mg under optimum reaction conditions (50°C and pH 5.0–5.5), was 17-fold higher than that measured in the culture supernatant. Xylan reaction products were identified as xylobiose, xylotriose, and xylotetraose. K_m values (mg ml^{-1}) for soluble oat spelt and birchwood xylan were 11.8 and 9.45, respectively. Although PXII-1 showed 85% activity retention upon incubation at 50°C and pH 5.0 for 20 days, incubation at pH 7.0 resulted in 50% activity loss within 3 days. PXII-1 stability at pH 7.0 was improved in the presence of 20 mM cysteine, which allowed for 85% activity retention for 25 days.

Yang *et al.* (2010) investigated an extracellular xylanase from *Aspergillus niger* C3486 grown on a medium containing D-xylose was purified to homogeneity as indicated by disc acrylamide gel electrophoresis with an apparent molecular mass of about 25 kDa using DEAE-Sephadex A-50 and Sephadex G-100 column chromatography. The xylanase was purified 14.79 fold with 29.88% recovery. Optimal temperature and pH for activity was observed at 55°C and 5.5, respectively. The extracellular purified xylanase had K_m value and V_{max} of 0.104 mg/ml and $24.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. The metal ions Hg^{2+} and Cu^{2+} showed some inhibition effects, while Mg^{2+} and Cr^{3+} had small stimulating effects on the activity.

Saha (2002) studied *Fusarium proliferatum* (NRRL 26517), was isolated by screening soil samples surrounding decaying corn and wood using corn fibre xylan as carbon source. The extracellular xylanase from this fungal strain was

purified 975-fold to homogeneity from the culture supernatant by ammonium sulphate treatment, DEAE Bio-Gel A column chromatography, octyl-Sepharose column chromatography and Bio-gel A-0.5 m gel filtration. The purified xylanase (specific activity 591 U mg⁻¹ protein) was a monomeric glycoprotein with a molecular weight (MW) of 22400 as determined by SDS-PAGE. The optimum pH and temperature for the action of the enzyme were at 5.0-5.5 and 55°C, respectively. The purified xylanase was fully stable at pH 5.0-7.5 and temperature up to 55°C.

Ratanachomsri *et al.* (2006) reported that total number of 32 strains producing extreme xylanases, the strain BCC7928, identified by using the internal transcribed spacer (ITS) sequence of rRNA to be a *Marasmius* sp., was chosen for further characterization because of its high xylanolytic activity at temperature as high as 90°C. The crude enzyme possessed high thermostability and pH stability. Purification of this xylanase was carried out using an anion exchanger followed by hydrophobic interaction chromatography, yielding the enzyme with >90% homogeneity. The molecular mass of the enzyme was approximately 40 kDa. The purified enzyme retained broad working pH range of 4-8 and optimal temperature of 90°C. When using xylan from birchwood as substrate, it exhibits K_m and V_{max} values of 2.6 ± 0.6 mg/ml and 428 ± 26 U/mg, respectively. The enzyme rapidly hydrolysed xylans from birchwood, beechwood, and exhibited lower activity on xylan from wheat bran, or celluloses from carboxymethylcellulose and Avicel. The purified enzyme was highly stable at temperature ranges from 50 to 70°C. It retained 84% of its maximal activity after incubation in standard buffer containing 1% xylan substrate at 70°C for 3h.

Knob and Carmona (2010) reported two xylanases from the crude culture filtrate of *Penicillium sclerotiorum* were purified to homogeneity by a rapid and efficient procedure, using ion-exchange and molecular exclusion chromatography. Molecular masses estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were 23.9 and 33.1 kDa for xylanase I and II, respectively. The native enzymes' molecular masses of 23.8 and 30.8 kDa were estimated for xylanase I and II, respectively, by molecular exclusion

chromatography. Both enzymes are glycoproteins with optimum temperature and pH of 50°C and pH 2.5 for xylanase I and 55°C and pH 4.5 for xylanase II. The reducing agents β -mercaptoethanol and dithio-treitol enhanced xylanase activities, while the ions Hg^{2+} and Cu^{2+} as well the detergent SDS were strong inhibitors of both enzymes, but xylanase II was stimulated when incubated with Mn^{2+} . The K_m value of xylanase I for birchwood xylan and for oat spelt xylan were 6.5 and 2.6 mg mL^{-1} , respectively, whereas the K_m values of xylanase II for these substrates were 26.61 and 23.45 mg mL^{-1} .

A thermostable xylanase was isolated from an *Aspergillus niger* wild type strain in a liquid Czapek Dox medium, containing oat spelts xylan as the sole carbon source. The molecular mass of the enzyme was estimated to be about 36 kDa by sodium dodecyl sulfate- polyacrylamide gel electrophoresis. The optimum pH for activity was found to be 7.5. The temperature optimum of the enzyme was found to be 60°C at pH 7.5. The enzyme remained stable up to 100°C, yet lost about 50% of its activity after 15 min at this temperature (Coral *et al.*, 2002).

Knob and Carmona (2008) purified two xylanases from the crude culture filtrate of *Penicillium sclerotium* to homogeneity by a rapid and efficient procedure, using ion exchange and molecular exclusion chromatography. Molecular masses estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were 23.9 and 33.1 kDa for xylanase 1 and 11, respectively. The native enzyme molecular masses of 23.8 and 30.8 kDa for were estimated for xylanase 1 and 11, respectively by molecular exclusion chromatography. The K_m value of xylanase for birchwood xylan and for oat spelt xylan were 6.5 and 2.6 mg mL^{-1} , respectively whereas the K_m values of xylanase 11 for the substrates were 26.61 and 23.45 mg mL^{-1} .

Shigeuuki and Yoshio (2009) purified three xylanases (designated as I, II and III) produced by *Penicillium janthinellum* Biourge from an extract of wheat bran koji culture by procedures including salting out with ammonium sulfate, SE-Sephadex column chromatography and gel filtration on Sephadex G-100. The

xylanase I was proved to be homogenous by ultra-centrifugal analysis and three xylanases were practically free from other xylanases such as mannanase, galactanase, arabanase and galactosidases. The xylanase I was most active at pH 5.3 and stable over a range of pH 5 to 8 at 30°C for 24 hr. The xylanase II and III were most active at pH 4.7 and were stable over a range of pH 4 to 9 and pH 5 to 9 at 30°C for 24 hr, respectively.

Characterization of a thermostable xylanase from a newly isolated thermophilic fungus *Talromyces thermophilus* was studied by Maallej *et al.* (2009). The enzyme was purified to homogeneity by ammonium sulfate precipitation, diethyl aminoethyl cellulose anion exchange chromatography, P-100 gel filtration and monoclone chromatography with a 23 fold increase in specific activity and 17.5% recovery. The molecular weight of the xylanase was estimated to be 25 kDa by sodium dodecyl sulfate – polyacrylamide gel electrophoresis and gel filtration. The enzyme showed high thermal stability at 50°C (7 days) and the half life of the xylanase at 100°C was 60 min. The enzyme was free from cellulase activity. K_m and V_{max} values at 50°C of the purified xylanase for birchwood xylan were 22.5 mg/ml and 1.235 μ mol min⁻¹ mg⁻¹, respectively

2.8 CELLULASE AND XYLANASE GENE FAMILIES

2.8.1 Cellulase Gene Families

Arai *et al.* (2003) reported *Clostridium thermocellum* CelJ is a modular enzyme containing a family 30 carbohydrate-binding module (CBM) and a family 9 catalytic module at its N-terminal moiety. To investigate the functions of the CBM and the catalytic module, truncated derivatives of CelJ were constructed and characterized. Isothermal titration calorimetric studies showed that the association constants (K_a) of the CBM polypeptide (CBM30) for the binding of cellopentaose and cellohexaose were 1.2×10^4 and 6.4×10^4 M⁻¹, respectively, and that the binding of CBM30 to these ligands is enthalpically driven. Qualitative analyses showed that CBM30 had strong affinity for cellulose and β -1,3-1,4-mixed glucan such as barley β -glucan and lichenan. Analyses of the hydrolytic action of the enzyme comprising the CBM and the catalytic module

showed that the enzyme is a processive endoglucanase with strong activity towards carboxymethylcellulose, barley -glucan and lichenan. By contrast, the catalytic module polypeptide devoid of the CBM showed negligible activity toward these substrates. These observations suggest that the CBM is extremely important not only because it mediates the binding of the enzyme to the substrates but also because it participates in the catalytic function of the enzyme or contributes to maintaining the correct tertiary structure of the family 9 catalytic module for expressing enzyme activity.

The enrichment from nature of novel microbial communities with high cellulolytic activity is useful in the identification of novel organisms and novel functions that enhance the fundamental understanding of microbial cellulose degradation. In this work we identify predominant organisms in three cellulolytic enrichment cultures with thermophilic compost as an inoculum. Community structure based on 16S rRNA gene clone libraries featured extensive representation of clostridia from cluster III, with minor representation of clostridial clusters I and XIV and a novel *Lutispora* species cluster. Our studies reveal different levels of 16S rRNA gene diversity, ranging from 3 to 18 operational taxonomic units (OTUs), as well as variability in community membership across the three enrichment cultures. By comparison, glycosyl hydrolase family 48 (GHF48) diversity analyses revealed a narrower breadth of novel clostridial genes associated with cultured and uncultured cellulose degraders. The novel GHF48 genes identified in this study were related to the novel clostridia *Clostridium straminisolvens* and *Clostridium clariflavum*, with one cluster sharing as little as 73% sequence similarity with the closest known relative. In all, 14 new GHF48 gene sequences were added to the known diversity of 35 genes from cultured species (Izquierdo *et al.*, 2010).

The wood decay fungus *Phanerochaete chrysosporium* has served as a model system for the study of lignocellulose conversions, but aspects of its cellulolytic system remain uncertain. Identified the gene that encodes glycoside hydrolase (GH) family 45 endoglucanase (EG) from the fungus, cloning the cDNA, determining its heterologous expression in the methylotrophic yeast

Pichia pastoris, and characterizing the recombinant protein. The cDNA consisted of 718 bp, including an open reading frame encoding a 19-amino-acid signal peptide, a 7-amino-acid presequence at the N-terminal region, and a 180-amino-acid mature protein, which has no cellulose binding domain. Analysis of the amino acid sequence revealed that the protein has a low similarity (<22%) to known fungal EGs belonging to the GH family 45 (EGVs). No conserved domain of this family was found by a BLAST search, suggesting that the protein should be classified into a new subdivision of this GH family. The recombinant protein has hydrolytic activity toward amorphous cellulose, carboxymethyl cellulose, lichenan, barley β -glucan, and glucomannan but not xylan. Moreover, a synergistic effect was observed with the recombinant GH family 6 cellobiohydrolase from the same fungus toward amorphous cellulose as a substrate, indicating that the enzyme may act in concert with other cellulolytic enzymes to hydrolyze cellulosic biomass in nature (Igarashi *et al.*, 2008).

2.8.2 Xylanase Gene Families

Fukuda *et al.* (2010) reported that *Paenibacillus* sp. W-61 is capable of utilizing water-insoluble xylan for carbon and energy sources and has three xylanase genes, *xyn1*, *xyn3*, and *xyn5*. *Xyn1*, *Xyn3*, and *Xyn5* are extracellular enzymes of the glycoside hydrolase (GH) families 11, 30, and 10, respectively. *Xyn5* contains several domains including those of carbohydrate-binding modules (CBMs) similar to a surface-layer homologous (SLH) protein. This study focused on the role of *Xyn5*, localized on the cell surface, in water-insoluble xylan utilization. Electron microscopy using immunogold staining revealed *Xyn5* clusters over the entire cell surface. *Xyn5* was bound to cell wall fractions through its SLH domain. A *xyn5* mutant grew poorly and produced minimal amounts of *Xyn1* and *Xyn3* on water-insoluble xylan. A *Xyn5* mutant lacking the SLH domain (*Xyn5*_SLH) grew poorly, secreting *Xyn5*_SLH into the medium and producing minimal *Xyn1* and *Xyn3* on water-insoluble xylan. A mutant with an intact *xyn5* produced *Xyn5* on the cell surface, grew normally, and actively synthesized *Xyn1* and *Xyn3* on water-insoluble xylan. Quantitative reverse transcription-PCR showed that xylobiose, generated from water-insoluble xylan

decomposition by Xyn5, is the most active inducer for *xyn1* and *xyn3*. Luciferase assays using a Xyn5-luciferase fusion protein suggested that xylotriose is the best inducer for *xyn5*. The cell surface Xyn5 appears to play two essential roles in water-insoluble xylan utilization: (i) generation of the xylo-oligosaccharide inducers of all the *xyn* genes from water-insoluble xylan and (ii) attachment of the cells to the substrate so that the generated inducers can be immediately taken up by cells to activate expression of the Xyn system.

Twelve members of the family 11 xylanases, including both mesophilic and thermophilic proteins, were studied using molecular dynamics (MD). Simulations of xylanases were carried out in an explicit water environment at four different temperatures, 300, 400, 500 and 600 K. A difference in thermotolerance between mesophilic and thermophilic xylanases became clear: thermophilic xylanases endured heat in higher simulation temperatures better than mesophilic ones. The unfolding pathways seemed to be similar for all simulations regardless of the protein. The unfolding initiates at the N-terminal region or alternatively from the α -helix region and proceeds to the 'finger region'. Unfolding of these regions led to denaturated structures within the 4.5 ns simulation at 600 K. The results are in agreement with experimental mutant studies by Purmonen *et al.* (2007).

Gallardo *et al.* (2010) reported a new bacterial xylanase belonging to family 5 of glycosyl hydrolases was identified and characterized. The xylanase, Xyn5B from *Bacillus* sp. strain BP-7, was active on neutral, nonsubstituted xylooligosaccharides, showing a clear difference from other GH5 xylanases characterized to date that show a requirement for methyl-glucuronic acid side chains for catalysis. The enzyme was evaluated on *Eucalyptus* kraft pulp, showing its effectiveness as a bleaching aid.

Xylanases are generally classified into glycosyl hydrolase families 10 and 11 and are found to frequently have an inverse relationship between their pI and molecular mass values. However, we have isolated a psychrophilic xylanase that belongs to family 8 and which has both a high pI and high molecular mass. This

novel xylanase, isolated from the Antarctic bacterium *Pseudoalteromonas haloplanktis*, is not homologous to family 10 or 11 enzymes but has 20-30% identity with family 8 members. NMR analysis shows that this enzyme hydrolyzes with inversion of anomeric configuration, in contrast to other known xylanases which are retaining. No cellulase, chitosanase or lichenase activity was detected. It appears to be functionally similar to family 11 xylanases. It hydrolyzes xylan to principally xylotriose and xylotetraose and is most active on long chain xylo-oligosaccharides. Kinetic studies indicate that it has a large substrate binding cleft, containing at least six xylose binding subsites. Typical psychrophilic characteristics of a high catalytic activity at low temperatures and low thermal stability are observed. An evolutionary tree of family 8 enzymes revealed the presence of six distinct clusters. Indeed classification in family 8 would suggest an (α/α) 6 fold, distinct from that of other currently known xylanases (Collins *et al.*, 2002).

2.9 MICROBIAL CELLULASES AND XYLANASES AND THEIR INDUSTRIAL APPLICATIONS

2.9.1 Microbial Cellulases and Their Industrial Applications

2.9.1.1 Pulp and Paper Industry

Interest in the application of cellulases in the pulp and paper industry has increased considerably during the last decade. The mechanical pulping processes such as refining and grinding of the woody raw material lead to pulps with high content of fines, bulk, and stiffness. While in contrast, biomechanical pulping using cellulases resulted in substantial energy savings (20-40%) during refining and improvements in hand-sheet strength properties by Singh *et al.* (2007). Mixtures of cellulases (endoglucanases I and II) and xylanases have also been used for bio modification of fiber properties with the aim of improving drainage and beat ability in the paper mills before or after beating of pulp. Mansfield *et al.* (1996) studied the action of a commercial cellulase preparation on different fractions of Douglas fir kraft pulp and observed that the cellulase treatment decreased the defibrillation reducing the fibre coarseness. While endoglucanases have the ability to decrease the pulp viscosity with a lower degree of hydrolysis

of cellulases have also been reported to enhance the bleach ability of softwood kraft pulp producing a final brightness score comparable to that of xylanase treatment.

Cellulases alone, or used in combination with xylanases, are beneficial for deinking of different types of paper wastes. Most applications proposed so far use cellulases and xylanases for the release of ink from the fiber surface by partial hydrolysis of carbohydrate molecules by Kuhad *et al.* (2010). It has been postulated that improvements in dewatering and deinking of various pulps result in the peeling of the individual fibrils and bundles, which have high affinity for the surrounding water and ink particles. The main advantages of enzymatic deinking are reduced or eliminated alkali usage, improved fiber brightness, enhanced strength properties, higher pulp freeness and cleanliness, and reduced fine particles in the pulp. Moreover, deinking using enzymes at acidic pH also prevents the alkaline yellowing, simplifies the deinking process, changes the ink particle size distribution, and reduces the environmental pollution. Although enzymatic deinking can lower the need for deinking chemicals and reduce the adverse environmental impacts of the paper industry the excessive use of enzymes must be avoided because significant hydrolysis of the fines could reduce the bond ability of the fibers.

Interestingly, the use of cellulases in improving the drainage has also been pursued by several mills with the objective to increase the production rate. Enzyme treatments remove some of the fines or peel off fibrils on the fiber surface and dissolved and colloidal substances, which often cause severe drainage problems in paper mills. In this aspect, cellulases have shown considerable improvement in the overall performance of paper mills by Bhat (2000). Enzymatic treatment also destabilizes the lipophilic extractives in the filtrates and facilitates their attachment to thermomechanical pulping fibers. These enzymes are also used in preparation of easily biodegradable cardboard, manufacturing of soft paper including paper towels and sanitary paper and removal of adhered paper.

2.9.1.1 Textile Industry

Cellulases are the most successful enzymes used in textile wet processing, especially finishing of cellulose-based textiles, with the goal of improved hand and appearance. Traditional stonewashing of jeans involves amylase-mediated removal of starch coating (desizing) and treatment (abrasion) of jeans with pumice stone (1-2 kg/pair of jeans) in large washing machines. Cellulases have been successfully used for the biostoning of jeans and biopolishing of cotton and other cellulosic fabrics. During the biostoning process, cellulases act on the cotton fabric and break off the small fiber ends on the yarn surface, thereby loosening the dye, which is easily removed by mechanical abrasion in the wash cycle. The advantages in the replacement of pumice stones by a cellulose-based treatment include less damage of fibers, increased productivity of the machines, and less work-intensive and environment benign by Karmakar and Ray (2011).

While the biopolishing is usually carried out during the wet processing stages, which include desizing, scouring, bleaching, dyeing, and finishing. The acidic cellulases improve softness and water absorbance property of fibres, strongly reduce the tendency for pill formation, and provide a cleaner surface structure with less fuzz. Cellulase preparations rich in endoglucanases are best suited for biopolishing enhancing fabric look, feel, and color without needing any chemical coating of fibers. The action of cellulases removes short fibers, surface fuzziness, creates a smooth and glossy appearance, and improves color brightness, hydrophilicity and moisture absorbance, and environmentally friendly process (Kuhad *et al.*, 2011). Similarly, endoglucanase activity-rich cellulase is also proved better for biofinishing. Most cotton or cotton-blended garments, during repeated washing, tend to become fluffy and dull, which is mainly due to the presence of partially detached microfibrils on the surface of garments. The use of cellulases can remove these microfibrils and restore a smooth surface and original color to the garments (Ibrahim *et al.*, 2011). The use of cellulase also helps in softening the garments and in removal of dirt particles trapped within the microfibril network.

Interestingly, there are several reports where the performance of the whole cellulase preparations was quite different from the enzyme rich in

endoglucanase activity, and that the latter offered better performance in applications where losses in fabric strength and weight were minimum. Depilling/cleaning and/or ageing effects are the result of the synergistic action of cellulases and mechanical action, simultaneously or sequentially by Baker *et al* (2011). Attempts have also been made via cellulases treatment to improve the dimensional stability of cellulosic fabrics and to upgrade the surface and dyeing properties of bleached cotton, mercerized cotton, and cotton/polyester blend fabric (50/50), using the pad-wet batch technique, followed by subsequent washing under mechanical action (Ibrahim *et al.*, 2011).

2.9.1.2 Bioethanol Industry

Enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corncob, rice straw, *Prosopis juliflora*, *Lantana camara*, switch grass, saw dust, and forest residues by cellulases for biofuel production is perhaps the most popular application currently being investigated. Bioconversion of lignocellulosic materials into useful and higher value products normally requires multistep processes (Kuhad *et al.*, 2010). These processes include; pretreatment (mechanical, chemical, or biological), hydrolysis of the polymers to produce readily metabolizable molecules (e.g., hexose and pentose sugars), bioconversion of these smaller molecules to support microbial growth and/or produce chemical products, and the separation and purification of the desired products. The utility cost of enzymatic hydrolysis may be low compared with acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4-6 and temperature 45-50°C) and does not have corrosion issues by Gupta *et al.* (2011). Technologies are currently available for all steps in the bioconversion of lignocellulosics to ethanol and other chemical products. However, some of these technologies must be improved to produce renewable biofuel and other byproducts at prices, which can compete with more conventional production systems. Not only the recalcitrance of the substrate, but also several other factors that also limit cellulase efficiency during the hydrolysis process including end product inhibition, thermal deactivation of the native protein, nonspecific binding to lignin and irreversible adsorption of the enzymes to the heterogeneous substrate by Kuhad *et al.* (2011).

To reduce the enzyme cost in the production of fuel ethanol from lignocellulosic biomass, two aspects are widely addressed: optimization of the cellulase production and development of a more efficient cellulase-based catalysis system. Protein engineering and directed evolution are powerful tools that can facilitate the development of more efficient thermophilic cellulases by Baker *et al.* (2005). Strategies for recycling and reuse of the enzymes may also be used to reduce enzymatic hydrolysis costs. The recovery of enzymes is largely influenced by adsorption of the enzymes onto the substrate, especially to lignin and enzyme inactivation. There are several reports where the nonspecific and irreversible adsorption of cellulase to lignin has been observed (Yang and Wyman, 2004). Besides, there are also reports where the compounds that mimic cellulose or the compounds have high affinity towards lignin have been used to prevent the adsorption of cellulases to lignin. Among different strategies to recover and reuse the cellulases are concentration of the cellulose fraction by ultrafiltration to remove sugars and other small compounds that may inhibit the action of the enzymes (Tu *et al.*, 2007) and recycling of immobilized enzymes, which enables separation of the enzymes from the process flow. However, the recycling techniques are mostly tested at laboratory scale. Therefore, the ability to scale up the techniques, the robustness, and feasibility still needs to be demonstrated.

2.9.1.3 Wine and Brewery Industry

Microbial glucanases and related polysaccharides play important roles in fermentation processes to produce alcoholic beverages including beers and wines by. These enzymes can improve both quality and yields of the fermented products. Glucanases are added either during mashing or primary fermentation to hydrolyze glucan, reduce the viscosity of wort, and improve the filterability. In wine production, enzymes such as pectinases, glucanases, and xylanases play an important role by improving color extraction, skin maceration, must clarification, filtration, and finally the wine quality and stability. B-glucosidases can improve the aroma of wines by modifying glycosylated precursors. Macerating enzymes also improve pressability, settling, and juice yields of grapes used for wine

fermentation. A number of commercial enzyme preparations are now available to the wine industry. The main benefits of using these enzymes during wine making include better maceration, improved color extraction, easy clarification, easy filtration, improved wine quality, and improved stability (Kuhad *et al.*, 2011).

Beer brewing is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination, which initiates the biosynthesis and activation of α - and β -amylases, carboxypeptidase, and glucanase that hydrolyze the seed reserves. Significant and reproducible improvements in grape press ability, settling rate, and total juice yield were achieved using a combination of macerating enzymes. Such improvements were noticeable only with a correct balance of pectinases, cellulases and xylanases. Using three varieties (Soave, Chardonnay and Sauvignon) of white grapes, Galante *et al.* (1998) assessed the performance of cytolase 219 (mixture of cellulase, pectinase, and xylanase) in wine making and reported a 10-35% increase in the extraction of the first wine must, 70-80% increase in the must filtration rate, 50-120 minutes decrease in pressing time, 30-70% decrease in must viscosity, 20-40% saving of energy during cooling of fermenter, and a significant improvement in wine stability. A range of improved enzymes like cellulase and pectinase that would be exogenously added to the process are expected to enhance the productivity of existing brewing processes in future.

2.9.1.4 Food Processing Industry

Cellulases have a wide range of potential applications in food biotechnology as well. The production of fruit and vegetable juices requires improved methods for extraction, clarification, and stabilization. Cellulases also have an important application as a part of macerating enzymes complex (cellulases, xylanases, and pectinases) used for extraction and clarification of fruit and vegetable juices to increase the yield of juices (Minussi *et al.*, 2002). The use of macerating enzymes increases both yield and process performance without additional capital investment. The macerating enzymes are used to improve cloud stability and texture and decrease viscosity of the nectars and purees from tropical fruits such as mango, peach, papaya, plum, apricot, and pear (Bhat 2000).

Texture, flavor, and aroma properties of fruits and vegetables can be improved by reducing excessive bitterness of citrus fruits by infusion of enzymes such as pectinase and β -glucosidases by Kuhad *et al.* (2011). Enzyme mixtures containing pectinases, cellulases and xylanases are also used for improved extraction of olive oil. Use of macerating enzymes not only improves the cloud stability and texture of nectars and purees, but also decreases their viscosity rapidly. Thus, the macerating enzymes, composed of mainly cellulase and pectinase, play a key role in food biotechnology and their demand will likely increase for extraction of juice from a wide range of fruits and vegetables. Furthermore, infusion of pectinases and β -glucosidases has also shown to alter the texture, flavor, and other sensory properties such as aroma and volatile characteristics of fruits and vegetables (Singh *et al.*, 2007; Bhat 2000).

2.9.1.5 Animal Feed Industry

Applications of cellulases and xylanases in the feed industry have received considerable attention because of their potential to improve feed value and performance of animals (Kuhad *et al.* 2011). Pretreatment of agricultural silage and grain feed by cellulases or xylanases can improve its nutritional value. The enzymes can also eliminate antinutritional factors present in the feed grains, degrade certain feed constituents to improve the nutritional value, and provide supplementary digestive enzymes such as proteases, amylases, and glucanases. For instance, the dietary fiber consists of nonstarch polysaccharides such as arabinoxylans, cellulose, and many other plant components including resistant dextrins, inulin, lignin, waxes, chitins, pectins, glucan, and oligosaccharides, which can act as anti-nutritional factor for several animals such as swine. In this case, the cellulases effectively hydrolyse the anti-nutritional factor, cellulose; in the feed materials into easily absorbent ingredient thus improve animal health and performance. Glucanases and xylanases have been used in the feed of monogastric animals to hydrolyze nonstarch polysaccharides such as β -3-glucans and arabinoxylans. Cellulases, used as feed additives alone or with proteases, can significantly improve the quality of pork meat. Glucanases and xylanases reduce viscosity of high fibre rye- and barley-based feeds in poultry and pig. These

enzymes can also cause weight gain in chickens and piglets by improving digestion and absorption of feed materials (Singh *et al.*, 2007; Bhat 2000).

Most low-quality feedstuffs contain higher concentrations of cellulose, small amounts of protein and fat, and relatively high ash contents when compared with high-quality feedstuffs. Cellulases can be used to improve silage production for cattle feeding, which involves enhancement of the digestibility of grasses containing large amounts of potentially total digestible nutrients and energy values together with only small amounts of water-soluble carbohydrates. The forage diet of ruminants, which contains cellulose, hemicellulose, pectin, and lignin, is more complex than the cereal-based diet of poultry and pigs. Enzyme preparations containing high levels of cellulase, xylanase, and pectinase have been used to improve the nutritive quality of forages by Kuhad *et al.* (2011). Nevertheless, the results with the addition of enzyme preparations containing cellulase, xylanase and pectinase to ruminant diet are somewhat inconsistent.

Animal feedstock production processes generally include heat treatments that inactivate potential viral and microbial contaminants. Application of thermophilic cellulase in feedstock production has the potential to reduce pathogens as well as to enhance digestibility and nutrition of the feed, thereby facilitating a combination of heat treatment and feed transformation in a single step (Bhat, 2000). The cellulases and xylanases are responsible for partial hydrolysis of lignocellulosic materials, dehulling of cereal grains, hydrolysis of glucans, and better emulsification and flexibility of feed materials, which results in the improvement in the nutritional quality of animal feed by Cowan (1996). Moreover, these enzymes can cause partial hydrolysis of plant cell wall during silage and fodder preservation.

The use of enzymes in animal nutrition became more important after the prohibition of using some nutritive ionophore antibiotics, which were previously used in the EU countries. There is a large variation in the digestibility of starch origins. The low digestibility of some starches promotes the appearance of some digestive tract diseases because the non digested and non absorbed starch

reaching the large intestine can act as a substrate for bacterial fermentation supporting the proliferation of some potentially hazardous pathogenic bacteria. Cellulases have a positive effect on the caecal fermentation processes by increasing the production of propionic acid, which act as a bacteriostatic material and thus can decrease the colonization of pathogenic bacteria (Kuhad *et al.*, 2011)

2.9.1.6 Agricultural Industries

Various enzyme preparations consisting of different combinations of cellulases and hemicellulases have potential applications in agriculture for enhancing growth of crops and controlling plant diseases (Bhat, 2000). Plant or fungal protoplasts produced using microbial hydrolases can be used to produce hybrid strains with desirable properties. Cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens in controlling the plant disease by Bhat, (2000). Fungal β -glucanases are capable of controlling diseases by degrading cell walls of plant pathogens. Many cellulolytic fungi including *Trichoderma* sp., *Geocladium* sp., *Chaetomium* sp., and *Penicillium* sp. are known to play a key role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system and increased crop yields by Kuhad *et al.* (2011). Although these fungi have both direct (probably through growth-promoting diffusible factor) and indirect (by controlling the plant disease and pathogens) effects on plants.

Cellulases have also been used for the improvement of the soil quality. Traditionally straw incorporation is considered an important strategy to improve soil quality and reduce dependence on mineral fertilizers by Ortiz Escobar and Hue (2008). Many studies have attempted to hasten straw decomposition via microbial routes. Cellulolytic fungi applications such as *Aspergillus*, *Chaetomium*, and *Trichoderma* and actinomycetes (Abdulla and El-Shatoury, 2007) have shown promising results.

2.9.1.7 Olive Oil Extraction

In recent years, extraction of olive oil has attracted the interest of international market because of its numerous health claims. Extraction of olive oil involves (1) crushing and grinding of olives in a stone or hammer mill; (2)

passing the minced olive paste through a series of malaxeurs and horizontal decanters; (3) high speed centrifugation to recover the oil. To produce high-quality olive oil, freshly picked, clean, and slightly immature fruits have been used under cold pressing conditions (Faveri *et al.*, 2008). However, high yields have been obtained with fully ripened fruit, when processed at higher than ambient temperatures, but this resulted in oil with high acidity, rancidity, and poor aroma. Hence, an improved method for the extraction of high-quality olive oil was needed to meet the growing consumer demand. The commercial enzyme preparation, Olivex (a pectinase preparation with cellulase and xylanase from *Aspergillus aculeatus*), was the first enzyme mixture used to improve the extraction of olive oil. Furthermore, the use of macerating enzymes increased the antioxidants in extra virgin olive oil and reduced the induction of rancidity. The main advantages of using macerating enzymes during olive oil extraction are (1) increased extraction (up to 2 kg oil per 100 kg olives) under cold processing conditions; (2) better centrifugal fractionation of the oily must; (3) oil with high levels of antioxidants and vitamin E; (4) slow induction of rancidity; (5) overall improvement in plant efficiency; (6) low oil content in the waste water. Likewise, the macerating enzymes could play prominent role in the extraction of oils from other agricultural oilseed crops.

These enzymes can also be used during olive paste malaxation. The presence of collateral activities of a cellulase and xylanase nature of the enzymatic formulation guarantees a rapid and intense disintegration of the cell walls and membranes of the olive fruits, thereby favoring the passage of noble substances (particularly the poly-phenols and aromatic precursors) into the final product. It is also used to decrease olive paste viscosity in olive oil production and to intensify the process of extracting the polyphenolic substances contained in the olive fruit by Kuhad *et al.* (2011). It is necessary to underline that the selected enzymes are naturally present inside the olive fruit, but they are strongly deactivated during the critical pressing step, probably because of oxidation phenomena (Chiacchierini *et al.*, 2007). So, the replacement of these enzymes is expected to be appropriate in relation with the role they play in determining the final product quality.

2.9.1.8 Carotenoid Extraction

Carotenoids are the main group of coloring substances in nature being responsible for many plant colors from red to yellow by Cinar (2005). There is a continuously growing market for carotenoids as food colorants due to their desirable properties, such as their natural origin, null toxicity, and high versatility, providing both lipo and hydro soluble colorants with colors ranging from yellow to red. In addition, provitamin A activity, a role in lipid oxidation, and anticarcinogenic properties are very important biological functions of these pigments. Usually a combination of cellulolytic and pectinolytic enzymes accelerates the rate of hydrolysis for achieving complete liquefaction. Cellulase randomly splits cellulose chains into glucose whereas commercial pectinase preparations from *Aspergillus niger* have pectinesterase (PE), polygalacturonase (PG), and pectin lyase (PL) activity by Kuhad *et al.* (2011). The use of pectinase and cellulase enzymes disrupts the cell wall of orange peel, sweet potato and carrot, and releases the carotenoids in the chloroplasts and in cell fluids. These pigments remain in their natural state still bound with proteins. This bonded structure prevents pigment oxidation and also affects color stability, whereas solvent extraction dissociates the pigments from the proteins and causes water insolubility and ease of oxidation.

2.9.1.9 Detergent Industry

Use of cellulases along with protease and lipase in the detergents is a more recent innovation in this industry. Cellulase preparations capable of modifying cellulose fibrils can improve color brightness, feel, and dirt removal from the cotton blend garments. The industrial application of alkaline cellulases as a potential detergent additive is being actively pursued with a view to selectively contact the cellulose within the interior of fibers and remove soil in the interfibril spaces in the presence of the more conventional detergent ingredients (Sukumaran *et al.*, 2005). Nowadays, liquid laundry detergent containing anionic or nonionic surfactant, citric acid or a water-soluble salt, protease, cellulose, and a mixture of propanediol and boric acid or its derivative has been used to improve the stability of cellulases. As most of the cellulose

fibers in the modern textile industry enzymes are used increasingly in the finishing of fabrics and clothes are arranged as long, straight chains of some small fibers can protrude from the yarn or fabric. The cellulases are applied to remove these rough protuberances for a smoother, glossier, and brighter-colored fabric by Karmakar and Ray (2011).

2.9.1.10 Waste Management

The wastes generated from forests, agricultural fields, and agro-industries contain a large amount of unutilized or underutilized cellulose, causing environmental pollution (Milala *et al.*, 2005). Nowadays, these so-called wastes are judiciously utilized to produce valuable products such as enzymes, sugars, biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds, and human nutrients by (Kuhad *et al.* 2011).

2.9.2 Microbial Xylanases and Their Industrial Applications

In recent years, the biotechnological use of xylans and xylanases has grown remarkably (Bhat 2000). Commercial xylanases are industrially produced in Japan, Finland, Germany, Republic of Ireland, Denmark, Canada and the USA. The microorganisms used to obtain these enzymes are *Aspergillus niger*, *Trichoderma* sp. and *Humicola insolens*. Nevertheless, commercial xylanases can also be obtained from bacteria. Xylanase began to be used in the 1980s: initially in the preparation of animal feed and later in the food, textile and paper industries. Currently, xylanase and cellulase, together with pectinases, account for 20% of the world enzyme market.

2.9.2.1 Paper and pulp

The main industrial application of the xylanases is in the bleaching of cellulose pulp. Enzymes began to be used in this sector during the last two decades, ever since peroxidases were applied to the degradation of lignin (Sandrim *et al.*, 2004). At present, in many countries, including Brazil, the chemical process, rather than enzymatic hydrolysis, is employed in paper manufacture. The usual method is known as the Kraft process, the name

signifying strength or force in German. Three species of *Eucalyptus* (*E. grandis*, *E. saligna* and *E. urophylla*) are particularly favoured as the raw material.

The method starts with the pretreatment of wood shavings with a combination of two reagents, sodium hydroxide and sodium sulphide, under 8 kgf/cm² and at 165°C, in a digester. The two reagents in the cooking liquor serve to accelerate the delignification, with recovery of the cellulose fibres. At this stage, the cellulose pulp is known as brown mass, appearing dark in colour because of the black liquor. It can be assumed that 90-95% of the hemicellulose and lignin are dissolved and partially degraded during this process. Summarizing, we have:

- Wood shavings (fibres and lignin) + reagents = "cellulose" and lignin.
- Using industrial terminology: wood + white liquor (NaOH+NaS₂) = "cellulose" + black liquor.

The deposited lignin confers a dark colour to the pulp and, after the brown mass is washed, pre-bleaching is carried out, consisting of the removal of minor impurities and a part of the lignin remaining from the cooking. Oxygen is used in this process, with the aim of reducing the cost of other reagents employed in the bleaching process. The pre-bleached pulp has a pale yellow colour and is not yet suitable for the production of good-quality paper for printing or writing. This staining is caused by residual lignin on the walls of the fibres. In the subsequent bleaching step, the paste attains a pure white colour, through the removal of light-absorbing substances (chromophores) formed by products of the breakdown of lignin. The bleaching process can be divided into three stages. In the first, ozone and chlorine dioxide are used and in the second stage, sodium hydroxide, oxygen and hydrogen peroxide. In the last stage another treatment with chlorine dioxide is carried out by Polizeli *et al.* (2005).

The main advantage of the Kraft process is the possibility of recovering the chemical products from the black liquor. However, not all industries recover the sodium hydroxide and other organic materials present in the black liquor. On the other hand, the disadvantages are the high initial costs, the strong smell of

gases emitted by the process, low yield (40-50%) and the high cost of bleaching. It can be seen that in the Kraft process polluting reagents are used in large amounts. The use of chlorine results in the production of organochlorines from the degradation products of lignin, which are highly toxic and mutagenic, requiring treatment of the effluents from the paper-making plant. Environmental regulations have restricted the use of chlorine compounds in bleaching processes in the paper and cellulose industries, especially in Western Europe and North America. Special attention has been given to using xylanase in pre-bleaching, which would lower the amount of chlorine compounds used by up to 30%, so that a 1520% reduction in organochlorines in the effluents could be achieved. The utilization of xylanases could lead to the replacement of 5-7 kg of chlorine dioxide per ton of Kraft pulp and an average fall of 2-4 units in the kappa number, a measure of the lignin content in the cellulose pulp. Xylanases employed in paper technology do not need to be purified, but must be active at high temperatures and alkaline pH, and must not contain cellulolytic enzymes in order to preserve the cellulose fibres.

2.9.2.2 Manufacture of Bread, Food and Drinks

Xylanases may be employed in bread-making, together with α -amylase, malting amylase, glucose oxidase and proteases. The xylanases, like the other hemicellulases, break down the hemicellulose in wheat-flour, helping in the redistribution of water and leaving the dough softer and easier to knead. During the bread-baking process, they delay crumb formation, allowing the dough to grow. With the use of xylanases, there has been an increase in bread volumes, greater absorption of water and improved resistance to fermentation (Camacho and Aguilar 2003). Also, a larger amount of arabinoxyloligosaccharides in bread would be beneficial to health. In biscuit-making, xylanase is recommended for making cream crackers lighter and improving the texture, palatability and uniformity of the wafers.

The juice and wine industries make up a good part of the enzyme market. The production of fruit and vegetable juices requires methods of extraction, clearing and stabilization. Nowadays, xylanases, in conjunction with cellulases,

amylases and pectinases, lead to an improved yield of juice by means of liquefaction of fruit and vegetables; stabilization of the fruit pulp; increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc., reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate. Xylanase, in combination with endoglucanase, takes part in the hydrolysis of arabinoxylan and starch, separating and isolating the gluten from the starch in the wheat flour. This enzyme is also used in coffee-bean mucilage. The main desirable properties for xylanases for use in the food industry are high stability and optimum activity at an acid pH. With the advances in the techniques of molecular biology, other uses of xylanases are being discovered. Recently, recombinant yeast of wine was constructed with the gene for xylanase of *Aspergillus nidulans*, *xlnA*, resulting in a wine with a more pronounced aroma than is conventional. During the manufacture of beer, the cellular wall of the barley is hydrolyzed releasing long chains of arabinoxylans which increase the beer's viscosity rendering it "muddy" in appearance. Thus, xylanases are used to hydrolyze arabinoxylans to lower oligosaccharides diminishing the beer's viscosity and consequently eliminating its muddy aspect (Dervilly *et al.* 2002).

2.9.2.3 Pharmaceutical and Chemical Applications

Xylanase and xylan are little used in the pharmaceutical industry. Xylanases are sometimes added in combination with a complex of enzymes (hemicellulases, proteases and others) as a dietary supplement or to treat poor digestion, but few medicinal products can be found with this formulation by Polizeli *et al.* (2005).

Hydrolytic products of xylan, such as p-D-xylopyranosyl residues, can be converted into combustible liquids (ethanol), solvents and artificial low-calorie sweeteners. The first steps are the delignification of hemicellulose material rich in xylan, followed by hydrolysis by xylanases and hemicellulases, to produce sugars such as p-D-xylopyranosyl units. Next, the products are fermented, mainly by yeasts (*Pichia stipitis* and *Candida shehatae*), to produce xylitol or ethanol (Screenath and Jeffries 2000). Among the sugars used in the production of ethyl

alcohol, p-D-xylopyranosyl residues represent between 5 and 20%. Xylitol is a poly-alcohol with a sweetening power comparable to that of sucrose. It is a non-cariogenic sweetener, suitable for diabetic and obese individuals and recommended for the prevention of osteoporosis and respiratory infections, lipid metabolism disorder, kidney and parenteral lesions. A variety of commercial products containing xylitol, such as chewing gum, can be found on the market. Although the enzymatic hydrolysis of xylan is a promising method of obtaining p-D-xylopyranosyl units, at present commercial xylitol is produced on a large scale by chemical catalysis. This is considered a high-cost process, mainly because the xylose has to be purified initially in several steps. Besides this, the chemical reactions often produce by-products toxic to fermentation; indeed, in the decomposition of lignocellulosic material, besides the liberation of sugars, products may be formed that are derived from the degradation of glucose (hydroxymethylfurfural), xylose (furfural) and lignin (aromatic and phenolic compounds and aldehydes). Substances liberated from the lignocellulose structure, such as acetic acid and extracted material (e.g. terpenes and their derivatives, tropolones and phenolic compounds such as flavonoids, stilbenes, quinones, lignans and tannins), or from the equipment (iron, chromium, nickel and copper), can be powerful inhibitors of microbial activity. The development of a more appropriate technology for xylitol production has generated great hope of its wider use in the food, pharmaceutical and odonto-logical industries.

2.9.2.4 Textiles

The xylanolytic complex can be used in the textile industry to process plant fibres, such as hessian or linen. For this purpose, the xylanase should be free of cellulolytic enzymes. One process consists of incubating dried ramee (China grass) stems with xylanase to liberate the long cellulose fibres intact. After using this method, there is no need to use the strong bleaching step, since the lignin does not undergo oxidation, which would lead to darkening of the fibres (Csiszar *et al.* 2001). Relatively little research has been done on the enzymatic preparation of textile fibres, and yet this appears to be a promising market demanding the development of new techniques.

2.10 SCALE UP OF CELLULASE AND XYLANASE ENZYMES IN A BIOREACTOR

Lee *et al.* (2011) studied the Novel design solid state bioreactor, FERMSOSTAT, had been evaluated in cellulase production studies using local isolate *Aspergillus niger* USM AI1 grown on sugarcane bagasse and palm kernel cake at 1:1 (w/w) ratio. Under optimised SSF conditions of 0.5 kg substrate; 70% (w/w) moisture content; 30°C; aeration at 4L/h/g fermented substrate for 5 min and mixing at 0.5 rpm for 5 min, about 3.4 U/g of Filter paper activity (FPase) was obtained. At the same time, comparative studies of the enzymes production under the same SSF conditions indicated that FPase produced by *A. niger* USM AI1 was about 35.3% higher compared to *Trichoderma reesei*. This shows that the performance of this newly designed SSF bioreactor is acceptable and potentially used as prototype for larger-scale bioreactor design.

The effect of agitation and aeration rates on xylanase activity of *Aspergillus niger* SS7 in 3-L stirred tank bioreactor was investigated by Bakri *et al.* (2011). The agitation rates tested were 100, 200 and 300 rpm at each airflow rates of 0.5, 1.0 and 1.5 vvm. The maximum xylanase activity in mono- agitator system was at the agitation speed of 200 rpm and aeration rate of 1.0 vvm. In bi-agitator system, at low agitation speed (100 rpm), the xylanase activity was enhanced by 13% compared to mono- agitator system for an aeration rate of 1.0 vvm. Xylanase productivity in continuous culture was higher by approximately 3.5 times than in batch culture.

Schell *et al.* (2001) investigated oxygen transfer during the growth of *Trichoderma reesei*, a cellulase producing microorganism; on soluble and insoluble substrates in vessel sizes from 7 to 9000 L. Oxygen uptake rates and volumetric mass transfer coefficients (kLa) were determined using mass spectroscopy to measure off gas composition. Experimentally measured kLa values were found to compare favorably with a kLa correlation available in the literature for a non- Newtonian fermentation broth during the period of heavy cell growth.

Effect of aeration (0.5, 1.0 and 1.5 vvm) and agitation rate (100, 300 and 500 rpm) on cellulase production in submerged culture of *Pyconoporus sanguineus* was studied in 2.5 L stirred tank bioreactor using Palm Oli Mill effluent as substrate. Maximum cell biomass (3.16 g L^{-1}) and cellulase activity ($0.1748 \text{ FPU mL}^{-1}$) was obtained at aeration rate of 1.0 vvm and agitation speed of 300 rpm. Volumetric mass transfer coefficient (k_La) was found to be dependent on aeration and agitation rate with maximum k_La (124.2 h^{-1}) attained at 300 rpm and 1.5 vvm by (Fadzilah and Mashitach, 2010).

Enhancement of thermophilic xylanase production by recombinant *Escherichia coli* DH5 α through suitable medium formulation was initially investigated using shake flask cultures by Basar *et al.* (2010). Thereafter the effect of dissolved oxygen tension (DOT) level on the performance of xylanase fermentation by *E. coli* DH5 α was investigated in 2L stirred tank bioreactor using the optimal medium. Among the two basal medium tested (complex medium of Luria Bertani and defined mineral medium), defined mineral medium gave the highest growth and xylanase production. The optimal glucose and $(\text{NH}_4)_2\text{SO}_4$ for xylanase production was obtained at 10 g L^{-1} and 2 g L^{-1} , respectively. Growth of *E. coli* DH5 α and xylanase production was inhibited in oxygen limited fermentation, where dissolved oxygen tension level was controlled at 0% saturation. On the other hand, xylanase production was enhanced at DOT level controlled at 20% saturation, though growth was not significantly improved. Substantially high xylanase production (1784.57 U/mL) was obtained in fermentation using optimal medium composition and DOT level.

Jo *et al.* (2008) reported the optimal conditions for pilot-scale production of the carboxymethylcellulase (CMCase) by *Bacillus amyloliquifaciens* DL-3 were investigated. The best carbon and nitrogen sources for the production of CMCase by *Bacillus amyloliquifaciens* DL-3 were found to be rice hull and peptone and their optimal concentrations were 5.0 and 0.20% (w/v), respectively. Optimal temperature and initial pH for the production of CMCase were 37°C and 6.8. Optimal agitation speed and aeration rate for the production of CMCase were

300 rpm and 1.0 vvm in a 7L bioreactor, which were different from those for the cell growth of *Bacillus amyloliquifaciens* DL-3. The highest productions of CMCase by *Bacillus amyloliquifaciens* DL-3 from 5.0% (w/v) rice hull as a carbon source under optimal conditions in a 7 or 100 L bioreactor were 220 and 367 U/mL, respectively.

Jhang and Chang (2005) studied that the thermostable cellulase was produced by *Streptomyces* sp. T3-1 grown in a 50L fermenter. Maximum cellulase activity was attained on the fourth day when agitation speeds and aeration rates were controlled at 300 rpm and 0.75 vvm, respectively. Maximum enzyme activities were: 148 IU CMCase mL⁻¹, 45 IU Avicelase mL⁻¹, and 137 IU β -glucosidase mL⁻¹ with productivity of 326 IU⁻¹ h⁻¹, which were 10–32% higher than the values obtained in shake-flask cultures.

Ilias and Hoq (1998) reported the cellulase-free xylanase production by *T. lanuginosus* MH4 was investigated in a 3 L stirred tank bioreactor under different agitation rates and an aeration rate of 1 v/v/m. The cultivation time in the bioreactor was reduced significantly over that in shake culture conditions. A xylanase productivity of 0.1 nkat 1⁻¹ h⁻¹ was achieved on xylan in the bioreactor. This was nearly double to that obtained in shake culture. The agitation rates influenced both growth and enzyme secretion in the bioreactor. The highest level of biomass concentration and activities of both xylanase and β -xylosidase were obtained at 150 rev min⁻¹.

Richana *et al.* (2007) studied the optimum conditions for the growth of *Bacillus pumilus* RXAIII-5 (a potential xylanase producer) were sought, these included temperature, pH, aeration, and agitation of the culture batch. The results of the study indicate that the optimum condition for processing in 50 ml Erlenmeyer flask are used temperature of 35°C (308°K), pH 7 and an agitation rate of 140 rpm. The highest xylanase activity and its specific activity are 297.132 U/mL⁻¹ and 655.32 U/g⁻¹ proteins, respectively. Subsequent experiments in a bioreactor using all of the experiment parameters mentioned above, except for the agitation rate, shows that the results are as follows. The highest specific

growth was at 0.082 hour^{-1} at an aeration and agitation rate of 0.5 vvm and 150 rpm, respectively. Based on the data of the cultivation kinetics, the optimum conditions for the fermentation in Biostat 2L-bioreactor are 1 vvm and 200 rpm of aeration and agitation, respectively. The efficiency of substrate (Y_p/s) and of cell biomass (Y_p/x) to produce xylanase is 50.744 U/g^{-1} and 43.906 U/g^{-1} , respectively. The efficiency of substrate to cell production (Y_x/s) is 1.178 g/g^{-1} . The liquid cultivation-medium has non-Newtonian properties. Based on a mathematical model it is found that the consistency index (k constant) and index of liquid behavior (n value) are $0.179 \text{ g/cm}^{-1}/\text{second}^{-1}$ and 0.3212, respectively. Based on the value of $0 < n < 1$ and the constant $k > 0$, the culture liquid is categorized as pseudo plastic one. The Rheynold number (NRe) is 6.9×10^3 which indicates it has turbulent characteristics. From a calculation it is found that the power required to run a suitably sized impeller is 0.228 HP (Horse Power) and the power consumption per unit volume is 0.2265 HP.m^{-3} .

The effects of various aeration (0.5 to 2.5 vvm) and agitation rates (300-700 rpm) on cell growth, glucose consumption and protease production by *B. licheniformis* UV-9 were studied in a 2L stirred tank bioreactor. Volumetric mass transfer coefficient values were measured by dynamics gassing-out techniques at each aeration and agitation rate (Nadeem *et al.*, 2009).

Production of extracellular alkaline protease using an industrial strain of *Bacillus licheniformis* in different industrial media was optimized. Of different media used, the production medium composed of glucose and soybean meal as carbon and nitrogen source, respectively, in addition to inorganic phosphate, calcium chloride and magnesium chloride showed the maximal enzyme production of about 2400 U mL^{-1} after 96 h. The effect of glucose concentration on the cell *growth* and the enzyme production was studied in shake flask culture. The maximal enzyme production was obtained on using glucose concentration of 60 g L^{-1} in the industrial medium. Further optimization in the production process was achieved on studying the effect of aeration rate in small scale 16L bioreactor. Whereas, the increase of airflow rate in the range between 0.5 to $2.0 \text{ v v}^{-1} \text{ min}$ showed a negative effect on cell growth, the maximal enzyme production of 4120

U mL⁻¹ was obtained at airflow 1 vv⁻¹ min after 84 h at agitation rate of 500 rpm. When the process was scaled up to 500 L bioreactor and cultivated at the same airflow rate, the enzyme production reached about 5100 U mL⁻¹ after 80h cultivation (El Enshasy *et al.*, 2008).

Ducros *et al.* (2008) studied the modified rotating simplex method has been successfully used to determine the best combination of agitation rate and aeration rate for maximum production of extracellular proteases by *Staphylococcus aureus* mutant RC128, in a stirred tank bioreactor operated in a discontinuous way. This mutant has shown altered exoprotein production, specially enhanced protease production. Maximum production of proteases (15.28 UP/ml), measured using azocasein as a substrate, was obtained at exponential growth phase when the bioreactor was operated at 300 rpm and at 2 vvm with a volumetric oxygen transfer coefficient (KLa) of 175.75 h⁻¹. These conditions were found to be more suitable for protease product.

Thermostable cellulase free xylanase produced by *Streptomyces* sp. Ab 106 using agricultural waste, sugarcane baggasse, as substrate at 50°C and pH 7.0. The central composite face centered Experimental design was applied to evaluate the optimal agitation and aeration rates in a five liter fermenter. The highest activity (16.0±0.51 IU/ml) was obtained at aeration rate of 1 vvm and an agitation rate of 150 rpm (k_{La}= 351⁻¹). Using repeated fed batch cultivation technique, the maximum xylanase activity of 32±1 IU/ml was obtained during the second cycle of repeated fed batch culture (Techapun *et al.*, 2003).

Gomes *et al.* (2000) reported the xylanase production by the Antarctic psychrophilic yeast *Cryptococcus adeliae* was increased 4.3 fold by optimizing the culture medium composition using statistical designs. The optimized medium containing 24.2g l⁻¹ xylan and 10.2g l⁻¹ yeast extract and having an initial pH of 7.5 yielded xylanase activity at 400 nkat (nanokatal) ml⁻¹ after 168^h shake culture at 4°C. In addition, very little endoglucanase, β-mannanase, β-xylosidase, β-glucosidase, β-l-arabinofuranosidase, and no filter paper cellulase activities were detected. Among 12 carbon sources tested, maximum xylanase activity was induced by xylan, followed by lignocelluloses such as steamed wheat straw and

alkalitreated bagasse. The level of enzyme activity produced on other carbon sources appeared to be constitutive. Among the complex organic nitrogen sources tested, the xylanase activity was most enhanced by yeast extract, followed by soymeal, Pharmamedia (cotton seed protein), and Alburex (potato protein). A batch culture at 10°C in a 5L fermenter (3.5L working volume) using the optimized medium gave 385nkat at 111 h of cultivation. The crude xylanase showed optimal activity at pH 5.0-5.5 and good stability at pH 4-9 (21h at 4°C). Although the enzyme was maximally active at 45°-50°C, it appeared very thermolabile, showing a half-life of 78min at 35°C. At 40°-50°C, it lost 71%–95% activity within 5 min.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled “**Purification of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost for industrial application**” was conducted in laboratory of the Microbiology section, Department of Basic Sciences, Nauni, Solan, Himachal Pradesh, India, during the years 2009-2012. The chapter provides insights into how the study was conducted, experimental designing, materials used and methodology adopted during the course of research period.

- 3.1 Isolation and screening of hypercellulolytic and hyperxylanolytic microorganisms from compost/degrading woods.
- 3.2 Identification of hypercellulolytic and hyperxylanolytic producing microbial isolates
- 3.3 Optimization and production of extracellular cellulase and xylanase
 - ❖ Under Submerged Fermentation
 - ❖ Statistical optimization under Submerged Fermentation
 - ❖ Under Solid State Fermentation
- 3.4 Purification and characterization of xylanase enzyme
- 3.5 Encoding of cellulase and xylanase gene from screened bacterial and fungal isolates
- 3.6 Scale up of enzyme in a bioreactor

3.1 ISOLATION AND SCREENING OF CELLULOLYTIC AND XYLANOLYTIC MICROORGANISMS FROM COMPOST

3.1.1 Isolation of Cellulolytic and Xylanolytic Microorganisms From Compost/Degrading Wood

3.1.1.1 Collection of Compost Sample

Degrading wood/ humus rich compost samples were collected and pooled in clean polythene bags from different sites of Himachal Pradesh Viz. Solan, Shimla, Mandi and Kangra district and brought to the laboratory.

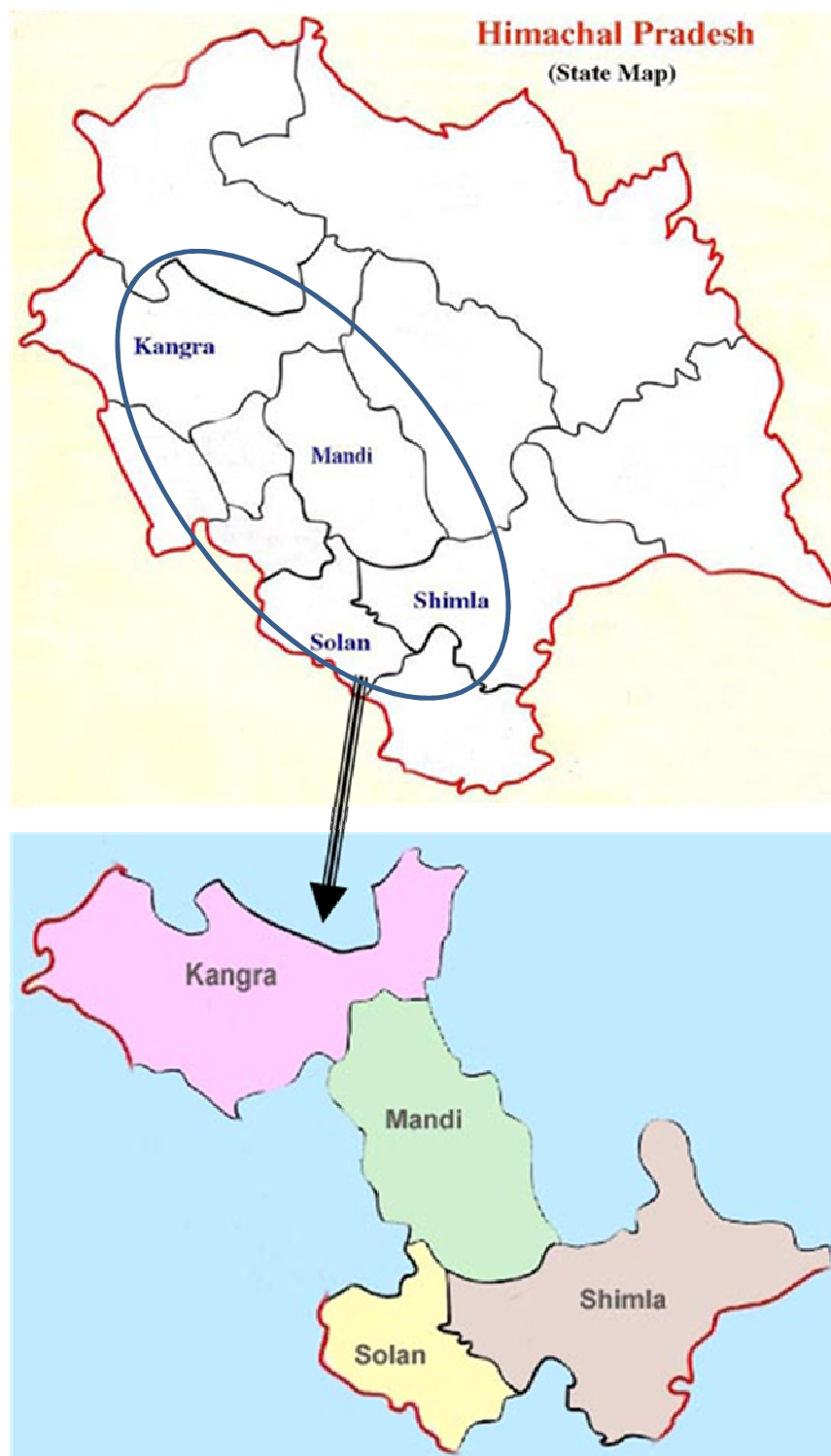


Fig.1 Isolation of cellulolytic and xylanolytic microorganisms from mid hills of Himachal Pradesh sub temperature zone of northern Himalayas

3.1.1.1.2 Enrichment of Sample

To 100g of compost sample in a petriplate, 2g of cellulose powder was added and water was sprinkled in the petriplate and mixed thoroughly. The petriplates containing above mixture were incubated at $28\pm 2^{\circ}\text{C}$ for one week.

3.1.1.1.3 Isolation of Cellulolytic and Hemicellulolytic Microorganisms

One gram of enriched compost sample was serially diluted from 10^{-2} to 10^{-8} times using sterilized distilled water. The dilutions were placed on Riviere medium. Diluted inoculum (0.1 ml) was spread on the surface of medium with the help of a spreader and the plates were incubated at $28\pm 2^{\circ}\text{C}$ for 5 days. The pure line cultures were obtained by streaking each microbial colony on the respective medium following 72h incubation at $28\pm 2^{\circ}\text{C}$. These pure line cultures were maintained on nutrient agar slants (pH 6.8) and preserved in refrigerator at 4°C . Further subculturing was done once in a month.

Chemical composition of Riviere (1961) medium: 1.0g NaNO_3 , 1.18g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.9g KH_2PO_4 , 0.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g KCl , 0.5g yeast extract, 1g cellulose powder/xylan, 20g agar. Final volume was made 1000ml and pH was adjusted to 6.8. This medium was used for the isolation of cellulolytic and xylanolytic microorganisms.

3.1.2 Screening of cellulolytic and hemicellulolytic microorganisms

3.1.2.1 Screening of Cellulolytic and Hemicellulolytic Bacterial Isolates

The bacterial isolates of section 3.1.1c were screened for the production of extracellular enzymes i.e. cellulase (FPase, CMCase, β -glucosidase and xylanase activity.

3.1.2.1.1 Production of Extracellular Cellulase and Xylanase by Bacterial Isolates

Each bacterial isolate was grown in 50 ml of nutrient broth and was incubated at $37\pm 2^{\circ}\text{C}$ for 24 h. As soon as the substantial growth of the isolate was observed in the broth, the optical density was set to 1.0 using sterilized distilled water.

5 ml of inoculum was added to each 45 ml of Riviere's broth containing 1% cellulose for cellulase and 1% xylan for xylanase production in 250 ml of Erlenmeyer flasks and the flasks were incubated at $37\pm 2^{\circ}\text{C}$ for 5 days at 120 rpm. After incubation the culture contents were centrifuged at 10000 rpm for 15 min (4°C). The supernatant was collected. The following quantitative tests were performed with the supernatant to find out the hypercellulolytic and hyperxylanolytic producers among different isolates.

3.1.2.1.2 Cellulase Assays

3.1.2.1.2.a (i) Filter Paperase (FPase) Activity (Grajek, 1987):

Reagents

- i) Strips of filter paper (Whatmann no. 1)
- ii) 0.05 M citrate buffer (pH 5.0)
- iii) Dinitrosalicylic acid (DNSA) reagent
- iv) Standard solution of glucose (0.4mg/ml)

Procedure

To 50 mg strips of filter paper (Whatmann no. 1), 0.5 ml of citrate buffer (0.05 M, pH 5.0) and 0.5 ml of culture supernatant was added. The control was also run with all the components except the substrate i.e. filter paper (Whatmann no. 1). The reaction mixture was incubated at 50°C for 30 min. After incubation 3 ml of DNSA reagent added to reaction mixture. Mixing was done on vortex mixer. The mixture was heated in a boiling water bath for 15 min. After cooling, the absorbance of reaction mixture was recorded at 540 nm against a reagent blank i.e. 1 ml of distilled water and 3 ml of DNSA reagent. The enzyme activity was expressed in terms of International units (IU) and Specific activity (SA).

- i) One International unit of enzyme activity represents μmoles of glucose released/ min/ ml of enzyme.
- ii) Specific activity represents μmoles of glucose released/min/mg of protein.

3.1.2.1.2.b (ii) CMCase Assay (Grajek, 1987):

Reagents

- i) 1.1% CMC in citrate buffer (0.055 M, pH 5.0)
- ii) Dinitrosalicylic acid (DNSA) reagent : 1.0g of NaOH , 0.2 g phenol, 20.0 g sodium potassium tartarate, 0.05g, sodium metabisulphite, 1.0g DNSA reagent were dissolved in distilled water and final volume was made 100 ml.
- iii) Standard solution of glucose (.4 mg/ ml)

Procedure

The reaction mixture contained 0.5 ml of 1.1% CMC in citrate buffer (0.055M, pH 5) and 0.5 ml of diluted enzyme. The reaction mixture was incubated at 50°C for 30 min. After incubation 3 ml of DNSA reagent added to reaction mixture and thorough shaking was done on vortex mixer. The tubes were immersed in boiling water bath and removed after 15 min when the colour development was complete. The tubes were cooled at room temperature. The optical density was read at 540 nm. The enzyme activity was expressed in terms of International units (IU) and Specific activity (SA).

- i) One International unit of enzyme activity represents μ moles of glucose released/ min/ ml of enzyme.
- ii) Specific activity represents μ moles of glucose released/min/mg of protein.

3.1.2.1.2.c (iii) β -glucosidase Activity (Grajek, 1987):

Reagents

- i) 1 mM p-nitrophenyl β -D-glucopyranoside in 0.05M sodium acetate buffer (pH 5.0)
- ii) 2 M sodium carbonate (Na_2CO_3) solution
- iii) Standard solution of p- nitrophenol (80 μ g/ml)

Procedure

The reaction mixture contained 0.9 ml of 1mM p-nitrophenol β -D-glucopyranoside in 0.05 M sodium acetate buffer (pH 5) and 100 μ l enzyme

solution. After incubation at 40°C for 10 min, 2 ml of 2 M Na₂CO₃ was added to the mixture to stop the reaction. The mixture was heated in boiling water bath for 15 min and after heating it was diluted with 10 ml of distilled water and p-nitrophenol liberated was determined from the absorbance at 400 nm against reagent blank. The standard curve was made from the stock solution of p-nitrophenol (80µl/ml). The enzyme activity was expressed in terms of International units (IU) and Specific activity (SA).

- i) One International unit of enzyme activity represents µmoles of p-nitrophenol released/ min/ ml of enzyme.
- ii) Specific activity represents µmoles of p-nitrophenol released/min/mg of protein.

3.1.2.1.2. d (iv) Xylanase Assay (Miller, 1959):

Reagents

- i) 1.0 g larch wood xylan in 100 ml of 0.055 M citrate buffer , pH 4.0
- ii) DNSA reagent
- iii) Standard solution of xylose (0.4mg/ml)

Procedure

To 0.5 ml of xylan solution (which was incubated overnight at 37°C, centrifuged and clear supernatant was used, 0.3 ml citrate buffer was added and 0.2 ml of enzyme. The control was run with all the components except substrate, the reaction mixture was incubated at 45°C for 10 min. after the incubation, the 3 ml of DNSA reagent was added to it. The mixture was heated on boiling water bath for 30 min. After cooling down to room temperature absorbance of reaction mixture was recorded at 540 nm. The enzyme activity was expressed in terms of International units (IU) and Specific activity (SA).

- i) One International unit of enzyme activity represents µmoles of xylose released/ min/ ml of enzyme.
- ii) Specific activity represents µmoles of xylose released/min/mg of protein.

3.1.2.1.2.e Protein Assay

Protein estimation (Lowry *et al.*, 1951):

Reagent

- i) Folin Ciocalteu's reagent (1N)
- ii) 2% sodium carbonate (Na_2CO_3) in 0.1 M sodium hydroxide solution
- iii) 1% sodium potassium tartarate solution
- iv) 1% copper sulphate (CuSO_4) solution
- v) Bovin Serum Albumin (BSA) as standard (10-100 $\mu\text{g/ml}$)
- vi) Lowry's alkaline reagent was prepared by mixing 98 ml of (2) with 1 ml of (3) and 1 ml of (4). It was made fresh at the time of use only.

Procedure

To 0.1 ml of the culture supernatant, 3.0 ml of Lowry's alkaline reagent was added, mixed and allowed to stand for 15 min. Diluted (1N) Folin - Ciocalteu reagent (0.3 ml) was added. The contents were shaken quickly on 37°C for maximum colour development. Absorbance of reaction mixture was measured at 670 nm against a reagent blank. The contents of protein in samples were estimated from standard curve which was prepared by using Bovine Serum Albumin (BSA) in concentration of 10-100 $\mu\text{g/ml}$.

3.1.2.2 Screening of Cellulolytic and Hemicellulolytic Fungal Isolates

The fungal isolates of section 3.1.1c were screened for the production of extracellular enzymes i.e. cellulase (FPase, CMCase, β -glucosidase and xylanase Activity.

3.1.2.2.1 Production of Extracellular Cellulase(s) and Xylanase by Fungal Isolates

Each fungal isolate was grown in Sabouraud dextrose agar medium in petri plate and incubated at 30 $\pm$ 2°C for full plate growth. As soon as the full plate growth of the isolate was observed in petri plate, Added 10% of full plate growth of culture scratched with 10 ml of autoclaved distilled water and fixing their inoculum size 1 $\times$ 10⁷ spores/ml.

10 ml of inoculum was added to each 90 ml of Riviere broth containing 1% cellulose for cellulase and 1% xylan for xylanase production in 250 ml of Erlenmeyer flasks and the flasks were incubated at $37\pm 2^{\circ}\text{C}$ for 7 days at 120 rpm. After incubation the culture contents were centrifuged at 10000 rpm for 15 min (4°C). The supernatant was collected. The following quantitative tests were performed with the supernatant to find out the hypercellulolytic and hyperxylanolytic producers among different isolates.

3.1.2.2.1 Cellulase Assays

3.1.2.2.1a (i) Filter Paperase (FPase) Activity (Grajek, 1987): as given in section 3.1.2.1.2.a (i)

3.1.2.2.1b (ii) CMCase Assay (Grajek, 1987): as given in section 3.1.2.1.2.b (ii)

3.1.2.2.1c (iii) β -glucosidase Activity (Grajek, 1987): as given in section 3.1.2.1.2.c (iii)

3.1.2.2.1d (iv) Xylanase Assay (Miller, 1959): as given in section 3.1.2.1.2.d (iv)

3.1.2.2.1e Protein Assay (Lowry *et al.*, 1951): as given in section 3.1.2.1.2.e

3.2 MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF CELLULASE AND XYLANASE PRODUCING ISOLATES

The cellulolytic and xylanolytic microorganisms isolated from different compost sample were differentiated and observed on the basis of morphological, cultural and physiological characteristics. Isolates were classified into bacteria and fungus.

3.2.1 Phenotypic Characterization

The Conventional morphological and biochemical tests as described by Aneja (2003) were performed, which include:

3.2.1.1 Morphological Test

Main morphological characteristics: cell morphology, Gram reaction, shape and culture conditions.

3.2.1.2 Physiological and Biochemical test

Main physiological test performed were catalase, urease, H₂S production, MR/VP test, gelatin hydrolysis, fermentation of carbohydrates, citrate and indole test.

3.2.2 Genotypic Identification

Bacterial isolates were identified at genomic level by using 16S r RNA gene technique as given below:

3.2.2.1 Isolation of Genomic DNA

Genomic DNA of BM-1, SH8 and SH0 were isolated by using standard protocol of DNA prep kit (Banglore Genei, India Pvt. Ltd. make). The bacterial strains BM-1, SH8 and SH0 were inoculated in 100 ml of nutrient broth separately and grown at 35±2°C for 18h. Isolation of total genomic DNA from each culture was carried out by the following procedure: 1ml of each culture was centrifuged at 10,000 rpm for 10 min. Supernatant was discarded. Bacterial pellets were resuspended in 100 µl of bacterial lysis buffer containing lysozyme @ 20mg/ml followed by incubation at 37°C for 30 min. 180 µl of lysis buffer I and 20 µl of proteinase k were added and mixed thoroughly by vortexing. Incubated at 55°C for 3h. The samples were vortexed at intervals for better lysis. The samples were spun at 10,000 rpm in a micro-centrifuge for 5 min and the collected supernatants decanted carefully to a fresh vial. 200 µl of lysis buffer II was added to the supernatants, mixed by vortexing and incubated at 70°C for 20 min. 4µl of RNase A (100 mg/ml) was added, mixed by vortexing and incubated at room temperature for 5 min. The sample was spun at 10,000 rpm in a micro-centrifuge for 5 min and the supernatant was decanted carefully to a fresh vial. 200 µl of absolute ethanol was added to the supernatant and was mixed thoroughly by vortexing. Spin column was kept in a 2 ml collection tube and the

sample ethanol mixture was added to the column. Centrifuged at 10,000 rpm for 1 min. Collection tube was discarded with flow through. The spin column was kept in a fresh 2 ml collection tube and 500 µl of wash buffer I was added. Spun at 10,000 rpm for 1 min. Collection tube was discarded with washed sample. The spin column was kept in a fresh 2 ml collection tube and 500 µl of wash buffer II was added. Centrifuged at 10,000 rpm for 5 min. Washed fraction was discarded and the collection tube was retained for further steps. The empty column was spun at 10,000 rpm for 2 min to ensure the removal of wash buffer. The spin column was placed in a 1.5 ml tube and 200 µl of prewarmed elution buffer was added. It was further incubated at room temperature for 5 min and centrifuged for 2 min to elute the DNA of each strain.

3.2.2.2 Agarose gel Electrophoresis

3.2.2.2.1 Material Required

- i) Horizontal gel electrophoresis
- ii) Power pack
- iii) Water bath
- iv) Microfuge
- v) Deep Freezer (-20°C)
- vi) Ice making machine
- vii) Gel documentation system

3.2.2.2.2 Solution/Reagents

1.	Agarose powder	1 %
2.	TAE buffer stalk	50 X
	i) Tris	242 g
	ii) Glacial acetic acid	57.1ml
	iii) EDTA (0.5 M, pH 8.0)	100 ml
	iv) Final volume	1000 ml
3.	6X loading dye	
	i) Sucrose	4g
	ii) Bromophenol blue	0.025 g

- iii) Xylene cyanol 0.025 g
 - iv) Volume 10 ml
 - v) Loading dye solution stored at 4°C
4. Ethidium bromide 10mg/ml

3.2.2.2.3 Protocol

1. 250 ml of TAE buffer (1X) was taken in a 500 ml Erlenmeyer flask.
2. 2.5 g of agarose was weighed and added in to the TAE buffer (1X).
3. The contents were heated on hot plate till agarose dissolves completely. Contents were cooled to 60°C. 5µl of ethidium bromide was mixed with precooled agarose.
4. Gel casting tray was fixed properly and kept in a electrophoretic tank.
5. Comb was inserted in the gel casting tray.
6. Agarose solution was poured into the casting tray and was allow to set (30 min at room temperature).
7. Comb was removed as gel polymerized completely.
8. Electrophoresis buffer i.e. TAE (1 X) was added to the buffer tank.
9. 5µl DNA sample was mixed with 2µl gel loading dye (Genei, make)
10. Samples were loaded in agarose gel slots.
11. The lid of the electrophoresis tank was closed and leads were attached.
12. A voltage of 1-5 V/cm was applied. Gel was run for 1.5 h.
13. Electric current was turned off.
14. Gel was examined under transmitted UV light.
15. Photograph was clicked by gel documentation system (Geneius Make)

3.2.2.2.3 Quantification of DNA (Sambrook and Russell, 1989)

1. 2 µl of DNA samples of each strain were taken and diluted with 1 ml of TE buffer.
2. The absorbance of DNA samples were measured at a wavelength of 260 nm against TE buffer blank in quartz cuvette.
3. DNA concentration was calculated as given below:

Calculations

$$\text{Total Quantity of DNA } (\mu\text{g}/\mu\text{l}) = \frac{\text{Measured OD}_{260} \times 50 \times \text{dilution factor}}{1000}$$

$$\text{Dilution factor} = \frac{\text{Total volume}}{\text{Volume of sample used}}$$

3.2.2.2.4 PCR Amplification of 16S rRNA Region

The PCR analysis was carried out with a volume of 25 μl mixture in a thermocycler. The procedure consisted of 35 cycles of 92°C for 1 min, 55°C for 1 min, 72 for 1 min.

PCR Reaction Mixture

Taq buffer (10X)	:	5.0 μl
dNTP 2mM	:	2.5 μl
Primer (F)	:	1.0 μl
Primer (R)	:	1.0 μl
Taq Polymerase	:	0.2 μl
Glycerol	:	0.5 μl
Water	:	12.8 μl
DNA	:	1 μl
MgCl ₂	:	1 μl

The universal primers of expected product size i.e. (1500 bp) used for amplification were BITS-1(5'AGAGTTTGATCCTGG) and BITS-4-(5'-TACCTTGTTACGACTT).

3.2.2.2.5 Agarose gel Electrophoresis

Same as section 3.2.2.2

3.2.2.2.6 PCR Clean up

PCR product so obtained after amplification with specific primers at standardized amplification temperature was clean up using PCR clean up kit (Real Genomics Hi yieldTM Make).

1. The PCR product (100 µl) was transfer to 1.5 ml of microcentrifuge tube.
2. 5 volume of DF buffer (provided with kit) was added to 1 volume of the sample and mixed by vortexing.
3. DF column (provided with kit) were placed on 2ml of collection tube. The sample mixture prepared in step 2 was applied into column followed by centrifugation at 13,000 rpm for 30 sec.
4. The flow though was discarded and DF column was placed back into 2 ml collection tube.
5. The dried DF column was transferred in new 1.5 ml microcentrifuge tube.
6. The PCR product was eluted by adding 25µl of elution buffer to the centre of column matrix. It was incubated for 2 min at room temperature followed by centrifugation at 13,000 rpm for 2 min.

Eluted PCR products of BM-1, SH8 and SH0 were sequenced by commercial available services of (Xcelaris, Ahmdabad, India). The sequences so obtained after sequencing were used to identify BM-1, SH8 and SH0 by BLASTN bioinformatics tool.

3.2.2.2.7 Identification

On the basis of 16S r RNA gene technique BM-1, SH8 and SH0 were identified as *B. subtilis*, *B. amyloliquefaciens* and *B. tequilensis*. The sequences so obtained were submitted in National Centre for Biotechnology Information (NCBI) to get an accession number. *B. subtilis* BM-1 was registered under the accession no. |JX129361.1| while, *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 were registered under the accession no. |JX129360.1| and |JX129359.1|, respectively.

3.2.3 Identification of Fungal Isolates by Using ITS 5.8S rRNA PCR Technique

Isolates W2 and T2 were further identified at genomic level using ITS 5.8S rRNA technique as given below.

3.2.3.1 Isolation of Genomic DNA (Genespin Plant Genomic DNA Preparation kit)

The genomic DNA extracted from approximately 100 mg of freeze dried fungal mycelia by crushing in 1.5 ml microcentrifuge tubes using micropestles. 400 µl buffer AP1 and 4 µl RNase A stock solution (100mg/ml) was added to a maximum of 100 mg (wet weight) or 20 mg (dried) disrupted plant of fungal tissue and vortex vigorously. The mixture was incubated for 10 min at 65 °C and mixed 2 or 3 times during incubation by inverting tube. 130 µl buffer was added to the lysate and incubated for 5 min on ice. Lysate was centrifuged for 5 min at 20,000 × g. After centrifugation supernatant was applied to QIAshredder Mni spin column and centrifuged for 2 min at 20,000 × g. Flow through fraction obtained from above step was placed into a new tube without disrupting the cell debris pellet. 1.5 volume of Buffer AP3/E was added to the cleared lysate and properly mixed by pipetting. 650 µl of the mixture so obtained from above step was kept into the DNeasy mini spin column placed in a 2 ml collection tube followed by centrifugation for 1 min at 6000 × g. flow through so obtained was discarded. Place the DNeasy Mini spin column into a new 2ml collection tube and of 500 µl buffer AW was added, followed by centrifugation for 1 min at ≥ 6000 × g. Flow through was discarded and collection tube was reused in next step. 500 µl of buffer AW was added to the DNeasy Mini spin column followed by centrifugation for 2 min at 20,000 × g to the dry membrane. DNeasy Mini spin column was placed to a 1.5 ml or 2 ml microcentrifuge tube and 100 µl of buffer AE was added onto the DNeasy membrane. Sample was incubated for 5 min at room temperature (15-25 °C) and then centrifuged for 1 min at 6000 × g to elute the DNA.

3.2.3.2 Agarose Gel Electrophoresis

Same as given in section 3.2.2.2

3.2.3.3 Quantification of DNA (Sambrook and Russell, 1989)

Same as given in section 3.2.2.2.3

3.2.3.4 PCR Amplification of 5.8S rRNA Region

Taq buffer (10X)	:	5.0µl
dNTP 2mM	:	2.5 µl
Primer (F)	:	1.0 µl
Primer (R)	:	1.0 µl
Taq Polymerase	:	0.2 µl
Glycerol	:	0.5 µl
Water	:	12.8 µl
DNA	:	1 µl
MgCl ₂	:	1 µl

PCR amplification was done to confirm the identity of the fungal strain W2 and T2, the small subunit 5.8 S rRNA genes were amplified from the genomic DNA with (ITS-1-5' TCCGTAGGTGAACCTGCGG 3') and (ITS-4-5' TCCTCCGCTTATTGATATGC-3') primers to get an amplicon size of 1500 bp. The amplified PCR product was cleaned up using PCR clean up kit (Real Genomics Hi yieldTM Make).

3.2.3.5 Agarose Gel Electrophoresis

Same as given in section 3.2.2.2

3.2.3.6 PCR Clean up

Same as given in section 3.2.2.2.6

Eluted PCR products of BM-1, SH8 and SH0 were sequenced by commercial available services of (Genei, Bangalore, India). The sequences so obtained after sequencing were used to identify T2 and W2 by BLASTN bioinformatics tool.

3.2.3.7 Identification

On the basis of 5.8S rRNA gene technique T2 and W2 were identified as *Trichoderma longibrachetum* and *Rhizopus oryzae*. The sequences so obtained were submitted in National Centre for Biotechnology Information (NCBI) to get an accession number. *Trichoderma longibrachetum* T2 was registered under the

accession no. IJX 213811.1| and *Rhizopus oryzae* W2 under the accession no. IJX 213812.1|.

3.3 OPTIMIZATION OF EXTRACELLULAR CELLULASE AND XYLANASE

Cellulase and xylanase were produced and scaled up under following modes of fermentation.

- (i) Submerged fermentation (SmF).
- (ii) Statistical optimization under (SmF).
- (iii) Solid state fermentation (SSF).

3.3.1 Optimization of Bioprocess Parameter For Extracellular Cellulase Production Under Submerged Fermentation Using Classical Approach One Factor At a Time (OFAT) by Hypercellulolytic Bacteria.

3.3.1.1 By *B. subtilis* BM-1

Different growth parameters were optimized for extracellular cellulase production for *B. subtilis* BM-1

3.3.1.1 A Effect of Media

3.3.1.1A.1a Inoculum Preparation

B. subtilis BM1 was grown in 50ml of nutrient broth and incubated at $37\pm 2^{\circ}\text{C}$ for 24 h. As soon as the substantial growth of the strain was observed in the broth, the optical density was set to 1.0 using nutrient broth as blank.

3.3.1.1A.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of each of different medium (given below) used in 250 ml of Erlenmeyer flask and were incubated for 5 days at $35\pm 2^{\circ}\text{C}$ with continuous shaking at 120 rpm. After 5 days, the culture contents of each medium were centrifuged at 10,000 rpm for 15 min at 4°C . Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.1.1A.1c Composition of Media Used

1. **Riviere Medium** (Riviere, 1961)

NaNO₃: 10g, Na₂HPO₄: 1.2g, KH₂PO₄: 0.50g, MgSO₄: 0.50g, KCl: 0.50g, Yeast Extract: 0.50g, Cellulose powder: 10g, in 1000 ml dW of distilled water, pH- 6.8

2. **Sarkar and Upadhay Medium** (Sarkar and Upadhay, 1993)

CH₃COONa: 3.0g, Na₂SO₄.7H₂O: 0.4g, MgSO₄: 0.2g, FeSO₄: 0.01g, EDTA: 0.05g, MgCl₂.6H₂O: 1.8g, CaCl₂.2 H₂O: 0.2g, K₂HPO₄: 0.25g, KH₂PO₄: 0.05, Yeast Extract: 2.0g, Carboxy Methyl Cellulose: 1g, in 1000 ml dW of distilled water, pH- 8.5, with NaOH.

3. **PYC Medium** (Kim *et al.*, 2005)

Polypeptone: 5.0g, Yeast Extract: 5.0g, K₂HPO₄: 1.0g, MgSO₄. 7H₂O: 0.2g, NaCl: 5.0g, Carboxy Methyl Cellulose: 10g, in 1000 ml dW of distilled water, pH-10, with Na₂CO₃, 20% w/v

4. **Basal Salt Medium** (Dhillon and Khanna, 2000)

(NH₄)₂SO₄-6.0g, KH₂PO₄-3.0g, NaCl- 0.5g, NH₄Cl-1.0g, 1M MgSO₄- 0.2ml, 1M CaCl₂- 0.1ml, Yeast Extract-0.5%/1000ml and 1% carbon source. (pH- 7.1)

5. **Basic Liquid Mineral Medium** (Heck *et al.*, 2002)

MgSO₄: 0.02g, KH₂PO₄: 0.1g, K₂HPO₄: 0.1g, NH₄NO₃: 0.1g, CaCl₂: 0.002g, FeCl₃: 0.005g, Cellulose: 1g, in 100 ml of distilled water, pH- 6.8

3.3.1.1A.1d Enzyme Assays

3.3.1.1A.1d (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.1.1A.1d (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.1.1A.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.1.1A.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1A.1.f Statistical Analysis

The data recorded for different parameters were subjected to Completely Randomized Design (Gomez and Gomez, 1976). The statistical analysis based on mean value of replications used for each treatment was made using analysis of variance (ANOVA) for Completely Randomized Design.

Different medium showing optimum cellulase production i.e. Basal salt medium was selected for further studies.

3.3.1.1. B Effect of Temperature

3.3.1.1. B. 1a Inoculum Preparation

Same as given in 3.3.1.1A.1a

3.3.1.1. B.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different temperatures ranging from 25, 35,....., 65°C at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.1.1 B.1c Enzyme Assays

3.3.1.1 B.1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.1.1 B.1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.1.1 B.1c (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.1.1 B.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1 B.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

Temperature showing optimum cellulase production i.e. 45°C was selected for further studies

3.3.1.1. C Effect of pH

3.3.1.1. C.1a Inoculum Preparation

Same as given in 3.3.1.1A.1a

3.3.1.1. C.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different pH ranging from 4, 5, 6, 7, 8 9 and 10 at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.1.1 C.1c Enzyme Assays

3.3.1.1 C.1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.1.1 C.1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.1.1 C.1c (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.1.1 C.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1 C.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

pH showing optimum cellulase production i.e. pH 6 was selected for further studies.

3.3.1.1. D Effect of Different Inoculum Size

3.3.1.1. D.1a Inoculum Preparation

Same as given in 3.3.1.1A.1a

3.3.1.1. D.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different inoculum size ranging from 1.0%, 2.5%, 5.0 %....15.0 % at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.1.1 D.1c Enzyme Assays

3.3.1.1 D.1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.1.1 D.1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.1.1 D.1c (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.1.1 D.1d Protein estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1 D.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

Inoculum size showing optimum cellulase production i.e. 10% was selected for further studies.

3.3.1.1. E Effect of Different Carbon Sources

3.3.1.1. E.1a Inoculum Preparation

Same as given in 3.3.1.1A.1a

3.3.1.1. E.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated with different substrates

(hexoses) ranging from Cellulose, Avicel, Cotton, Whatman No.1, News paper, Wheat bran and Carboxymethyl Cellulose at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.1.1. E.1c Enzyme Assays

3.3.1.1. E.1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.1.1 E.1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.1.1 E.1c (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.1.1 E.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1. E.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

Different carbon substrate for optimum cellulase production i.e. wheat bran was selected for further studies.

3.3.1.1.F Effect of Different Concentrations of Carbon Source

3.3.1.1. F.1a Inoculum Preparation

Same as given in 3.3.1.1A.1a

3.3.1.1. F.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of Basal salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different concentrations of wheat bran ranging from 0.25%, 0.50%, 0.75%....2.0 % at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

- 3.3.1.1. F.1c Enzyme Assays**
- 3.3.1.1. F.1c (i) Filter Paperase (FPase) Activity (Grajek, 1987)** as given in section 3.1.2.1.2.a (i)
- 3.3.1.1 F.1c (ii) CMCase Assay (Grajek, 1987)** as given in section 3.1.2.1.2.b (ii)
- 3.3.1.1 F.1c (iii) β -Glucosidase Activity (Grajek, 1987)** as given in section 3.1.2.1.2.c (iii)
- 3.3.1.1 F.1d Protein Estimation** (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1. F.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

Different carbon substrate for optimum cellulase production i.e. wheat bran was selected for further studies.

3.3.1.1. G Effect of Different Concentrations of Carbon Source

3.3.1.1. G.1a Inoculum Preparation

Same as given in 3.3.1.1A.1a

3.3.1.1. G.1a Production of Cellulase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different concentrations of wheat bran ranging from 0.25%, 0.50%, 0.75%....2.0 % at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

- 3.3.1.1 G.1c Enzyme Assays**
- 3.3.1.1 G.1c (i) Filter Paperase (FPase) Activity (Grajek, 1987)** as given in section 3.1.2.1.2.a (i)
- 3.3.1.1 G.1c (ii) CMCase Assay (Grajek, 1987)** as given in section 3.1.2.1.2.b (ii)
- 3.3.1.1 G.1c (iii) β -Glucosidase Activity (Grajek, 1987)** as given in section 3.1.2.1.2.c (iii)

3.3.1.1 G.1d Protein estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1 G.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

Different carbon substrate for optimum cellulase production i.e. wheat bran was selected for further studies.

3.3.1.1.H Effect of Incubation Time

3.3.1.1. H.1a Inoculum Preparation

Same as given in 3.3.1.1A.1a

3.3.1.1. H.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different incubation time ranging from 12h, 24h, 36h.....120h at 120 rpm. After mentioned incubation time, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.1.1 H.1c Enzyme Assays

3.3.1.1 H.1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.1.1 H.1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.1.1 H.1c (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.1.1 H.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.1.1 H.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

Best incubation time showing optimum xylanase production i.e. 72 h for BM-1 was selected for further studies.

3.3.2 Optimization of Bioprocess Parameter for Extracellular Cellulase Production under Submerged Fermentation Using Classical Approach One Factor at a Time (OFAT) by Hyperxylanolytic Bacteria

3.3.2.1 By *Bacillus amyloliquefaciens* SH₈

By *Bacillus tequilensis* SH₀

Different growth parameters were optimized for extracellular xylanase production for *Bacillus amyloliquefaciens* SH8 and *Bacillus tequilensis* SH0.

3.3.2.1.A Effect of Media

3.3.2.1.A.1a Inoculum Preparation

B. amyloliquefaciens SH8 and *B. tequilensis* SH0 was grown in 50ml of nutrient broth and incubated at 37±2°C for 24 h. As soon as the substantial growth of the strain was observed in the broth, the optical density was set to 1.0 using nutrient broth as blank.

3.3.2.1.A.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of each medium (given below) used in 250ml of Erlenmeyer flask and were incubated for 5 days at 37±2°C with continuous shaking at 120 rpm. After 5 days, the culture contents of each medium were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.2.1.A.1c Composition of Media Used

1. TGY-Medium (Garg *et al.*, 2009)

Tryptone-0.5%, K₂HPO₄-0.1%, glucose-0.1%, Yeast Extract-0.5% (pH 7.1)

2. Emerson Medium (Garg *et al.*, 2009)

Yeast extract-0.55%, K₂HPO₄-0.1%, MgSO₄· 7H₂O: 0.02%, peptone-0.5% (pH 7.1)

3. Mineral Medium (Bocchini *et al.*, 2008)

MgSO₄- 0.2g, KH₂PO₄-0.5g, K₂HPO₄-0.5g, NH₄NO₃-0.5g, (NH₄)₂SO₄-0.5g, FeCl₃.7H₂O- 0.001g, KCl-0.5g, Na₂CO₃-5ml (separately sterilized) /1000ml (pH- 7.1)

4. Basal Salt Medium (Dhillon and Khanna, 2000)

(NH₄)₂SO₄-6.0g, KH₂PO₄-3.0g, NaCl- 0.5g, NH₄Cl-1.0g, 1M MgSO₄-0.2ml, 1M CaCl₂- 0.1ml, Yeast Extract-0.5%/1000ml (pH- 7.1)

5. Mandel's Medium (Kheng and Omar, 2005)

Peptone-1.0g, KH₂PO₄-2.0g, Urea- 0.3g, MgSO₄. 7H₂O- 0.3g, CaCl₂-0.3g, (NH₄)₂SO₄-1.4g, FeSO₄. 7H₂O-5.0mg, MnSO₄.H₂O-1.6mg, CoCl₂-2.0mg, ZnSO₄.7H₂O-1.4mg./1000ml (pH- 7.1)

3.3.2.1.A.1d Enzyme Assays

3.3.2.1.A.1d (i) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.2.1.A.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.2.1.A.1f Statistical Analysis

Same as given in 3.3.1.1.A.1f

Best medium showing optimum cellulase production i.e. basal salt medium was selected for further studies.

3.3.2.1.B Effect of Temperature

3.3.2.1.B.1a Inoculum Preparation

Same as given in section 3.3.2.1.A.1a

3.3.2.1.B.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different temperatures ranging from 25, 35,....., 65°C at 120 rpm. After 5 days, the culture contents were

centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.2.1.B.1c Enzyme Assays

3.3.2.1.B.1c (i) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.2.1.B.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.2.1.B.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

Temperature showing optimum xylanase production i.e. 45°C was selected for further studies

3.3.2.1.C Effect of pH

3.3.2.1.C.1a Inoculum Preparation

Same as given in section 3.3.2.1.A.1a

3.3.2.1.C.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different pH ranging from 4, 5, 6, 7, 8 9 and 10 at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.2.1.C.1c Enzyme Assays

3.3.2.1.C.1c (i) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.2.1.C.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.2.1.C.1e Statistical Analysis

Same as given in 3.3.1.1A.1f

pH showing optimum xylanase production i.e. 5 and 5.5 pH was selected of *B. tequilensis* SH0 and *B. amyloliquefaciens* SH8 for further studies, respectively.

3.3.2.1.D Effect of Different Inoculum Size

3.3.2.1.D.1a Inoculum Preparation

Same as given in section 3.3.2.1.A.1a

3.3.2.1.D.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different inoculum size ranging from 1.0%, 2.5%, 5.0 %....15.0 % at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.2.1.D.1c Enzyme Assays

3.3.2.1.D.1c (i) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.2.1.D.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.2.1.D.1e Statistical Analysis

Same as given in 3.3.1.1.A.1f

Temperature showing optimum xylanase production i.e. 45°C was selected for further studies.

3.3.2.1.E Effect of Different Carbon Sources

3.3.2.1. E.1a Inoculum Preparation

Same as given in section 3.3.2.1.A.1a

3.3.2.1.E.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated with different substrates that are xylan, xylose, wheat bran, lactose, fructose, dextrose, sucrose, arabinose and D- ribose at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.2.1.E.1c Enzyme Assays

3.3.2.1.E.1c (i) Xylanase Assay (Miller, 1959): as given in section 3.1.2.1.2.d (iv)

3.3.2.1.E.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.2.1.E.1e Statistical Analysis

Same as given in 3.3.1.1.A.1f

Best carbon source showing optimum xylanase production i.e. wheat bran was selected for further studies

3.3.2.1. F Effect of Different Carbon Concentrations

3.3.2.1. F.1a Inoculum Preparation

Same as given in section 3.3.2.1.A.1a

3.3.2.1.F.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different concentrations of xylan ranging from 0.5%, 1.0%, 1.5%....3.0 % at 120 rpm. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.2.1.F.1c Enzyme Assays

3.3.2.1.F.1 (i) Xylanase Assay (Miller, 1959): as given in section 3.1.2.1.2.d (iv)

3.3.2.1.F.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.2.1.F.1e Statistical Analysis

Same as given in 3.3.1.1.A.1f

1.25% wheat bran concentration showing optimum xylanase production i.e. was selected for further studies.

3.3.2.1.G.1 Effect of Incubation Time

3.3.2.1G.1a Inoculum Preparation

Same as given in section 3.3.2.1.A.1a

3.3.2.1.G.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different incubation time ranging from 12h, 24h, 36h.....120h at 120 rpm. After mentioned incubation time, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.3.2.1.G.1c Enzyme Assays

3.3.2.1.G.1 (i) Xylanase Assay (Miller, 1959): as given in section 3.1.2.1.2.d (iv)

3.3.2.1.G.1d Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.2.1.2.e

3.3.2.1.G.1e Statistical Analysis

Same as given in 3.3.1.1.A.1f

Best incubation time showing optimum xylanase production i.e. 72h for SH8 and 94h for SH0 was selected for further studies.

3.3.3 Statistical Optimization of Nutrients for Bioprocess Parameters and Xylanase Production SH8 Using Response Surface Methodology (RSM)

3.3.3.1 By *B. amyloliquefaciens* SH8

3.3.3.1.1 Plackett- Burman Design

The Plackett- Burman Design (PBD) is a special variation of a two level fraction factorial design based on the incomplete equilibrium piece principle. It can pick up the main factors with the least number of experiments from a list of candidate factors. Nitrogen sources, salt, pH, inoculum size and Incubation time were screened by a one factor at a time strategy and the best nutrient was included in the design. The possible factors that affect the xylanase yield included NH_4Cl , KH_2PO_4 , NH_4SO_4 , tryptone, NaCl , yeast extract, peptone soybean meal, inoculum size, MnSO_4 , FeSO_4 , MgCl_2 , CaCl_2 , CoCl_2 , pH, temperature, incubation time, tween 80, Zn SO_4 . The 20-run of PBD was used to estimate to study the 19 factors.

Table 1. Plackett Burman Experimental Design for Nutrient Optimization for Xylanase Production

Numeric	Name	Units	Low	High
A	NH_4Cl	g/L	0	1.0
B	KH_2PO_4	g/L	0	1.0
C	NH_4SO_4	g/L	0	5.0
D	NaCl	g/L	0	1.5
E	Yeast Extract	g/L	0	5.0
F	Peptone	g/L	0	5.0
G	Soybean meal	g/L	0	5.0
H	Inoculum size	ml/L	5	15.0
J	Tryptone	g/L	0	5.0
K	MnSO_4	g/L	0	0.10
L	FeSO_4	g/L	0	0.10
M	MgCl_2	g/L	0	0.20
N	CaCl_2	g/L	0	0.15
O	CoCl_2	g/L	0	0.15
P	pH	-	4	6
Q	Temperature	$^{\circ}\text{C}$	40	50
R	Incubation Time	In Days	3	5
S	Tween 80	ml/L	0	1.0
T	ZnSO_4	g/L	0	0.1

3.3.3.1.1 Central Composite Design (CCD) and Response Surfaces Analysis

Table 2. Central Composite Experimental Design for Screened Nutrient Optimization for Xylanase Production

Numeric	Name	Units	Low	High	-alpha(α)	+alpha(α)
A	Tryptone	g/l	3.0	11.0	-0.1	15
B	NH ₄ Cl	g/l	0.5	2.5	-0.5	3.5
C	CaCl ₂	g/l	0.10	0.5	-0.1	0.7
D	ZnSO ₄	g/l	0.1	0.25	0.025	0.325

The Design-Expert Software (Version 8.05) was used for the statistical design of experiments and for data analysis.

3.3.4 Optimization of Bioprocess Parameter for Extracellular Cellulase and Xylanase Production under SSF Using Classical Approach One Factor At a Time (OFAT) by Hypercellulolytic and Xylanolytic Fungi By *T. longibrachetum* T2 By *R. oryzae* W2

Different growth parameters were optimized for extracellular cellulase and xylanase production for *T. longibrachetum* T2 and *R. oryzae* W2 from various lignocellulosic wastes.

3.3.4.1 Selection of Lignocellulosic Agricultural and Forest Waste as Substrate

The following lignocellulosic agricultural and forest residues were selected as carbon substrates for enzyme production studies depending upon their wide abundance and easy availability in local forests/fields /adjoining areas.

- : Wheat (*Triticum aestivum*) straw
- : Rice (*Oryzae sativa*) straw
- : *Pinus roxburghii* (Pine) needle
- : *Pinus roxburghii* (Pine) wood
- : *Dandracalamus strictus* (Bamboo) wood

The wood of these species were collected. Wood chipping of biomass was done to get small pieces. The chipped biomass was dried in an oven at 50°C for 72 h. After drying, the chips of each species were ground to fine particles of 1.0-1.5 mm mesh size while wheat straw, rice straw and pine needles were crushed

with hand to an average size of 1 to 10 cm. The completely dried biomass was stored in air tight containers for further use.

3.3.4.1.1 Pretreatments of Lignocellulosic Biomass

3.3.4.1.1 a) Steam Pretreatment

200 g of each selected biomass soaked in water and subjected to steam pretreatment at 121°C at 15 psi for 1 h in an autoclave. After drying at 60°C overnight, it was stored in air tight containers.

3.3.4.1.1 b) Alkali (2.0% NaOH) Pretreatment

200 g of each selected biomass was dipped in 2.0% NaOH. After 2 h, it was thoroughly washed with tap water until it became neutral. After drying at 60°C overnight, it was stored in air tight containers.

3.3.4.1.1 c) Acid (2.0% H₂SO₄) Pretreatment

200 g of each selected biomass was dipped in 2.0% H₂SO₄. After 2 h it was thoroughly washed with tap water until it becomes neutral, dried at 60°C and kept in air tight containers.

3.3.4.1.1A Production of Cellulase and Xylanase Enzymes

3.3.4.1.1A1 Inoculum Preparation

The bit of *T. longibracheatum* T2 and *R. oryzae* W2 was placed in SDA medium and incubated at 28±2°C for 7 days and 3 days, respectively.

3.3.4.1.1A1a Production of Cellulase and Xylanase Using Lignocellulosic Agricultural and Forest Wastes

To 5 g of each untreated and pretreated biomass, 15 ml of moistening agent viz. Vogel medium was added (in the ratio of 1:5 i.e. substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated separately with full plate growth culture scratched with 10ml of *R. oryzae* W₂ and *T. longibracheatum* T₂ having 1×10⁷ spores/ml and incubated at 30±2°C for 10 days in static phase.

3.3.4.1.1A1b Extraction of Cellulase and Xylanase by Repeated Extraction Method (Bollag and Edelstein, 1991)

To 5 g of each untreated and pretreated biomass, 50 ml of phosphate buffer (0.1M, pH 6.9) with 0.1% Tween-80 was added in 250 ml Erlenmeyer flask. The contents were kept in the shaker for 1 h at 120 rpm and then filtered through muslin cloth. The process was repeated twice with 25 ml of phosphate buffer. After filtration, contents were centrifuged at 5,400 rpm for 10 min at 4°C. The supernatant was collected for further studies.

3.3.4.1.1A1c Enzyme Assays

- 3.3.4.1.1A1c (i) Filter Paperase (FPase) Activity (Grajek, 1987)** as given in section 3.1.2.1.2.a (i)
- 3.3.4.1.1A1c (ii) CMCase Assay (Grajek, 1987)** as given in section 3.1.2.1.2.b (ii)
- 3.3.4.1.1A1c (iii) β -glucosidase Activity (Grajek, 1987)** as given in section 3.1.2.1.2.c (iii)
- 3.3.4.1.1A1c (iv) Xylanase Assay (Miller, 1959)** as given in section 3.1.2.1.2.d (iv)
- 3.3.4.1.1A1c (v) Protein Assay (Lowry *et al.*, 1951)** as given in section 3.1.2.1.2.e

3.3.4.1.1A1d Statistical Analysis

Same as given in 3.3.1.1.A.1f

Among all pretreated lignocellulosic biomass, the best substrate i.e. alkali pretreated *wheat straw* (in terms of showing highest enzyme units) was selected for further optimization studies for *T. longibrachearum* T₂ and untreated and steam pretreated wheat straw was the best lignocellulosic substrate selected for further optimization studies for *R. oryzae* W₂.

3.3.4.1.1B Effect of Different pH

3.3.4.1.1B1 Inoculum Preparation

Same as given in 3.3.4.1.1A1

3.3.4.1.1B1a Production of Cellulase and Xylanase Using Lignocellulosic Wheat Straw Waste

To 5 g of each mild steam treated and pretreated biomass, 15 ml of moistening agent *viz.* Vogel medium was added (in the ratio of 1:5 i.e. substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 10ml of *T. longibrachaeum* T₂ and *R. oryzae* W₂ having 1×10^7 spores/ml and incubated at different moistening agent pH ranging from 3.5, 4.0,..... 6.5 for 10 days in static phase.

3.3.4.1.1B1b Extraction of Cellulase and Xylanase by Repeated Extraction Method (Bollag and Edelstein, 1991)

Extraction of cellulase and xylanase enzymes as mentioned in section 3.3.4.1.1A1b

3.3.4.1.1B1c Enzyme Assays

3.3.4.1.1B1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.4.1.1B1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.4.1.1B1c (iii) β -glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.4.1.1B1c (iv) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.4.1.1B1c (v) Protein Assay (Lowry *et al.*, 1951) as given in section 3.1.2.1.2.e

3.3.4.1.1B1d Statistical Analysis

Same as given in 3.3.1.1.A.1f

pH showing optimum cellulase and xylanase production i.e. 5.0 was selected for further studies

3.3.4.1.1C Effect of Different Temperature

3.3.4.1.1C1 Inoculum Preparation

Same as given in 3.3.4.1.1A1

3.3.4.1.1C1a Production of Cellulase and Xylanase Using Lignocellulosic Wheat Straw Waste

To 5 g of each untreated and pretreated biomass, 15 ml of moistening agent *viz.* Vogel medium was added (in the ratio of 1:5 i.e. substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 10ml of *T. longibrachaeum* T₂ and *R. oryzae* W₂ having 1×10^7 spores/ml and incubated at different temperature ranging from 20, 25.....45°C for 10 days in static phase.

3.3.4.1.1C1b Extraction of Cellulase and Xylanase by Repeated Extraction Method (Bollag and Edelstein, 1991).

Extraction of cellulase and xylanase enzymes as mentioned in section 3.3.4.1.1A1b.

3.3.4.1.1C1c Enzyme Assays

3.3.4.1.1C1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.4.1.1C1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.4.1.1C1c (iii) β -glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.4.1.1C1c (iv) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.4.1.1C1c (v) Protein Assay (Lowry *et al.*, 1951) as given in section 3.1.2.1.2.e

3.3.4.1.1C1d Statistical Analysis

Same as given in 3.3.1.1.A.1f

Temperature showing optimum cellulase and xylanase production i.e. 30°C was selected for further studies

3.3.4.1.1D Effect of Different Moisture Ratio

3.3.4.1.1D1 Inoculum Preparation

Same as given in 3.3.4.1.1A1

3.3.4.1.1D1a Production of Cellulase and Xylanase Using Lignocellulosic Wheat Straw Waste

To 5 g of each mild steam treated and pretreated biomass, 15 ml of moistening agent *viz.* Vogel medium was added (in the ratio of 1:5 i.e. substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 10ml of *Rhizopus W₂* and *Trichoderma longibrachiatum* T₂ having 1×10^7 spores/ml and incubated at different moisture ratio ranging from 1:3:0, 1:3:5, ...1:6:5 for 10 days in static phase.

3.3.4.1.1D1b Extraction of Cellulase and Xylanase by Repeated Extraction Method (Bollag and Edelstein, 1991).

Extraction of cellulase and xylanase enzymes as mentioned in section 3.3.4.1.1A1b.

3.3.4.1.1D1c Enzyme Assays

3.3.4.1.1D1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.4.1.1D1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.4.1.1D1c (iii) β -glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.4.1.1D1c (iv) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.4.1.1D1c (v) Protein Assay (Lowry *et al.*, 1951) as given in section 3.1.2.1.2.e

3.3.4.1.1D1d Statistical Analysis

Same as given in 3.3.1.1.A.1f

Moisture ratio showing optimum cellulase and xylanase production i.e. 1.5:0 was selected for further studies

3.3.4.1.1E Effect of Different Incubation Time

3.3.4.1.1E1 Inoculum Preparation

Same as given in 3.3.4.1.1A1

3.3.4.1.1E1a Production of Cellulase and Xylanase Using Lignocellulosic Wheat Straw Waste

To 5 g of each mild steam treated and pretreated biomass, 15 ml of moistening agent *viz.* Vogel medium was added (in the ratio of 1:5 i.e. substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 10ml of *T. longibrachaeum* T₂ and *R. oryzae* W₂ and having 1×10^7 spores/ml and incubated at different days ranging from 3th, 4th, ...10th in static phase.

3.3.4.1.1Eb Extraction of Cellulase and Xylanase by Repeated Extraction Method (Bollag and Edelstein, 1991)

Extraction of cellulase and xylanase enzymes as mentioned in section 3.3.4.1.1A1b.

3.3.4.1.1E1c Enzyme Assays

3.3.4.1.1E1c (i) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.2.1.2.a (i)

3.3.4.1.1E1c (ii) CMCase Assay (Grajek, 1987) as given in section 3.1.2.1.2.b (ii)

3.3.4.1.1E1c (iii) β -glucosidase Activity (Grajek, 1987) as given in section 3.1.2.1.2.c (iii)

3.3.4.1.1E1c (iv) Xylanase Assay (Miller, 1959) as given in section 3.1.2.1.2.d (iv)

3.3.4.1.1E1c (v) Protein Assay (Lowry *et al.*, 1951) as given in section 3.1.2.1.2.e

3.3.4.1.1E1d Statistical Analysis

Same as given in 3.3.1.1.A.1f

Different incubation time for optimum cellulase and xylanase production i.e. 5th day for W₂ and 7th day for T₂ was selected for further purification studies.

3.3.4.2 Scanning Electron Microscopy (SEM) of Pretreated and Untreated Wheat Straw

SEM pictures were taken of untreated and pretreated wheat straw (dried) of *T. longibrachaeum* T₂ and *R. oryzae* W₂ at magnifications of 400X times

with a working distance of 50 μm using Large Field Detector with an aperture size of 500 μm at 5.0 kV and pressure 90 pascal of EFI company, Model No. D9393.

3.4 PURIFICATION OF XYLANASE FROM MICROORGANISMS

3.4.1 Purification of Xylanase of Bacteria

By *B. amyloliquefaciens* SH8

By *B. tequilensis* SH0

3.4.1.1 Inoculum Preparation and Production of Xylanase

Each of SH8 and SH0 culture having OD 1.0 were separately inoculated in Basal salt medium broth (pH 5.5) @ 10%. Inoculated flasks were kept at $45 \pm 2^\circ\text{C}$ at 120 rpm for 72 and 94 h. The culture contents of both organisms were centrifuged at 10,000 rpm for 15 min at 4°C . Supernatants were collected and stored at 4°C in refrigerator for further use.

3.4.1.2 Lyophilization of Xylanase

Culture supernatants of *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 (20 ml each) were taken separately in round bottomed flasks for concentrating xylanase enzyme. Supernatants were lyophilized in a lyophilizer (Allied Frost, make) for 5 h. Lyophilized supernatants were assayed to check its xylanase activity by well diffusion method.

3.4.1.2.1 Xylanase Assay: as given in section 3.1.2.1.2.d (iv)

3.4.1.2.2 Protein Estimation (Lowry *et al.*, 1951)

Proteins were estimated in culture supernatants as well as lyophilized preparation of *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 by Lowry's method as given in section 3.1.2.1.2.e

3.4.1.3 Ion Exchange Chromatography (Bollag, 1991)

DEAE-cellulose (2 g) was suspended in 200 ml of distilled water for 48 hours and packed in a glass column (10x1.0 cm). Concentrated supernatant containing enzyme was loaded on ion exchange column. The column was first eluted with 2 bed volumes of distilled water to wash out unbound proteins. The

bound proteins were eluted with linear salt gradients (2 bed volumes) of 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M KCl in distilled water. Fractions of 3 ml each were collected and monitored for xylanase Activity and the protein content was measured at 280 nm. The active fractions were pooled and used for electrophoresis and gel filtration chromatography. The enzyme Activity and soluble protein was estimated after each step of purification and purification fold was calculated.

3.4.1.4 Gel Filtration Chromatography (Bollag, 1991)

Sephadex G-100 (5g) was suspended in 500 ml of distilled water for 48 h. It was packed into the glass column having dimensions of (31x2.5 cm) avoiding entrapment of any air bubble in the gel bed. It was then equilibrated with three bed volumes of 0.1M Phosphate buffer (pH 6.9). To check the uniformity of packed gel column and for calculating the void volume, blue dextran (2mg/ml) was loaded on the column and 2 ml of fractions were collected. The absorbance was recorded at 600 nm. Lyophilized protein sample (2 ml) was loaded on the Sephadex G-100 column. It was then eluted with three bed volumes of 0.1M phosphate buffer (pH 6.9) and 3 ml fractions were collected. A flow rate of 3 ml in 6 min was maintained. The protein content of collected fractions were measured at 280 nm and fractions showing maximum absorbance were analyzed for xylanase Activity. The most active fractions were pooled and stored at 4°C. Purity of sample was checked out by SDS polyacrylamide slab gel electrophoresis.

3.4.1.5 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

3.4.1.5.1 Equipment

1. Minigel apparatus
2. Power supply (200 V, 500mA)
3. Boiling water bath
4. Eppendorf centrifuge (Sigma)
5. Hamilton syringes (50 µl and 100 µl capacity)
6. Small glass or plastic container with lid
7. Eppendorf tubes
8. Rocking or rotary shaker

3.4.1.5.2 Pouring of Gel

Reagents

1. Acrylamide, Electrophoresis grade
2. Bis - acrylamide (N, N' methylene bisacrylamide)
3. Tris (2 hydroxymethyl- 2- methyl-1, 3-propanediol)
4. SDS (Sodium dodecyle sulphate)
5. TEMED (N, N, N', N' - tetramethylene- ethylenediamine)
6. Ammonium persulfate
7. 2-mercaptoethanol
8. Glyrerol
9. Bromophenol blue
10. Glycine
11. Hydrochloric acid (HCl)
12. Dithiotheitol (DTT)

3.4.1.5.3 Stock Solutions

1. 2 M Tris HCl (pH 8.8) 100 ml
2. 1 M Tris HCl pH 6.8, 100 ml
3. 10 % (w/v) SDS 100 ml, stored at room temperature
4. 50 % (v/v) glycerol, 100 ml
5. 1 % (w/v) bromophenol blue, 10 ml

3.4.1.5.4 Working Solution

1. Solution A (Acrylamide stock solution). 100 ml 30 % w/v acrylamide, 0.8 % (w/v) bisacrylamide
2. Solution B (4 x separating gel buffer), 100 ml
 - a. 75 ml 2 M Tris HCl pH 8.8 -1.5 M
 - b. 4 ml 10 % SDS -0.4 %
 - c. 21 ml H₂O

Stable for months in the refrigerator.

3. Solution C (4 x stacking gel buffer), 100 ml
 - a. 50 ml 1 M Tris HCl (pH 6.8) - 0.5 M

- b. 4 ml 10 % SDS -0.4 %
- c. 46 ml H₂O

Stable for months in refrigerator.

- 4. 10 % ammonium persulfate, 5 ml
 - a. 0.5 g ammonium persulfate
 - b. 5 ml H₂O

Stable for months in a capped tube in a refrigerator.

- 5. Electrophoresis buffer, 1 litre
 - a. 3 g Tris - 25 mM
 - b. 14.4 g Glycine - 92 mM
 - c. 1g SDS - 0.1 %
 - d. H₂O to make 1 litre
 - e. pH should be approximately 8.3

Stable indefinitely at room temperature

- 6. 5 x sample buffer, 10 ml
 - a. 0.6 ml 1 M Tris -HCl pH 6.8 - 60 mM
 - b. 5 ml 50 % glycerol - 25 %
 - c. 2 ml 10 % SDS - 2 %
 - d. 0.5 ml 2 mercaptoethanol - 14.4 mM
 - e. 1 ml 1% bromophenol blue - 0.1 %
 - f. 0.9 ml H₂O

Stable for weeks in the refrigerator for months at -20°C.

3.4.1.5.5 Calculation for 12.5 % Separating Gel

Solution A	:	X/3 ml, i.e. 4.16 ml
Solution B	:	2.5 ml, i.e. 2.5 ml
H ₂ O	:	(7.5 – X/3) ml, 3.34 ml
10 % ammonium persulfate	:	50 µl
TEMED	:	10 µl

Total volume	:	10 ml

3.4.1.5.6 Assembling of Gel Sandwich

For minigel, bottom of both gel plates and spacer were perfectly flushed against a flat surface before tightening lamp assembly. Solutions A and B and water was combined in a small Erlenmeyer flask or a test tube. Ammonium persulfate and TEMED were added and mixed by swirling or inverting container gently. Gel solution was introduced into gel sandwich using a pipette. When appropriate amount of separating gel solution had been added, 1 cm of water was gently layered on top of separating gel solution to keep the gel surface flat. Gel was allowed to polymerize (30 – 60 min).

When the gel has polymerized, a distinct interface appeared between the separating gel.

3.4.1.5.7 Pouring the Staking Gel

5 % stacking gel (4 ml)
2.3 ml H₂O
0.67 ml solution A
1.0 ml solution C
30 µl 10 % ammonium persulfate
5 µl TEMED

Water covering the separating gel was poured off. Solution A, C and water were combined in a test tube. Ammonium persulfate and TEMED was added and mixed by gently swirling or inverting the container. Stacking gel solution was pipette onto separating gel until solution reached top of front plate. The comb was inserted into gel sandwich until bottom of teeth reached top of front plate. Care was taken in order to avoid making of any bubbles. Staking gel was allowed to polymerize (about 30 minutes). After staking gel polymerized, the comb was removed carefully. The gel was placed into electrophoresis chamber. Electrophoresis buffer was added into the buffer tank making sure that both top and bottom of gel were immersed in buffer. Air bubbles clinging to bottom of gel were removed to ensure even current flow.

3.4.1.5.8 Preparation and Loading of Samples

Protein sample and 5x sample buffer (20µl + 5µl) were combined in an eppendorf tube and heated at 100°C for 2-10 min. This sample solution was spun for 1 sec in microfuge. Sample solution was introduced into well using Hamilton syringe. Molecular weight standards were run in one side of the well.

3.4.1.5.9 Running of a Gel

Electrode plugs were attached to proper electrodes. Current was allowed to flow towards anode. Power supply was turned on to 200 V. The dye front was allowed to migrate to 1 cm from the bottom of the gel in 30 - 40 min for two 0.75 mm gels. Power supply was turned off. Electrode plugs were removed from electrodes. Gel plates were removed from electrode assembly. A spacer was removed carefully and the space inserted in one corner between the plates gently used to apart the gel plates. The gel stuck to one of the plate.

3.4.1.5.10 Staining of a Gel with Coomassie Blue

This method of staining was used to detect as little as 0.1 µg of protein in a single band.

Stock Solution

Coomasie gel stain 1 litre

1.0 g coomassie blue R-250

450 ml methanol

450 ml H₂O

100 ml glacial acetic acid

Coomasie Gel Destain 1 Litre

100 ml methanol - 5 ml methanol

100 ml glacial acetic acid - 5 ml glacial acetic acid

800 ml H₂O - 50 ml distilled water

3.4.1.5.11 Staining Procedure

Gel was picked up and transferred to a small container containing coomassie staining (approx. 20 ml). It was agitated for 5-10 min on slow rotatory

shaker. Stain was poured out. Coomassie destain was added about 50 ml. To destain completely, destain solution was changed 3 times after 20-20 min and agitated overnight.

3.4.1.5.12 Inference

After destaining clear bands of both the isolates appeared on the gel indicating homogeneity of protein.

3.4.1.5.13 Molecular Weight Determination

The molecular weight of partially purified xylanase was determined with the help of molecular marker ranging between 14.3 kDa- 97.4 kDa.

3.4.1.6 Characterization of Xylanase of Bacteria

By *B. amyloliquefaciens* SH8

By *B. tequilensis* SH0

3.4.1.6.1 Effect of pH

The partially purified xylanase was tested for the optimum pH for xylanase Activity. The pH profile of partially purified xylanase was evaluated by incubating the enzyme for 10 min in the presence of appropriate buffer i.e. 0.05M HCl-glycine (pH 3.0), 0.05M citrate buffer (pH 4.0-6.0), 0.05M sodium phosphate buffer (pH 7.0-8.0) and 0.05M NaOH-glycine buffer (pH 9.0-10.0, 11.0, 12.0).

3.4.1.6.1a Xylanase Assays

Same as given in section 3.1.2.1.2.d (iv)

3.4.1.6.2 Effect of Temperature

The optimum temperature for partially purified xylanase for the hydrolysis of oat spelt xylan (1%) in 0.05M of citrate buffer at pH 6.0 was determined by incubating the enzyme for 10 min at different temperature ranging from 30°C, 40°C 100°C.

3.4.1.6.2a Xylanase Assays

Same as given in section 3.1.2.1.2.d (iv)

3.4.1.6.3 Effect of Metal Ions

Partially Purified xylanase in 0.05M citrate buffer (pH 6.0) with various metal ions (5 mM) K^+ , Hg^{2+} , Na^+ , Ca^{2+} , Cu^{2+} , Mn^{2+} , Zn^{2+} , Mg^{2+} were incubated at 90°C for 10 min.

3.4.1.6.3a Xylanase Assays

Same as given in section 3.1.2.1.2.d (iv)

3.4.1.6.4 Substrate Specificity

The hydrolytic Activity against 1% birch wood xylan, oat spelt xylan, avicel, cellulose and carboxymethyl cellulose in 0.05M citrate buffer (pH 6.0) was analysed by incubating the samples at 90°C for 10 min. to evaluate the substrate specificity of partially purified xylanase.

3.4.1.6.4a Xylanase Assays

Same as given in section 3.1.2.1.2.d (iv)

3.4.1.6.5 Effect of Different Concentration of Birch Wood Xylan

The different concentrations of substrate ranging from 0.2%, 0.4%..... 2.0% in 0.05M citrate buffer (pH 6.0) were incubated at 90°C for 10 min.

3.4.1.6.5a Xylanase Assays

Same as given in section 3.1.2.1.2.d (iv)

3.4.1.6.6 Determination of Enzyme Kinetics

The effect of birch wood xylan concentration ranging from 2.0, 4.0..... 20.0 mg/ml on partially purified xylanase Activity in 0.05M citrate buffer (pH 6.0) were incubated at 90°C for 10 min. under optimal Assay conditions. The kinetic parameters of Michaelis- Menton constant, K_m and maximal reaction velocity, V_{max} were determined by linear regression according to Lineweaver and Burk double-reciprocal plot

3.4.1.6.4a Xylanase Assays

Same as given in section 3.1.2.1.2.d (iv)

3.4.2 Purification of Xylanase enzyme of fungi by *T. longibracheatum* T2

3.4.2.1 Production of Xylanase

10 g of alkali pretreated, *wheat straw*, moistened with 30ml of Vogel medium pH (5.0) was taken and autoclaved. Added seven days old *T. longibracheatum* T2 culture scratched with 10 ml of autoclaved distilled water and fixing their inoculum size 1×10^7 spores/ml and kept for 7 days at 30°C. The crude enzyme (200ml) was harvested by Repeated Extraction Method, centrifuged and stored at 4°C.

3.4.2.2 Lyophilization of Xylanase

Same as section 3.4.1.2

3.4.2.2a Xylanase Activity

Same as given in section 3.1.2.1.2.d (iv)

3.4.2.2b Protein Estimation

Same as given in section 3.1.2.1.2.e

3.4.2.3 Ion Exchange Chromatography

Same as section 3.4.1.3

3.4.2.4 Gel Filtration Chromatography

Same as section 3.4.1.4

3.4.2.5 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

Same as section 3.4.1.5 except

3.4.2.5.5 Calculation for 15 % Separating Gel

Solution A	:	X/3 ml, i.e. 5 ml
Solution B	:	2.5 ml, i.e. 2.5 ml
H ₂ O	:	(7.5 – X/3) ml, 2.5 ml
10 % ammonium persulfate	:	50 µl
TEMED	:	10 µl

Total volume	:	10 ml

3.4.2.6 Characterization of Partially Purified Xylanase of Fungi by *T. longibracheatum* T2

3.4.2.6.1 Effect of pH

Same as section 3.4.1.6.1

3.4.2.6.1a Xylanase Assays

Same as section 3.1.2.1.2.d (iv)

3.4.2.6.2 Effect of Temperature

The optimum temperature for partially purified xylanase for the hydrolysis of oat spelt xylan (1%) in 0.05M of citrate buffer at pH 5.0 was determined by incubating the enzyme for 10 min at different temperature ranging from 30°C, 40°C 80°C.

3.4.2.6.2a Xylanase Assays

Same as section 3.1.2.1.2.d (iv)

3.4.2.6.3 Effect of Metal Ions

Partially Purified xylanase in 0.05M citrate buffer (pH 5.0) with various metal ions (5 mM) K^+ , Hg^{2+} , Na^+ , Ca^{2+} , Cu^{2+} , Mn^{2+} , Zn^{2+} , Mg^{2+} were incubated at 60°C for 10 min.

3.4.2.6.3a Xylanase Assays

Same as section 3.1.2.1.2.d (iv)

3.4.2.6.4 Substrate Specificity

The hydrolytic activity against 1% birch wood xylan, oat spelt xylan, avicel, cellulose and carboxymethyl cellulose in 0.05M citrate buffer (pH 5.0) was analysed by incubating the samples at 60°C for 10 min. to evaluate the substrate specificity of partially purified xylanase.

3.4.2.6.4a Xylanase Assays

Same as section 3.1.2.1.2.d (iv)

3.4.2.6.5 Effect of Different Concentration of Birch Wood Xylan

The different concentrations of substrate ranging from 0.2%, 0.4%..... 2.0% in 0.05M citrate buffer (pH 6.0) were incubated at 60°C for 10 min.

3.4.2.6.5a Xylanase Assays

Same as section 3.1.2.1.2.d (iv)

3.4.2.6.6 Determination of Enzyme Kinetics

The effect of birch wood xylan concentration ranging from 2.0, 4.0..... 20.0 mg/ml on partially purified xylanase Activity in 0.05M citrate buffer (pH 5.0) were incubated at 60°C for 10 min. under optimal Assay conditions. The kinetic parameters of Michaelis- Menton constant, K_m and maximal reaction velocity, V_{max} were determined by linear regression according to Lineweaver and Burk double-reciprocal plot

3.4.2.6.6a Xylanase Assays

Same as section 3.1.2.1.2.d (iv)

3.5 ENCODING OF CELLULASE AND XYLANASE GENE FROM SCREENED BACTERIAL AND FUNGAL ISOLATES

3.5.1 Isolation of Genomic DNA of Bacteria

Same as in section 3.2.2.1

3.5.1.1 Quantification of Gnomic DNA

Same as in section 3.2.2.2.3

3.5.1.2 Agarose Gel Electrophoresis

Same as in section 3.2.2.2

3.5.1.3 Primer Selection

The primers were selected by taking into account nucleotide sequences of cellulase producing genes from *B. subtilis* BM-1 and xylanase producing genes from *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 bacterial isolates available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected

sets of primers were synthesized from Sigma-Genosys. Following sets of primers were used to amplify chromosome encoded cellulase and xylanase producing genes of bacteria.

3.5.1.4 Amplification of Cellulase and Xylanase Producing Genes

The PCR analysis of chromosomal DNA of *B. subtilis* BM-1, *B. amyloliquefaciens* and *B. tequilensis* SH0 was carried out with a volume of 25 µl mixture in a thermocycler (Eppendorf, make).

Table 4. Primer designing of bacteria

Bacteria	Gene	Sequence (5'-3')	MW	Tm	µg/OD	nmol	GC%	mer	Product Size (bp)
<i>Bacillus subtilis</i> BM-1	Cellulase gene	GGTCTGTGCCCAAATCAG	6068	63.2	32.69	50.7	50.0	20 20	1404
		GCGAAGGAGGAAAAGATCAG	6273	62.7	28.73	43.0	50.0		
		GGTCTGTGCCCAAATCAG	6068	63.2	32.69	50.7	50.0	20 18	1432
<i>Bacillus Amyloliquefaciens</i> SH8	Xylanase gene	ATAATCGCCGCTTTGTGC	5465	63.7	33.28	64.7	50.0		
		CATGTGTATGTGCTTCCTCGTC	6683	64.4	34.09	64.4	50.0	22 20	718
		AATGTCCGGTATGGCTTCAG	6148	63.7	31.87	56.5	50.0		
<i>Bacillus tequilensis</i> SH0	Xylanase gene	GCAGTCATGTGTATGTGCTTCC	6732	64.4	33.05	60.8	50.0	22 19	709
		CTCAGCCGTCCATTTTCG	5706	65.6	34.60	58.7	53.0		
		GGGGAAGGCTGGCTTATATC	6213	63.4	31.82	57.2	55.0	20 19	559
		ATGGTGTGCCTTCACTTTCC	6050	63.9	34.41	65.0	50.0		
		AGGGCAACCTCATGTCAAAG	6135	64.0	30.62	57.4	50.0	20 22	2020
		GTAGCGGCAGTTCTTCGAGAG	6164	63.9	32.42	59.7	55.0		

PCR Reaction Mixture

Taq buffer (10X)	5.0µl
dNTP 2mM	2.5 µl
Primer (F)	1.0 µl
Primer (R)	1.0 µl
Taq Polymerase	0.2 µl
Glycerol	0.5 µl
Water	12.8 µl
Chomosomal DNA	1 µl
MgCl ₂	1 µl

The procedure for amplification consisted of 35 cycles of 92°C for 1 min, 55.0°C for 1 min, 72 for 1 min. The primers given above were used for amplification. The amplified PCR product was cleaned up as given below:

3.5.1.5 PCR Clean up

Same as section 3.2.2.2.6

3.5.1.6 Sequencing

Eluted PCR products of BM-1 and SH8 were sequenced by commercial available services of (Xcelaris, Ahmdabad, India). The sequences so obtained from cellulase gene from *B. subtilis* BM-1 and *B. amyloliquefaciens* SH8 were submitted in National Centre for Biotechnology Information (NCBI) to get an accession number.

3.5.2 Isolation of Genomic DNA of Fungi

Same as in section 3.2.3.1

3.5.2 .1Quantification of Gnomic DNA

Same as in section 3.2.2.2.3

3.5.2 .2Agarose Gel Electrophoresis

Same as in section 3.2.2.2

3.5.2 .3 Primer Selection

The primers were selected by taking into account nucleotide sequences of cellulase and xylanase producing genes from *T. longibracheatum* T2 and cellulase producing genes from *R. oryzae* W2 fungal isolates available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma-Genosys. Following sets of primers were used to amplify chromosome encoded cellulase and xylanase producing genes of bacteria.

3.5.2.4 Amplification of Cellulase and Xylanase Producing Genes

The PCR analysis of chromosomal DNA of *T. longibracheatun* T2 and *R. oryzae* W2 was carried out with a volume of 25 µl mixture in a thermocycler (Eppendorf, make)

Table 5. Primer designing of fungi

Bacteria	Gene	Sequence (5'-3')	MW	Tm	µg/OD	nmol	GC%	mer	Product Size (bp)
<i>Trichoderma longibrachaeum</i> T2	Endoglucanase-gene-1	TCAGCTACACTGCCGTTGAC	6053	64.1	33.25	54.8	55.0	19 20	1522
		GGCATTGCGAGTAGTCTGTG	6204	63.4	32.14	55.8	55.0		
		CATCTCAAACGCCTCCATC	5966	64.5	32.76	54.3	50.0	20 21	1553
		AGGCATTGCGAGTAGTCTGTG	6517	64.3	31.56	57.4	52.0		
	Cellobiohydrolase	ATTCTCACCACGCTGGCTAC	6013	64.1	33.51	55.6	55.0	20 19	1571
		TACAGGAACGATGGGTTGC	6197	64.7	31.37	60.6	50.0		
		GCTGCACAAAGTAGGCTTGG	6182	64.7	31.84	54.0	55.0	20 20	1540
		ATTGTGCGCATTCTCACCAC	6013	64.1	33.51	60.6	55.0		
	β-glucosidase	TCAAGCTCTTTGCGCTCTTC	6010	64.7	35.60	40.8	50.0	20 19	1400
		GCCCAAGGACTTTCAATGG	5813	64.3	31.92	50.9	53.0		
		AACGCTTCTGCCATTCTC	5675	64.2	34.60	29.8	53.0	18 20	1414
		GACTTTCAATGGGGTTTGC	7179	65.3	32.92	59.0	50.0		
	Xylanase	GACAGCAAGCTCAACTGCATAG	6737	63.9	31.82	49.5	50.0	20 19	747
		GGTAGTTCATCTGCCAAGG	6133	63.3	34.41	58.9	55.0		
		CAGCAAGCTCAACTGCATAG	6095	61.3	30.62	59.0	50.0	20 20	704
		GCATTGAAGTGGTCTGCAC	6148	63.3	32.42	53.6	50.0		
<i>Rhizopus oryzae</i> W2	Cellulase gene	CAGCTCGCCCTTTCTGTATC	6118	63.8	32.66	56.2	60.0	20 19	646
		CAGCTCGCCCTTTCTGTATC	5995	63.7	35.05	66.6	55.0		
		ACCAACTTGACCGAGCTCAC	6031	64.3	32.11	70.0	55.0	19 20	654
		TTCTGTATCCCGGGTCTGAG	6115	63.9	33.39	55.4	55.0		

PCR Reaction Mixture

Taq buffer (10X)	5.0µl
dNTP 2mM	2.5 µl
Primer (F)	1.0 µl
Primer (R)	1.0 µl
Taq Polymerase	0.2 µl
Glycerol	0.5 µl
Water	12.8 µl
Chomosomal/ Plasmid DNA	1 µl
MgCl ₂	1 µl

The procedure for amplification consisted of 35 cycles of 92°C for 1 min, 46.6°C for 1 min, 72 for 1 min. The primers given above were used for amplification. The amplified PCR product was cleaned up as given below:

3.5.2.5 PCR Clean up

Same as section 3.2.2.2.6

3.6 SCALE UP OF XYLANASE ENZYME IN A BIOREACTOR

By *B. amyloliquefaciens* SH8

3.6.1 Inoculum Preparation

B. amyloliquefaciens SH8 was grown in 300 ml of nutrient broth and incubated at $37\pm 2^{\circ}\text{C}$ for 24 h. As soon as the substantial growth of the strain was observed in the broth, the optical density was set to 1.0 using nutrient broth as blank.

3.6.2 Production of Xylanase

Batch cultivation for the production of xylanase was carried out in a 7.5 L stirred tank bioreactor (New Brunswick Scientific, New Jersey USA) with a 3.0 L working volume at 45°C and pH 5.5. Carbon and nitrogen sources; for batch fermentations were 1.0% wheat bran, tryptone 9.0 g/L, NH_4Cl 2.0 g/L, ZnSO_4 0.20/L and CaSO_4 0.20 g/L. Foaming was controlled with addition of 2-3 drops of polypropylene glycol. The medium was sterilized in situ for 20 min at 121°C . The different bioprocess parameters optimized in bioreactor were as given below:

3.6.3 Effect of Inoculum Age

The different inoculum ages 2, 4 and 6h were evaluated for the production of xylanase in a 7.5 L stirred tank bioreactor. The bioreactor was inoculated with a 10% (v/v) of inoculum under aseptic conditions. The agitation speed of a 7.5 L was 150 rpm and aeration rate 1.0 vvm, respectively. The sample was withdrawn regularly at different intervals ranging from 2, 4,..... 10h.

3.6.3a Xylanase Assay:

Same as given in section 3.1.2.1.2.d (iv)

3.6.3b Protein Assay

Same as given in section 3.1.2.1.2.e

3.6.4 Effect of Inoculum Size

The different inoculum sizes 5, 10 and 15% were evaluated for the production of xylanase in a 7.5 L stirred tank bioreactor. The bioreactor was

inoculated with 4h inoculum age and inoculum size varied under aseptic conditions. The agitation speed of a 7.5 L was 150 rpm and aeration rate 1.0 vvm, respectively. The sample was withdrawn regularly at different intervals ranging from 2, 4..... 10h.

3.6.4a Xylanase Assay

Same as given in section 3.1.2.1.2.d (iv)

3.6.4b Protein Assay

Same as given in section 3.1.2.1.2.d e

3.6.5 Effect of Aeration Rate

The different aeration rates 0.5, 1.0 and 1.5 vvm were evaluated for the production of xylanase in a 7.5 L stirred tank bioreactor. The bioreactor was inoculated with a 10% (v/v) of inoculum and 4h inoculum age under aseptic conditions. The agitation speed of a 7.5 L was 150 rpm and aeration rate varied, respectively. The sample was withdrawn regularly at different intervals ranging from 2, 4.... 10h.

3.6.5a Xylanase Assay

Same as given in section 3.1.2.1.2.d (iv)

3.6.5b Protein Assay

Same as given in section 3.1.2.1.2.e

3.6.6 Effect of Agitation Rate

The different agitation rates 100 rpm, 200 rpm and 300 rpm were evaluated for the production of xylanase in a 7.5 L stirred tank bioreactor. The bioreactor was inoculated with a 10% (v/v) of inoculum and 4h inoculum age under aseptic conditions. The aeration rate was 1.0 vvm and agitation rates varied, respectively. The sample was withdrawn regularly at different intervals ranging from 2, 4... 10h.

3.6.6a Xylanase Assay

Same as given in section 3.1.2.1.2.d (iv)

3.6.6b Protein Assay

Same as given in section 3.1.2.1.2.e

3.6.7 Determination of Growth Kinetics in a Bioreactor

Cell biomass in the fermentation broth was quantified by dry-cell weight analysis and by measurement of the optical density of the broth. For dry weight determinations, the cells were recovered by centrifugation at 4°C, 8000 rpm and washed twice with distilled water. The recovered biomass was dried to constant weight in an oven at 80°C for 24 h. Absorbance was read at 540 nm using a UV/Vis spectrophotometer for optical density.

3.6.7a Xylanase Assay

Same as given in section 3.1.2.1.2.d (iv)

3.6.7b Protein Assay

Same as given in section 3.1.2.1.2.e

3.6.8 Mathematical and Kinetic Modeling for Xylanase Enzyme

Microbial fermentation, particularly that relating to enzyme production does not essentially follow the conventional kinetic model of substrate-limiting biomass growth and product formation proposed by Monod Eq. (1). Therefore, the logistic equation is used as an alternative empirical function (Luedeking and Piret 1959) for microbial cell growth

$$\text{Monod equation} = \mu = \mu_m S/(K_s+S) \text{-----(1)}$$

$$1/\mu = K_s/K_m \cdot 1/S + 1/\mu_{\max}$$

Where μ = specific growth rate, $\mu_{\max}$ = maximum specific growth rate, S = substrate concentration, K_s = substrate, saturation constant (i.e. substrate concentration at half $\mu_{\max}$).

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate through the parameters $\mu_{\max}$ and K_s .

$$Y_{x/s} = dx/ds \text{-----(2)}$$

$$Y_{x/s} = X_m - X_o/S_o - S_m$$

$$\mu = dx/dt \cdot 1/x \text{-----(3)}$$

In addition to this, Monod also related the yield coefficient ($Y_{x/s}$) (equation 2) to the specific rate of biomass growth (μ) and the specific rate of substrate utilization (q) (equation 3).

$$q_p = \alpha\mu + \beta$$

$$q_p = dp/dt = \alpha dx/dt + \beta x \text{-----(4)}$$

where α = growth associated constant, β = non growth associated constant.

Luedeking-Piret equation (Eq. 4) was applied for comparison between experimental (actual) results and the model prediction for xylanase production. During this modeling, rate of xylanase production is linearly proportional to the biomass growth rate.

Chapter-4

RESULTS AND DISCUSSION

The results emanating from the present investigation entitled **“Purification of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost for industrial application”** were conducted in Microbiology section, Department of Basic Sciences, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, during the year of 2008-12. The facts and statistics used for analysis pertaining to different parameters and characters were statistically analyzed and significance of results was verified. The results have been discussed after interpretation of the hypotheses of the research undertaken by me. This chapter deals with the experimental results and discussion and most likely causes and effects, underlying the patterns, resulting in the predictions, evidence or even a line of reasoning, which supports each interpretation, agree or perhaps contradict previous work, in the light of the available literature. The salient findings obtained during the course of investigation are presented under following main headings:

- 4.1 Isolation and screening of hyper cellulolytic and xylanolytic microorganisms
- 4.2 Phenotypic and genotypic identification of screened hyper cellulolytic and xylanolytic microorganisms
- 4.3 Production and optimization of cellulase and xylanase enzyme from hyper cellulolytic and xylanolytic microorganisms.
- 4.4 Purification and characterization of xylanase enzyme from hyper xylanolytic microorganisms.
- 4.5 Encoding of cellulase and xylanase gene from cellulolytic and xylanolytic microorganisms.
- 4.6 Scale up of xylanase enzyme of *B. amyloliquefaciens* SH8

4.1 ISOLATION AND SCREENING OF HYPER CELLULOLYTIC AND XYLANOLYTIC MICROORGANISMS

4.1.1 Isolation of Cellulolytic and Xylanolytic Bacteria from Compost

In total, 93 bacteria isolated from pooled samples in Rivieres medium having pH 6.8 have been enlisted in Fig 1 a, b, c, d, e, f, g and h with their morphological properties. Out of 93 bacteria, 30 were isolated from Solan district, 24 from Shimla district, 22 from Mandi district and 17 bacteria were isolated from Kangra district of Himachal Pradesh.

Bacteria isolated from compost showed a great variation in their morphological characteristics i.e. colour, texture and shape as shown in Table 1. The colour of the colonies also exhibited a variation amongst the different strains. They varied from cream, pink, yellow, red, brown white to off white. Gram staining of all these bacterial isolates differentiated them into gram +ve and gram-ve. Amongst these 93 isolates, 74% were found to be gram +ve and the rest 26% were found to be gram -ve. Among all isolates, 45% were rod shaped while 55% were spherical in shape.

Fig. 2 a, b, c, d, e, f, g, h, i, j, k and l represents the biochemical characteristics of 93 bacterial isolates. Different biochemical tests were performed such as cellulase test, catalase test, H₂S production test, urease test, citrate utilization test, fermentation of carbohydrates, hydrolysis of gelatin, casein test and indole test for the characterization of all the 93 bacterial isolates. Out of 93 bacterial isolates, urea test was positive for 35% isolates and 65% were negative for the bacterial isolates. Out of 93 bacterial isolates, 39% showed positive H₂S production test, 51% for citrate utilization test, 42% for MR, 26% for VP test, 70% gelatin hydrolysis test and 36% showed positive test for fermentation of carbohydrates. 26% strains showed positive test for casein hydrolysis. For catalase test, all strains showed positive test, while for indole test all strains showed negative test. Whereas 98% showed positive test for cellulase test and rest were negative. Depending upon their morphological, physiological and biochemical characteristics, these bacteria were tentatively identified as *Bacillus* sp. and *Coccus* sp.

Table 1. Biochemical characteristics of screened cellulolytic and xylanolytic bacterial isolates

Biochemical tests	SH0	SH8	BM-1
Growth on MacConkey	nlf	nlf	nlf
Indole test	-	-	-
Methyl red test	-	-	-
Voges Prauskauer test	-	-	-
Citrate utilization test	-	-	-
H ₂ S production	-	-	-
Gas production from glucose	-	-	-
Casein hydrolysis test	+	+	+
Gelatin hydrolysis test	+	+	+
Starch hydrolysis test	-	(+)	-
Nitrate reduction	+	+	+
Catalase test	+	+	+
Oxidase test	(+)	(+)	(+)
Urea hydrolysis	-	-	-
Esculin hydrolysis	-	+	+
Arginine dihydrolase	+	-	-
Tween 20 hydrolysis	+	-	+
Tween 40 hydrolysis	+	-	+
Tween 60 hydrolysis	+	-	+
Tween 80 hydrolysis	+	-	+
Acid production			
Raffinose	+	-	(+)
Maltose	-	(+)	+
Galactose	-	-	-
Sucrose	-	+	+
Dextrose	-	+	+
Arabinose	-	(+)	+

+: Postive; -: Negative; (+): Weak positive; nlf: non lactose fermenting

Compost is a rich habitat of potential biodegrading microorganisms as it passes though a succession of phases including drastic temperature variations to complete the process. Thus, isolation of hypercellulolytic and hyperxylanolytic microorganisms has been done in the present study from the compost considering it a valuable niche of microorganisms.

Various potential enzyme producing microorganisms have already been isolated from compost samples as cited in the literature. Baharuddin *et al.* (2010)

isolated and characterized thermophilic bacterial strain, *Geobacillus pallidus* from empty fruit bunch (EFB) and palm oil mill effluent (POME) compost. Nik and Rosario (2010) isolated two-hundred and twenty-five mangrove bacterial strains from several mangrove areas in the Philippines and were qualitatively screened for cellulase production and 154 were found to be capable of cellulase production. Among them, ten strains showed very strong positive cellulase activities and these were identified as *Cellulomonas* sp., *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus* sp. Krairitthichai and Thongwai (2007) isolated one hundred and twenty-five isolates of bacteria from soils, decomposing logs and composts collected from the Northern part of Thailand. McCaig *et al.* (2001) and Song *et al.* (2001) isolated compost microorganisms which included *Bacillus* sp., etc. Mayende *et al.* (2006) isolated thermophilic microorganisms from a compost system followed by screening for cellulase and polyphenol oxidase activity.

Roy and Abedin (2002) isolated xylanase-producing strains from drain water. The strains were isolated on xylan-agar media and were screened out by β -xylanolysis method. The strains were further confirmed by DNS-method. The isolated strains were identified as *Aeromonas* on the basis of catalase, oxidase, and gram staining and morphological and cultural characteristics as well as by different biochemical tests. Roy and Rowshanul (2009) obtained twelve isolates from soil samples of different areas in the Rajshahi University campus and studied for detection of xylanase activity. One of the strains was identified as *Bacillus cereus* which produces xylanase extracellularly. Interest in these microorganisms has been increased recently due to their potential commercial applications, which include biodegradation and the production of bioactive compounds such as enzymes, biofuels and antibiotics (Malherbe and Cloete, 2002).

4.1.2 Isolation of Cellulolytic and Xylanolytic Fungi from Compost

Different types of fungi from pooled samples in Rivieres' medium having pH 4.8 have been enlisted in Table 2 and 3 with their morphological, physiological and biochemical properties. These showed different mycelial

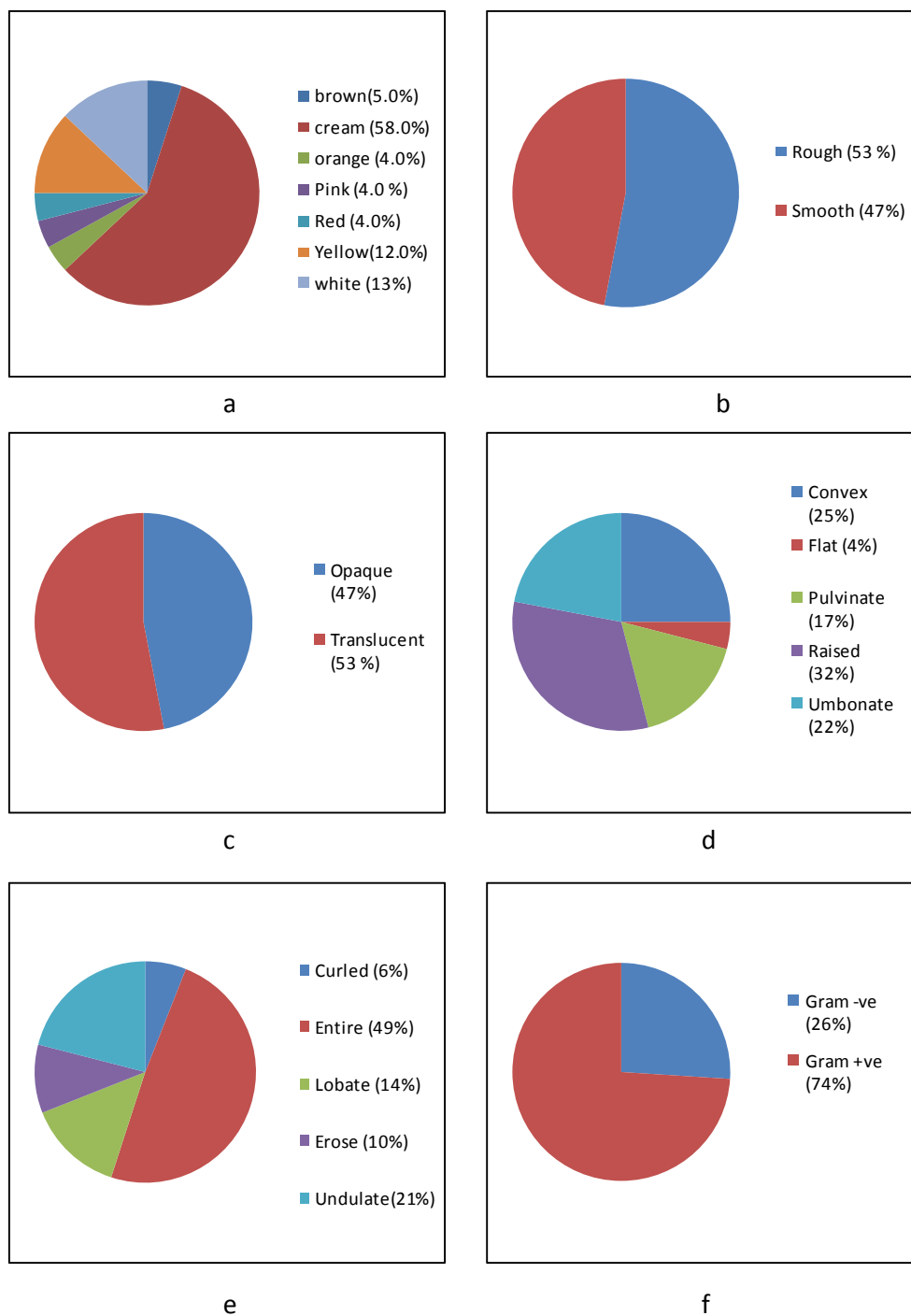


Fig. 1 Morphology of cellulolytic and xylanolytic bacterial isolates on the basis of (a) colour (b) surface (c) opacity (d) elevation (e) margin (f) Gram's reaction

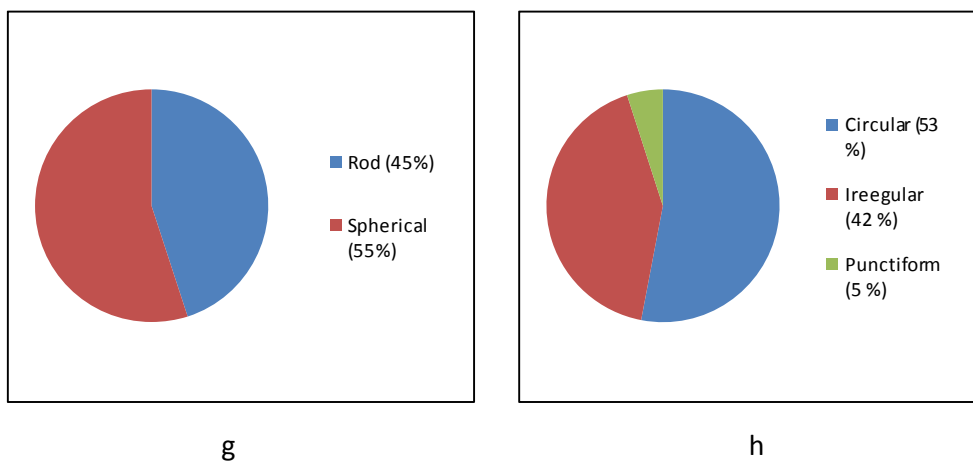


Fig. 1 continued.... (g) shape (h) configuration

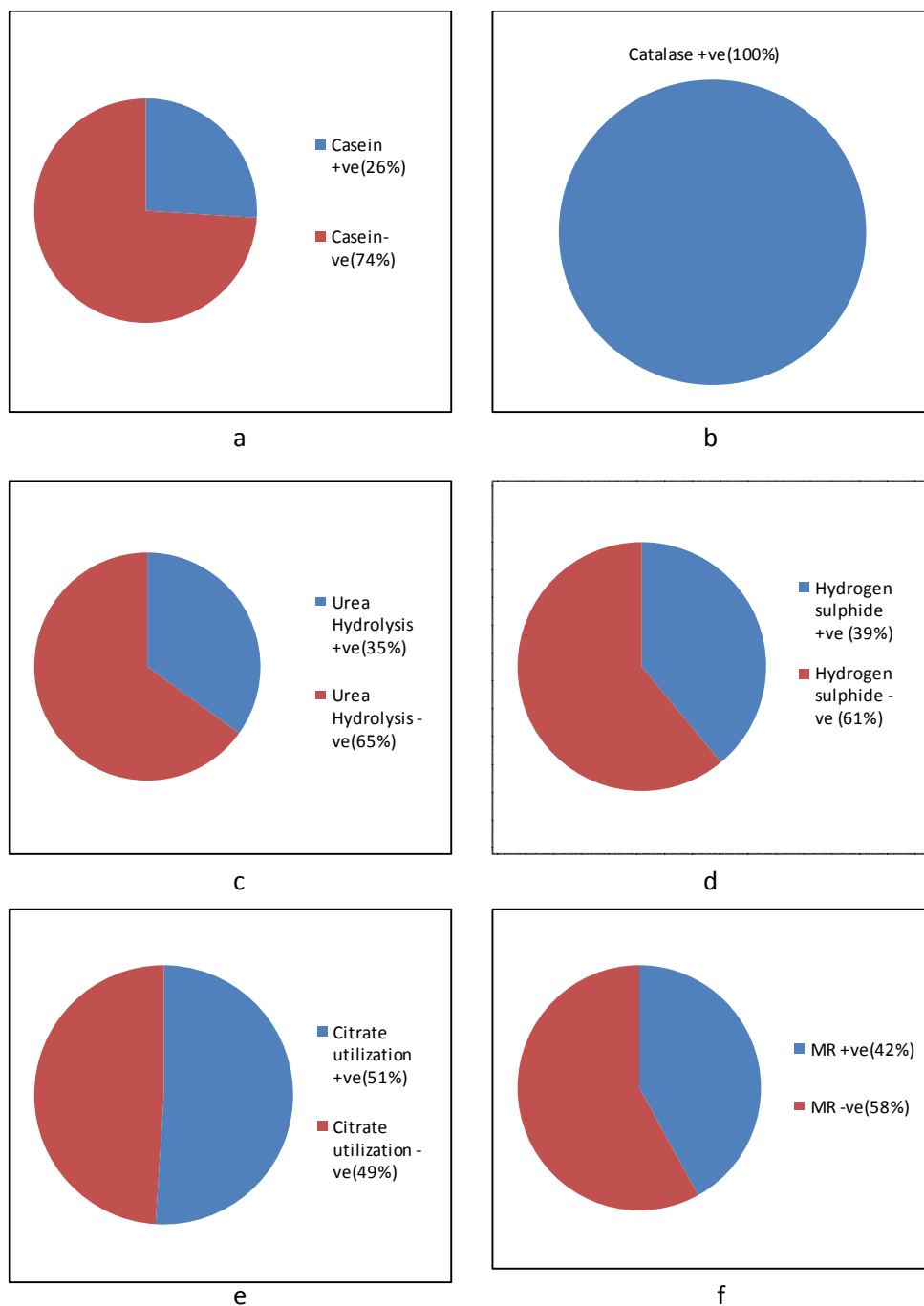


Fig. 2 Biochemical characteristics of cellulolytic and xylanolytic bacterial isolates (a) cellulase test (b) catalase test (c) urea hydrolysis (d) hydrogen sulphide (e) citrate utilization (f) MR test

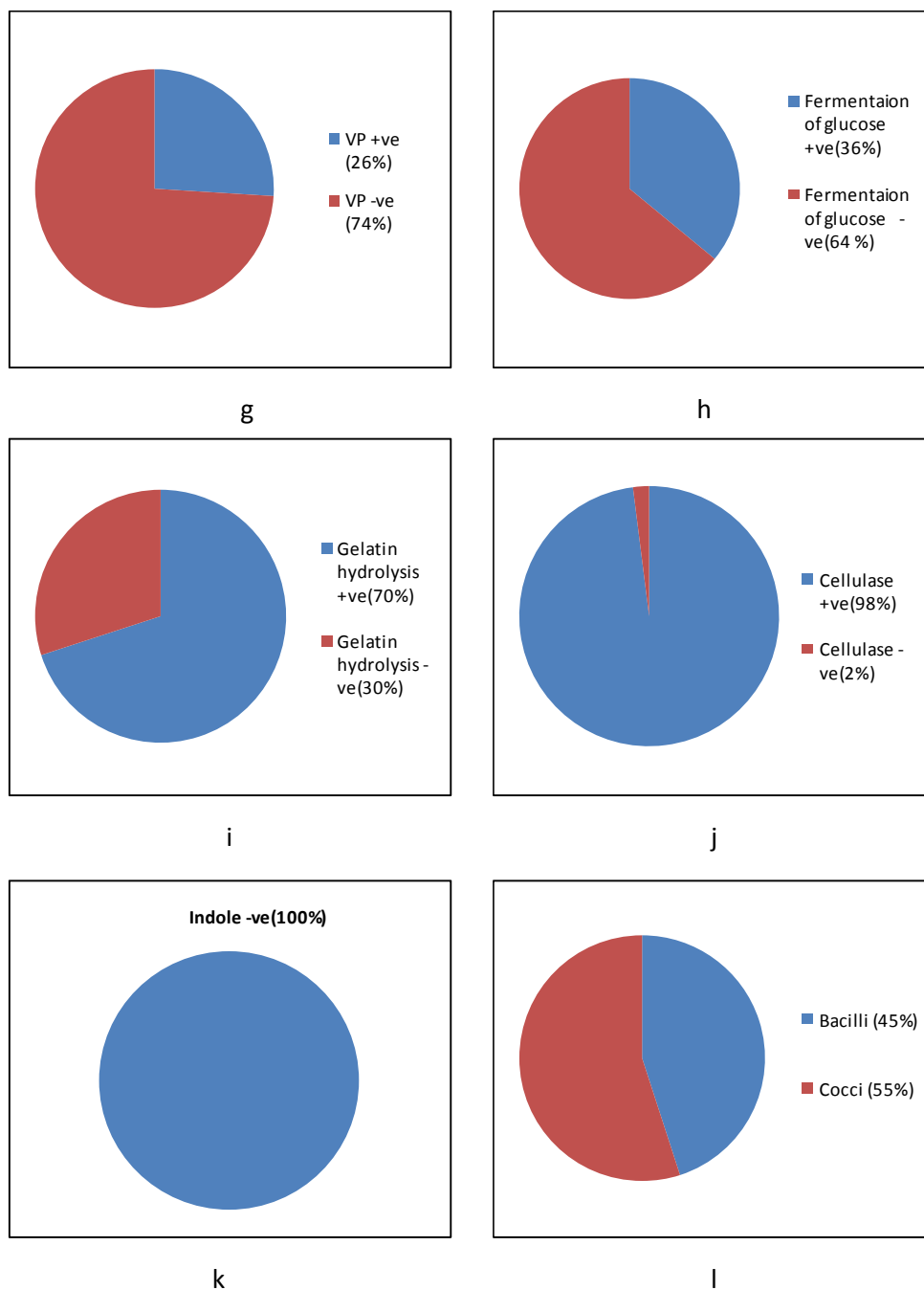


Fig.2 Continued.... (g) VP test (h) fermentation of glucose (i) gelatin hydrolysis (j) casein test (k) indole test (l) tentative identification

characteristics and spore morphology which includes the spore colour and its texture. The fungal isolate T2 showed yellowish green colour and feathery mycelium and was identified as *Trichoderma longibrachiatum*, W2 had non-septate, abundant cottony hyphae, blackish-brown sporangia, fast growing and was identified as *R. oryzae*, Isolate S1 showed white green and rough mycelium and it was identified as *Penicillium* sp., Isolate S2 with whitish mycelium, coenocytic hyphae, feathery texture was identified as *Pythium* sp. M1 showed green short hyphae and rough mycelium and was identified as *Penicillium chrysogenum*. Both the Isolate M2 and K3 had septate hyphae, blackish colour and rough, globular and tightly packed conidia and was identified as *Aspergillus niger*. Isolate K1 was having purple coloured, sickle-shaped macroconidium and had been identified as *Fusarium oxysporum*. Isolate K2 showed grey brownish spore, non-septate, sporangiospore, long and abundant cottony mycelium, fast growing and was identified as *Mucor* sp.

Table 2. Isolation of cellulolytic and xylanolytic fungi from compost and their morphological and cultural characteristics

Fungal Isolate	Source	Mycelium	Spore		
			Colour	Texture	Tentative Identification
T2	Solan	Short hyphae	Yellowish green	Rough	<i>Trichoderma longibrachiatum</i>
W2	Solan	Hyphae (long)	White	Rough	<i>Rhizopus oryaze</i>
S1	Shimla	Short Hyphae	Green	Rough	<i>Penicillium</i> sp.
S2	Shimla	Filamentous	White	feathery	<i>Phytium</i> Sp.
M1	Mandi	Short hypahe	Light green	Rough	<i>Penicillium chrysogenum</i>
M2	Mandi	Hyphae (long)	Black	Rough	<i>Aspergillus niger</i>
K1	Kangra	Filamentous	Purple	Feathery	<i>Fusarium oxysporium</i>
K2	Kangra	Filamentous	Grey brown	Rough	<i>Mucor</i> Sp.
K3	Kangra	Hyphae (long)	Black	Rough	<i>Aspergillus niger</i>

Table 3. Biochemical characteristics of cellulolytic and xylanolytic fungal isolates from compost

S. No.	Isolate No.	Cellulase Test	Catalase Test	H ₂ S Test	Urea Hydrolysis	Citrate Utilization	MR Test	VP Test	Fermentation of glucose	Gelatin Hydrolysis	Casein Test	Indole Test	Tentative Identification
1.	T2	+	-	-	-	+	+	+	+	+	+	-	<i>Trichoderma longibrachiatum</i>
2.	W2	+	+	-	-	+	+	+	+	+	+	-	<i>Rhizopus oryzae</i>
3.	S1	+	-	-	-	+	-	-	+	+	+	-	<i>Penicillium sp.</i>
4.	S2	+	-	-	-	-	+	+	-	-	-	-	<i>Phytium Sp.</i>
5.	M1	+	-	-	-	-	-	-	+	+	-	-	<i>Penicillium chrysogenum</i>
6.	M2	+	+	-	-	+	+	+	+	+	+	-	<i>Aspergillus niger</i>
7.	K1	+	-	-	-	+	+	-	-	-	+	-	<i>Fusarium oxysporium</i>
8.	K2	+	-	-	-	-	-	-	-	-	-	-	<i>Mucor Sp.</i>
9.	K3s	+	+	-	-	+	+	+	+	+	+	-	<i>Aspergillus niger</i>

Myat *et al.* (2008) isolated wild type *Aspergillus niger* strains from different vegetable sources. All local strains were studied by cultural, morphological characteristics and microscopic examinations. Among these, four strains were found cellulolytic exhibiting a clear zone on Czapeck-Dox medium supplemented with carboxymethyl cellulose (1%) as well as cellulose by using in vitro screening method for cellulase enzyme activity.

Nair *et al.* (2008) isolated seventy fungal strains from soil collected from different parts of Southern Kerala in India. Most of them were screened and identified as *Aspergillus niger*, *A. flavus*, *A. fumigatus* and *A. ochraceus*. *Penicillium chrysogenum* PCL 501 was isolated from wood waste and found to be the best producer of xylanase (Okafor *et al.*, 2007). Many fungal strains secrete higher amounts of cellulases than bacterial ones, with *Trichoderma* as the leading one. Most commercial cellulases are mesophilic enzymes produced by the filamentous fungus *Trichoderma reesei* and *Aspergillus niger* (Amouri and Gargouri, 2006).

Palaniswamy *et al.* (2008) reported that fifty-three litter degrading fungal species were isolated by nylon litterbag technique. The production of endo-p-1,4-xylanase (1,4-p-D-xylan xylanohydrolase, E.C. 3.2.1.8), p-D-xylosidase (1,4-p-xylan xylanohydrolase, E.C. 3.2.1.37) and protease was studied using oat spelt xylan as carbon source. Results showed that all fifty-three fungal species isolated from various litter samples produced fairly good xylanolytic enzyme activity. The xylanase and p-D-xylosidase activity ranges from 4.41 to 132.20 U and 48.72 to 1510.32 U, respectively. Growth was determined in terms of mycelial dry weight, which ranged between 0.209 and 1.047 mg/ml.

Nwodo-Chinedu *et al.* (2005) cellulolytic microfungi were isolated from the Saw-mills wastes using minimal salt agar medium containing 0.2% (w/v) crystalline cellulose, sugarcane pulp, corn cob or saw-dust as sole carbon/energy source. The colonies of cellulolytic microfungi which appeared on the plates increased in size and number as the incubation period (days) increased. Among the fungal isolates were two pathogenic *Aspergilli* (*A. flavus* and *A. fumigatus*),

three different black *Aspergilli* (herein designated as *A. niger* I, *A. niger* II and *A. niger* III), *Botrytis cinerea*, *Fusarium* sp. and *Penicillium* sp. Cell-free filtrates of 7 day cultures of *A. flavus*, *A. niger* I, *A. niger* II, *B. cinerea* and *P.* sp. grown on the minimal salt broth supplemented with crystalline cellulose as sole carbon/energy source showed very significant CM–cellulase activity.

4.1.3 Screening of Cellulolytic and Xylanolytic Bacteria from Compost

The production of extracellular enzymes i.e. cellulase and xylanase from different bacterial isolates are depicted in Table 4. Among them, BM-1 showed the highest total cellulase activity of 1.33 IU/ml (CMCase 0.61 IU/ml, FPase 0.09 IU/ml and 0.63 IU/ml β -glucosidase) followed by BM-9 showing enzyme activity of 1.21 IU/ml (CMCase 0.59 IU/ml, FPase 0.08 IU/ml and β -glucosidase 0.54 IU/ml) whereas SH8 showed the lowest cellulase activity i.e. 0.043 IU/ml. Whereas cellulase free maximum xylanase activity was exhibited by SH8 14.40 IU/ml closely followed by SH0 - 13.30 IU/ml and the least xylanase activity was noted in M₆ - 0.40 IU/ml.

Sajani *et al.* (2010) isolated four bacterial colonies (*Bacillus* sp., two *Pseudomonas* sp. and *Proteus* sp.) with the help of spread plate technique and were checked for cellulase production (zone formation) by congo red and NaCl/NaOH. High level of cellulase production was achieved by *Bacillus* sp. (0.09 IU/ml and 0.08 IU/ml) for coir waste and saw dust as substrate. Tae *et al.* (2000) isolated a bacterium producing extracellular cellulase from cow dung compost and has been identified as *Bacillus* sp. When the isolate was cultured in CMC media at 37°C for 24 h, CMCase, FPase and Avicelase activity was 1.65 IU/ml, 0.13 IU/ml and 0.18 IU/ml whereas β -glucosidase activity was not detected. The optimum pH and temperature for enzyme induction was 7.5 and 50°C. The maximum CMCase activity was observed at pH 7.5 and 75°C. Annamalai *et al.* (2009) isolated a bacterium, *Bacillus subtilis* from the estuarine environment and was grown in shake flasks to derive the optimum culture conditions and also cultured in lab scale fermenter to obtain more xylanase. Maximum enzyme production (128 IU/ml) was recorded in stationary phase (36 h) of the culture.

Table 4. Screening of bacterial isolates for hypercellulase and xylanase production

S. No	Isolate Name	Protein (mg/ml)	Cellulase								Protein (mg/ml)	Xylanase	
			FPase activity		CMCase activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase)			Enzyme activity	Specific Activity
			Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity			
1.	SO ₁	0.23	0.01	0.05	0.31	1.35	0.25	1.09	0.57	2.48	0.31	3.40	10.97
2.	SO ₂	0.31	0.03	0.11	0.32	1.03	0.56	1.81	0.91	2.95	0.24	2.30	9.58
3.	SO ₃	0.30	0.03	0.11	0.34	1.13	0.54	1.80	0.91	3.04	0.58	7.10	12.24
4.	SO ₄	0.32	0.07	0.20	0.43	1.34	0.43	1.34	0.93	2.89	0.46	5.20	11.30
5.	SO ₅	0.27	0.07	0.25	0.11	0.41	0.56	2.07	0.74	2.73	0.41	4.80	11.71
6.	SO ₆	0.26	0.02	0.08	0.18	0.69	0.51	1.96	0.71	2.73	0.67	9.40	14.03
7.	SO ₇	0.27	0.03	0.13	0.23	0.85	0.48	1.78	0.74	2.76	0.65	9.20	14.15
8.	SO ₈	0.29	0.03	0.11	0.34	1.17	0.45	1.55	0.82	2.83	0.21	2.10	10.00
9.	SO ₉	0.35	0.04	0.13	0.53	1.51	0.49	1.40	1.06	3.04	0.15	1.30	8.67
10.	SO ₁₀	0.24	0.04	0.15	0.28	1.17	0.34	1.42	0.66	2.73	0.17	1.80	10.59
11.	SO ₁₁	0.35	0.07	0.19	0.45	1.29	0.56	1.60	1.08	3.07	0.36	3.70	10.28
12.	SO ₁₂	0.35	0.03	0.09	0.42	1.20	0.59	1.69	1.04	2.98	0.54	6.90	12.78
13.	SO ₁₃	0.34	0.02	0.05	0.47	1.38	0.60	1.76	1.09	3.20	0.37	4.00	10.81
14.	SO ₁₄	0.36	0.02	0.05	0.60	1.67	0.54	1.50	1.16	3.22	0.39	4.40	11.28
15.	SO ₁₅	0.35	0.03	0.08	0.43	1.23	0.61	1.74	1.07	3.05	0.48	5.20	10.83
16.	SO ₁₆	0.25	0.01	0.05	0.16	0.64	0.59	2.36	0.76	3.05	0.51	6.40	12.55
17.	SO ₁₇	0.35	0.01	0.03	0.44	1.26	0.62	1.77	1.07	3.06	0.67	9.40	14.03
18.	SO ₁₈	0.30	0.01	0.05	0.39	1.30	0.44	1.47	0.84	2.81	0.45	4.90	10.89
19.	SO ₁₉	0.31	0.03	0.11	0.42	1.35	0.54	1.74	0.99	3.21	0.44	4.80	10.91

Table continued.....

20.	SO ₂₀	0.29	0.04	0.12	0.48	1.66	0.32	1.10	0.84	2.88	0.46	5.10	11.09
21	SO ₂₁	0.18	0.05	0.25	0.13	0.72	0.29	1.61	0.47	2.58	0.21	2.30	10.95
22	SO ₂₂	0.23	0.04	0.19	0.23	1.00	0.34	1.48	0.61	2.67	0.40	4.20	10.50
23	SO ₂₃	0.31	0.08	0.24	0.19	0.61	0.61	1.97	0.88	2.82	0.30	3.30	11.00
24	SO ₂₄	0.22	0.06	0.28	0.17	0.77	0.34	1.55	0.57	2.60	0.39	4.10	10.51
25	SO ₂₅	0.25	0.05	0.18	0.12	0.48	0.45	1.80	0.62	2.46	0.24	2.30	9.58
26	SO ₂₆	0.30	0.09	0.30	0.25	0.83	0.54	1.80	0.88	2.93	0.71	10.00	14.08
27	SO ₂₇	0.31	0.06	0.19	0.27	0.87	0.52	1.68	0.85	2.74	0.61	8.20	13.44
28	SO ₂₈	0.31	0.03	0.11	0.35	1.13	0.58	1.87	0.96	3.11	0.64	9.10	14.22
29	SO ₂₉	0.27	0.03	0.10	0.27	1.00	0.43	1.59	0.73	2.69	0.48	5.20	10.83
30	SH ₀	0.11	0.00	0.00	0.03	0.27	0.02	0.18	0.05	0.45	0.80	13.30	16.63
31	SH ₁	0.28	0.02	0.06	0.38	1.36	0.41	1.46	0.81	2.89	0.54	6.90	12.78
32	SH ₂	0.31	0.03	0.09	0.47	1.52	0.34	1.10	0.84	2.70	0.37	3.80	10.27
33	SH ₃	0.29	0.03	0.11	0.37	1.28	0.56	1.93	0.96	3.32	0.74	11.20	15.14
34	SH ₄	0.30	0.07	0.22	0.41	1.37	0.57	1.90	1.05	3.48	0.76	12.10	15.92
35	SH ₅	0.28	0.05	0.18	0.39	1.39	0.43	1.54	0.87	3.11	0.19	2.00	10.53
36	SH ₆	0.26	0.03	0.13	0.22	0.85	0.49	1.88	0.74	2.86	0.28	2.90	10.36
37	SH ₇	0.25	0.06	0.22	0.37	1.48	0.23	0.92	0.66	2.62	0.67	9.70	14.48
38	SH ₈	0.13	0.00	0.00	0.01	0.07	0.02	0.15	0.03	0.23	0.83	14.40	17.35
39	SH ₉	0.32	0.08	0.25	0.42	1.31	0.59	1.84	1.09	3.41	0.26	2.70	10.38
40	SH ₁₀	0.32	0.09	0.28	0.39	1.22	0.56	1.75	1.04	3.25	0.44	4.80	10.91
41	SH ₁₁	0.29	0.07	0.23	0.43	1.48	0.45	1.55	0.95	3.27	0.60	8.00	13.33
42	SH ₁₂	0.30	0.09	0.28	0.43	1.43	0.55	1.83	1.07	3.55	0.65	9.00	13.85
43	SH ₁₃	0.33	0.03	0.10	0.56	1.70	0.59	1.79	1.18	3.59	0.74	11.20	15.14
44	SH ₁₄	0.24	0.03	0.12	0.28	1.17	0.32	1.33	0.63	2.62	0.45	4.90	10.89

Table continued.....

45	SH ₁₅	0.27	0.02	0.08	0.53	1.96	0.33	1.22	0.88	3.26	0.54	6.90	12.78
46	SH ₁₆	0.26	0.04	0.15	0.37	1.42	0.35	1.35	0.76	2.92	0.35	3.60	10.29
47	SH ₁₇	0.27	0.07	0.24	0.28	1.04	0.45	1.67	0.80	2.94	0.57	7.30	12.81
48	SH ₁₈	0.30	0.07	0.24	0.31	1.03	0.60	2.00	0.98	3.27	0.58	7.50	12.93
49	SH ₁₉	0.29	0.08	0.29	0.22	0.76	0.62	2.14	0.92	3.19	0.46	4.90	10.65
50	SH ₂₀	0.26	0.04	0.17	0.29	1.12	0.31	1.19	0.64	2.47	0.48	5.20	10.83
51	SH ₂₁	0.29	0.04	0.14	0.37	1.28	0.47	1.62	0.88	3.03	0.41	4.30	10.49
52	SH ₂₂	0.30	0.02	0.06	0.49	1.63	0.41	1.37	0.92	3.06	0.52	6.30	12.12
53	SH ₂₃	0.26	0.07	0.28	0.30	1.15	0.34	1.31	0.71	2.74	0.57	7.30	12.81
54	SH ₂₄	0.30	0.04	0.13	0.33	1.10	0.60	2.00	0.97	3.23	0.67	9.40	14.03
55	M ₁	0.36	0.07	0.20	0.59	1.64	0.54	1.50	1.20	3.34	0.56	6.20	11.07
56	M ₂	0.26	0.03	0.13	0.37	1.42	0.23	0.88	0.63	2.44	0.71	10.30	14.51
57	M ₃	0.29	0.08	0.26	0.30	1.03	0.45	1.55	0.83	2.84	0.46	5.00	10.87
58	M ₄	0.26	0.07	0.25	0.10	0.38	0.62	2.38	0.79	3.02	0.17	1.20	7.06
59	M ₅	0.23	0.09	0.38	0.29	1.26	0.21	0.91	0.59	2.55	0.16	1.10	6.88
60	M ₆	0.22	0.05	0.20	0.19	0.86	0.49	2.23	0.73	3.30	0.10	0.40	4.00
61	M ₇	0.28	0.07	0.23	0.27	0.96	0.53	1.89	0.87	3.09	0.28	3.00	10.71
62	M ₈	0.29	0.08	0.29	0.26	0.90	0.58	2.00	0.92	3.19	0.45	4.90	10.89
63	M ₉	0.32	0.05	0.17	0.55	1.72	0.51	1.59	1.11	3.48	0.43	4.60	10.70
64	M ₁₀	0.27	0.08	0.28	0.28	1.04	0.43	1.59	0.79	2.91	0.59	7.80	13.22
65	M ₁₁	0.29	0.02	0.08	0.52	1.79	0.40	1.38	0.94	3.25	0.38	3.90	10.26
66	M ₁₂	0.21	0.03	0.16	0.18	0.86	0.34	1.62	0.55	2.64	0.53	6.80	12.83
67	M ₁₃	0.28	0.04	0.14	0.37	1.32	0.43	1.54	0.84	2.99	0.67	9.30	13.88
68	M ₁₄	0.28	0.06	0.23	0.42	1.50	0.33	1.18	0.81	2.91	0.78	12.90	16.54

Table continued.....

69	M ₁₅	0.28	0.07	0.25	0.38	1.36	0.39	1.39	0.84	3.00	0.25	2.70	10.80
70	M ₁₆	0.29	0.03	0.11	0.54	1.86	0.40	1.38	0.97	3.35	0.61	8.30	13.61
71	M ₁₇	0.28	0.08	0.27	0.20	0.71	0.56	2.00	0.84	2.99	0.27	2.90	10.74
72	M ₁₈	0.27	0.08	0.29	0.29	1.07	0.50	1.85	0.87	3.21	0.47	5.10	10.85
73	M ₁₉	0.30	0.03	0.09	0.41	1.37	0.54	1.80	0.98	3.25	0.28	3.00	10.71
74	M ₂₀	0.32	0.05	0.17	0.52	1.63	0.55	1.72	1.12	3.51	0.56	7.20	12.86
75	M ₂₁	0.26	0.06	0.21	0.22	0.85	0.47	1.81	0.75	2.87	0.45	4.90	10.89
76	M ₂₂	0.24	0.04	0.16	0.01	0.04	0.49	2.04	0.54	2.25	0.61	8.30	13.61
77	BM ₀	0.36	0.04	0.12	0.28	0.78	0.36	1.00	0.68	1.89	0.19	2.00	10.53
78	BM ₁	0.39	0.09	0.24	0.61	1.56	0.63	1.62	1.33	3.42	0.49	5.90	12.04
79	BM ₂	0.25	0.06	0.23	0.02	0.08	0.60	2.40	0.68	2.71	0.22	2.20	10.00
80	BM ₃	0.22	0.04	0.20	0.28	1.27	0.21	0.95	0.53	2.42	0.51	6.30	12.35
81	BM ₄	0.29	0.03	0.10	0.54	1.86	0.34	1.17	0.91	3.13	0.24	2.40	10.00
82	BM ₅	0.28	0.06	0.22	0.39	1.39	0.43	1.54	0.88	3.15	0.76	11.80	15.53
83	BM ₆	0.29	0.03	0.12	0.43	1.48	0.48	1.66	0.94	3.26	0.69	9.80	14.20
84	BM ₇	0.29	0.05	0.19	0.41	1.41	0.49	1.69	0.95	3.29	0.66	9.10	13.79
85	BM ₈	0.29	0.03	0.11	0.49	1.69	0.39	1.34	0.91	3.14	0.58	7.40	12.76
86	BM ₉	0.35	0.08	0.22	0.59	1.69	0.54	1.54	1.21	3.45	0.54	6.60	12.22
87	BM ₁₀	0.27	0.08	0.28	0.51	1.89	0.28	1.04	0.87	3.21	0.42	4.20	10.00
88	BM ₁₁	0.31	0.02	0.07	0.56	1.81	0.42	1.35	1.00	3.24	0.60	8.20	13.67
89	BM ₁₂	0.25	0.04	0.17	0.31	1.24	0.32	1.28	0.67	2.69	0.68	9.90	14.56
90	BM ₁₃	0.26	0.07	0.25	0.42	1.62	0.38	1.46	0.87	3.33	0.73	10.80	14.79
91	BM ₁₄	0.24	0.05	0.20	0.21	0.88	0.26	1.08	0.52	2.15	0.76	12.30	16.18
92	BM ₁₅	0.23	0.03	0.12	0.51	2.22	0.47	2.04	1.01	4.38	0.52	6.20	11.92
93	BM ₁₆	0.25	0.04	0.17	0.48	1.92	0.56	2.24	1.08	4.33	0.38	3.70	9.74

* Enzyme activity (IU) : μ moles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

Baharuddin *et al.* (2010) reported that single isolated bacteria species was successfully isolated from Empty Fruit Bunch (EFB)-Palm Oil Mill Effluent (POME) compost at thermophilic stage. Restriction fragment length polymorphism profiles of the DNAs coding for the 16S rRNAs with the phylogenetic analysis showed that the isolated bacteria from EFB-POME thermophilic compost gave the highest homology (99%) with similarity to *Geobacillus pallidus*. The strain was spore forming bacteria and able to grow at 60°C with pH 7.

Chang *et al.* (2009) isolated five strains of thermophiles from processed *Brassica* waste, and the hydrolytic activity on various cellulosic biomass substrata and their temperature profiles were determined. These strains were identified as *Thermoactinomyces* and *Bacillus* spp. Maximal cellulase activity corresponded to 2.3 IU/ml of enzyme. Julio *et al.* (2002) isolated 87 bacterial strains among which five strains were selected for their ability to produce either celullases or xylanases and were cultivated on soyabean (*Glycine max*) residue. *Bacillus subtilis* BL62 showed the highest specific cellulase activity, 1.08 IU/mg protein within 24 h of growth. Whereas, *Bacillus subtilis* BL53 showed the highest specific activity for xylanase, 5.19 IU/mg protein within 72 h of cultivation.

New substrates, 2-(2'-benzothiazolyl)-phenyl (BTP) cellooligosaccharides with degree of polymerization (d.p) 2-4(BTPG2-4) were synthesized for the screening of microbial cellulolytic activity in plate assays. The substrates were very efficient that was shown for several cellulolytic bacteria, including yeast-like isolates from Kamchatka hot springs. Three tested bacterial strains and eighteen of 30 of the yeast isolates showed ability to degrade cellulose with cellobiohydrolase, β -glucosidase and endo-cellulase activities measured with standard substrates. The structures of 2-(2-benzothiazolyl)-phenyl oligosaccharides were solved by NMR and mass spectrometry. The usefulness of the 2-(2-benzothiazolyl)-phenyl substrates were also shown during purification of the *B. polymyxa* cellulolytic complex, which consists of at least three types of the

enzymes: cellobiohydrolase, endo- β -D-glucanase and β -glucosidase by Ivanen *et al.* (2009).

Depending upon highest cellulase activity of BM-1 and cellulase free xylanase activity of SH8 and SH0 were selected for further enzyme production and optimization study.

4.1.4 Screening of Hypercellulolytic and Hyperxylanolytic Fungi from Compost

Table 5 depicted the screening of hypercellulolytic and hyperxylanolytic fungi from compost. It was found that *R. oryzae* W2 was the highest cellulase producer (4.20 IU/ml) i.e. FPase (0.54 IU/ml), CMCase (1.61IU/ml) and β -glucosidase (2.05 IU/ml) followed by *T. longibrachiaetim* T2 (3.91 IU/ml) i.e. FPase (0.48 IU/ml), CMCase (1.52 IU/ml) and β -glucosidase (1.91 IU/ml), respectively. The lowest cellulase activity was shown by *Pythium* sp. S2 i.e. (1.69 IU/ml) while other isolates produced cellulase between these two extremes. As for xylanase enzyme was concerned same trend was observed for highest enzyme producers i.e. xylanase activity was also found maximum in *R. oryzae* W2 i.e. (15.56 IU/ml) followed by *T. longibrachiaetim* T2 (14.87 IU/ml) and the least enzyme activity was shown by *Pythium* sp. S2 i.e. 6.23 IU/ml.

Kar *et al.* (2006) isolated a xylanase producing fungi from soil and identified as *Trichoderma reesei* SAF3. Maximum growth of the organism was found at 48 h under submerged condition in xylan containing enriched medium, whereas highest enzyme production (4.75 IU/mL) was recorded at 72h. No detectable cellulase activity was noted during whole cultivation period.

Chidi *et al.* (2008) isolated *Aspergillus terreus* UL 4209 strain, from soil of South Africa and were used to produce an extracellular cellulase-free xylanase in shake flask cultures containing oat spelt and/or birchwood xylan. Maximum xylanase activity (35 IU/ml) was observed after 96 h at 35°C and pH 6 in 1% oat spelt xylan. Khan *et al.* (2003) isolated about 104 different strains of *Aspergillus niger* from soil collected from different areas of Lahore by serial dilution method on xylene agar malt extract medium. The cultures were incubated at 30°C for 3-5

Table 5. Screening of fungal isolates for hypercellulase and hyperxylanase production

S. no.	Isolate name	Protein (mg/ml)	FPase activity		CMCase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)		Protein (mg/ml)	Xylanase	
			Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity		Enzyme activity	Specific activity
1.	T2	0.49	0.48	0.98	1.52	3.10	1.91	3.90	3.91	7.98	0.52	14.87	28.60
2.	W2	0.53	0.54	1.02	1.61	3.04	2.05	3.87	4.20	7.92	0.48	15.56	32.42
3.	S1	0.43	0.34	0.79	1.29	3.00	1.70	3.95	3.33	7.74	0.37	11.62	31.41
4.	S2	0.39	0.10	0.26	0.75	1.92	0.84	2.15	1.69	4.33	0.47	6.23	13.26
5.	M1	0.46	0.31	0.67	1.00	2.17	1.23	2.67	2.54	5.52	0.42	12.21	29.07
6.	M2	0.51	0.40	0.78	1.29	2.53	1.89	3.71	3.58	7.02	0.36	13.93	38.69
7.	K1	0.40	0.27	0.68	0.75	1.88	1.17	2.93	2.19	5.48	0.41	6.76	16.49
8.	K2	0.34	0.23	0.68	0.86	2.53	1.10	3.24	2.19	6.44	0.33	7.90	23.94
9.	K3	0.44	0.43	0.98	1.38	3.14	1.85	4.20	3.66	8.32	0.49	13.26	27.06

* Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

days for maximal sporulation. Similarly, *Aspergillus* strains were screened for xylanase production by submerged fermentation. Xylanase activity ranged from 30-225 IU/ml and dry cell mass from 6.48-26.56 g/l. The mycelia dry weight was 9.54 g/l while mycelia were small in size and rounded in shape.

Jahangeer *et al.* (2005) isolated 115 fungal cultures from environmental sources including soil, air and infected plant, 78 (67.83%) were found to possess cellulose degrading ability. Cellulytic fungi belonged to *Aspergillus* sp., *Trichoderma* sp., *Fusarium* sp., *Alternaria* sp., *Penicillium* sp., and *Rhizopus* sp. Cellulase production by fungi was measured by using enzyme assays. Highest yield of enzyme was noted at 37°C while maximum activity in the range of pH 4 -4.8. Kinetics of enzyme production was also studied on selected *Aspergillus* isolates and mostly high enzyme production was observed after 7 days.

Based on highest cellulase and xylanase activity, fungal isolate W2 and T2 were screened for further optimization under solid state fermentation.

4.2 PHENOTYPIC AND GENOTYPIC IDENTIFICATION OF SCREENED HYPER CELLULOLYTIC AND XYLANOLYTIC MICROORGANISMS

4.2 Phenotypic and Genotypic Identification of Screened Hypercellulolytic and Xylanolytic Bacteria

4.2.1 Phenotypic Identification of Bacterial Isolates

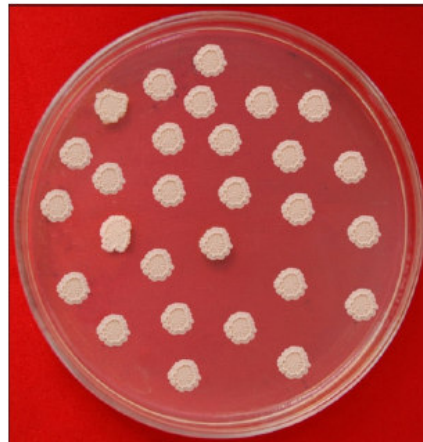
Initially, the bacterial isolates, SH8, SH0 and BM-1 were tentatively identified on the basis of their phenotypic and biochemical characteristics in our research laboratory (Plate 1, 2 and 3). On the basis of phenotypic and biochemical characters SH8, SH0 and BM-1 were placed under the group of Genus *Bacillus* sp. as per Bergey's Manual of Determinative Bacteriology (7th Edn.).

4.2.2 Genotypic Characterization

These three strains were identified at genomic level by using 16S rRNA gene technique. Genomic DNA of both isolates was isolated by using DNA



a



b

Plate 1. Morphology of *Bacillus amyloliquifaciens* SH8 (24 h)



a



b

Plate 2. Morphology of *Bacillus tequilensis* SH0 (24h)

purification kit (Banglore Genei, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The genomic DNA obtained from SH8 isolate had a concentration of 41 ng/ μ l, SH0 72 ng/ μ l, while genomic DNA obtained from BM-1 had a concentration 140 ng/ μ l. The isolated genomic DNA was used in PCR to amplify small subunit of 16S rRNA using universal primer having product size of approximately, 1500 bp. The PCR product so obtained after amplification was visualized using ethidium bromide on 2% agarose gel (Plate 4). Amplified PCR products were purified and got sequenced by the services provided by Bioserve, India. Pvt. Ltd to confirm the results.

4.2.2.1 Nucleotide Sequencing

Following sequences of isolates SH8, SH0 and BM-1 were obtained after sequence analysis.

Sequence of SH8

Forward Primer

CGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTT
AGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCC
GGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAG
GTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGT
ACCTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCT
GATGTGAAAGCCCCCGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACCTTGAG
TGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGA
GGAACACCAAGTGGCGAAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAA
AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
GCC

>P1_ub3 sequence exported from P1_ub3_A05_2011-07-15.ab1

GGAAGGCTGGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTG
CTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGAC
TGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTT
CAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
TTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTTCAATAGGGCG
GTACCTTGACGGTACCTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCG

GTTCCTTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAAACT
GGGGAACCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAGGAACACCAAGTGGCGAAAGCGACTCTCTGGTCTGTAAGTACGCT
GAGGAGCGAAGCGTGTGGAGCGAACAGATTAGATACCCTGGTAGTCACGCGTAACG
ATGATGCTAGTGCTAAGGGGTGTCGCCCTATGCTGCAGCTACGCAATTAAGTCACTA
CGCCCTGGGGGAG

Reverse primer

>PRIMER2_UB1 sequence exported from PRIMER2_UB1_D09_2011-09-12.ab1

CATGCAAGTTCGAGCGGACAGATGGAAGCCTGGCTTCCTTGATGTAAGCGGCGGACG
GTGAGTACAACGTGGTAACTGCTGTAAGACTGGGATAACTCCGGGGAAACCTGGGGC
TAATACCGGGATGGTTGTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTA
CCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTA
GGGAAGAACAAGTACCGTTTCAATAGGGCGGTACCTTGACGGTACCTAACCAGAAA
GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC
GGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC
CCGGCTCAACCGGGGAGGGTCATTGGAAGTGGGGAACCTGAGTGCAGAAGAGGAG
AGTGGAATTCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAAGTGG
CGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGGGGAGCGA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGG
GGTTTCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGT
CCC

Sequence of SH0

Forward Primer

TGGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGA
TGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAA
CTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATA
AAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATC
GTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTTCAATAGGGCGGTACCTTGAC
GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTGTCCGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTG
AGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTG
GAGGAACACCAAGTGGCGAAAGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGA
AGCGTGTGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTTCCGCCCTTAGTGCTGCAGCTA
ACGCATTAAGCACTCCG

>P1_ub4 sequence exported from P1_ub4_B05_2011-07-15.ab1

GCAAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTG
CTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGAC
TGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTT
CAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCT

AGTTGGTGAGGTAAACGGCTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
TTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTTCAATAGGGCG
GTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
TAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCG
GTTTCTTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAACT
GGGGAACCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAGGAACACCAAGTGGCGAAGCGACTCTCTGGTCTGTAAGTACGCTG
AGAGCGAAGCGTGGGGAGCGAACAGATTAGATACCCTGGTAGTCCCACGCCGTAA
CGATGAATGCTAAGTTGTTAGGGGGTTGCCGGCCCCCTTAGTTGCCTGCCAGGCCTA
ACCGCCATT

Reverse Primer

>PRIMER2_UB3 sequence exported from PRIMER2_UB3_H09_2011-09-12.ab1

GCTTGGCTCCTGATGATAGCGGGCGGGACGGGGTGAGTAAACACGTGGGGTAACCTG
GCTGTAAGACTGGGATACTCCGGGGAAC TGGGGCTAATACCGGGATGGTTGTTGAAC
CGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGC
GCATAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTTCAA
TAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTC
GCAGGCGGTTTTCTTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATT
GGAACTGGGGAACCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAAGTGGCGAAGGCGACTCTCTGGTCTGTAAC
TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTCCGCCCTTAGTGCTGCAGC
TAACGCATTAAGCATCCGCCTGGGGAGTACCGTCC

Sequence of BM-1

Forward Primer

>PRIMER1_UB6 sequence exported from PRIMER1_UB6_A10_2011-09-12.ab1

CGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTT
AGCGGCGGACGGGTGAGTAACACGTGGGTAACTGCCTGTAAGACTGGGATAACTCC
GGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAG
GTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTTCAATAGGGCGGTACCTTGACGGT
ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTTCTTAAGTCT
GAGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAACTGGGGAACCTTGAG
TGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGA
GGAACACCAAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAAA
GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAAGTGTTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCATCCG

Reverse Primer

>PRIMER2_UB6 sequence exported from PRIMER2_UB6_A10_2011-09-12.ab1

```
AATGACGTGCCTTGCACTGATTTTCAACAATGAAGCGAGTGGCGAACTGGTGAGTAA
CACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGGTGCTAATAC
CGTATAACAACAAAATCCGCATGGATTTTGTGTTGAAAGGTGGCTTCGGCTATCACTTC
TGGATGATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAGAGGCCCACCAAGACGATG
ATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAA
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGA
GCAATGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTAAAGAAG
AACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGGCGGTTTAAAGTCTGATGTGAAAGCCTTCGGCTTA
ACCGGAGAAGTGCATCGGAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAAC
TCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCG
GCTGTCTAGTCTGTAAGTACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTA
GATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTGGAGGGTTCCGC
CCTTCAGTGCTGCAGCTAACGCATTAAGCATCCGCTGGGGAGACGACCCAC
```

Sequences of three isolates so obtained were submitted to NCBI database and matched with already existing sequences. Sequence similarity search (mBLAST, NCBI) for the SH8 showed 99 % homology with nucleotide sequence of *Bacillus amyloliquefaciens* strain XS-1 with accession no. JX129360.1 (Table 6), whereas SH0 showed 98 % species specific alignment with *Bacillus tequilensis* strain VITJAAM2 with accession no. JX307679.1 (Table 7) while BM-1 showed 99 % homology with nucleotide sequence of *Bacillus subtilis* AP254 with accession no. JX094283.1 (Table 8).

The 16SrRNA sequences of these isolates have been registered to genebank databases. *Bacillus amyloliquefaciens* SH8 is registered under the accession number JX29360.1 whereas *Bacillus tequilensis* SH0 is registered under the accession number JX129359.1 and *Bacillus subtilis* BM-1 is registered under the accession number JX129361.1 (Table 6, 7 and 8). Phylogenetic trees of *Bacillus amyloliquefaciens* SH8 JX29360.1, *Bacillus tequilensis* JX129359.1 SH0 and *Bacillus subtilis* BM-1 JX129361.1 with respect to other *Bacillus* sp. bacteria as inferred by neighbor joining method have been presented in (Fig 3, 4 and 5). Fig 6 depicted the phylogenetic relationship among SH8, SH0 and BM-1.

Table 6. Sequence alignment using BLAST and genotyping of finally screened SH8 by 16 S rRNA gene Technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
<u>JX129360.1</u>	<i>Bacillus amyloliquefaciens</i> strain SH8 16S ribosomal RNA gene, partial sequence	<u>1576</u>	1576	100%	0.0	100%
<u>JX069970.1</u>	<i>Bacillus amyloliquefaciens</i> strain XS-1 16S ribosomal RNA, partial sequence	<u>1568</u>	1568	100%	0.0	99%

Table 7. Sequence alignment using BLAST and genotyping of finally screened SH0 by 16 S rRNA gene Technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
<u>JX129359.1</u>	<i>Bacillus tequilensis</i> strain SH0 16S ribosomal RNA gene, partial sequence	<u>1604</u>	1604	95%	0.0	100%
<u>JX307679.1</u>	<i>Bacillus tequilensis</i> strain VITJAAM2 16S ribosomal RNA gene, partial sequence	<u>1504</u>	1504	95%	0.0	98%

Table 8. Sequence alignment using BLAST and genotyping of finally screened BM-1 by 16 S rRNA gene Technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
<u>JX129361.1</u>	<i>Bacillus subtilis</i> strain BM-1 16S ribosomal RNA gene, partial sequence	<u>1568</u>	1568	95%	0.0	100%
<u>JX094283.1</u>	<i>Bacillus subtilis</i> strain AP254 16S ribosomal RNA gene, partial sequence	<u>1563</u>	1563	95%	0.0	99%

In literature also genera specific identification of cellulase and xylanase producing bacteria have been done on the basis of their physiological and biochemical characteristics as described by Bergay's manual of determinative bacteriology while species specific confirmed identification is mediated using PCR with universal primers and species specific primer. The Bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains. As is well known that 16S ribosomal RNA is a

component of the 30S small subunit of ribosomes. It is approximately 1.5kb (or 1500 nucleotides) in length. The 16S rRNA gene is used for phylogenetic studies because it is highly conserved between different species of bacteria and archaea and always yields confirmed identification. 16S rRNA gene analysis has been adapted globally as it is an authenticated approach to identify the different isolates and to characterize their morphological and phylogenetic position (Fontana *et al.*, 2005).

Kim *et al.* (2012) reported that among the isolates, three strains, SL9-9, C5-16, and S52-2, showing higher potential for practical uses were purified on carboxymethyl cellulose (CMC) agar plates and identified as *Bacillus subtilis* strains by morphological, physiological, and biochemical characterization and 16S rRNA gene analysis.

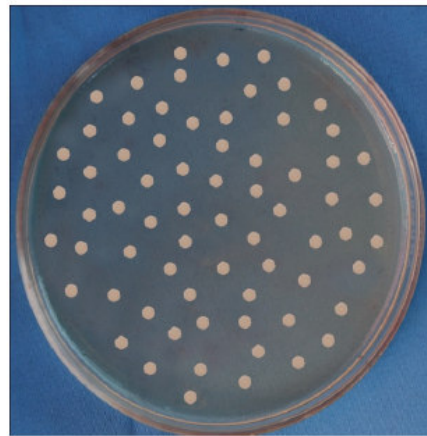
Isolate C1 was identified by Saman *et al.* (2010) through morphological, physiological and 16S rRNA characteristics. Morphological characterization included Gram staining, physiology included biochemical activity (catalase, gelatin hydrolysis, utilization of citrate), fermentation of sugars and enzyme activity. Isolate C1 was found to be endospore forming Gram +ve rod. Colonies were white and feathery in appearance and grew after 22h. Phylogenetic analysis based on 16S rRNA gene sequences indicated that C1 was identified as *Bacillus cereus*.

Mitra and Roy (2010) first carried out morphological and biochemical tests with the strain 2479. PCR amplification of 16S rRNA gene sequence was done in 0.8% agarose gel electrophoresis~1500bp band was obtained by PCR amplification. BLASTN analysis of the sequence data revealed that strain 2479 showed closest similarity (99%) with *Bacillus* sp. JDM-2-1 (EF 584539). Strain 2479 belongs to the genus *Bacillus* by 16S rRNA gene sequence.

Baharuddin *et al.* (2010) conducted morphological as well as genetic analysis of microbes isolated from compost. Total genomic DNA were extracted from approximately 1g of mixed compost and amplified by using PCR primers. The PCR products were subsequent to identify the nearest relatives of 16S rRNA



a



b

Plate 3. Morphology of *Bacillus subtilis* BM1 (24 h)

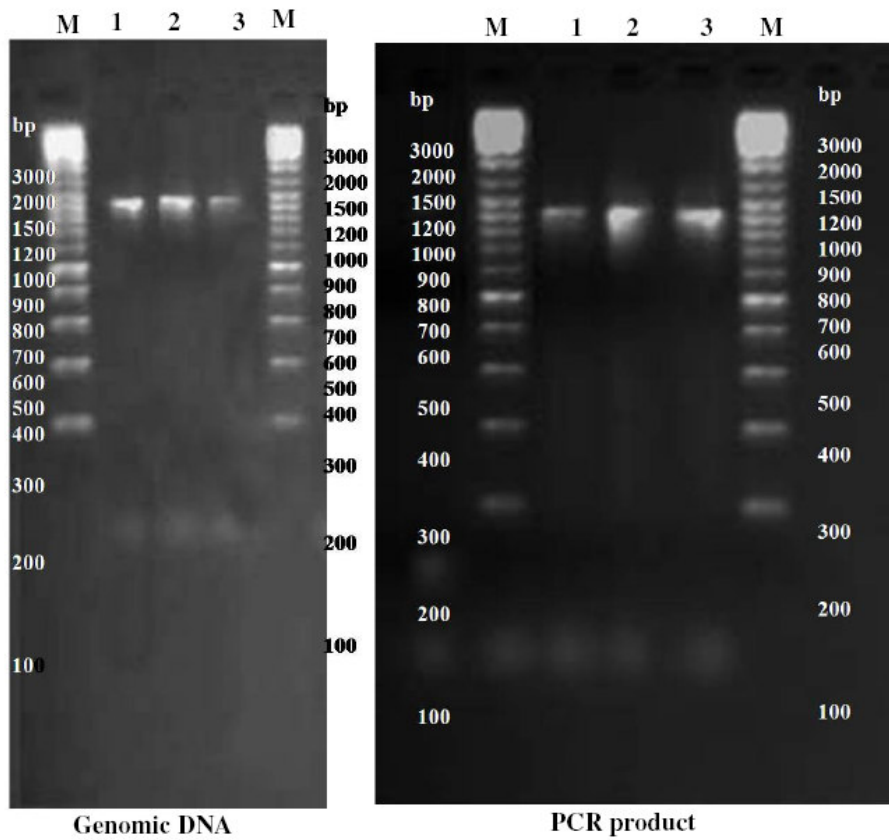


Plate 4. Molecular identification of screened isolates by 16 S rRNA gene technique

Lane 1. = *Bacillus Subtilis* BM-1
 Lane 2. = *Bacillus amyloliquifacins* SH8
 Lane 3. = *Bacillus tequilensis* SH0
 M = Marker

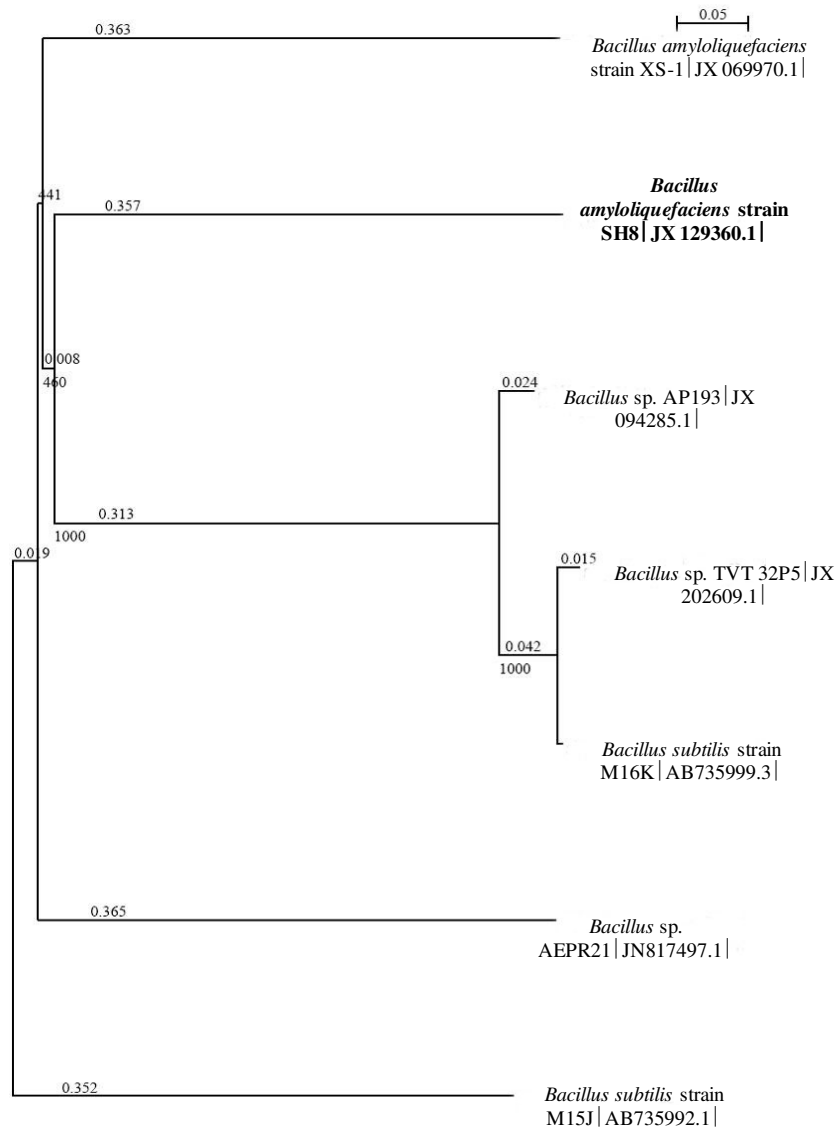


Fig. 3 Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *B. amyloliquefaciens* SH8 among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

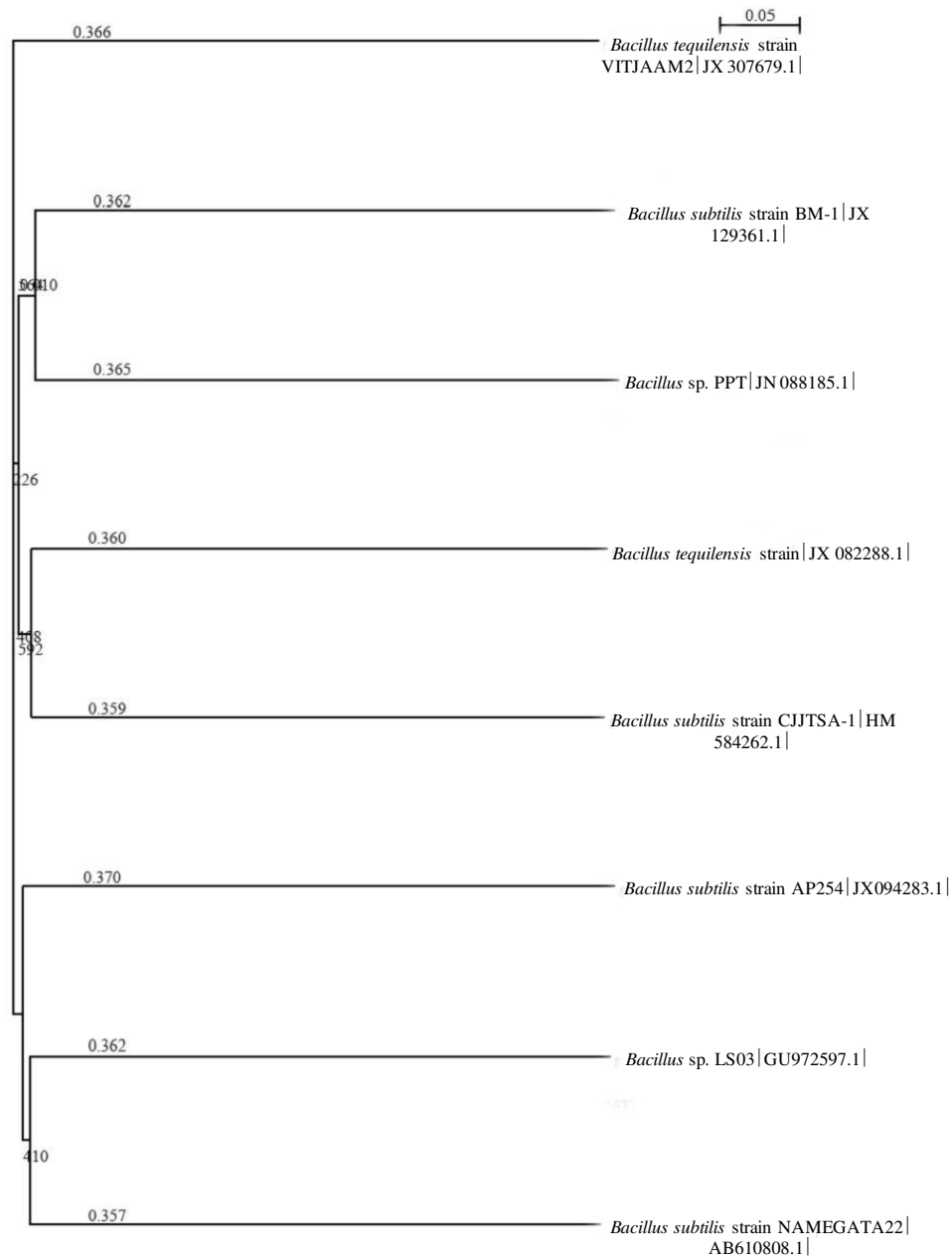


Fig. 4 Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *B. tequilensis* SH0 among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

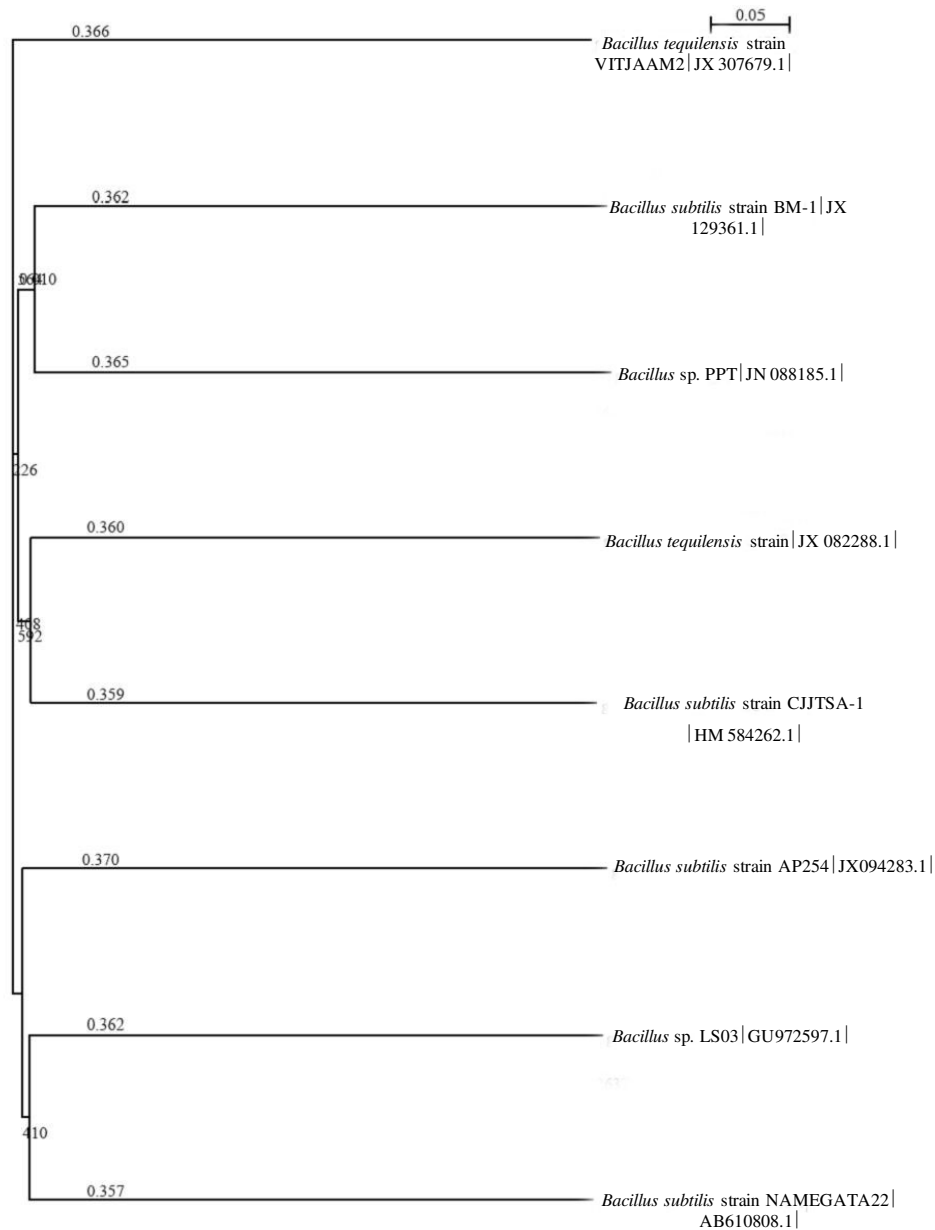


Fig.5 Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *B. subtilis* BM-1 among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

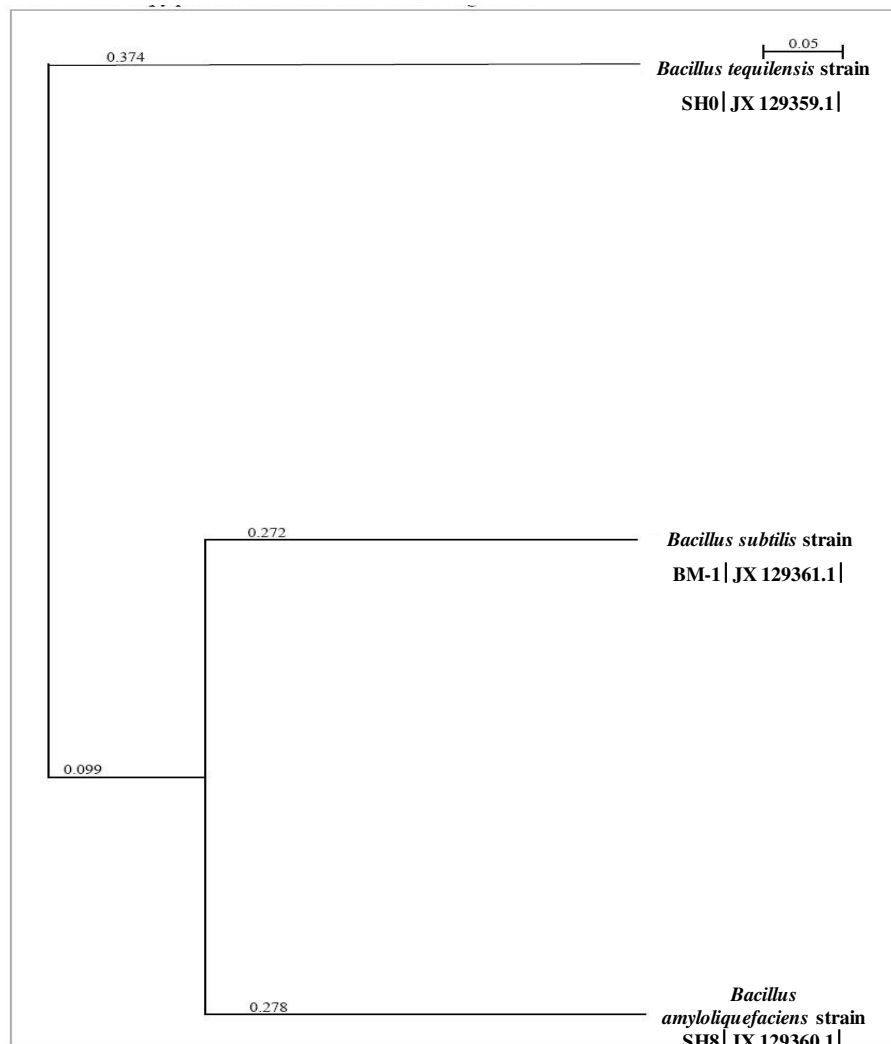


Fig. 6 Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *B. amyloliquefaciens* SH8, *B. tequilensis* SH0 and *B. subtilis* BM-1 based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

genes. The PCR product showed highest homology (99%) to *Geobacillus pallidus*.

4.2 PHENOTYPIC AND GENOTYPIC IDENTIFICATION OF SCREENED HYPERCELLULOLYTIC AND XYLANOLYTIC FUNGI

4.2.3 Identification of Screened Fungi

4.2.3.1 Phenotypic Identification of Fungal Isolates

Primarily the fungal isolates, T2 and W2 were tentatively identified on the basis of their phenotypic and biochemical characteristics in our research laboratory (Plate 5 and 6). On the basis of phenotypic and biochemical characters isolates T2 and W2 were placed under the group of Genus *Trichoderma* sp. and *Rhizopus* sp.

4.2.3.2 Genotypic characterization

Both the fungal isolates were identified at genomic level by using 18S rRNA gene technique. Genomic DNA of both isolates was isolated by using DNA purification kit (Plant Genomic DNA extraction kit, Quagen Make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The genomic DNA obtained from T2 isolate had a concentration of 50 ng/ μ l while genomic DNA obtained from W2 had a concentration 220 ng/ μ l. The isolated genomic DNA was used in PCR to amplify small subunit of 18S rRNA using universal primer having product size of approximately 650 bp. The PCR product so obtained after amplification was visualized using ethidium bromide on 2% agarose gel (Plate 7). Amplified PCR products were purified and got sequenced by the services provided by Bioserve, India. Pvt. Ltd to confirm the results.

4.2.3.2.1 Nucleotide Sequencing

Following sequences of isolates T2 and W2 were obtained after sequence analysis.

Sequence of T2

Forward Primer

>ITS1_UHF1 sequence exported from ITS1_UHF1_H09_2011-06-07.ab1

GTGTGCGAGTTAGCTCCCAACCCCATGTGAACGTTACCAATCTGTTGCCTCGGCGGG
ATTCTCTTGCCCCGGGCGCGTCGCAGCCCCGGATCCCATGGCGCCCGCCGAGGACC
AACTCCAAACTCTTTTTTCTCTCCGTCGCGGCTCCCGTCGCGGCTCTGTTTTATTTTT
GCTCTGAGCCTTTCTCGGCGACCCTAGCGGGCGTCTCGAAAATGAATCAAAACTTTC
AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCC
AGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCCTCGAACCCCTCCGGGG
GGTCCGGCGTTGGGGA

>ITS4_UHF1 sequence exported from ITS4_UHF1_B10_2011-06-07.ab1

AGAGAGGGTCATGATACCATACAAAATATATACGCAGGGCGGGCCCTCGGGGAGAA
AAAAAGAGTTGGGAGTGGTCTTTTCGACGGGCGCCCATGGATCCGGGGGCTGCGACGCG
CCCGGGGCAAGAGAATCCCGCCGAGGCAACAGATTGGTAACGTTACATTGGGGTTT
GGGAGTTGTAAACTCGGAAATTTCCGTAGGGGTGACCTGCGGAGGGATCATTACCG
AGTTTACAACCTCCCAAACCCCAATGTGAACGTTACCAATCTGTTGCCTCGGCGGGATT
CTCTTGCCCCGGGCGCGTCGCAGCCCCGGATCCCATGGCGCCCGCCGAGGACCAAC
TCCAAACTCTTTTTTCTCTCCGTCGCGGCTCCCGTCGCGGCTCTGTTTTATTTTTGCTC
TGAGCCTTTCTCGGCGACCCTAGCGGGCGTCTCGAAAATGAATCAAAACTTTCAACA
ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTA
TTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCCTCGAACCCCTCCGGGGGGTC
GGCGTTGGGGATCGGCCCTCACCGGGCCGCCCGGAAATACAGTGGCGGTCTCGCC
GCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCACCGGGAGCGCGGCGCGGCCACA
GCCGTAACACCCCAAACTTCTGAAATGTGACCTCGGATCCAGGTAGATTCCG

>UHF1

CCTGCGGAGGGATCATTACCGAGTTTACAACCTCCCAAACCCCAATGTGAACGTTAC
CAATCTGTTGCCCTCGGCGGGATTCTCTTGCCCCGGGCGCGTCGCAGCCCCGGATCCCA
TGCGCCCCCGCCGAGGACCAACTCCAAACTCTTTTTTCTCTCCGTCGCGGCTCCCGT
CGCGGCTCTGTTTTATTTTTGCTCTGAGCCTTTCTCGGCGACCCTAGCGGGCGTCTCG
AAAATGAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG
CAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCA
ACCCCTCGAACCCCTCCGGGGGGTTCGGCGTTGGGGATCGGCCCTCACCGGGCCGCC
CCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCG
CACCGGGAGCGCGGCGCGGCCACAGCCGTAAACACCCCAAACTTCTGAAATGTGA
CCTC

Sequence of W2

Forward Primer

>ITS1_UHF2 sequence exported from ITS1_UHF2_A10_2011-06-07.ab1

TTTTTTTGGTAAAGCGCCTTACCTTAGGGTTTCCTCTGGGGTAAGTGATTGCTTCTACA
CTGTGAAAATTTGGCTGAGAGACTCAGACTGGTCATGGGTAGACCTATCTGGGGTTT
GATCGATGCCACTCCTGGTTTCAGGAGCACCCCTCATAATAAACCTAGAAATTCAGT
ATTATAAAGTTTAATAAAAAACAACCTTTTAACAATGGATCTCTTGGTTCTCGCATCGA
TGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCAGTGAATCATCG

AGTCTTTGAACGCAGCTTGCACCTCTATGGTTTTCTATAGAGTACGCCTGCTTCAGTA
 TCATCACAAACCCACACATAACATTTGTTTATGTGGTAATGGGTCGCATCGCTGTTTT
 ATTACAGTGAGCACCTAAAATGTGTGTGATTTTCTGTCTGGCTTGCTAGGCAGGAATA
 TTACGCTGGTCTCAGGATCTTTTTCTTTGGTTTCGCCCAGGAAGTAAAGTACAAGAGTA
 TAATCCAGCAACTTTCAAACCTATGATCTGAAGTCAGGTGGGATTACCCGCTGAACCT
 AAGCATATCAAAACCGGAAAAAGAAAAA

Reverse primer

>ITS4_UHF2 sequence exported from ITS4_UHF2_C10_2011-06-07.ab1

TCACCCACTAGGTTACTCCGCACTTGTTAACTTTTTATCATGCGAGAATCCAAAATCC
 ATGTAAAGTGTTTTTAATAAACTTTATAACTGAATTCTAGTTTATATGAAGGGTGC
 TCCTGAAACCCGGAGTGATCGATCAAACCCAGATAGTCTACCCATGACCCGTCTG
 AGTCTCTCAGCCAAATTTTACAGTGTAGAAGCAATCACTTACCCAGAGGAAACCC
 TAAGGTAAGGCGCTTTAACATAATTAATGATTCCTTTCTTCCGTAAGGGGTGACCTGC
 GGAAGGATCATTAAATTATGTTAAAGCGCCTTACCTTAGGGTTTCCTCTGGGGTAAGTG
 ATTGCTTCTACACTGTGAAAATTTGGCTGAGAGACTCAGACTGGTCATGGGTAGACC
 TATCTGGGGTTTGATCGATGCCACTCCTGGTTTCAGGAGCACCCCTCATAATAAACCT
 AGAAATTCAGTATTATAAAGTTTAATAAAAAACAACTTTTAACAAATGGATCTCTTGGT
 TCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCA
 GTGAATCATCGAGTCTTTGAACGCAGCTTGCACCTATGGTTTTCTATAGAGTACGC
 CTGCTTCAGTATCATCACAAACCCACACATAACATTTGTTTATGTGGTAATGGGTCGC
 ATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGTGTGATTTTCTGTCTGGCTTGCTA
 GGCAGGAATATTACGCTGGTCTCAGGATCTTTTTCTTTGGTTTCGCCCAGGAAGTAAAG
 TACAAGAGTATAATCCAGCAACTTTCAAACCTATGATCGAAGTCAGGGGGGATTCCCA

>UHF-2

CCTGCGGAAGGATCATTAAATTATGTTAAAGCGCCTTACCTTAGGGTTTCCTCTGGGG
 TAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGAGAGACTCAGACTGGTCATGGG
 TAGACCTATCTGGGGTTTGATCGATGCCACTCCTGGTTTCAGGAGCACCCCTCATAAT
 AAACCTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAACTTTTAACAAATGGATC
 TCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGC
 ATATTCAGTGAATCATCGAGTCTTTGAACGCAGCTTGCACCTATGGTTTTCTATAG
 AGTACGCCTGCTTCAGTATCATCACAAACCCACACATAACATTTGTTTATGTGGTAAT
 GGGTCGCATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGTGTGATTTTCTGTCTG
 GCTTGCTAGGCAGGAATATTACGCTGGTCTCAGGATCTTTTTCTTTGGTTTCGCCCAGG
 AAGTAAAGTACAAGAGTATAATCCAGCAACTTTCAAACCTATGATCTGAAGTCAGGTG
 GGATTACCCGCTGAACCTTAAGCATATCA

Sequences of both the isolates so obtained were submitted to NCBI database and matched with already existing sequences. Sequence similarity search (m BLAST, NCBI) for T2 showed 99% homology with nucleotide sequence of *T. longibrachetum* T36 with accession no. GU176487.1 (Table 9) and W2 showed 99% alignment with nucleotide sequence of *R. oryzae* strain RO-KSU-09 with accession no. HM753603.1 (Table 10).

The 18SrRNA sequences of these isolates have been lodged to genebank databases. *Trichoderma longibrachetum* T2 is lodged under the accession number JX29360.1 (Table 9) whereas *R. oryzae* W2 is registered under the

accession number JX129359.1 (Table 10). Phylogenetic trees of *Trichoderma longibrachiatum* T2 JX29360.1 and *R. oryzae* W2 JX129361.1 with respect to other *Trichoderma* and *Rhizopus* sp. as inferred by neighbor joining method have been presented in (Fig. 7 and 8).

Table 9. Sequence alignment using BLAST and genotyping of finally screened T2 by ITS 5.8S/18S rRNA gene technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
<u>JX213811.1</u>	<i>Trichoderma longibrachiatum</i> isolate T2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	<u>737</u>	737	89%	0.0	99%
<u>GU176487.1</u>	<i>Trichoderma longibrachiatum</i> strain T36 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	<u>745</u>	745	90%	0.0	99%

Table 10. Sequence alignment using BLAST and genotyping of finally screened W2 by ITS 5.8S/18S rRNA gene technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
<u>JX213812.1</u>	<i>R. oryzae</i> isolate W2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	<u>1068</u>	1068	95%	0.0	100%
<u>HM753603.1</u>	<i>R. oryzae</i> strain RO-KSU-09 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	<u>1066</u>	1066	96%	0.0	99%

The nuclear ribosomal RNA (rRNA) gene complex is a tandem repeat unit of from one to several thousand copies. This complex has several domains that evolve at varying rates (Jorgenson and Cluster 1988), and thus have different phylogenetic utilities. The 18S and 28S rRNA genes evolve relatively slowly and are useful in addressing broad phylogenetic hypotheses involving a broad range

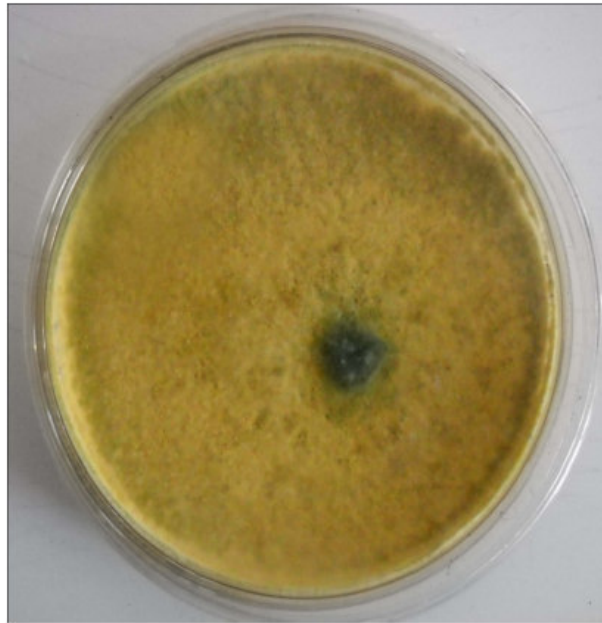


Plate 5. Morphology of *Trichoderma longibrachiatum* T2 (7 days)



a



b

Plate 6. Morphology of *Rhizopus oryzae* W2 (3 days)

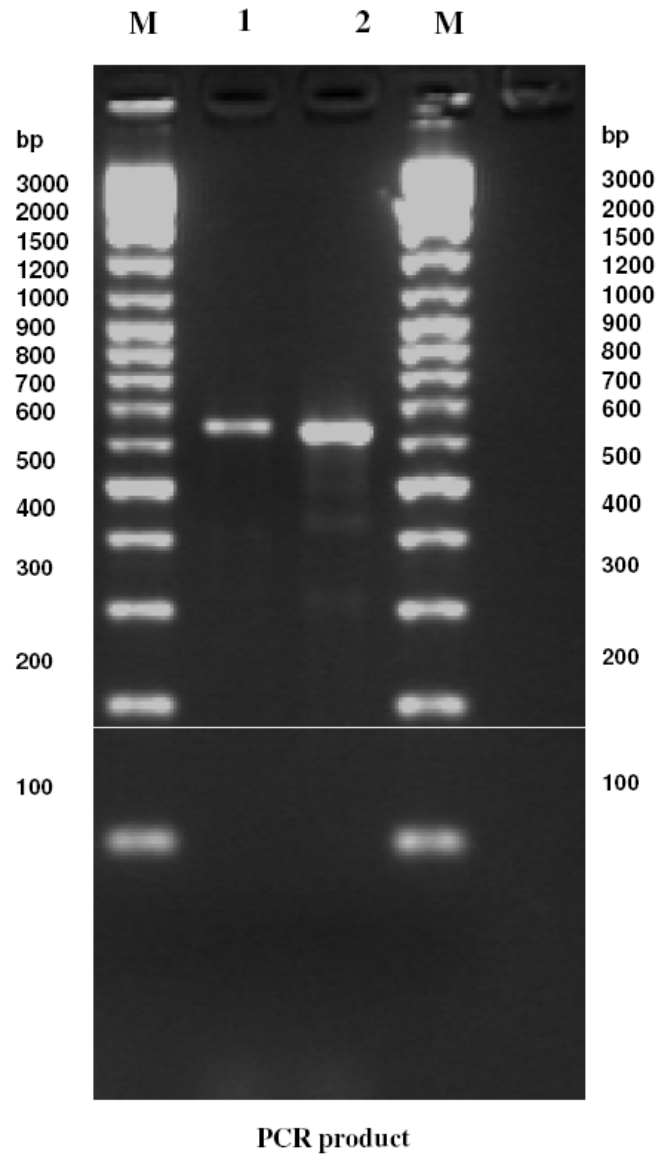


Plate 7. Molecular identification of screened isolates by ITS 5.8 S rRNA gene technique

Lane 1. = *Trichoderma longibracheatum* T2
 Lane 2. = *Rhizopus oryzae* W2
 M = Marker

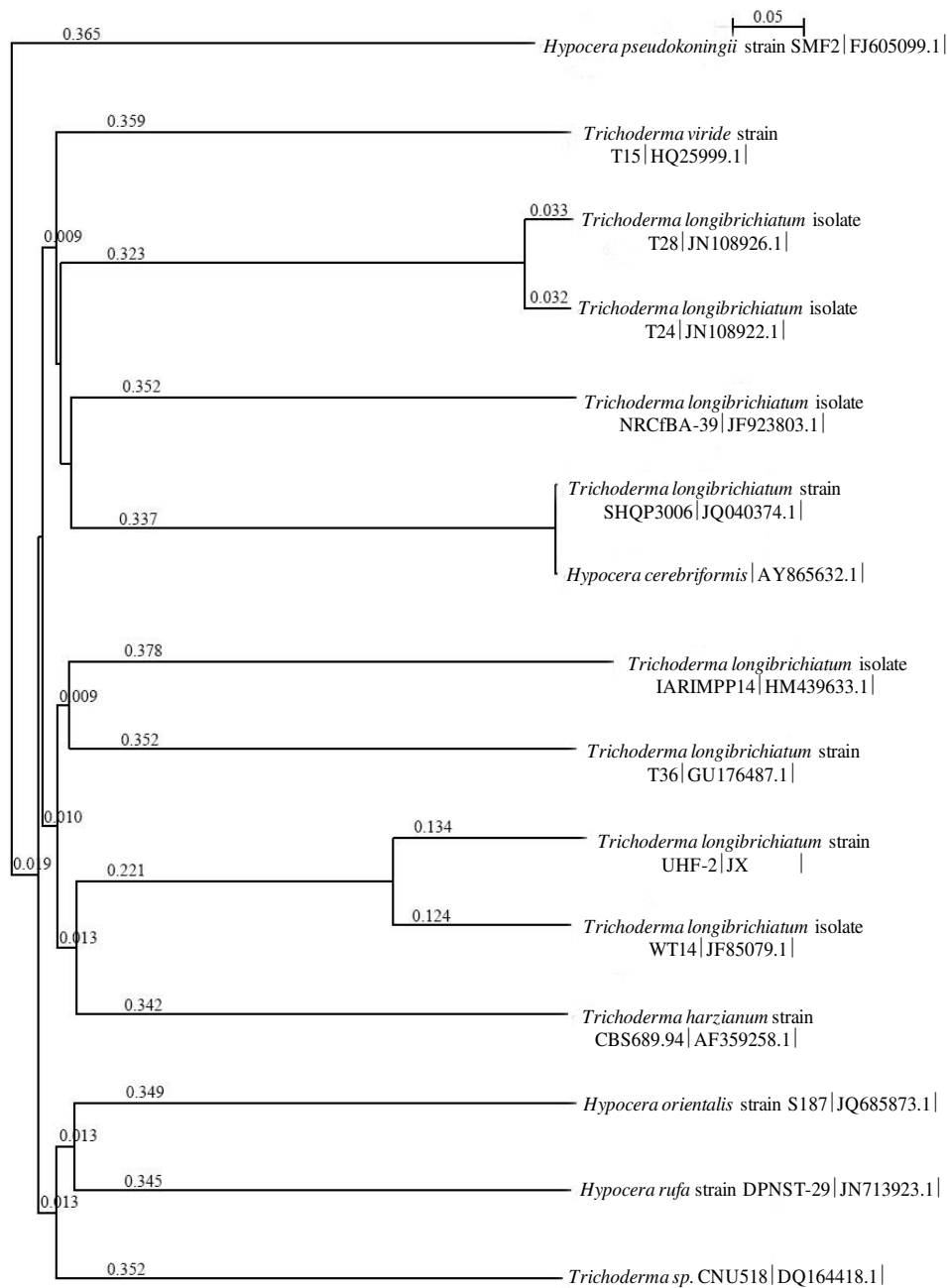


Fig. 7. Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *T. longibracheatum* among the genus *Trichoderma* based on a distance matrix analysis of 18S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

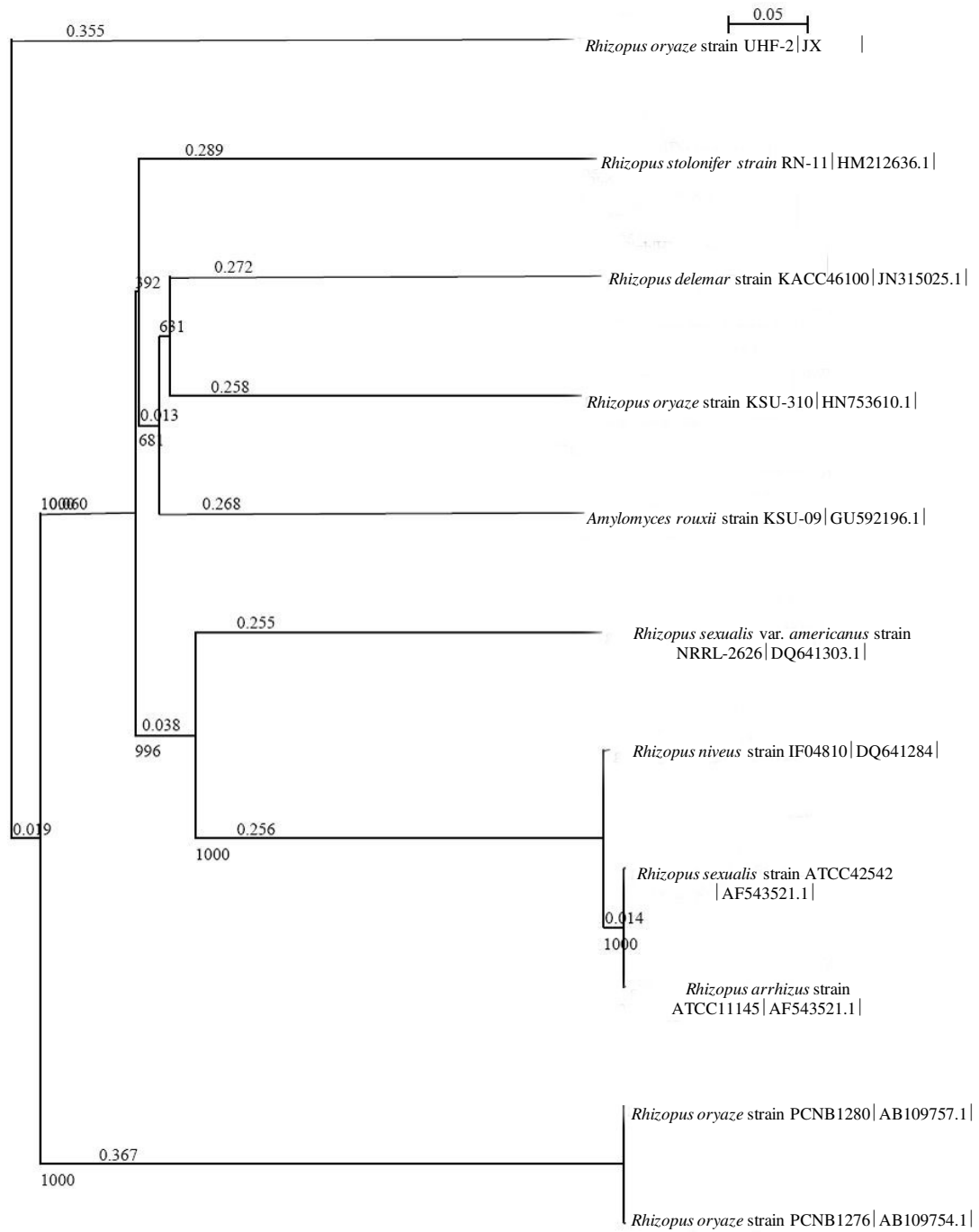


Fig. 8. Neighbor-joining tree constructed from clustal X showing phylogenetic relationship of *R. oryzae* W2 among the genus *Rhizopus* based on a distance matrix analysis of 18S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

of organisms (Bruns *et al.* 1991; Cullings, 1994; Maidak *et al.* 1997). The internal transcribed spacer (ITS) evolves relatively quickly and can be useful in determining interspecies (Jorgenson and Cluster 1988; Cullings *et al.* 1996; Vogler and Bruns 1998) and sometimes intraspecies relationships (Baura *et al.* 1992). The intergenic spacer (IGS), a noncoding region, evolves at a rate suitable for population studies (Jorgenson and Cluster 1988; Appel and Gordon 1996). Within the ITS is a largely ignored coding region, the 5.8S rRNA gene. Similar to the other coding regions, the 5.8S region evolves relatively slowly but, because of its location within the ITS, it is generally used only as an alignment tool. Recently, however, this region of DNA was shown to contain considerable phylogenetic information, particularly with respect to deep basal branches (HersHKovitz and Lewis 1996).

The ITS region includes the ITS1 and ITS2 regions, separated by the 5.8S gene, and is situated between the 18S (SSU) and 28S (LSU) genes in the nr DNA repeat unit. The large number of ITS copies per cell (up to 250) makes the region an appealing target for sequencing environmental substrates where the quantity of DNA present is low by Vilgalys R and Gonzalez (1990). The entire ITS region has commonly been targeted with traditional Sanger sequencing approaches and typically ranges between 450 and 700 bp. Either the ITS1 or the ITS2 region have been targeted in recent high-throughput sequencing studies, because the entire ITS region is still too long for 454 sequencing or other high-throughput sequencing methods. Using high-throughput sequencing, thousands of sequences can be analysed from a single environmental sample, enabling in-depth analysis of the fungal diversity by Bellemain *et al.* (2010).

Ahlawat *et al.* (2010) amplified two parents and 15 monosporus isolates of *Volvariella volvacea* in polymerase chain reaction of internal transcribed spacer (ITS) region of 5.8S rDNA, an amplicon of same length of 720 bp revealed that all the samples belonged to same species. The isolates also showed variation in enzyme activity profile with respect to exoglucanase, endoglucanase, xylanase, laccase and polyphenol oxidase.

Bakri *et al.* (2010) reported that one hundred and thirty-six fungal isolates were screened for xylanase production. Two xylanase producing isolates, FSS117 and FSS129, were identified on the basis of analyses of 5.8S gene sequencing. The closest phylogenetic neighbors according to 5.8S gene sequence data for the two isolates were *Aspergillus tubingensis* and *Aspergillus terreus*, respectively. When birchwood xylan or corn cob hulls was used as a substrate for 5 days under submerged culture cultivation, xylanase production from *A. terreus* FSS129 was 113 and 174IU/ml, respectively. pH and temperature for optimum xylanase activity were 8 and 65°C.

4.3 OPTIMIZATION OF CELLULASE AND XYLANASE BY POTENTIAL MICRORGANISMS

4.3.1 Cellulase and Xylanase from Three Potential Bacteria was Optimized Using Two Different Approaches under SmF

4.3.1.1 Classical Approach One Factor at a Time (OFAT)

4.3.1.2 Response Surface Methodology (RSM)

In industrial biotechnology, most investigations into submerged aerobic cultures, especially the development of media are conducted using shake flask cultures. This is because the scale is convenient and replication can be easily achieved. Production variables such as autoclaving procedure, inoculum size, temperature, water activity, medium composition, pH, different substrate and their concentration, incubation time and degree of mixing can be identified and their effect on fermentation productivity commonly determined by the conduct of sequential cultures where only one variable at a time is altered. Such investigations provide a basis for the manipulation of production variables in order to optimize as essential parameters to maximize the extracellular enzyme production. In the present study, the screened and identified hyperenzyme producing bacterial isolates i.e. *Bacillus subtilis* BM-1 for cellulase optimization whereas *Bacillus amyloliquefaciens* SH8 and *Bacillus tequilensis* SH0 were used for xylanase optimization.

4.3.1.1.1 Effect of Different Media

The data pertaining to effect of different media on cellulase and xylanase production enlisted in Table 11 and 12 and had revealed that highest cellulase activity was found in the Basal Salt medium (BSM) i.e. 1.52 IU/ml for *B. subtilis* BM-1 while least enzyme production 0.86 IU/ml was observed in PYC medium (Fig. 9). A perusal of the data revealed that maximum production in BSM was statistically significant over Riverys (1.33 IU/ml) followed by SU medium (1.28 IU/ml), Basic liquid mineral medium (1.14 IU/ml) and PYC (0.86 IU/ml). Similarly, the maximum xylanase activity was observed in Basal Salt medium (BSM) i.e. 22.8 IU/ml for *B. amyloliquefaciens* SH8 and 21.2 IU/ml of *B. tequilensis* SH0 which were significantly higher than others. Least enzyme production i.e. 1.62 IU/ml and 0.33 IU/ml by *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 was observed in Emersion and mineral salt medium (Fig. 10 and 11). Data pertaining to highest xylanase production in *B. amyloliquefaciens* SH8 revealed that BSM was statistically superior over Mineral salt (3.24 IU/ml), followed by TGY (2.40 IU/ml), Mandel medium (2.03 IU/ml) and Emersion medium (1.62 IU/ml), whereas also in *Bacillus tequilensis* SH0 Basal salt medium was statistically significant Mandel medium (5.84 IU/ml) followed by TGY (5.84 IU/ml), Emersion medium (2.79 IU/ml) and mineral salt medium (0.336 IU/ml).

Table 11: Effect of different media on the production of extracellular cellulase from *Bacillus subtilis* BM-1 [JX129361.1] under SmF

Media	Protein (mg/ml)	Cellulase							
		FPase activity		CMCase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
BSM	0.44	0.12	0.25	0.68	1.41	0.74	1.52	1.54	3.21
SU	0.41	0.08	0.19	0.52	1.26	0.68	1.65	1.28	3.12
Mineral Medium	0.36	0.02	0.06	0.54	1.63	0.58	1.75	1.14	3.45
Riverys	0.37	0.09	0.23	0.61	1.56	0.63	1.61	1.33	3.41
PYC	0.17	0.05	0.28	0.43	2.38	0.43	2.38	0.86	4.77
CD_{0.05}	0.047	0.0116	0.029	0.035	0.068	0.059	0.071	0.058	0.069

Enzyme activity (IU) : μ moles of reducing sugars released/min/ml of enzyme.

Specific activity : enzyme activity/mg of protein

Table 12. Effect of different media on xylanase production from *B. amyloliquefaciens* SH8 [JX129360.1] and *B. tequilensis* SH0 [JX129359.1] under SmF

Media	<i>B. amyloliquefaciens</i> SH8		<i>B. tequilensis</i> SH0	
	Protein conc. (mg/ml)	Xylanase activity	Protein conc. (mg/ml)	Xylanase activity
Emersion	0.42	1.62* (3.85)**	0.32	2.03* (6.34)**
TGY	0.63	2.40 (1.20)	0.48	2.79 (5.81)
Mandel's Medium	0.58	2.03 (3.80)	0.58	5.84 (10.07)
Mineral salt	0.12	3.24 (27.00)	0.11	0.33 (3.00)
BSM	1.23	22.80 (18.53)	1.23	21.20 (17.24)
CD_{0.05}	1.01	1.14 (1.69)	0.036	1.21 (0.94)

* Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

Medium formulation is the foremost step for designing successful laboratory experiments for yields enhancement. Medium components must satisfy the elemental requirement for cell biomass and metabolite production; hence there should be adequate energy supply for biosynthesis and cell maintaince. Nutrient concentration in the medium had influenced the production of cellulase and xylanase by the bacterial isolates. All the bacterial isolates showed maximum growth in the Basal salt medium containing ammonium sulphate (0.6%), ammomium chloride (0.1%), sodium chloride (0.05%), yeast extract (0.5%). Potassium hydrogen phosphate (0.3%), magnesium chloride and calcium chloride as growth supplements in the medium and seemed to have promoted extracellular cellulase and xylanase production. Sodium chloride present in the medium probably helped maintaining the osmotic balance of the medium while magnesium sulfate was a cofactor for a variety of metabolic reactions. Basal salt medium containing $(\text{NH}_4)_2\text{SO}_4$ and NH_4Cl as its major ingredients and both being rich source of nitrogen (Briggs *et al.*, 2005) had caused highest cellulase as well as xylanase production from bacteria. Basal medium also contained yeast extract which was the most important source of nitrogen in the medium in terms of accessibility and composition to get the higher

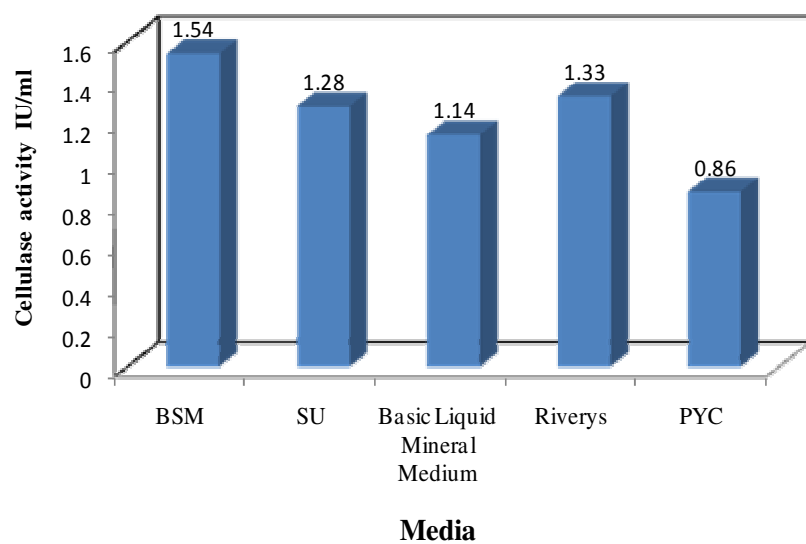


Fig. 9. Effect of media on cellulase production from *B. subtilis* BM-1 under SmF

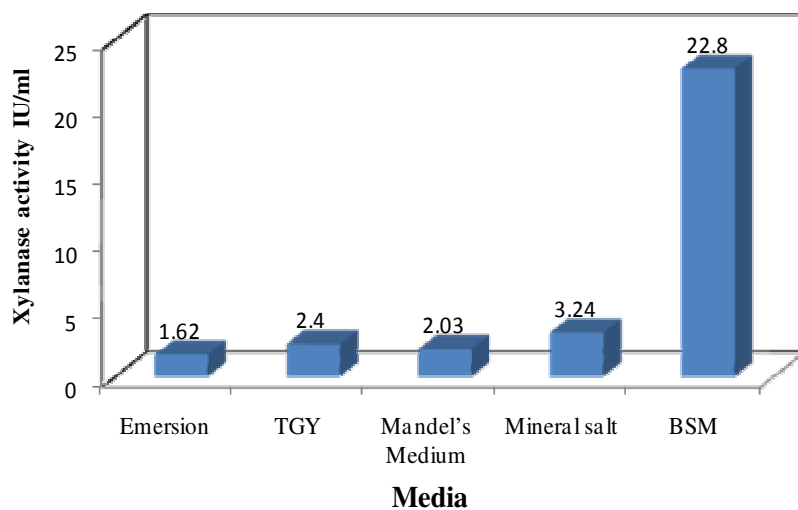


Fig. 10. Effect of media on xylanase production from *B. amyloliquefaciens* SH8 under SmF

yield of cellulase and xylanase (Sharma and Bajaj, 2005). Since nitrogen is a major constituent of protoplasm and building block of the enzymes, the above mentioned three nitrogen sources have helped directly in higher amount of enzyme synthesis.

In the present study, though effect of different nutrients has not been tested separately but the significant role of nitrogen sources on enzyme production has been already cited in literature. Ammonium sulphate has been found best for exoglucanase and endoglucanase activity (Vyas *et al.*, 2005). The CaCl_2 and MgSO_4 have stimulated growth promotory role as far as production of extracellular cellulase and xylanase is concerned.

Gupta *et al.* (2012) reported that the eight isolates of cellulose-degrading bacteria (CDB) were isolated from four different invertebrates (termite, snail, caterpillar, and bookworm) by enriching the basal salt medium with filter paper as substrate for cellulose degradation. The extracellular cellulase activities ranged from 0.012 to 0.196 IU/mL for FPC and 0.162 to 0.400 IU/mL for endoglucanase assay. All the cultures were also further tested for their capacity to degrade filter paper by gravimetric method. The maximum filter paper degradation percentage was estimated to be 65.7 for CDB 8. Sharma *et al.* (2007) recorded maximum xylanase production using basal medium supplemented with wheat bran as a carbon source by *G. thermoleovorans*.

Maximum cellulase productivity was exhibited when molasses broth medium was supplemented with cellulose as a carbon source and $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source (Shabeb *et al.*, 2010). Poorna and Prema (2007) and Chellapandi and Himanshu (2008) reported that maximum cellulase activity was obtained by *Bacillus pumilus*, *Ruminococcus albus*, etc., when ammonium sulphate was added to the production media. Similarly, peptone and tryptone have shown to promote xylanase as cited in the literature. Dhillon and Khanna (2000) recorded that the best nitrogen source for xylanase production by *B. circulans* AB16 was tryptone and also for *Geobacillus thermoleovorans* (Sharma *et al.*, 2007). Yeast extract, beef extract, peptone or soybean meal have been reported as most

balanced and conditioned nitrogen sources in terms of accessibility and composition for getting high yield of xylanase from different bacteria (Shabeb *et al.*, 2010).

Verma *et al.* (2011) observed that 30°C and pH 5.0 are the most favourable for conditions for cellulase production by *Trichoderma reesei*. FPase activity significantly increases by incorporation of whey as well as wheat starch hydrolyaste in basal salt media.

Dhillon *et al.* (2000) isolated *Bacillus circulans* AB16 from garbage dump, the highest xylanase activity was in basal medium of 55 IU/ml. *B. pumilus* showed a 3.4 fold increase in the xylanase production was achieved using the optimized culture medium consisting of (g/l): K₂HPO₄, MgSO₄.7H₂O, CaCl₂.2H₂O, NaCl, and peptone, yeast extract and wheat bran. Similarly, a thermophilic *Arthrobacter* sp. MTCC 5214 produced optimal extracellular xylanase in medium supplemented with modified salt medium of pH 9 supplemented with 0.5% birch wood was isolated from locally from a sediment sample collected from Mandovi estuary, west coast of India (Khandeparker and Bhosle, 2006). Kamble and Jadhav (2012) studied the xylanase production in Erlenmeyer flasks containing fresh basal salt medium supplemented with 1% oat spelt xylan.

4.3.1.1.2 Effect of Temperature

Effect of temperature on cellulase and xylanase production from hypercellulolytic and xylanolytic bacteria has been shown in Tables 13 and 14, respectively. Maximum cellulase titres were produced at 45°C of *B. subtilis* BM-1 (1.91 IU/ml) whereas; least cellulase production was ascertained at 65°C for both (0.4 IU/ml) as shown in Fig. 12.

B. amyloliquefaciens SH8 and *B. tequilensis* SH0 exhibited optimum temperature of 45°C (25.3 IU/ml) and (23.8 IU/ml), respectively for maximum xylanase production which are significantly higher than others. Minimum

xylanase was produced at 65°C for both of bacterial isolates (4.5 and 3.9 IU/ml) as demoed in Fig. 13 and 14.

Table 13: Effect of different temperature on the production of extracellular cellulase from *Bacillus subtilis* BM-1 [JX129361.1] under SmF

Temp.	Cellulase								
	Protein (mg/ml)	FPase activity		CMCase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
25°C	0.41	0.08	0.19	0.59	1.43	0.63	1.53	1.30	3.17
35°C	0.48	0.12	0.25	0.68	1.41	0.74	1.52	1.54	3.21
45°C	0.55	0.15	0.28	0.83	1.50	0.93	1.69	1.91	3.47
55°C	0.47	0.08	0.18	0.48	1.02	0.53	1.12	1.09	2.31
65°C	0.18	0.01	0.06	0.18	1.00	0.21	1.16	0.40	2.22
CD_{0.05}	0.057	0.0094	0.041	0.075	0.169	0.038	0.043	0.132	0.150

Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

Specific activity : enzyme activity/mg of protein

Table 14. Effect of different temperature on xylanase production from *B. amyloliquefaciens* SH8 [JX129360.1] and *B. tequilensis* SH0 [JX129359.1] under SmF

Temperature	<i>B. amyloliquefaciens</i> SH8		<i>B. tequilensis</i> SH0	
	Protein conc. (mg/ml)	Xylanase activity	Protein conc. (mg/ml)	Xylanase activity
25°C	0.59	14.16* (24.0)**	0.42	14.20 (33.80)
35°C	0.81	22.60 (27.9)	0.89	19.50 (21.90)
45°C	1.14	25.30 (22.10)	1.13	23.80 (21.00)
55°C	0.42	13.30 (31.66)	0.43	0.43 (26.00)
65°C	0.36	4.50 (12.50)	0.39	0.39 (5.80)
CD_{0.05}	0.026	1.72 (0.31)	0.032	2.01 (1.16)

* Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

Optimum temperature range obtained for potential enzyme producing bacteria in the present study has reflected their thermophilic nature, thus suggesting the strong possibility of their being an asset for commercial use.

Temperature is one of the most important parameter essential for the success of fermentation reaction. Microorganisms grow slowly at a temperature below or above the normal temperature because of reduced rate of cellular production. If the growth temperature is too high but not lethal, there may be permanent induction of target protein expression (Immanuel *et al.*, 2006). At lower temperature, substrate transport across the cells is suppressed and lowest product yield are attained. At higher temperature, the maintaince energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway resulting in maximum production (Aiba *et al.*, 1973). Incubation temperature is a critical factor in enzymatic productivity (Seyis and Aksoz, 2003). Maximum enzyme production is produced at optimum temperature and the decrease in enzyme production at lower or higher temperatures is due to the fact that at these temperatures, growth of the organisms was inhibited, causing a decrease in the synthesis of the enzymes (Simoes *et al.*, 2009). Production of more activity at optimum temperature may be due to the faster metabolic activity and increase in protein content and extracellular enzyme production in culture supernatant. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity (Willey *et al.*, 2008).

Thermophiles can tolerate high temperature by using increased interaction than non-thermotolerant organisms, because of the presence of the hydrophobic, electrostatic and disulphide interaction (Kumar and Nusinov, 2001). Temperature variation was a special feature because it can penetrate physical barrier and can have dramatic effects on the structure of macromolecules and also affects all levels of biological adaptation (Hickey and Singer, 2004). Specialized proteins known as chaperonins are produced by these organisms, which help, after their denaturation to refold the proteins to their native form and restore their functions (Everly and Alberto, 2000). The cell membrane of thermophiles is made up of saturated fatty acids. The fatty acid provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures. The DNA of thermophiles contains a reverse DNA gyrase which produces positive super coils in the DNA (Lopez, 1999). This raises the melting point of the DNA (the

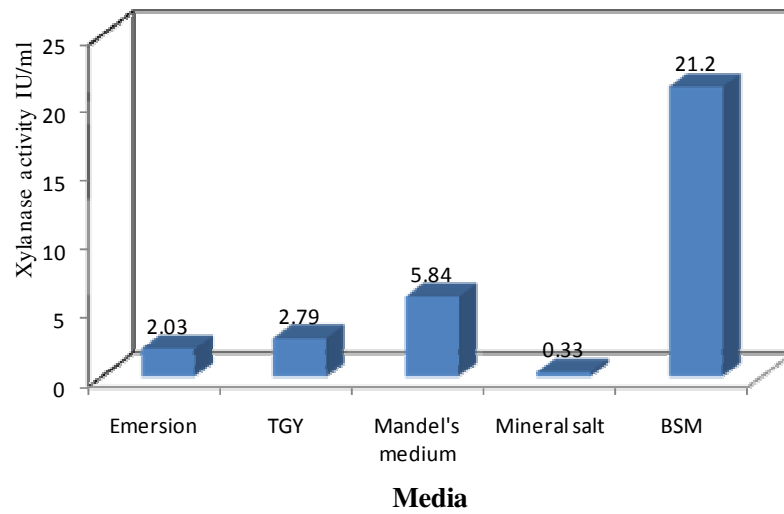


Fig. 11. Effect of media on xylanase production from *B. tequilensis* SH0 under SmF

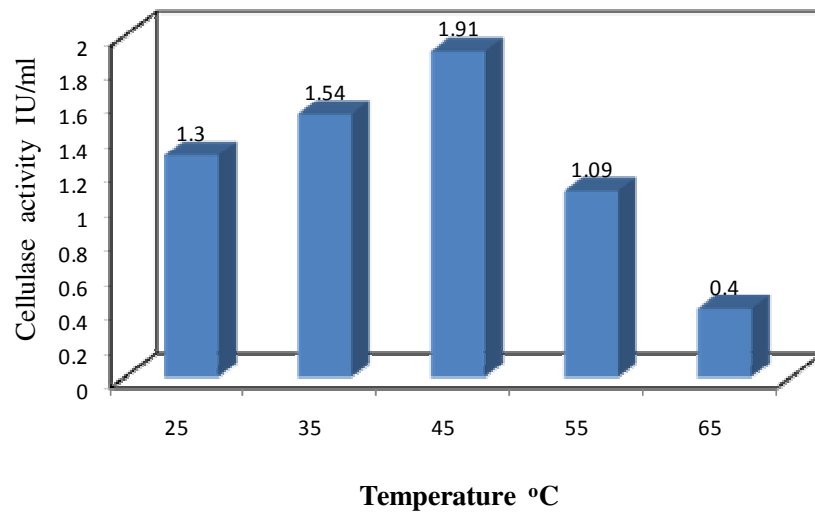


Fig. 12. Effect of temperature on cellulase production from *B. subtilis* BM-1 under SmF

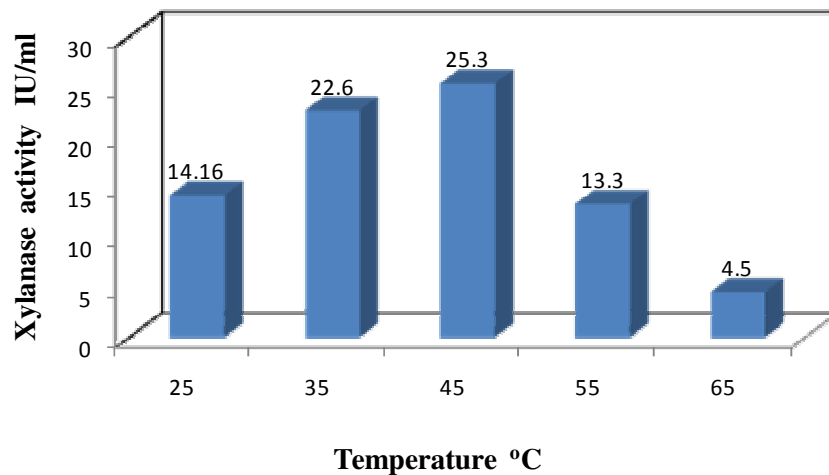


Fig. 13. Effect of temperature on xylanase production from *B. amyloliquefaciens* SH8 under SmF

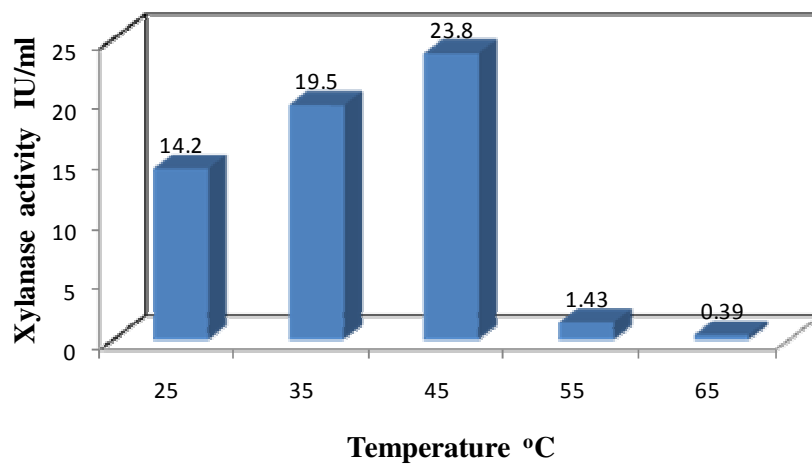


Fig. 14. Effect of temperature on xylanase production from *B. tequilensis* SH0 under SmF

temperature at which the strands of the double helix separate) to at least as high as the organisms maximum temperature for growth. Thermophilic microorganisms are the potential source of the thermostable enzymes. An increase in Gibbs free energy changes hydration energy (Gromiha *et al.*, 1999) and increase in number of salt bridges, side chain-side chain interactions, aromatic clusters (Salenminde *et al.*, 2009), contacts between the residue of hydrogen bonds (Saraboji *et al.*, 2005), ion pairs (Mauguinin *et al.*, 2009), electrostatic interactions of charged residues (Yao *et al.*, 2002), amino acid coupling pattern, hydrophobic free energy and hydrophobic free residues in thermophilic protein have shown enhanced protein stability (Saraboji *et al.*, 2005). Thermal stability of cellulase and xylanase is an important property for its potential application in several industrial processes because use of such enzymes has been expected to greatly reduce the need for temperature adjustment before the enzyme addition.

Similar finding has been reported by Verma *et al.* (2012) reported that maximum cellulase production was found at 45°C (15 IU/ml) from *Bacillus* sp. Nirmala and Sindhu (2011) that maximum endoglucanase activity (6.6 U/ml) was achieved at optimum conditions pH 9.0, at 45°C for 72h from *Bacillus circulans*. Abou-Taleb *et al.* (2009) abided by effect of some nutritional and environmental factors on growth and cellulase production by *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C2 was investigated. Incubation temperature at 30 and 45°C achieved the highest activity of cellulase for *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C2, respectively and the suitable shaking rate was 150 and 200 rpm and *Bacillus* strain M-9 (Bajaj *et al.* 2009) also exhibited maximum endoglucanase activity at 45°C. Odeniyi *et al.* (2009) reported that temperature for cellulase and polygalacturonase production in culture media by *Bacillus coagulans* was 37°C. Wahyuntari *et al.* (2009) observed the effect of pH and temperature on *Bacillus* sp. AQ-1 growth and xylanase production. The best conditions observed for the production were Luria broth supplemented with 0.5% oat spelt xylan medium was at 40°C at pH 7. The maximum enzyme production was 0.23 IU/ml after 20 h of fermentation. Roy and Rowshanul (2009) examined the effect of temperature on activity of xylanase against oat spelt xylan from

Bacillus cereus and observed that xylanase activity showed maximum activity at 40°C. Wahyuntari *et al.* (2009) reported xylanase production from *Bacillus* sp. AQ1 at 40°C and Kumar *et al.* (2010) reported the xylanase production from *Bacillus pumilus* at 40°C.

4.3.1.1.3 Effect of pH

Table 15 and 16 limned the effect of pH on the medium for cellulase and xylanase production of *B. subtilis* BM-1, *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0. Cellulase production was estimated at 4.0, 5.0.....10.0 whereas; xylanase production was estimated at 4.0, 4.5, 5.0..... 10.0. A perusal of the data revealed that maximum production of cellulase in *B. subtilis* BM-1 was observed in pH 6.0 i.e. 2.10 IU/ml and minimum was observed in pH 10.0 i.e. 0.48 IU/ml which is statistically significant higher than others (Fig. 15). The highest xylanase titers was observed in pH 5.5 for both, *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 i.e. 31.0 IU/ml and 26.1 IU/ml which is statistically significant higher than others and least xylanase titers was observed at pH 10.0 i.e. 0.46 IU/ml and 0.12 IU/ml respectively, and all the pH observations were at with each other (Fig. 16 and 17). All the three isolates have been found acidophilic in nature thus can have an edge in industry for specific applications oriented around low pH.

Since the pH of the medium influences the growth of microorganisms as enzyme production. If the cultivation of the organisms is carried out at an unfavourable pH, it may limit the growth and xylanase production by substrate inaccessibility. pH is an important parameter that influence enzyme activities and production (Odeniyi *et al.*, 2009). The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganism. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus lowering their overall metabolic activity (Willey *et al.*, 2008).

Table 15: Effect of different pH on the production of extracellular cellulase from *Bacillus subtilis* BM-1 [JX129361.1] under SmF

pH	Cellulase								
	Protein (mg/ml)	FPase activity		CMCase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
3	-	-	-	-	-	-	-	-	-
4	0.31	0.05	0.18	0.21	0.77	0.26	0.96	0.52	1.19
5	0.48	0.12	0.25	0.68	1.41	0.74	1.52	1.54	3.23
6	0.61	0.18	0.29	0.92	1.00	1.00	1.60	2.10	3.70
7	0.55	0.15	0.28	0.83	1.50	0.93	1.69	1.95	3.49
8	0.46	0.10	0.21	0.57	1.23	0.61	1.32	1.28	2.78
9	0.31	0.06	0.19	0.39	1.25	0.47	1.51	0.92	2.96
10	0.17	0.04	0.23	0.23	1.35	0.21	1.23	0.48	2.82
CD_{0.05}	0.070	0.025	0.030	0.067	0.35	0.142	0.29	0.141	0.64

Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.
 Specific activity : enzyme activity/mg of protein

Enzymes from microorganisms that can survive under extreme pH could be particularly useful for applications under highly acidic or highly alkaline reaction conditions for example, in the production of detergents. However, one of the striking properties of acidophilic and alkaliphilic microorganisms is their ability to maintain a neutral pH internally and so the intracellular enzymes from these microorganisms do not need to be adapted to extreme growth conditions. However, this does not account for extracellular proteins which have to function in low or high pH environments in the case of acidophiles and alkaliphiles, respectively. Proteases, amylases, lipases and other enzymes that are resistant to and active at high pH and high chelator concentrations of modern detergents are desirable by (Kumar *et al.*, 2011). One of the most striking properties of acidophilic and alkaliphilic microorganisms is their use of proton pumps to maintain a neutral pH internally and so the intracellular enzymes from these microorganisms do not need to be adapted to extreme growth conditions. However, the extracellular enzyme proteins of acidophiles have to function at low pH whereas those of alkaliphiles function at alkaline pH. How these extracellular proteins operate at high or low pH values is yet poorly understood. In order for the cells to survive in the aggressive conditions of pH, alkaliphiles and

acidophiles utilize several strategies. Alkaliphiles have negatively charged cell wall polymers in addition to peptidoglycan which may reduce the charge density at the cell surface and help to stabilize the cell membrane. Cellular fatty acids in alkaliphilic bacterial strains contain predominantly saturated and mono-unsaturated straight-chain fatty acids (Ma *et al.*, 2004). In order to withstand low pH, acidophiles employ a range of mechanisms such as: a positively charged membrane surface a high internal buffer capacity, over-expression of H⁺ exporting enzymes and unique transport systems (Wiegel and Kevbrin, 2004; Horikoshi, 1999).

Table 16. Effect of different pH on xylanase production from *B. amyloliquefaciens* SH8 [JX129360.1] and *B. tequilensis* SH0 [JX129359.1] under SmF

pH	<i>B. amyloliquefaciens</i> SH ₈		<i>B. tequilensis</i> SH ₀	
	Protein conc. (mg/ml)	Xylanase activity	Protein conc. (mg/ml)	Xylanase activity
4.0	0.41	12.20* (29.70)**	0.14	10.10* (25.25)**
4.5	0.69	18.70 (27.10)	0.45	16.10 (35.77)
5.0	1.32	24.40 (17.18)	1.43	28.30 (19.70)
5.5	1.42	31.00 (21.80)	1.39	26.10 (18.77)
6.0	1.35	26.60 (19.70)	1.34	25.00 (18.60)
6.5	1.32	25.00 (18.90)	1.29	24.60 (19.00)
7.0	1.26	23.20 (18.40)	1.21	23.60 (19.5)
7.5	0.92	21.80 (23.60)	0.71	20.20 (28.40)
8.0	0.65	17.30 (26.6)	0.48	16.30 (33.90)
8.5	0.38	10.00 (27.10)	0.42	13.20 (31.40)
9.0	0.33	3.20 (9.60)	0.39	10.10 (25.89)
9.5	0.25	2.10 (8.40)	0.21	4.20 (20.00)
10.0	0.12	0.46 (3.60)	0.18	0.89 (4.90)
CD_{0.05}	0.018	0.69 (0.27)	0.087	0.29 (2.01)

* Enzyme activity (IU): μmoles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

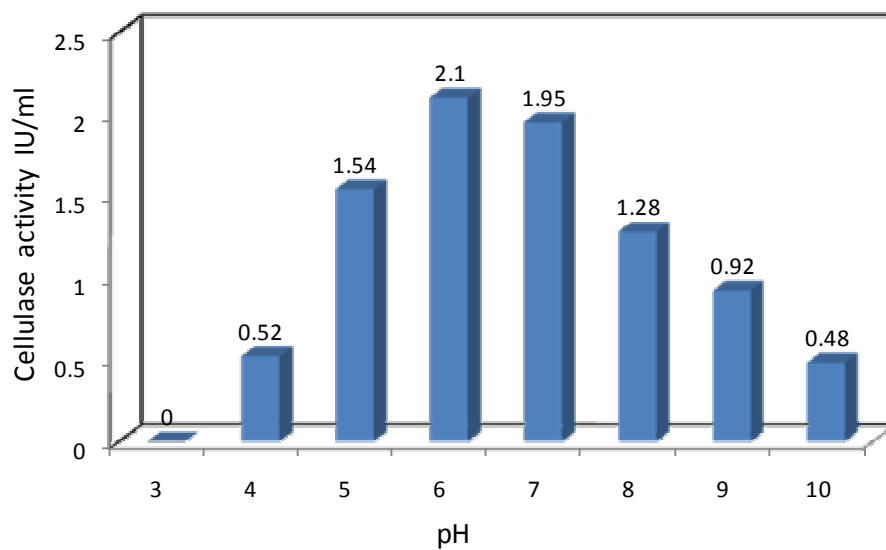


Fig. 15. Effect of pH on cellulase production from *B. subtilis* BM-1 under SmF

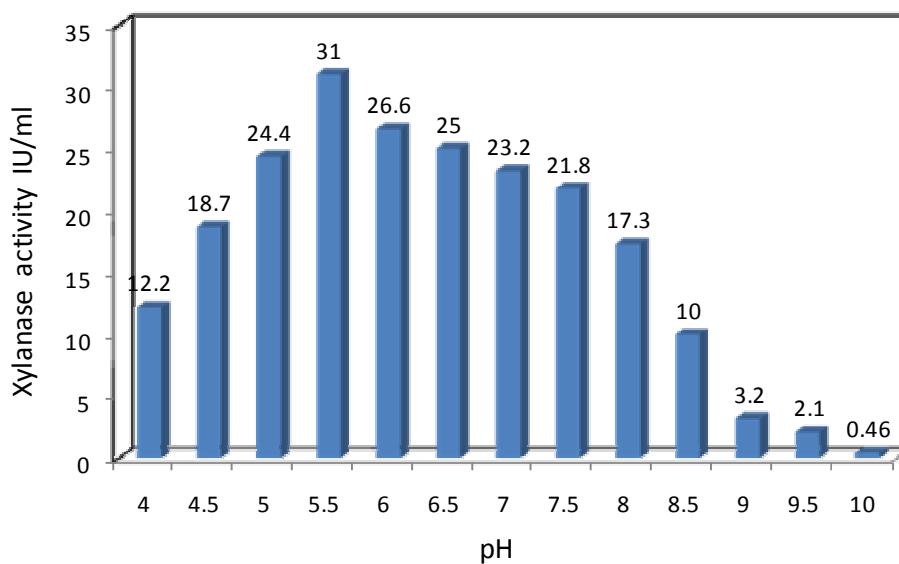


Fig. 16. Effect of pH on xylanase production from *B. amyloliquefaciens* SH8 under SmF

As the pH changes, there is a variation in enzyme activity which is logical because the optimum pH for growth rate may be different from that for growth yield and entirely different from the optimum for product formation. Changes of pH of 0.5-1.0 units around the optimum for growth usually have little effect on growth rate or efficiency but may affect product formation (Ellwood and Tempest, 1972).

Earlier studies revealed that optimum conditions for production of cellulase enzyme by *Bacillus* sp. RP1 were obtained at initial pH 6.0, incubation temperature 37°C and CMC as carbon source with an endoglucanase activity of 0.907 U/ml by Moeis *et al.* (2010). Otajavwo and Aluyi (2011) recorded that *Clostridium thermocellum* produced optimum cellulolytic activities at 50°C and pH 6.0. Sakthivel *et al.* (2010) reported that pH 7.0 was ideal for cellulase production from *Cornebacterium lipophiloflavum*. Shankar and Isaiarasu (2011) abided by that maximum cellulase activity was found at pH 6.0 and 37°C from *Bacillus pumilus* EWBCM1 and *Bacillus pumilus* showed highest activity of CMCase at pH 6.0 (Ariffin *et al.*, 2006). Tae-II *et al.* (2000) isolated *Bacillus licheniformis* NLRI-X33 from cowdung (cattle manure) and observed that optimum pH for cellulase production was 7.5. Kumar *et al.* (2012) ascertained that initial enzyme optimization showed pH 8.0 and 32°C, xylan and yeast extract favouring cellulase production.

Abou- Dohara *et al.* (2011) evinced that maximum xylanase production was achieved after 48 h, at 50°C and pH 6 for *Bacillus coagulans* and 60h, at 50°C and pH 7.0 by *Bacillus licheniformis*. Oakley *et al.* (2003) reported that xylanase production by *B. subtilis* B230 was best at pH 8.0, 37°C whereas St John *et al.* (2006) observed that xylanase production by *B. subtilis* 168 was best at pH 7.0 and 37°C. Sanghi *et al.* (2007) examined that *Bacillus* sp. produced maximum cellulase-free-xylanase enzyme titre of 283 IU/ml at pH 7.0.

Kapoor *et al.* (2008) optimized cultural conditions for xylanase production by an alkalophilic *Bacillus pumilus* MK001 strain under submerged fermentation. A initial pH 9.0 (610.0IU/ml), agitation 200 rpm, inoculum size

1.25% and inoculum age of 2h were found optimal for xylanase production. Harchand and singh (1997) also investigated a profound effect of pH on cellulase activity of *S. albaduncus*. Similar to present results, the *Bacillus* sp.NZ (Sheikh and Bajaj, 2009) and *Bacillus* strain M-9 (Bajaj *et al.* 2009) also exhibited maximum endoglucanase activity at 45°C with initial pH of 9.0. Kumar *et al.* (2010) reported the xylanase production from *Bacillus pumilus* at 40°C and pH 6.0. Sharma and Chand (2012) reported maximum xylanase activity at pH 7.5 from *Pseudomonas* sp.

4.3.1.1.4 Effect of Inoculum Size

Effect of inoculum size on cellulase and xylanase production was evaluated in Table 17 and 18. Different inoculum size used were 1.0%, 2.5%, 5.0 %, 7.5%, 10.0%, 12.5% and 15.0% (V/V). The data revealed that optimum inoculum size found to be 10.0% (V/V) for cellulase production from *B. subtilis* BM-1 (2.3 IU/ml) as depicted in Fig 18. Similarly, highest xylanase production from *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 i.e. 31.0 IU/ml and 26.1 IU/ml was obtained @ 10% inoculum having statistically significant differences over other inoculum sizes. The least cellulase and xylanase titers were recorded at 1% inoculum size. Thus, it is evident from the data that the enzyme titers were significantly affected by different inoculum size as shown in Fig 19 and 20.

In the present experiment, inoculum size ranging from 1.0% to 7.5 % probably was very low in turn resulting in lower number of cells in production medium thus causing lesser production of enzyme. A further increase in the amount of the inoculum i.e. 10.0% had improved the titers of cells enzyme reaching to maximum 10.0% showing equilibrium between the number of cells and substrate concentration at this level and enhanced the production of cellulase and xylanase. Beyond this concentration i.e. @ (15%) enzymatic activity had dipped due to overload in the number of cells causing nutrient starvation. It seems that an inoculum size of 1% to 7.5% in BSM medium was under loaded in terms of lesser number of cells leaving most of the nutrients unutilized while size @ 15% did not cause an overload of cell facing nutrient limitation whereas inoculum size of 15% was so high that the nutrient were consumed faster and

resulting in less cell growth and leading to lower enzyme yield whereas at inoculum size of 10%(V/V), a balance between proliferating biomass and available nutrient was seen. Enzyme activity is maximum at optimal level because at this point because equilibrium is maintained between inoculum size and availability of substrates while the decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks. With subsequent increase in inoculum size, competition for carbon source increases and results in rapid depletion of macro and micro nutrients and thus inhibits their growth and enzyme production (Omojasola *et al.*, 2008).

Table 17. Effect of different inoculum size on the production of extracellular cellulase from *Bacillus subtilis* BM-1 [JX129361.1] under SmF

Inoculum size	Cellulase								
	Protein (mg/ml)	FPase activity		CMCase activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase)	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
1%	0.28	0.08	0.30	0.56	2.15	0.68	2.63	1.32	5.00
2.5%	0.32	0.11	0.34	0.68	2.12	0.73	2.28	1.52	4.75
5.0%	0.46	0.12	3.80	0.72	1.56	0.87	1.89	1.64	3.50
7.5%	0.51	0.16	0.26	0.84	1.40	0.93	1.82	1.87	3.20
10.0%	0.61	0.28	0.45	0.91	1.50	1.00	1.63	2.19	3.59
12.5%	0.54	0.18	0.33	0.86	1.59	0.90	1.66	1.92	3.55
15.0%	0.50	0.14	0.28	0.79	1.58	0.84	1.68	1.77	3.54
CD _{0.05}	0.117	0.068	0.165	0.073	0.096	0.097	0.30	0.176	0.76

Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

Specific activity : enzyme activity/mg of protein

The culture used to inoculate the fermentation medium must be in a healthy, in active state and of optimum size, possibly minimizing the length of log phase, thus in its high rate for substrate conversion. The inoculum quantity generally used is between 3% and 10% of the medium volume. An adequate inoculum volume is used to generate the maximum production in a short time as possible, thus increasing the vessel productivity. The physiological conditions of the inoculum, when it transferred to the fermentation medium can have major effect on product formation. Ray *et al.* (2007) stressed that bacterial inocula

should be transferred in the lag/logarithmic growth phase when the cells are still metabolically active.

Table 18. Effect of different inoculum size on xylanase production from *B. amyloliquefaciens* SH8 [JX129360.1] and *B. tequilensis* SH0 [JX129359.1] under SmF

Inoculum size	<i>B. amyloliquefaciens</i> SH8		<i>B. tequilensis</i> SH0	
	Protein conc. (mg/ml)	Xylanase activity	Protein conc. (mg/ml)	Xylanase activity
1 %	0.32	9.70* (30.30)**	0.26	8.90* (3.40)**
2.5%	0.59	14.60 (24.70)	0.44	12.10 (27.50)
5.0%	0.67	18.30 (27.31)	0.65	16.20 (24.90)
7.5%	1.33	26.00 (19.54)	1.24	22.30 (17.98)
10.0%	1.42	31.00 (21.8)	1.42	26.10 (40.40)
12.5%	0.89	21.00 (23.50)	0.69	16.90 (24.40)
15.0%	0.57	14.10 (24.70)	0.36	9.30 (25.80)
CD_{0.05}	0.023	0.78 (0.26)	0.035	0.30 (0.75)

* Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

Similar studies evaluated by various authors revealed that maximum inoculum 10 % was used for cellulase production from *Bacillus* sp. JS14 under SSF by Singh and Kaur (2012). Das *et al.* (2010) examined inoculum size for cellulase production from *Bacillus* sp. isolated from cowdung compost and found that 7% inoculum size resulted in maximum enzyme activity $92.747 \mu\text{mg}^{-1} \text{min}^{-1}$. Ray *et al.* (2007) optimized 3% (v/v) inoculum of *Bacillus circulans* TP3 for optimum cellulase production. Moeis *et al.* (2010) reported that on variation of inoculum size 1 to 10%, maximum endoglucanase activity was obtained with 5% inoculum (0.473 U/ml) from *Bacillus* sp. RP1. Taleb *et al.* (2009) varied inoculum size ranging from 1 to 7% and maximum cellulase production was observed at 3% inoculum size. Shankar and Isaiarasu (2011) reported that in *Bacillus pumilus* EWBCM1 maximum cellulase production was attained at 2% inoculum size. Omojasola *et al.* (2008) revealed that optimum glucose from the waste substrates using *T. longibracheatum* was produced at 10% inoculum size at 45°C on day 7. In general, optimal spore concentration is between 10^6 and 10^7

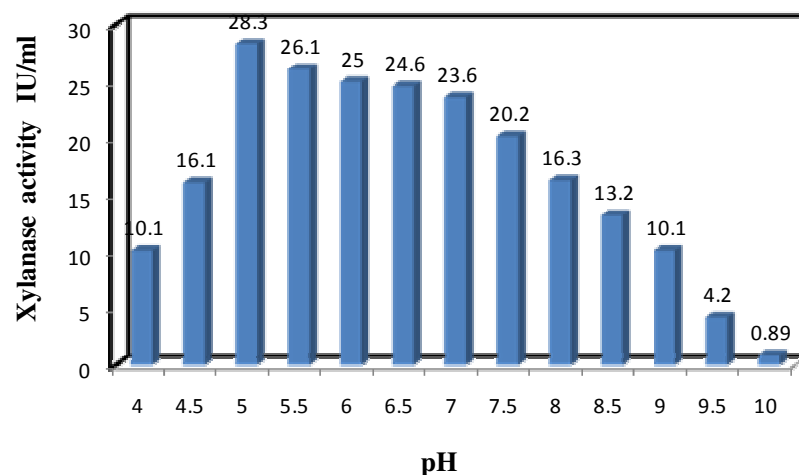


Fig. 17. Effect of pH on xylanase production from *B. tequilensis* SH0 under SmF

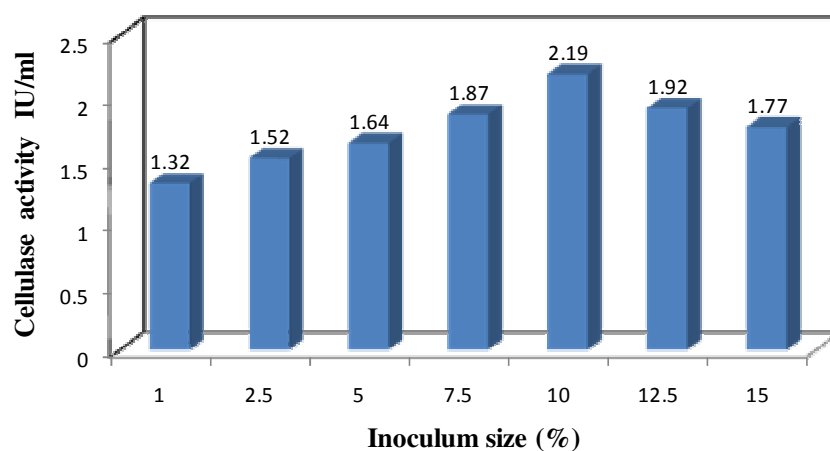


Fig. 18. Effect of inoculum size on cellulase production from *B. subtilis* BM-1 under SmF

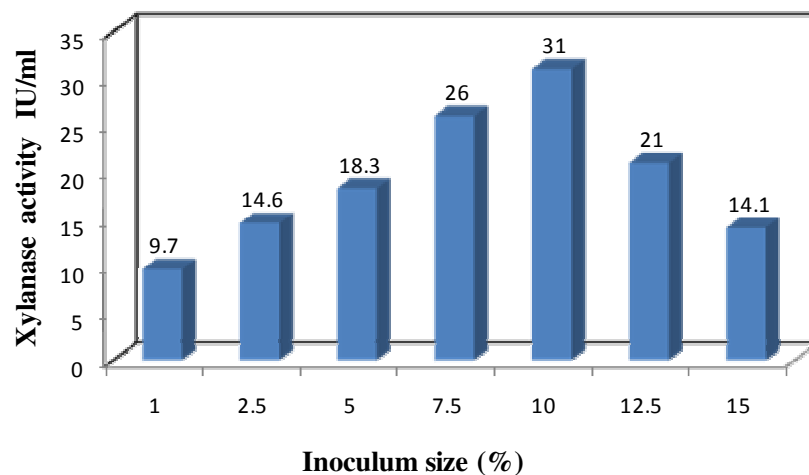


Fig 19. Effect of inoculum size on xylanase production from *B. amyloliquefaciens* SH8 under SmF

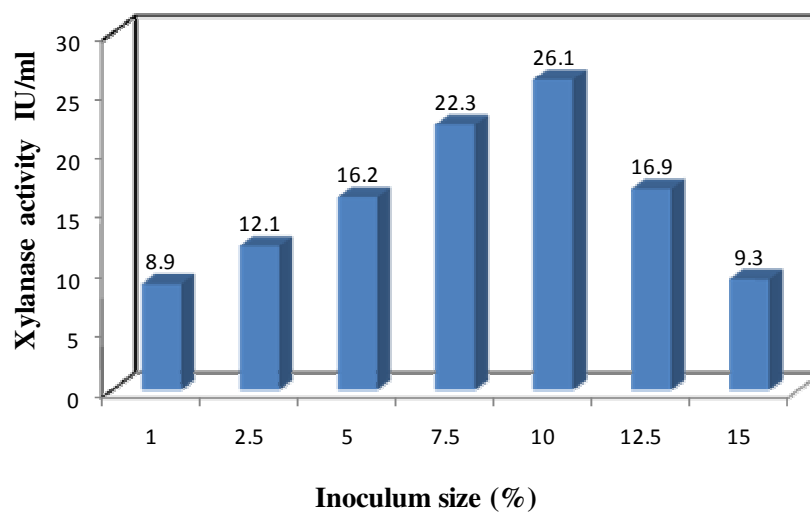


Fig. 20. Effect of inoculum size on xylanase production from *B. tequilensis* SH0 under SmF

spores/ml; out of this range, a decrease in xylanase activity occurs (Qinnghe *et al.*, 2004). 5% inoculum size has been found to be optimum for cellulase production (Zhang *et al.*, 2001 and Alam *et al.*, 2005).

Kulkarni and Rao (1996) described that 10% inoculum size was optimum for xylanase production by *Bacillus* sp. NCIM 59. Sindhu *et al.* (2006) depicted that 10% inoculum size was found to be optimum for production of xylanase by *B. megaterium*. Sephay *et al.* (2011) accounted that inoculum size 2% (V/V) was optimum for xylanase production from indigenous *Bacillus mojaensis* AG137. Similarly, Kapoor *et al.* (2008) the maximum xylanase production from an alkalophilic *Bacillus pumilis* strain MK001 at inoculum size of 1.25% (v/v) under submerged fermentation. However, Sanghi *et al.* (2007) detected that alkalophilic *Bacillus subtilis* ASH resulted in maximum xylanase production at inoculum size of 15 %.

4.3.1.1.5 Effect of Carbon Source

Table 19 and 20 depicted the effect of different substrates for cellulase production i.e. cellulose, avicel, cotton, whatman No. 1, news paper, wheat bran and carboxymethyl cellulose whereas xylose, arabinose, ribose, mannose, xylan, dextrose, fructose, sucrose, lactose and wheat bran for hyperxylanase production. Statistical analysis showed significant variation among all the substrates for enzyme titers. The cellulase titers varied from 1.90 to 2.62 IU/ml in *B. subtilis* BM-1 whereas, xylanase titer varied from 5.61 to 34.1 IU/ml and 4.56 to 32.3 in *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0, respectively as evinced in Fig 21, 22 and 23. Optimal level of cellulase and xylanase were recorded when wheat bran was used as a carbon source in all the three selected hyperenzyme producers.

The diverse pattern found in enzyme production along the different carbon sources used in the present experiment is directly linked to the specific carbon substrates used in the present study. This clearly indicates that carbon source used in the production medium has direct impact on enzyme units (Seyis and Aksoz, 2005). Some of the carbon source used in the medium supports the good growth as well as good enzyme synthesis, while others supports the good growth as well as good enzyme synthesis (Satyanarayanan, 2007). Carbon

sources are also essential elements growth and metabolism of microorganisms thus ultimately effect enzyme production (Nager *et al.*, 2010).

Table 19. Effect of different carbon source on the production of extracellular cellulase from *Bacillus subtilis* BM-1 [JX129361.1] under SmF

Substrate	Cellulase								
	Protein (mg/ml)	FPase activity		CMCase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
Cellulose	0.61	0.18	0.29	0.92	1.50	1.00	1.60	2.10	3.40
Avicel	0.52	0.16	0.30	0.86	1.65	0.96	1.86	1.90	3.80
Cotton	0.41	0.15	0.36	0.68	1.65	0.73	1.72	1.56	3.80
Whatman No. 1	0.48	0.14	0.29	0.84	1.75	0.85	1.77	1.83	3.80
News paper	0.39	0.10	0.25	0.54	1.64	0.60	1.53	1.24	3.10
Wheat bran	0.67	0.20	0.34	1.20	1.64	1.30	1.94	2.62	3.91
Carboxy methyl Cellulose	0.57	0.16	0.28	1.00	1.75	0.97	1.70	2.13	3.73
CD_{0.05}	0.118	0.038	0.082	0.171	0.35	0.23	0.29	0.135	0.97

Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

Specific activity : enzyme activity/mg of protein

Table 20. Effect of different carbon source on xylanase production from *B. amyloliquefaciens* SH8 [JX129360.1] and *B. tequilensis* SH0 [JX129359.1] under SmF

Substrate	<i>B. amyloliquefaciens</i> SH8		<i>B. tequilensis</i> SH0	
	Protein conc. (mg/ml)	Xylanase activity	Protein conc. (mg/ml)	Xylanase activity
Xylose	1.41	28.10* (19.90)**	1.23	26.60* (21.60)**
Arabinose	1.27	25.00 (19.60)	1.15	23.90 (20.70)
Ribose	1.21	24.30 (20.00)	1.1	21.80 (19.80)
Xylan	1.43	31.10 (21.70)	1.44	28.80 (20.00)
Wheat bran	1.49	34.10 (22.88)	1.48	32.30 (21.82)
Dextrose	0.84	20.11 (22.94)	0.76	17.78 (23.39)
Fructose	0.67	15.24 (22.74)	0.70	16.23 (23.19)
Sucrose	0.32	5.61 (17.53)	0.29	4.56 (15.72)
Lactose	0.78	18.20 (23.33)	0.80	19.46 (24.33)
CD_{0.05}	0.152	1.68 (3.72)	0.210	2.56 (3.86)

* Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

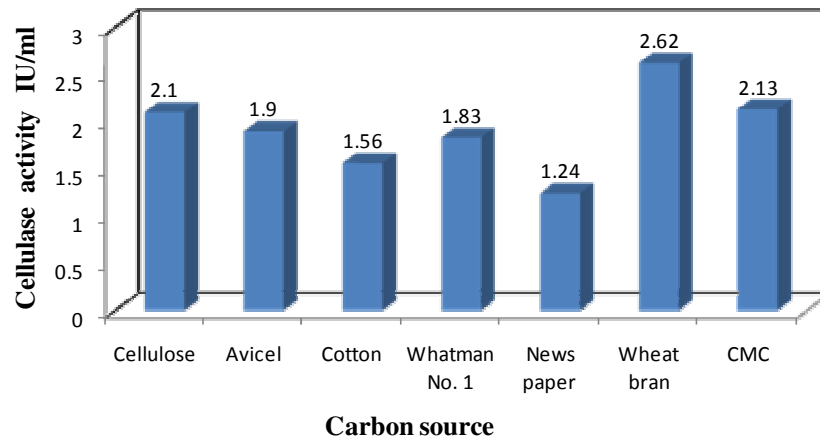


Fig. 21. Effect of carbon source on cellulase production from *B. subtilis* BM-1 under SmF

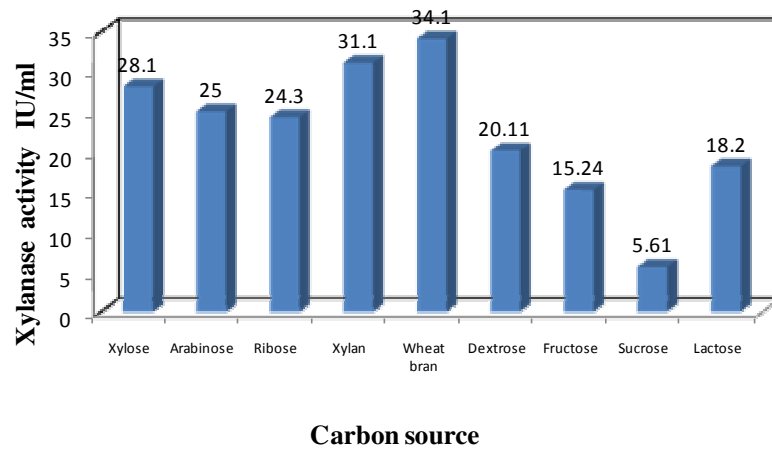


Fig. 22. Effect of carbon source on xylanase production from *B. amyloliquefaciens* SH8 under SmF

Sugars serve as important nutrients for microbial growth. Xylan containing residue may serve as inducer for promoting the xylanase production (Beg *et al.*, 2001). It appears that low molecular mass fragments of xylan play a key role in the regulation of xylanase biosynthesis that include xylose, xylobiose, xylooligosaccharide, heterodisaccharide of xylose and glucose and their positional isomers. Gigi and Bahrim (2010) examined carbon source of different origins was used in the production medium for determining the highest yield of xylanase production. Results indicated the role of carbon compounds in maximizing the production of xylanase enzyme. The highest activity was obtained when wheat bran was used as the carbon source, while the activity was minimum with CMC. The universal suitability of wheat bran may be due to its insolubility in water, and due to the nature of cellulose and hemicellulose.

Shankar and Isaiarasu (2011) studied the different carbon sources and maximum cellulase activity (0.6 U/ml) was found when galactose was used as carbon source. Singh and Kuar (2012) reported cellulase production from wheat bran using response surface methodology. Irfan *et al.* (2012) reported that various agriculture wastes were evaluated for xylanase production and maximum xylanase activity was found sugarcane bagasse (439.5±2.84 IU). Hong *et al.* (1999) used wheat bran as an effective inducer for carboxy methylcellulase production in both parent and mutant strain of *Bacillus* s. 7923. Bai *et al.* (2012) recorded the cellulase production by *Bacillus subtilis* from wheat bran. Kumar *et al.* (2009) ascertained the maximum cellulase production from rice husk from *Bacillus* sp. FME. Poorna and Prema (2006) limned the endoxylanase production from wheat bran. Kiddinamoorthy *et al.* (2007) showed the production of extracellular xylanase from *Bacillus* sp. GRE 7 using a bench top bioreactor and solid state fermentation (SSF) was attempted. SSF using wheat bran as a substrate resulted in an enzyme productivity of 3,950 U/g of wheat bran. This was highest value obtained by similar studies made earlier and closely comparable to that of *Bacillus* sp. JB-99 (3,644 U/g) SSF cultivated on rice bran (Virupakshi *et al.*, 2005) and *Bacillus pumilus* ASH (5,300 U/g) SSF cultivated on rice bran (Battan *et al.*, 2006). Sanghi *et al.* (2007) revealed that under optimized conditions, xylanase production by *Bacillus subtilis* ASH under SSF (8,964 U/g dry wheat

bran) was about twofold greater than in submerged fermentation. Thus, *B. subtilis* produced a very high level of xylanase in SSF using inexpensive agro-residues, a level which is much higher than that reported by any other bacterial isolate. Geeta and Gunasekaran (2010) used wheat bran as carbon source for optimization of xylanase production. Gessesse and Mamo (1999) reported that higher levels of xylanase (600 U/g) were produced by *Bacillus* sp. AR-009 with wheat bran containing medium. Gupta and Kar (2009) recorded maximum xylanase production from *Bacillus* sp. in wheat bran residue supplemented with mineral salt medium.

4.3.1.1.6 Effect of Substrate Concentration

Statistical analysis of Table 21 and 22 showed significant variation among different substrate concentrations used for cellulase production ranging from 0.25%, 0.5%.....to 2.0% from hypercellulolytic *B. tequilensis* BM-1 and 0.25%, 0.5%....to 3.0% for xylanase production from hyperxylanolytic *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0, respectively. Optimum level of substrate concentration was found 0.75% for *B. subtilis* BM-1 showing significantly higher enzyme activity of 2.94 IU/ml whereas least enzyme activity was recorded in 2.0% substrate concentration as depicted in Fig 24. Increase in substrate concentration level other than optimum showed a consistent decrease in cellulase production. Whereas effect of substrate concentration on xylanase production from potential bacteria revealed that *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 exhibited statistically significant higher xylanase activity at 1.25% substrate concentration i.e. 39.2 IU/ml and 36.8 IU/ml, respectively as shon in Fig. 25 and 26. Minimum xylanase production i.e. 5.1 IU/ml and 4.2 IU/ml was observed at 3.0 % substrate concentration for both hyperxylanolytic bacteria.

Substrate concentration is an influencing factor that affects the yield and initial rate of hydrolysis of cellulose (Da Silva *et al.*, 2005). Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. Optimum substrate concentration normally results in an

increase in the yield and reaction rate of the hydrolysis (Regina *et al.*, 2008). However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme production (Liu and Yang, 2007 and Singhania *et al.*, 2007). Gbemeloluwa and Moo-young (1991) reported the inhibitory effect of accumulated cellobiose and cellodextrin of low degree of polymerisation. The decrease may also be due to depletion of the other nutrients (mineral-salt) other than the energy source or due to the specific binding of the enzymes with the substrate (Grey and Neclakantan, 1983 and Gilkes *et al.*, 1984).

Table 21. Effect of different concentration of carbon source on the production of extracellular cellulase from *Bacillus subtilis* BM-1 [JX129361.1] under SmF

Concent.	Protein (mg/ml)	Cellulase							
		FPase activity		CMCase activity		β -glucosidase		Cellulase (CMCase + FPase + β - glucosidase)	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
0.25%	0.52	0.17	0.32	0.80	1.50	0.91	1.75	1.88	3.60
0.50%	0.61	0.19	0.31	1.00	1.60	1.20	1.96	2.39	3.90
0.75%	0.69	0.24	0.34	1.30	1.88	1.40	2.00	2.94	4.20
1.00%	0.67	0.22	0.32	1.10	1.64	1.30	1.94	2.62	3.91
1.25%	0.66	0.21	0.31	1.00	1.51	1.10	1.66	2.31	3.50
1.50%	0.62	0.18	0.29	0.90	1.45	0.92	1.48	2.00	3.20
1.75%	0.56	0.15	0.26	0.80	1.42	0.81	1.44	1.76	3.14
2.0%	0.48	0.13	0.27	0.75	1.56	0.69	1.43	1.57	3.27
CD_{0.05}	0.142	0.100	0.125	0.44	0.49	0.35	0.87	0.63	1.18

Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.
Specific activity : enzyme activity/mg of protein

Saheb *et al.* (2010) reported that different concentration of molasses varying from 2.0 to 20 % were evaluated for cellulase production and maximum production was found in 5.0% of substrate. Singh and Kaur (2012) observed those different wheat bran concentration, pH and incubation periods were optimized for cellulase production from *Bacillus* sp. JS14 using response surface methodology. Canonical analysis revealed that maximum CMCase activity of 2040 IU/L was achieved at wheat bran concentration 400g/L; pH6.5, temperature 40°C and incubation period of five days. Abou-Taleb *et al.* (2009) ascertained that 1.0% of carboxymethylecllulose and 0.7% yeast extract were most effective as a

carbon and nitrogen source for cellulase production from *B. alcalophilus* S39 and *B. amyloliquefaciens* C2₃. Haapela *et al.* (1995) reported that maximum endoglucanase activity was recovered on the medium with cellulose at 1% (10g/l). Sanghi *et al.* (2007) used 2% wheat bran as a carbon source for xylanase production (8,964 U/g). Kapoor *et al.* (2008) evaluated different agro-residues for xylanase production and among them maximum production was observed in 1.0% wheat bran (1220±56.0 IU/ml).

Table 22. Effect of different substrate concentration on xylanase production from *B.amyloliquefaciens* SH8 [JX129360.1] and *B. tequilensis* SH0 [JX129359.1] under SmF

Concentration	<i>B. amyloliquefaciens</i> SH8		<i>B. tequilensis</i> SH0	
	Protein conc. (mg/ml)	Xylanase activity	Protein conc. (mg/ml)	Xylanase activity
0.25%	0.41	12.30* (30.00)**	0.35	9.40* (26.80)**
0.5%	0.67	18.20 (27.16)	0.42	14.20 (33.80)
0.75%	1.12	25.10 (22.41)	0.91	21.30 (23.40)
1.0%	1.49	34.10 (22.88)	1.48	32.30 (21.82)
1.25%	1.56	39.20 (25.1)	1.54	36.80 (23.89)
1.50%	1.47	34.00 (23.10)	1.48	31.00 (1.48)
1.75%	1.43	29.00 (20.20)	1.19	24.20 (20.33)
2.0%	1.38	24.30 (17.60)	0.73	20.40 (27.90)
2.25%	0.72	19.10 (26.50)	0.42	15.30 (36.40)
2.50%	0.57	16.30 (28.50)	0.37	11.40 (2.50)
2.75%	0.36	11.20 (31.10)	0.26	6.10 (23.40)
3.0%	0.28	5.10 (28.90)	0.18	4.20 (23.30)
CD_{0.05}	0.029	0.33 (0.25)	0.026	0.28 (0.52)

* Enzyme activity (IU): μmoles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

Immanuel *et al.* (2006) evinced the cellulolytic enzyme-endoglucanase activity against coir fibre, a major biowaste by bacteria such as *Cellulomonas*, *Bacillus* and *Micrococcus* sp. isolated from coir retting effluents of estuarine

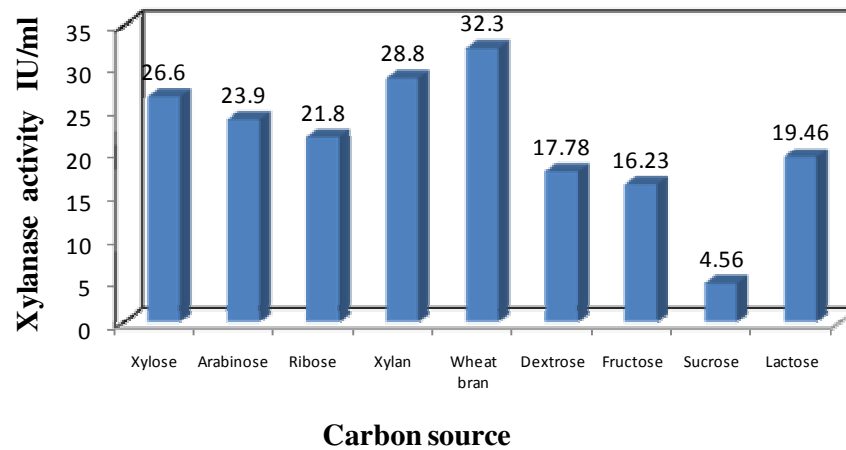


Fig. 23. Effect of carbon source on xylanase production from *B. tequilensis* SH0 under SmF

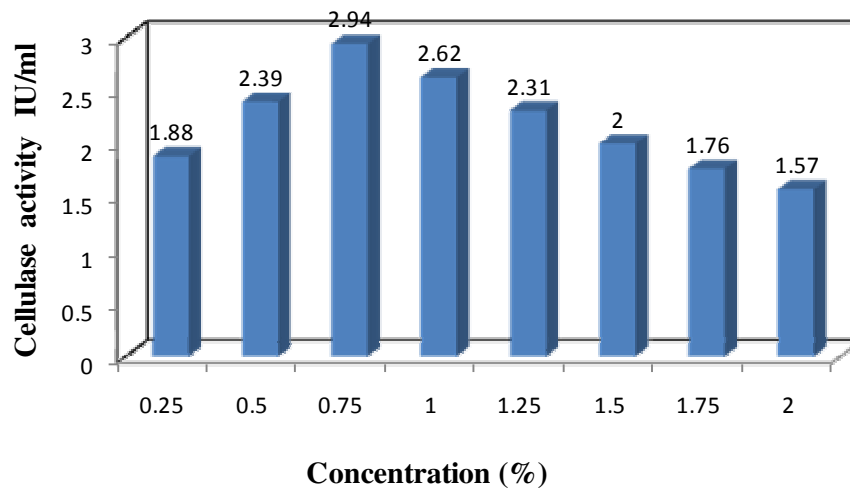


Fig. 24. Effect of substrate concentration on cellulase production from *B. subtilis* BM-1 under SmF

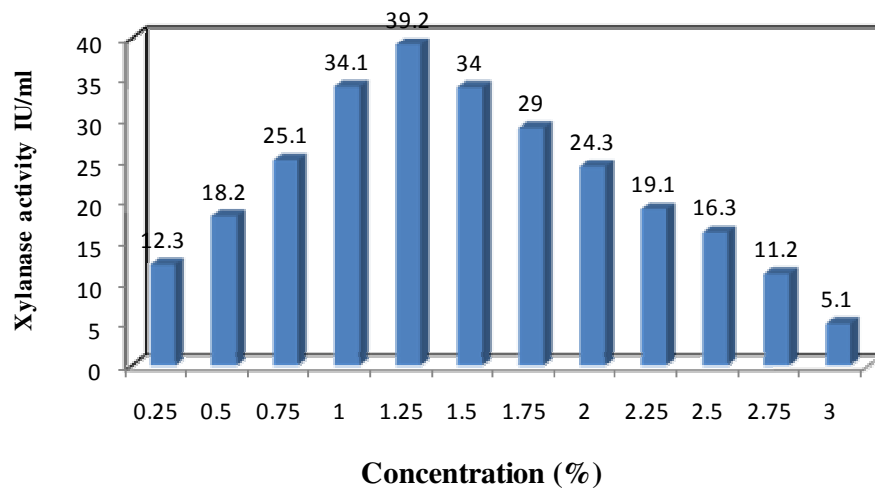


Fig. 25. Effect of substrate concentration on xylanase production from *B. amyloliquefaciens* SH8 under SmF

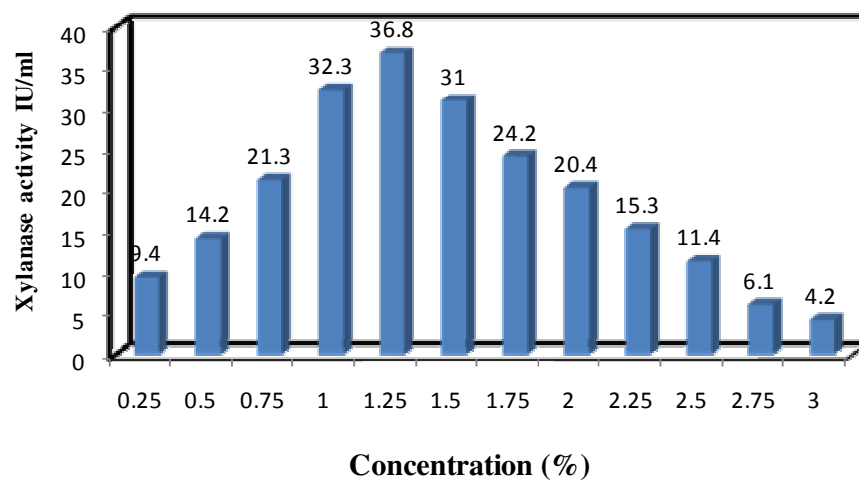


Fig. 26. Effect of substrate concentration on xylanase production from *B. tequilensis* SH0 under SmF

environment was studied. The enzyme assay was carried out by using various concentrations (0.5-2%) of substrate of coir powder as a carbohydrate in different pH (5-9) and temperature (20-50°C). The enzyme activity was minimum in 0.5% substrate concentration at lower pH 5 (0.0087, 0.0143 and 0.0071 U/mL) and at 20°C temperature (0.0151, 0.0154 and 0.0122 U/mL) by the bacterial strains such as *Cellulomonas*, *Bacillus* and *Micrococcus* sp. respectively. Then this level was increased and reached maximum at the neutral pH (0.0172, 0.0165 and 0.0121 U/mL) and at 40 °C (0.0336, 0.0196 and 0.0152 U/mL) by the selected bacterial species. Further increase of pH and temperature, the enzyme activity reduced considerably to 0.0083, 0.0143 and 0.0037 U/mL at pH 9 and 0.0154, 0.0197 and 0.0121 U/mL at 50°C by the tested bacterial strains. The same trend was also obtained in other substrate concentrations such as 1.0, 1.5 and 2.0 %. Within the four substrate concentrations, the endoglucanase enzyme activity was more in 1.5% concentration at the tested pH and temperatures.

4.3.1.1.7 Effect of Incubation Time

The effect of incubation time on cellulase and xylanase production has been evaluated in Table 23 and 24. Enzyme activity was measured at regular intervals up to periods of 120h. Highest cellulase activity was measured on 72h (4.05 IU/ml) from *Bacillus subtilis* BM-1 followed by gradual decline with increase in incubation time as depicted in Fig 27. Least enzyme production was observed on 48 h (1.74 IU/ml) of fermentation time. Statistically enzyme production on 72 h was found significantly higher than others incubation time. The effect of incubation time on xylanase production has been evaluated. Least xylanase production was observed in 48 h of fermentation time by *B. amyloliquefaciens* and *B. tequilensis* i.e. (17.10 IU/ml) and (14.13 IU/ml) while maximum xylanase production was noticed at 72 h and 96 h of fermentation time by *B. amyloliquefaciens* and *B. tequilensis* i.e. (46.50 IU/ml) and (41.30 IU/ml), respectively as shown in Fig. 28 and 29.

When growth of all the three selected bacterial isolates was studied, it showed exponential phase extending from 12 to 48 h and then entering into stationary phase at 72 h. Enzyme production when compared with growth profile,

peak in yield was noticed at 72 h for *B. subtilis* and *B. amyloliquefaciens* while 96 h for *B. tequilensis* i.e. stationary phase as shown in Fig 30. Similar reports were shown in the literature where highest yield of enzyme was found in exponential/stationary phase. A decline in enzyme activity afterwards was probably due to proteolysis or due to depletion of nutrients available to microorganisms, causing a stressed microbial physiology resulting in inactivation of enzyme (Flores *et al.*, 1997).

Table 23. Effect of different incubation time on the production of extracellular cellulase from *Bacillus subtilis* BM-1 [JX129361.1] under SmF

Incubation time	Cellulase								
	Protein (mg/ml)	FPase activity		CMCase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
48h	0.45	0.14	0.31	0.70	1.56	0.90	2.00	1.74	3.87
60h	0.62	0.20	0.32	1.00	1.61	1.30	2.10	2.50	4.03
72h	1.11	0.25	0.23	1.80	1.62	2.00	1.80	4.05	3.65
84h	0.96	0.37	0.39	1.60	1.67	1.80	1.88	3.77	3.93
96h	0.86	0.32	0.37	1.40	1.63	1.60	1.86	3.32	3.86
108h	0.76	0.29	0.38	1.20	1.58	1.50	1.97	2.99	3.93
120h	0.70	0.24	0.34	1.30	1.86	1.40	2.00	2.94	4.20
CD_{0.05}	0.182	0.075	0.080	0.43	0.35	0.29	0.36	0.60	0.70

Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

Specific activity : enzyme activity/mg of protein

Nirmala and Sindhu (2011) recorded maximum cellulase production from *Bacillus* strain at 72 h (5.0 IU/ml) of fermentation time. Shankar and Isaarasu (2011) reported maximum cellulase production at 72 h (0.5851 $\pm$ 0.006 IU/ml) from *Bacillus pumilus* EWBCM1 under varying cultural conditions.

Shafique *et al.* (2004) reported that maximum exoglucanase production by *Bacillus subtilis* under SSF was attained at 72 h of fermentation time (1.90 U/ml). Heck *et al* (2002) reported the maximum production of cellulase enzyme from *B. pumilus* and *B. subtilis* at 72 h of incubation time. Shu-bin *et al.* (2012) reported that maximum carboxymethyl cellulase and cellobiase productivities

were 48.42 ± 0.87 and 2.15 IU/g of dried okara, respectively from *Bacillus subtilis* Pa5 at 48 h of fermentation time.

Table 24. Effect of different incubation time on xylanase production from *B. amyloliquefaciens* SH8 [JX129360.1] and *B. tequilensis* SH0 [JX129359.1] under SmF

Incubation time	<i>B. amyloliquefaciens</i> SH8		<i>B. tequilensis</i> SH0	
	Protein conc. (mg/ml)	Xylanase activity	Protein conc. (mg/ml)	Xylanase activity
48h	1.21	17.10* (14.10)**	0.42	14.13* (36.10)**
60h	1.36	28.40 (20.80)	1.17	23.40 (20.00)
72h	1.64	46.50 (28.53)	1.51	30.10 (19.90)
84h	1.61	43.00 (26.71)	1.59	35.20 (22.10)
96h	1.58	41.20 (26.08)	1.68	41.30 (24.50)
108h	1.56	39.90 (25.58)	1.62	38.90 (24.00)
120h	1.54	38.00 (24.68)	1.54	36.80 (23.80)
CD _{0.05}	0.0175	3.25 (2.70)	0.0209	0.23 (0.86)

* Enzyme activity (IU): μ moles of reducing sugars released/min/ml of enzyme.

**Specific activity : enzyme activity/mg of protein.

Bai *et al.* (2012) reported optimum production of cellulase at 72 h of fermentation time from *B. subtilis*. Irfan *et al.* (2012) recorded maximum xylanase production at 48 h under shake conditions while under static conditions at 72 h (408 ± 2.9 IU) of fermentation time from *B. subtilis* using various agricultural wastes. Abou Dohara *et al.* (2011) showed that optimum incubation time for xylanase production was 48 h and 60 h for *B. coagulans* and *B. licheniformis*, respectively. Maximum xylanase production 125 IU/ml was recorded in the stationary phase (36 h) culture by *B. subtilis* isolated from estuarine environment by optimization of cultural conditions (Annamalai *et al.*, 2009). Kumar *et al.* (2009) recorded optimum cellulase production after 6 days under submerged fermentation in different substrates from newly isolated *Bacillus* sp. FME.

Maximum titers of xylanase (2600 IU/ml) were obtained by *Streptomyces* sp. SU9 in the medium containing oat spelt as a substrate with pH of 9 in shake

flask condition after 96 h of fermentation (Bajaj *et al.* 2010). Gupta and Kar (2009) recorded maximum xylanase production from *Bacillus* sp. in wheat bran residue at 72 h (9.0 IU/ml) of fermentation time supplemented with mineral salt medium.

The optimization of different fermentation factors particularly physical and chemical parameters are of primary importance in the development of any fermentation process owing their impact on the yield practicability and economy of the process (Wenster-Botz, 2000). The significant increase in extracellular cellulase activity from *B. subtilis* BM-1 and xylanase activity from *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 are the main highlight of submerged fermentation. The stepwise increase in cellulase titers of *B. subtilis* BM-1 from 1.33 IU /ml to 4.05 IU/ml, xylanase titers of *B. amyloliquefaciens* SH8 ranging from 14.40 IU/ml to 46.50 IU/ml, while in *B. tequilensis* SH0 from 13.30-41.20 IU/ml with an impressive hike of 204.51, 222.91 and 210.52%, respectively (Fig 31, 32 and 33) which is an outcome of optimizing the various bioprocess parameters i.e. nutrients, temperature, pH, inoculum size, different carbon source, concentration of carbon source and incubation time using classical approach OFAT. The optimal conditions derived for *B. subtilis* BM-1 for cellulase production and *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0 for xylanase production indicates these isolates as an acidophilic and thermophilic in nature.

4.3.1.2 OPTIMIZATION OF XYLANASE PRODUCTION UNDER SUBMERGED FERMENTATION BY *Bacillus amyloliquefaciens* SH8 USING RESPONSE SURFACE METHODOLOGY (RSM)

In order to check the potential of *Bacillus amyloliquefaciens* SH8 isolate to synthesize xylanase production for biotechnological applications, determination of cumulative effect of multiple parameters regulating the rate of enzyme production became warranted. Bioprocess technologies require effective problems solving methods as they involve multiple parameters to adjust. RSM is a collection of statistical techniques for designing an experiment, building models, evaluating the effects of factors, and searching for optimum conditions of

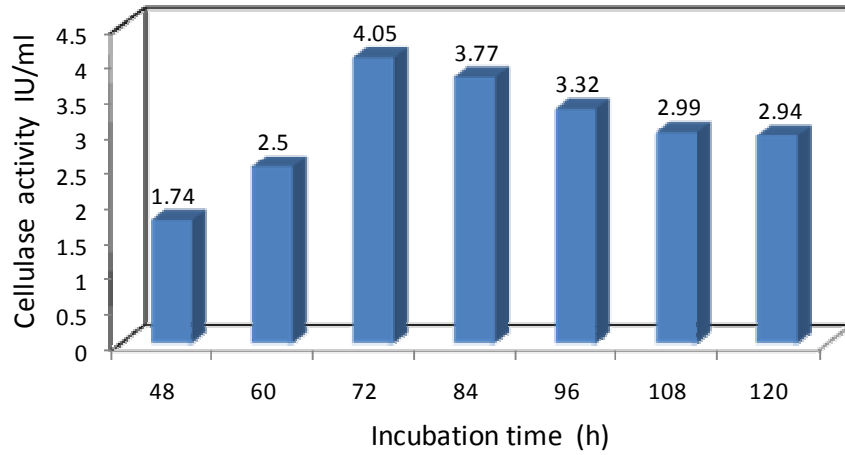


Fig. 27. Effect of incubation time on cellulase production from *B. subtilis* BM-1 under SmF

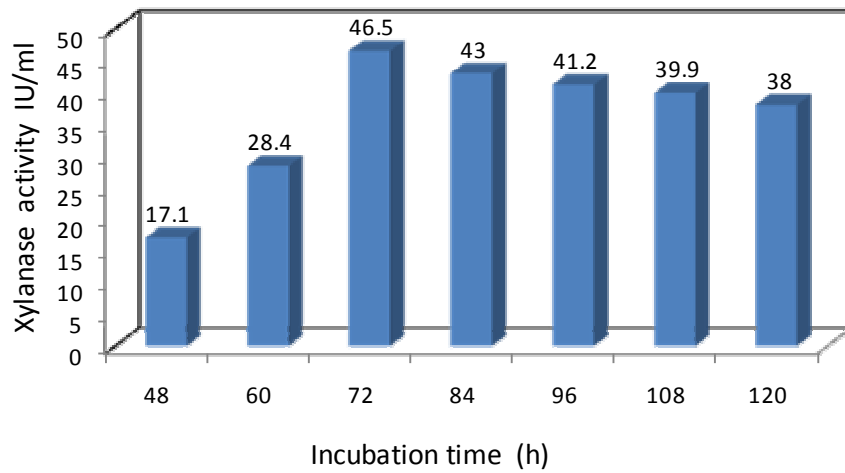


Fig. 28. Effect of incubation time on xylanase production from *B. amyloliquefaciens* SH8 under SmF

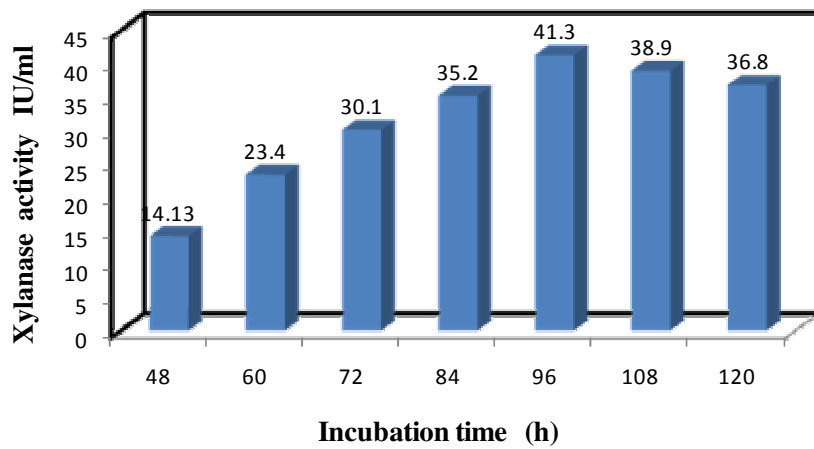


Fig. 29. Effect of incubation time on xylanase production from *B. tequilensis* SH0 under SmF

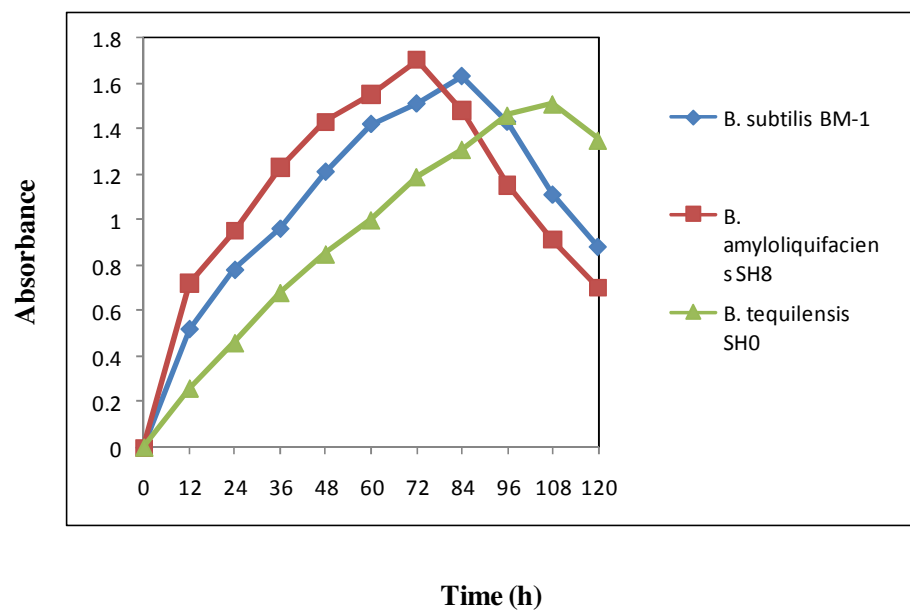


Fig. 30. Growth curve of *B. subtilis* BM-1, *B. amyloliquefaciens* and *B. tequilensis* SH0

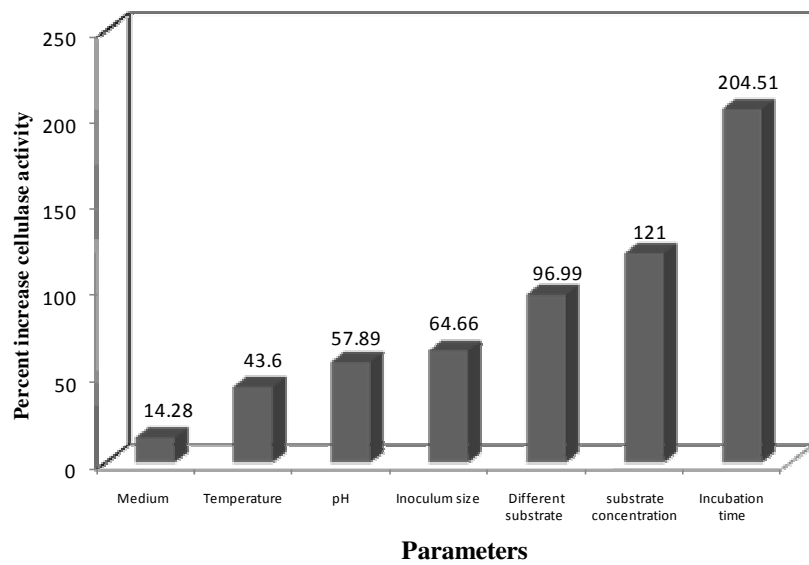


Fig. 31. An overview of percent increase in cellulase activity from *B. subtilis* BM-1 after optimization of different parameters under SmF

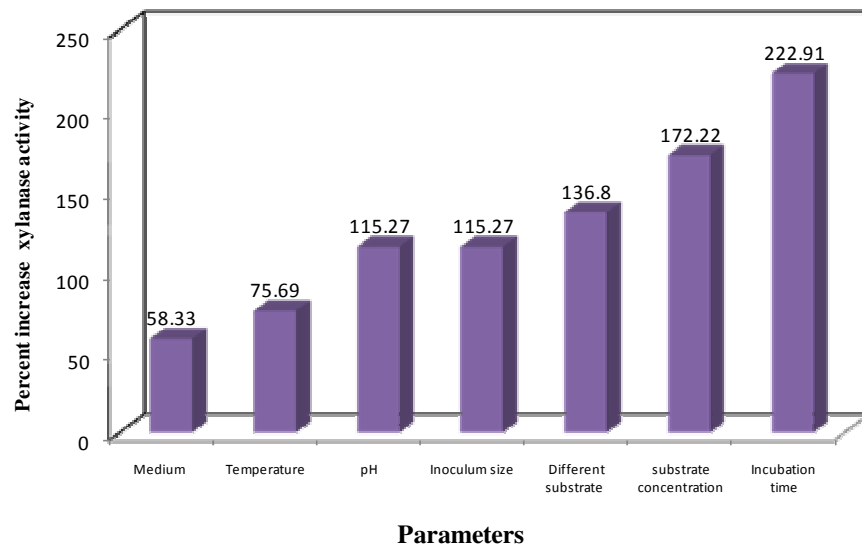


Fig. 32. An overview of percent increase in xylanase activity from *B. amyloliquefaciens* SH8 after optimization of different parameters under SmF

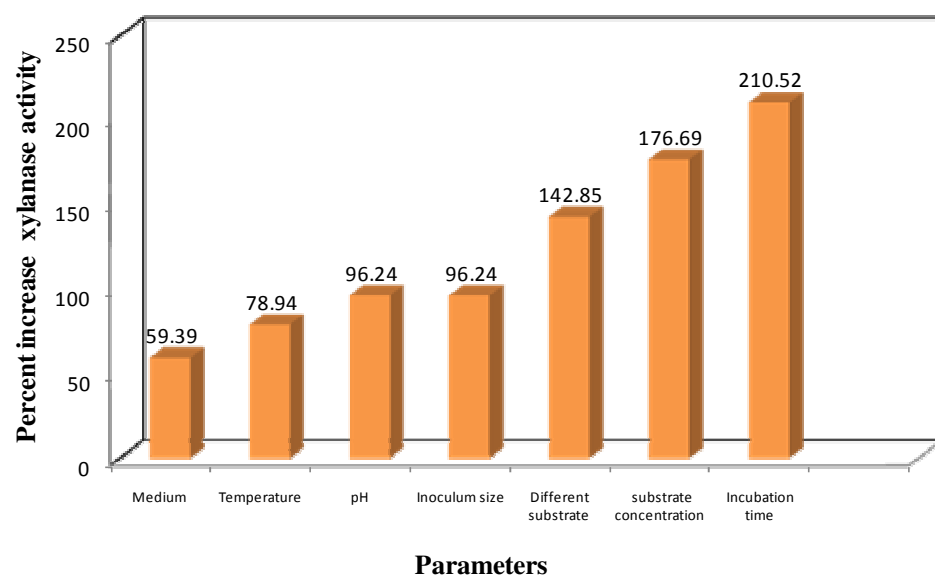


Fig.33. An overview of percent increase in xylanase activity from *B. tequilensis* SH0 after optimization of different parameters under SmF

factors for desirable responses. Because Response Surface Methodology is more satisfactory and effective than others methods such as classical one-factor-at-a-time (OFAT) or mathematical models because it can study many variables simultaneously with low number of observations, saving time and costs (Bezerra *et al.* 2008; Deepak *et al.*, 2008), thereafter optimization of xylanase from *B. amyloliquefaciens* SH8 has been analysed by using RSM.

This method has successfully optimized composition and growth of the medium for the production of different enzymes (Cui *et al.* 2006; Zhang *et al.*, 2006; Li *et al.*, 2007; Vohra and Satynaryana 2002; Berecka *et al.*, 2006). The present study illustrates the optimum conditions for xylanase production by *B. amyloliquefaciens* SH8 applying response surface methodology. A Plackett-Burman design (PBD) (Plackett and Burman, 1946) was firstly used to identify the medium components which had significant effect on xylanase production. A central composite design (CCD) (Box and Wilson, 1951) was then employed to optimize the factors that had a significant influence on xylanase production (Xu *et al.*, 2008; Maciel *et al.*, 2008; Karmakar and Ray, 2011; Singh and Kaur, 2012; Mullai *et al.*, 2010).

4.3.1.2.1 Plackett Burman Design

Plackett-Burman (PB) Design was used in this research to select the most significant culture conditions that would influence the xylanase production as depicted in Table 25. This design was used to analyze effect of 20 variables on xylanase production.

Plackett Burman design was employed to characterize the individual and interactive effects of nitrogen sources, salt additives, pH, temperature, and inoculum size and incubation time on xylanase production. Table 26 and 27 depicted the second order model was hypothesized and statistically evaluated by analysis of variance ANOVA showed that the value of R^2 for the response was in reasonable agreement with adjusted R^2 . The "Pred R^2 " of 0.8462 is in reasonable agreement with the "Adj R^2 " of 0.9562. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 17.060

Table 25. Experimental design using the Plackett Burman design for screening culture conditions affecting xylanase yield by *Bacillus amyloliquefaciens* SH8

Run	A	B	C	Dl	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	T	Xylanase IU/ml	Square root Transformation	
	g/l	g/l	g/l	g/l	g/l	g/l	g/l	%	g/l	g/l	g/l	g/l	g/l	g/l		°C	Days	ml/l	g/l	Experimental value	Experimental value	Predicted value
1	0.00	1.00	5.00	0.00	5.00	5.00	0.00	5.00	5.00	0.10	0.10	0.20	0.00	0.15	4.00	50.00	3.00	0.00	0.00	0.00	0.00	0.05
2	1.00	1.00	0.00	1.50	0.00	5.00	0.00	5.00	0.00	0.00	0.10	0.20	0.00	0.15	6.00	40.00	3.00	1.00	0.10	29.80	5.46	5.56
3	0.00	1.00	0.00	1.50	0.00	0.00	0.00	5.00	5.00	0.10	0.00	0.20	0.15	0.00	4.00	50.00	5.00	1.00	0.10	26.00	5.10	5.00
4	1.00	0.00	0.00	0.00	0.00	5.00	5.00	5.00	5.00	0.10	0.00	0.00	0.15	0.15	6.00	50.00	3.00	1.00	0.00	31.39	5.60	6.29
5	0.00	0.00	0.00	1.50	5.00	0.00	5.00	15.00	0.00	0.00	0.10	0.20	0.15	0.15	4.00	50.00	3.00	1.00	0.00	14.24	3.77	3.61
6	1.00	1.00	5.00	0.00	5.00	0.00	5.00	5.00	0.00	0.00	0.00	0.20	0.15	0.00	6.00	50.00	3.00	0.00	0.10	32.40	5.69	5.55
7	0.00	1.00	5.00	0.00	0.00	5.00	5.00	15.00	5.00	0.00	0.10	0.00	0.15	0.00	4.00	40.00	3.00	1.00	0.10	34.40	5.87	6.17
8	1.00	1.00	5.00	1.50	0.00	5.00	0.00	15.00	0.00	0.00	0.00	0.00	0.15	0.15	4.00	50.00	5.00	0.00	0.00	21.30	4.62	4.43
9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	0.00	0.00	0.00	0.00	0.00	4.00	40.00	3.00	0.00	0.00	27.20	5.22	5.38
10	0.00	0.00	0.00	0.00	5.00	5.00	0.00	15.00	5.00	0.00	0.00	0.20	0.15	0.15	6.00	40.00	5.00	0.00	0.10	57.00	7.55	7.42
11	0.00	1.00	0.00	0.00	0.00	0.00	5.00	15.00	0.00	0.10	0.10	0.00	0.00	0.15	6.00	50.00	5.00	0.00	0.10	0.00	0.00	0.18
12	1.00	0.00	5.00	0.00	0.00	0.00	0.00	15.00	5.00	0.00	0.10	0.20	0.00	0.00	6.00	50.00	5.00	1.00	0.00	0.00	0.00	0.08
13	1.00	0.00	0.00	1.50	5.00	5.00	5.00	5.00	5.00	0.00	0.10	0.00	0.00	0.00	4.00	50.00	5.00	0.00	0.10	27.69	5.26	4.98
14	0.00	1.00	5.00	1.50	5.00	0.00	5.00	5.00	5.00	0.00	0.00	0.00	0.00	0.15	6.00	40.00	5.00	1.00	0.00	18.60	4.31	4.55
15	1.00	1.00	0.00	0.00	5.00	5.00	5.00	15.00	0.00	0.10	0.00	0.20	0.00	0.00	4.00	40.00	5.00	1.00	0.00	33.99	5.83	5.83
16	1.00	0.00	5.00	0.00	5.00	0.00	0.00	5.00	0.00	0.10	0.10	0.00	0.15	0.15	4.00	40.00	5.00	1.00	0.10	0.00	0.00	0.60
17	1.00	0.00	5.00	1.50	0.00	0.00	5.00	15.00	5.00	0.10	0.00	0.20	0.00	0.15	4.00	40.00	3.00	0.00	0.10	27.80	5.27	4.87
18	1.00	1.00	0.00	1.50	5.00	0.00	0.00	15.00	5.00	0.10	0.10	0.00	0.15	0.00	6.00	40.00	3.00	0.00	0.00	30.90	5.56	5.10
19	0.00	0.00	5.00	1.50	5.00	5.00	0.00	15.00	0.00	0.10	0.00	0.00	0.00	0.00	6.00	50.00	3.00	1.00	0.10	0.00	0.00	-0.14
20	0.00	0.00	5.00	1.50	0.00	5.00	5.00	5.00	0.00	0.10	0.10	0.20	0.15	0.00	6.00	40.00	5.00	0.00	0.00	0.00	0.00	-0.42

A: NH₄Cl, B: KH₂PO₄, C: NH₄SO₄, D: NaCl, E: Yeast extract F: Peptone, G: Soybean meal, H: Inoculum size, J: Tryptone, K: MnSO₄, L: FeSO₄, M: MgCl₂, N: CaCl₂, O: CoCl₂, P: pH, Q: Temperature, R: Time, S: Tween 80, T ZnSO₄

Table 26. ANOVA of response surface for Placket-Burman (PB) Design

Source	Sum of squares	df	Mean square	F value	p-value prob>F	
Model	128.84	13	9.91	32.88	0.0002	significant
A-NH ₄ CL	6.59	1	6.59	21.85	0.0034	
B-KH ₂ PO ₄	4.76	1	4.76	15.79	0.0073	
C-NH ₄ SO ₄	27.83	1	27.83	92.34	< 0.0001	
F-Peptone	1.38	1	1.38	4.59	0.0759	
G-Soybean	3.29	1	3.29	10.92	0.0163	
J-Tryptone	9.71	1	9.71	32.23	0.0013	
K-MnSO ₄	20.77	1	20.77	68.92	0.0002	
L-FeSO ₄	27.08	1	27.08	89.84	< 0.0001	
N-CaCl ₂	7.69	1	7.69	25.53	0.0023	
P-pH	2.28	1	2.28	7.58	0.0332	
Q-Temperature	11.28	1	11.28	37.42	0.0009	
R-Time	4.77	1	4.77	15.84	0.0073	
T-ZnSO ₄	1.40	1	1.40	4.64	0.0746	
Residual	1.81	6	0.30			
Cor Total	130.65	19				

$R^2=0.9862$, Adj $R^2=0.9562$

SS; sum of square, Df ; degree of freedom; MS, mean sum of square

Table 27. Levels of the variables and statistical analysis of Plackett-Burman design

Factor	Coefficient Estimate	df	Standard Error	Low	High	VIF
Intercept	3.76	1	0.12	3.46	4.06	
A-NH ₄ CL	0.57	1	0.12	0.27	0.87	1.00
B-KH ₂ PO ₄	0.49	1	0.12	0.19	0.79	1.00
C-NH ₄ SO ₄	-1.18	1	0.12	-1.48	-0.88	1.00
F-Peptone	0.26	1	0.12	-0.037	0.56	1.00
G-Soybean	0.41	1	0.12	0.11	0.71	1.00
J-Tryptone	0.70	1	0.12	0.40	1.00	1.00
K-MnSO ₄	-1.02	1	0.12	-1.32	-0.72	1.00
L-FeSO ₄	-1.16	1	0.12	-1.46	-0.86	1.00
N-CaCl ₂	0.62	1	0.12	0.32	0.92	1.00
P-pH	-0.34	1	0.12	-0.64	-0.038	1.00
Q-Temperature	-0.75	1	0.12	-1.05	-0.45	1.00
R-Time	-0.49	1	0.12	-0.79	-0.19	1.00
T-ZnSO ₄	0.26	1	0.12	-0.036	0.56	1.00

indicates an adequate signal. This model can be used to navigate the design space. Our Model *F*-value of 32.88 implies that the model was significant. There is only a 0.02% chance that a “Model *F*-value” could occur due to noise. The *P* value; that is, the probability that the magnitude of contrast coefficient is due to random process variability, served as a tool for checking the significance of each of the coefficient. A regression model having an R^2 value higher than 0.9 is considered to have very high correlation (Haaland, 1989). The closer the value of *R* (Correlation coefficient) to 1, the better the correlation between the experimental results and predicted values. The values of R^2 (0.9862) indicates a close agreement between the experimental results and the theoretical values predicted by the model equation. The square root of the residual sum of square is 0.5, dip suddenly, with a minimum of the best optimal value 0.46 (Fig 34). The data require the square root transformation, as current value of confidence interval it contains (λ) is very close to the optimum value (Box and Cox, 1964). The model shows the minimum and maximum confidence interval value is 0.16 and 0.74 (Fig 34).

Table 25 showed that NH_4Cl , tryptone, CaCl_2 , and ZnSO_4 have significant positive effect on the xylanase production at the 5% level of significance, whereas KH_2PO_4 , peptone, Soybean meal, pH, and temperature and incubation time have insignificant effect on xylanase production. The factors NH_4SO_4 , MnSO_4 and FeSO_4 resulting in a negative effect on xylanase production were no longer taken into account, while the positive factors were included in the next CCD optimization. It was postulated that the positive effect of NH_4Cl , Peptone, Ammonium sulphate, tryptone, yeast extract and, soybean meal attributed to the requirement of nitrogen sources during fermentation. The optimal nitrogen source for xylanase production was diversified depending upon the microorganisms tested.

Design-Expert® Software
Spt(R1)

Lambda
Current = 0.5
Best = 0.46
Low C.I. = 0.16
High C.I. = 0.74

Recommend transform:
Square Root
(Lambda = 0.5)

k = 0.057
(used to make
response values
positive)

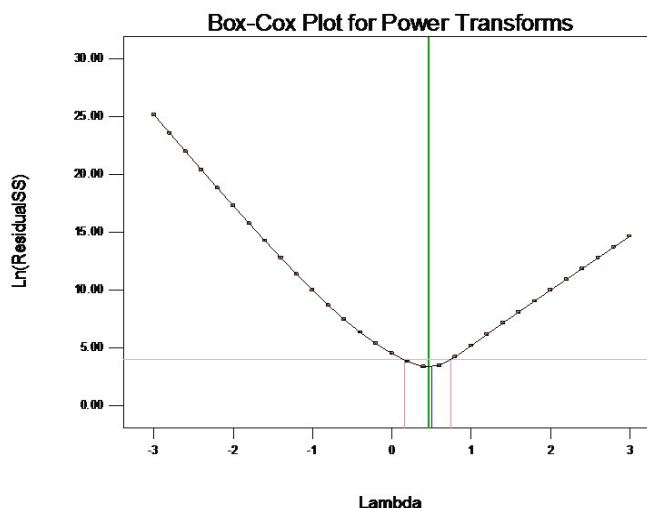
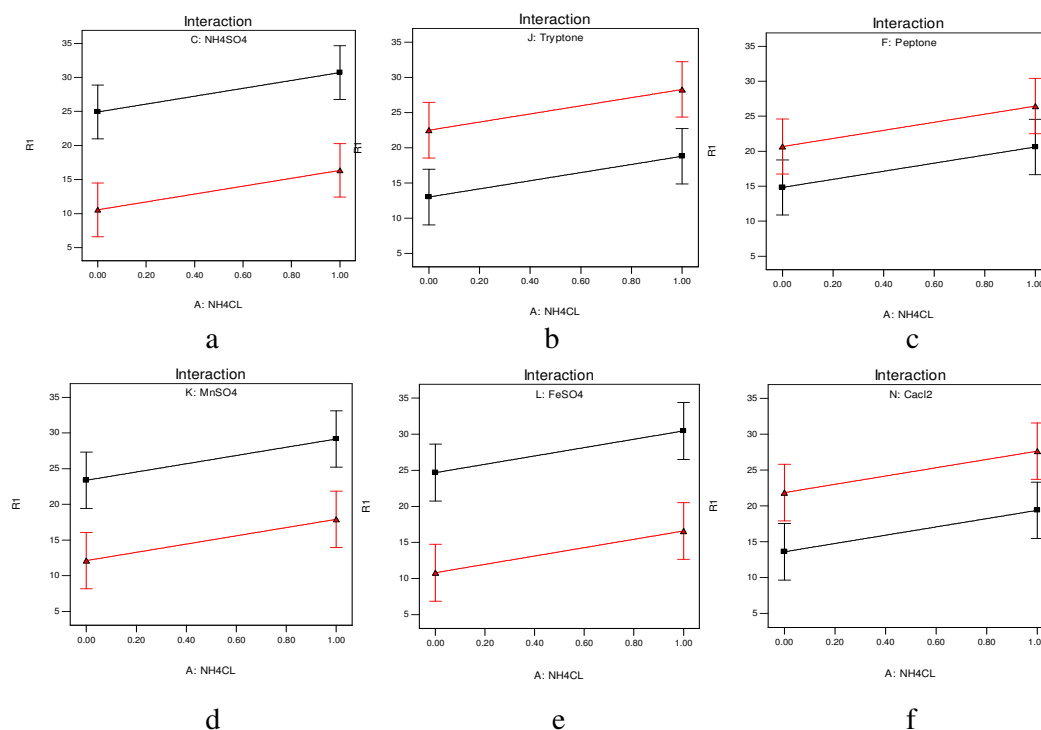


Fig 34. Box-Cox plot for power transforms of xylanase production in PBD by *Bacillus amyloliquefaciens* SH8

Validation of the Model:

The statistical optimal values of variables were obtained. The Plackett Burman design and the response at the different point yield maximum xylanase production as shown in Fig 35 and 36.



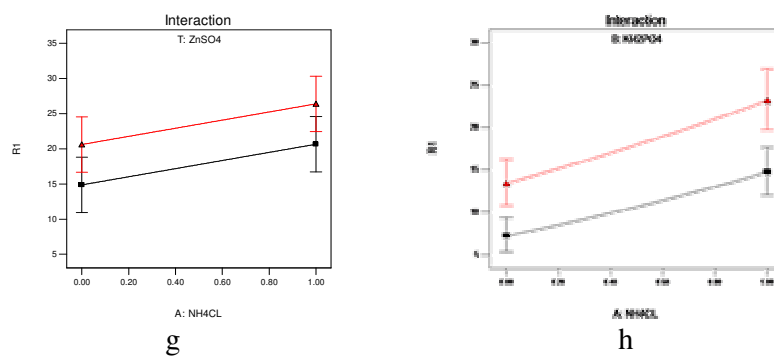


Fig 35. Interaction plot for xylanase production in PBD by *Bacillus amyloliquefaciens* SH8

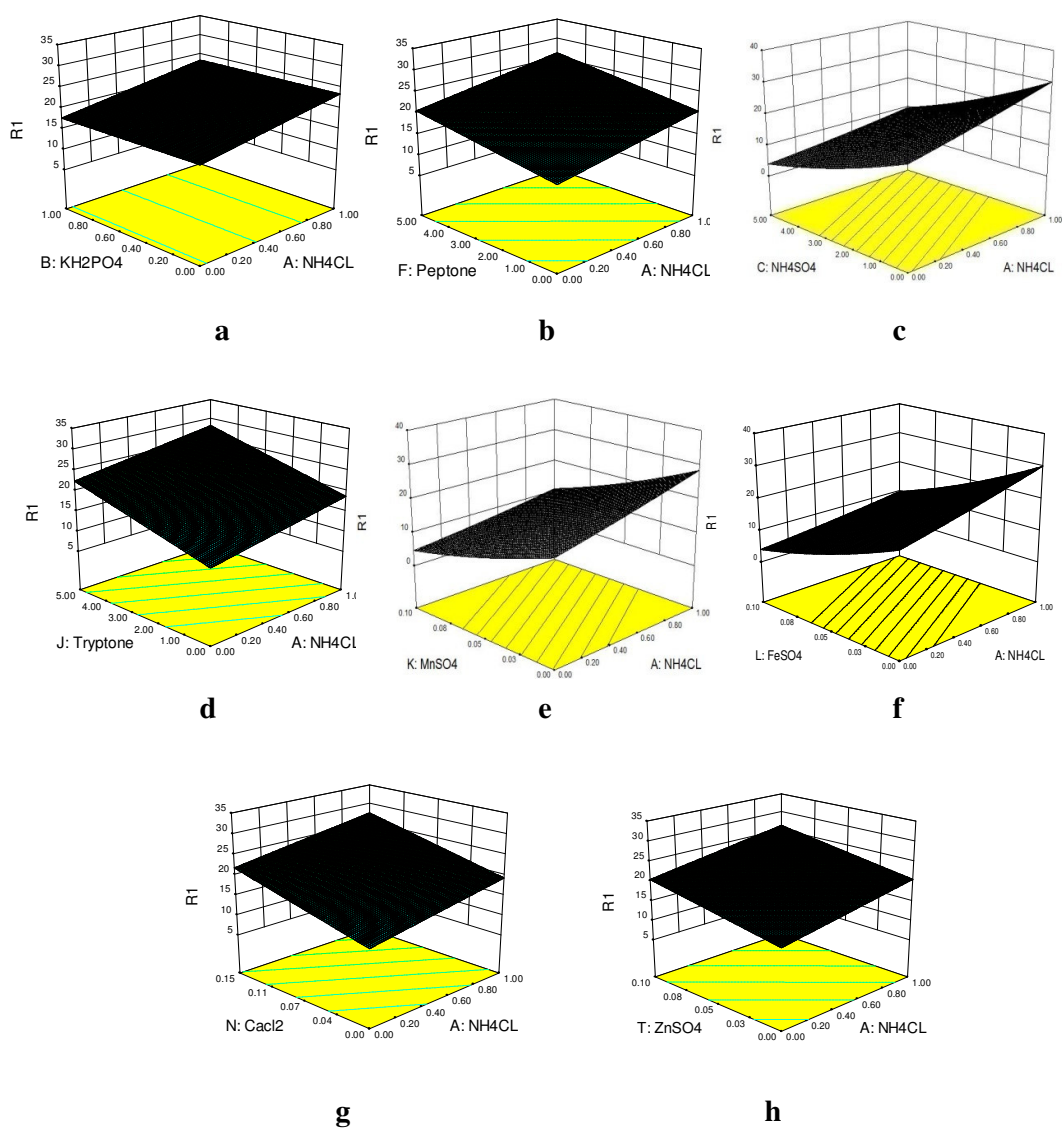


Fig 36. Response surfaces for xylanase production in PBD by *Bacillus amyloliquefaciens* SH8

4.3.2.2 Optimization of medium components by Central composite design (CCD)

CCD is a useful tool deterring the optimal of medium constituents and their interactions. Based on the resulted from PBD and nitrogen sources etc were selected for further evaluation other effect xylanase production by CCD. Based on the results from PBD and optimal Nitrogen and salt additives experiments, tryptone, NH_4Cl , CaCl_2 and ZnSO_4 were selected for further evaluation of their effects on xylanase production. The following Table shows the actual and predicted responses. Table 28 depicted the results for RSM analysis based on CCD, 30 experiments were carried out and there response values with different combinations of four factors were considered. From multiple regression analysis we found that the second order polynomial equation can explain xylanase production regardless of the significance of coefficients. If Y is the response value than the fitted response surface model is

$$Y=2.14+0.75A+0.10B+0.65C+1.2D-0.03AB-0.7AC-0.41AD-0.05BC-0.008BD+2.45CD-0.08A^2-1.65B^2+0.47C^2+0.10D^2$$

RSM was employed to characterize the individual and interactive effects of nutrient concentration on the xylanase production. Table 29 and 30 depicted the second order model was hypothesized and statistically evaluated by analysis of Variance (ANOVA). ANOVA results showed that the value of R^2 for the response was in reasonable agreement with the adjusted R^2 . This insured a satisfactory adjustment of quadratic model of to the experimental data. The Model F-value of 55.61 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, D, AB, AC, A^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 3.27 implies the Lack of Fit is not significant relative to the pure error. There is a 10.12% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of

fit is good, we want the model to fit. The model presented a high determination coefficient ($R^2=0.9811$) explaining the 98.11% variability in the response. The "Pred R^2 " of 0.9019 is in reasonable agreement with the "Adj R^2 " of 0.9635. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 27.461 indicates an adequate signal. This model can be used to navigate the design space. The coefficient of variation (CV) indicates that the degree of precision with which the treatments were compared. Usually the higher the value of CV (4.089) indicated a better precision and reliability of the experiment (Box *et al.*, 1978). The 2D contour plots showed the interactive effect of nutrients on xylanase activity as depicted in Fig 47. The three dimensional response surface from the four (Fig. 48) variables showed non linear relationship between the independent variables. It is necessary to check the fitted model to ensure that it provides an adequate approximation of the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of fitted response give poor or misleading results. The residuals from the least squares fit play an important role in judging the model adequacy (Myers and Montgomery, 2002). By constructing a normal probability plot of the residuals, a check was made for the normality assumption as given in Fig 40a). The normality assumption was satisfied as the residual plot approximated along a straight line. The plot (Fig. 40a) is satisfactory so we conclude that quadratic model is adequate to describe the xylanase activity by response surface. Normally, a regression model having an R^2 value higher than 0.9 is considered to have very high correlation (Haaland, 1989). The closer the value of R (Correlation coefficient) to 1, the better the correlation between the experimental results and predicted values. The values of R^2 (0.9811) indicates a close agreement between the experimental results and the theoretical values predicted by the model equation. The inverse of the residual sum of square is -1, dip suddenly, with a minimum of the best optimal value -1.18 (Fig 37). The data require the inverse transformation, as current value of confidence interval it contains (λ) is very close to the optimum value (Box and Cox, 1964). The model shows the minimum and maximum confidence interval value is -2.49 and 0.52 (Fig 37).

Table 28. Experimental design of central composite for four factors and the production of xylanase

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response		Inverse	
	A:NH ₄ Cl	B:tryptone	C:CaCl ₂	D:ZnSO ₄	Xylanase IU/ml		Transformation	
					Experimental value	Predicted value	Experimental value	Predicted value
1	1.5	7.0	0.3	0.15	45.10	45.55	0.022	0.022
2	1.5	7.0	0.3	0.15	45.00	45.55	0.022	0.022
3	1.0	9.0	0.2	0.10	39.34	39.01	0.025	0.026
4	1.5	7.0	0.3	0.15	45.21	45.55	0.022	0.022
5	1.5	7.0	0.3	0.15	44.89	45.55	0.022	0.022
6	1.5	11.0	0.3	0.15	53.98	55.72	0.019	0.018
7	0.5	7.0	0.3	0.15	29.61	31.11	0.034	0.033
8	1.0	9.0	0.2	0.20	45.18	45.24	0.022	0.022
9	2.0	9.0	0.4	0.20	61.23	61.84	0.016	0.016
10	1.5	7.0	0.1	0.15	46.00	47.42	0.022	0.021
11	2.0	5.0	0.2	0.10	44.90	47.83	0.022	0.021
12	1.0	5.0	0.4	0.10	32.10	31.62	0.031	0.032
13	1.0	5.0	0.2	0.20	35.00	34.12	0.029	0.029
14	2.0	9.0	0.4	0.10	51.65	53.34	0.019	0.019
15	1.0	9.0	0.4	0.20	49.60	47.48	0.020	0.021
16	1.5	3.0	0.3	0.15	35.87	34.47	0.028	0.028
17	1.5	7.0	0.3	0.15	44.98	45.55	0.022	0.022
18	2.0	9.0	0.2	0.20	65.10	66.39	0.015	0.015
19	1.5	7.0	0.3	0.05	37.67	39.59	0.027	0.026
20	1.0	5.0	0.2	0.10	31.23	29.47	0.032	0.033
21	1.5	7.0	0.3	0.15	48.12	45.55	0.021	0.022
22	2.5	7.0	0.3	0.15	65.00	63.84	0.015	0.016
23	1.5	7.0	0.5	0.15	46.10	45.03	0.022	0.022
24	2.0	9.0	0.2	0.10	64.13	59.96	0.016	0.017
25	2.0	5.0	0.2	0.20	53.00	52.69	0.019	0.019
26	1.0	9.0	0.4	0.10	40.01	39.17	0.025	0.025
27	1.0	5.0	0.4	0.20	44.41	43.20	0.023	0.023
28	2.0	5.0	0.4	0.10	35.34	38.36	0.028	0.027
29	1.5	7.0	0.3	0.25	48.99	50.13	0.020	0.020
30	2.0	5.0	0.4	0.20	54.32	52.75	0.018	0.019

Table 29. ANOVA of response surface for Central Composite Design

Source	Sum of squares	df	Mean square	F value	p-value prob>F	
Model	6.672E-004	14	4.766E-005	55.61	< 0.0001	significant
A-NH ₄ Cl	4.068E-004	1	4.068E-004	474.71	< 0.0001	
B-tryptone	1.678E-004	1	1.678E-004	195.82	< 0.0001	
C-CaCl ₂	3.489E-007	1	3.489E-007	0.41	0.5330	
D-ZnSO ₄	6.490E-005	1	6.490E-005	75.74	< 0.0001	
AB	6.139E-006	1	6.139E-006	7.16	0.0173	
AC	6.337E-006	1	6.337E-006	7.40	0.0158	
AD	1.997E-006	1	1.997E-006	2.33	0.1477	
BC	1.792E-007	1	1.792E-007	0.21	0.6540	
BD	1.179E-008	1	1.179E-008	0.014	0.9082	
CD	3.758E-007	1	3.758E-007	0.44	0.5179	
A ²	1.009E-005	1	1.009E-005	11.78	0.0037	
B ²	1.890E-006	1	1.890E-006	2.21	0.1582	
C ²	3.265E-007	1	3.265E-007	0.38	0.5463	
D ²	1.821E-007	1	1.821E-007	0.21	0.6514	
Residual	1.285E-005	15	8.569E-007			
Lack of Fit	1.115E-005	10	1.115E-006	3.27	0.1012	Not significant
Pure Error	1.703E-006	5	3.405E-007			
Cor Total	6.801E-004	29				

R² =0.9811, Adj R²=0.9635

SS; sum of square, Df ; degree of freedom; MS, mean sum of square

Table 30. Levels of variables and statistical analysis of Central Composite design

Factor	Coefficient Estimate	df	Standard Error	Low	High	VIF
Intercept	0.022	1	3.779E-004	0.021	0.022773	
A-NH ₄ Cl	-4.117E-003	1	1.890E-004	-4.520E-003	-0.00371	1
B-tryptone	-2.644E-003	1	1.890E-004	-3.047E-003	-0.00224	1
C-CaCl ₂	1.206E-004	1	1.890E-004	-2.822E-004	0.000523	1
D-ZnSO ₄	-1.644E-003	1	1.890E-004	-2.047E-003	-0.00124	1
AB	6.194E-004	1	2.314E-004	1.262E-004	0.001113	1
AC	6.293E-004	1	2.314E-004	1.361E-004	0.001123	1
AD	3.533E-004	1	2.314E-004	-1.400E-004	0.000847	1
BC	1.058E-004	1	2.314E-004	-3.875E-004	0.000599	1
BD	2.714E-005	1	2.314E-004	-4.661E-004	0.00052	1
CD	-1.532E-004	1	2.314E-004	-6.465E-004	0.00034	1
A ²	6.066E-004	1	1.768E-004	2.299E-004	0.000983	1.05
B ²	2.625E-004	1	1.768E-004	-1.143E-004	0.000639	1.05
C ²	-1.091E-004	1	1.768E-004	-4.858E-004	0.000268	1.05
D ²	8.148E-005	1	1.768E-004	-2.953E-004	0.000458	1.05

Design-Expert® Software
1/(Xylanase)

Lambda
Current = -1
Best = -1.18
Low C.I. = -2.49
High C.I. = 0.52

Recommend transform:
Inverse
(Lambda = -1)

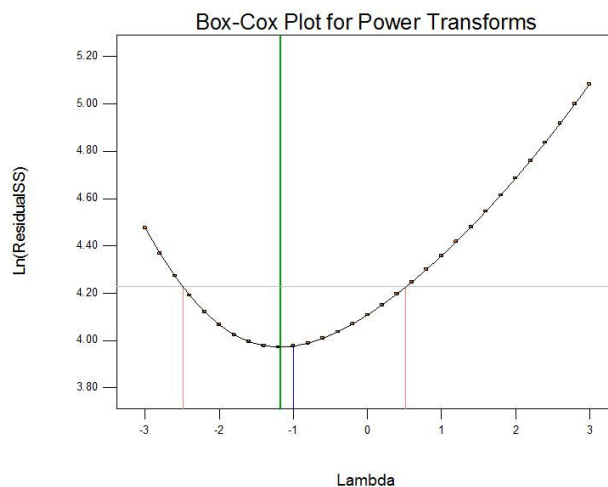


Fig 37. Box-Cox plot for power transforms of CCD for xylanase production by *Bacillus amyloliquefaciens* SH8

4.3.2.2.3 Validation of the model

The statistical optimal values of variables were obtained. The central composite design and the response at the different point yield maximum xylanase.

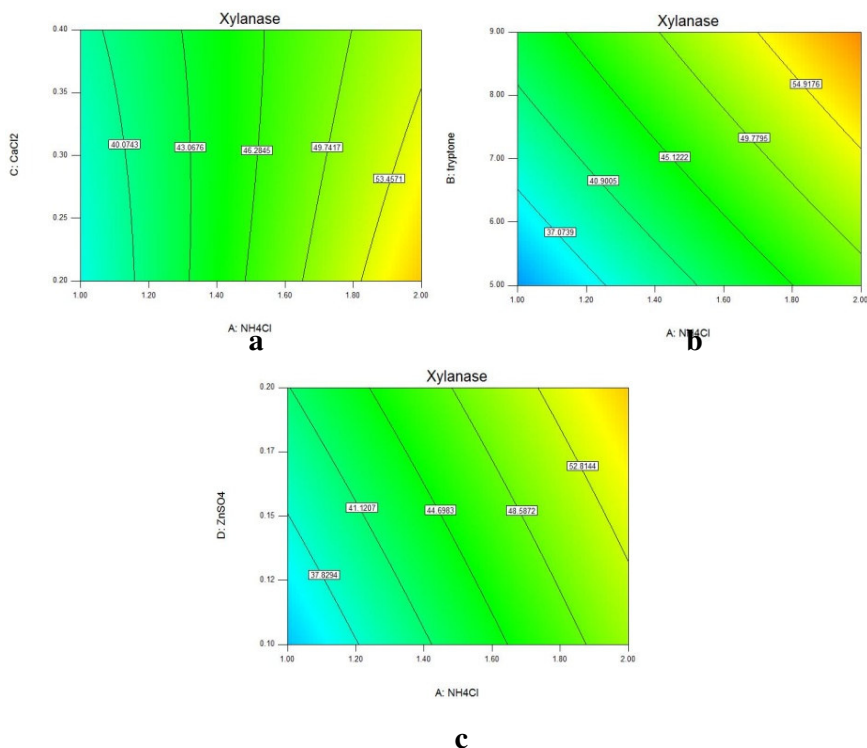


Fig: 38. 2D Contour plot Design for xylanase production in CCD by *Bacillus amyloliquefaciens* SH8

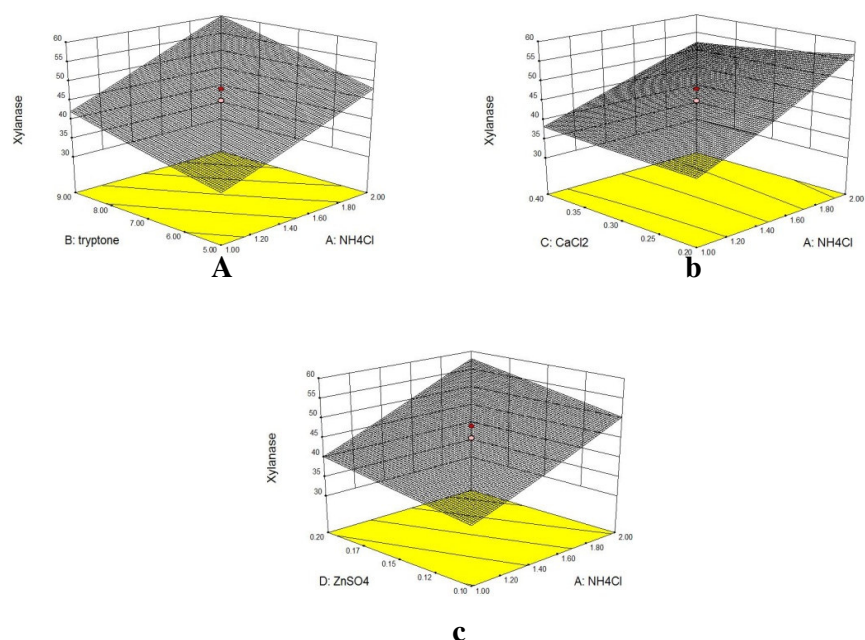


Fig 39. 3D surface for xylanase production in CCD by *Bacillus amyloliquefaciens* SH8

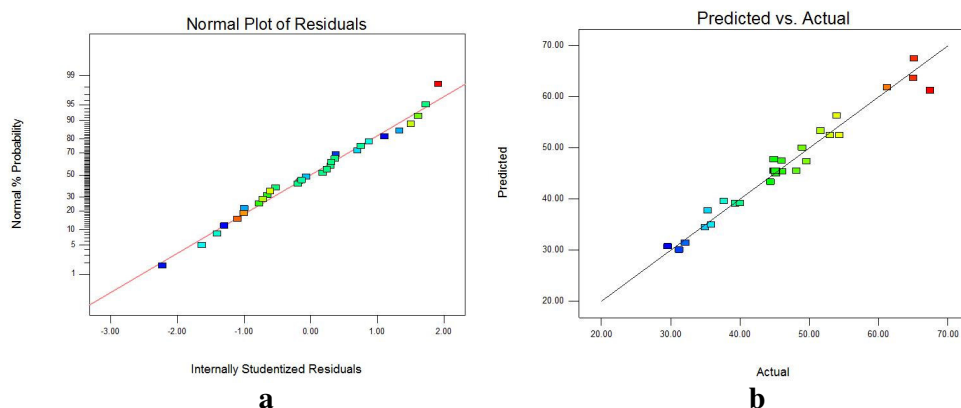


Fig 40. a) Plot of internally studentized residulas Vs. predicted response b) Actual Vs Predicted vaules for xylanase production in CCD by *Bacillus amyloliquefaciens* SH8.

In literature concentration of NH_4Cl in production medium for xylanase production has been predicted. In present study NH_4Cl , tryptone, CaCl_2 and ZnSO_4 have been found the best combination though PBD for highest production of xylanase from *Bacillus amyloliquefaciens* SH8. This combination has been found unique in assance that four of these nitrogen and salt additives have been found to play a direct role in promotery role in enzyme synthesis from this isolates (65.10 IU/ml). When literature was surveyed for the similar studies, the

same combination was found rare for others organisms. The effect of tryptone, CaCl_2 and ZnSO_4 has been found to supplements the nutritive rich medium for extracellular enzyme production. Usually tryptone has increased enzyme production for this bacterium by Bajaj and Singh (2010). However, Dhillon and Khanna (2000) reported that best nitrogen source for the xylanase production for *B. cirulans* AB-16 and Sharma *et al.* (2007) for *G. thermoleovorans* was tryptone while different concentration of NH_4Cl were used for xylanase production in *Bacillus* sp. for studing the stastical anlysis of main and interaction effects by Mullali *et al.* (2010). Pham *et al.* (1998) have supplemented NH_4Cl for xylanase production by *Bacillus* sp. using statistical design. Similarly, the role of CaCl_2 and ZnSO_4 has been found to enhance xylanase production in under Submerged fermentaion (Su *et al.*, 2011). Cui and Zhano (2012) reported that CaCl_2 was employed for the improvement of xylanase production using tesponse surface menthოდology. Sakthiselvan *et al.* (2012) used CaCl_2 and ZnSO_4 for xylanase production in *T. harzianum* MTCC 4358 on a novel substrate by Plackett Burman Design.

It proves the significant effect of vital componets for higher synthesis of xyalanse. Thus, rendering them an important constituents of any production medium.

4.3.2 CELLULASE AND XYLANASE OPTIMIZATION FROM HYPERCELLULOLYTIC AND XYLANOLYTIC FUNGI UNDER SSF USING CLASSICAL APPROACH ONE FACTOR AT A TIME (OFAT)

Solid state fermentation (SSF) involves the cultivation of microorganisms on a solid substrate in the near absence of free flowing liquid. The moisture involved in the fermentation process facilitates the growth and metabolic functions of the microorganism, and is typically retained within or complexed to the growth matrix. The characteristics of filamentous fungi, most notably hyphal growth, tolerance of low water activity, protein secretion, and an ability to grow on a variety of low-value lignocellulosic materials provide an unique adaptability to SSF. SSF is generally regarded as a more appropriate process for enzyme production, considering the natural growth environment of most filamentous

fungi by Gaffney *et al.* (2009). The range of enzymes produced during SSF is typically mediated by both the characteristics of the microorganism and the composition of the growth media.

For the commercial scale, conversion of cellulosic substrate requires the use of cellulases including endoglucanases or 1,4- β -D-glucan-4-glucanohydrolases (EC 3.2.1.4), exoglucanases, including 1,4- β -D-glucan glucanohydrolases (EC 3.2.1.91) and β -glucosidases or β -glucoside glucosidases (EC 3.2.1.21), which act synergistically for complete depolymerization into glucose. The commercial cellulases and xylanases are relatively costly; hence, their production from a wide range of microorganisms has been studied extensively to make the enzyme production process feasible (Lynd *et al.*, 2005). Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity. Fungal cellulases have been the major subjects of investigation over the years, as these catalyze the decay of lignocellulosic material in our ecosystem and some fungi produce extracellular cellulases in significant amounts. Commercial cellulase preparations from *Trichoderma reesei* are popular, as they contain high activities of both exo-glucanase and endo-glucanase, but have low levels of β -glucosidases. Attention has recently been diverted to other microorganisms, including the members of genus *Aspergillus*, which possess all the three essential components of cellulase system (Sohail *et al.*, 2009) including endo- β -1,4- glucanase, exo- β -1,4-glucanase, and β -1,4-glucosidase. Therefore, to obtain maximum yields of cellulase enzymes using abundantly available and the cost effective lignocellulosic substrates, fermentation studies are necessary and are demanding attention worldwide as reported by Mekala *et al.* (2008).

4.3.2.1 Physicochemical Pretreatment of Lignocellulosic Substrate

The particle size of substrate, especially in case of hard surface, is important for SSF. The particle size increases the porosity of the cell surface in providing better respiration/aeration efficiency (Murthy *et al.* 2009). On the other hand, the small particle (0.6-0.8 mm) may result in substrate accumulation, poor

growth, and enzyme production by interfering in microbial aeration (Krishna and Chandrasekaran 1996). The alteration of the substrate using pretreatment techniques leads a to change in physical nature of lignin, increase in the available surface area, increase pore sizes, partial depolymerization of cellulose and deacetylation of hemicelluloses which enhance the availability of the substrate (Murthy and Naidu, 2010).

Extracellular cellulase and xylanase production from potential hypercellulolytic and xylanolytic *T. longibracheatum* T2 and *R. oryzae* W2 were evaluated under solid state mode of fermentation utilizing lignocellulosic agricultural and forest waste (untreated and pretreated) as carbon source. Agricultural and forest residue used in the present study include *Triticum aestivum* straw, *Oryza sativa* straw, *Dandrocalamus strictus* wood, *Pinus roxburghii* needle and *Pinus roxburghii* wood. Chipping of bamboo and pine wood were done to get small pieces. Dried wood chips were grounded to fine particles of 1.0-1.5 mm mesh size while wheat straw, rice straw and pine needles were crushed with hand to an average size of 1 to 10 cm. As pretreatment of lingocellulosic substrates is prerequisite to open up the complex structure of native substrate, different substrate have been evaluated for further use.

4.3.2.2 Effect of Alkali Pretreatment on Lignocellulosic Substrate

The different lingocellulosic substrate namely, pine needle (T₁), pine wood (T₂), bamboo (T₃), wheat straw (T₄) and rice straw (T₅) were treated with alkali for assessing the activity of cellulase and xylanase enzymes. Data pertaining to cellulase titers viz., Fpase, CMCase and β -glucosidase and xylanase are presented in Table 31 to 34.

A perusal of Table 31 revealed that the significant difference in cellulase production was observed when different lingocellulosic substrates and pretreatment alone and in combination were used by *T. longibracheatum* T2. The significantly maximum cellulase titers (82.10 U/gds) were estimated in wheat straw, which was followed by rice straw (65.80 U/gds), bamboo (65.50 U/gds) and pine wood (60.50 U/gds) in descending order. The significantly minimum

Table 31. Cellulase production from *Trichoderma longibrachetum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste as substrate under SSF

Biomass	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
Pine needles (T ₁)	1.60* (0.08)** (3.40)***	2.40 (0.12) (4.80)	2.00 (0.10) (4.10)	14.20 (0.710) (30.21)	16.80 (0.84) (34.20)	15.50 (0.77) (32.20)	24.00 (1.20) (51.00)	34.00 (1.70) (69.30)	29.00 (1.45) (60.15)	39.80 (1.99) (84.68)	53.20 (2.66) (108.50)	46.50 (2.32) (96.59)	0.47	0.49	0.48	33.66
Pine wood (T ₂)	2.60 (0.13) (4.10)	3.60 (0.18) (5.30)	3.10 (0.15) (4.70)	16.40 (0.82) (26.45)	18.40 (0.92) (27.40)	17.40 (0.87) (26.91)	34.00 (1.70) (54.81)	46.00 (2.30) (68.65)	40.00 (2.00) (61.74)	53.00 (2.65) (85.48)	68.00 (3.40) (101.49)	60.50 (3.02) (93.48)	0.62	0.67	0.64	28.30
Bamboo wood (T ₃)	3.20 (0.16) (4.15)	4.20 (0.21) (5.18)	3.70 (0.18) (4.60)	19.60 (0.98) (25.44)	24.00 (1.20) (29.62)	21.80 (1.09) (27.53)	36.00 (1.80) (46.75)	44.00 (2.60) (54.30)	40.00 (2.20) (50.52)	58.8 (2.94) (76.36)	72.40 (3.62) (89.13)	65.50 (3.28) (82.74)	0.77	0.81	0.79	22.78
Wheat straw (T ₄)	7.00 (0.35) (6.66)	9.20 (0.35) (8.00)	8.10 (0.35) (7.30)	22.00 (1.10) (20.95)	32.00 (1.60) (27.80)	27.33 (1.35) (24.37)	42.00 (2.10) (40.00)	52.00 (2.60) (45.20)	47.00 (2.35) (42.60)	71.00 (3.55) (67.61)	93.20 (4.66) (81.04)	82.10 (4.10) (74.32)	1.05	1.15	1.10	31.26
Rice straw (T ₅)	5.20 (0.26) (5.97)	7.80 (0.26) (8.29)	6.50 (0.26) (7.10)	16.60 (0.83) (19.08)	28.00 (1.40) (24.34)	22.30 (1.11) (21.88)	32.00 (1.60) (36.78)	42.00 (2.10) (36.52)	37.00 (1.85) (36.65)	53.80 (2.69) (61.83)	77.80 (3.89) (67.65)	65.80 (3.29) (64.74)	0.87	0.94	0.90	44.6
Mean	3.92 (0.19) (4.85)	5.44 (0.22) (6.31)		17.76 (0.88) (24.43)	23.84 (1.19) (28.67)		33.60 (1.68) (45.87)	43.60 (2.26) (54.79)		55.28 (2.76) (75.19)	72.92 (3.64) (89.56)		0.76	0.81		
CD _{T 0.05}	0.27 (0.021) (0.34)			0.81 (0.139) (0.50)			0.13 (0.30) (0.59)			0.87 (0.43) (1.15)			0.2			
CD _{B 0.05}	0.42 (00.013) (0.54)			1.2 (0.088) (0.80)			0.20 (0.193) (0.93)			1.21 (0.27) (0.76)			0.39			
CD _{TxB0.05}	0.56 (0.029) (0.72)			1.74 (0.192) (1.08)			1.20 (0.21) (1.26)			1.62 (0.60) (1.04)			0.04			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Treated- untreated/untreated x100

cellulase titers (46.50 U/gds) were observed in pine needle. Similarly in case of pretreatment, significantly maximum cellulase production was observed in alkali treated substrate (72.92 U/gds) than untreated substrate (55.28 U/gds). Interaction study (TxB) revealed that significantly maximum cellulase titers (93.2 U/gds) were estimated; when alkali pretreated substrate of wheat straw was used as a substrate and minimum in untreated substrate of pine needle (39.80 U/gds).

An appraisal of data in Table 32 showed that the significant differences in xylanase titers were observed when different lingocellulosic substrate and pretreatment alone and in combination was used for the study. Significantly highest xylanase titers (329.40 U/gds) were estimated in wheat straw, which was followed by rice straw (299.3 U/gds), pine wood (273.1 U/gds). The significantly lowest xylanase titers were observed in pine needles (226.4 U/gds). The xylanase production was also significantly influenced by pretreatment. The significantly highest xylanase production was observed in alkali treated substrate (301.04 U/gds) and minimum in untreated substrate (250.32 U/gds). Interaction effect (TxB) unveiled that significantly highest xylanase titers (356.60 U/gds) were estimated; when alkali pretreated substrate of wheat straw was utilized as a substrate. The significantly least xylanase titers were found in untreated substrate of pine needle (204.60 U/gds).

A scrutiny of data in Table 33 revealed that the significant difference in cellulase production was noticed when different lingocellulosic substrate and pretreatment were employed alone and in combination for study in *R. oryzae* W2. The significantly maximum cellulase titers (92.90 U/gds) was estimated in wheat straw, which was followed by rice straw (80.30 U/gds), bamboo (59.15 U/gds) and pine wood (56.15 U/gds). The significantly minimum cellulase titers (41.15 U/gds) were noticed in pine needles. In case of pretreatment, the significantly more cellulase production was recorded in untreated substrate (74.86 U/gds) in comparison to alkali pretreated substrate (57.00 U/gds). Interaction analyses (TxB) showed that significantly maximum cellulase titers (106.4 U/gds) were estimated; when an untreated wheat straw was used as a substrate. This was succeeded by untreated substrate of rice straw (87.60 U/gds), alkali pretreated

Table 32. Xylanase production from *Trichoderma longibracheatum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste as substrate under SSF

Biomass	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
Pine needles (T₁)	204.60* (10.23)** (435.31)***	248.20 (12.41) (506.53)	226.4 (11.32) (470.92)	0.47	0.49	0.48	21.30
Pine wood (T₂)	252.80 (12.64) (407.74)	293.40 (14.67) (437.91)	273.1 (13.65) (482.83)	0.62	0.67	0.64	16.06
Bamboo wood (T₃)	222.60 (11.13) (289.09)	277.80 (13.89) (342.96)	250.2 (12.51) (316.03)	0.77	0.81	0.79	24.79
Wheat straw (T₄)	302.20 (15.11) (289.71)	356.60 (17.83) (310.95)	329.4 (16.47) (300.33)	1.05	1.15	1.10	17.55
Rice straw (T₅)	269.40 (13.47) (309.65)	329.20 (16.46) (350.21)	299.3 (14.96) (329.93)	0.87	0.94	0.90	22.19
Mean	250.7 (12.51) (346.3)	301.04 (15.05) (389.71)		0.76	0.81		
CD _{T 0.05}	278.55 (2.03) (1.28)			0.20			
CD _{B 0.05}	6.8 (1.28) (1.34)			0.39			
CD _{TxB0.05}	9.22 (1.76) (1.84)			0.04			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = $\frac{\text{Treated} - \text{untreated}}{\text{untreated}} \times 100$

substrate of wheat straw (79.40 U/gds) and alkali pretreated substrate of rice straw (73.00 U/gds) in descending order. The significantly least cellulase titers were recorded in alkali pretreated substrate of pine needles (34.20 U/gds).

Table 34 depicted that significantly highest xylanase titers (323.35 U/gds) were figured in wheat straw, which was followed by rice straw (278.20 U/gds) and pine wood (223.10 U/gds). The significantly lowest xylanase titers were observed in pine needles (156.80 U/gds). The xylanase production was not significantly influenced by alkali pretreatment and more xylanase production was observed in untreated substrate (256.18 U/gds) than alkali pretreated substrate (209.60 U/gds). Interaction study (Tx B) revealed that significantly highest xylanase titers (362.50 U/gds) were recorded; when untreated wheat straw was used as a substrate. The significantly lowest xylanase titers were noticed in alkali pretreated substrate of pine needle (143.4 U/gds) and Plate 8-17.

Alkaline pretreatment is basically a delignification process, in which a significant amount of hemicellulose is solubilized as well. The action mechanism is believed to be saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. It also removes acetyl and various uronic acid substitutions on hemicelluloses which are responsible to reduce the accessibility of cellulose and hemicellulose to enzymes. This pretreatment of lignocellulosic materials causes swelling, leading to decreased degree of polymerization and crystallinity, increased surface area, disruption of the lignin structure and separation of structural linkages between lignin and carbohydrate (Zheng *et al.*, 2009). The effectiveness of alkaline pretreatment varies, depending on the substrate and treatment conditions. In general, alkaline pretreatment is more effective on hardwood, herbaceous crops, and agricultural residues with low lignin content than on softwood with high lignin content (Bjerre *et al.*, 1996). Generally alkaline pretreatment is more effective on hardwood with low lignin content (digestibility increase from 14% to 55%) than on softwood with high lignin content (digestibility decrease from 24-55 to 20%) as cited by Mtui, (2009).

Table 33. Cellulase production from *Rhizopus oryzae* W2 [JX 213812.1] using alkali pretreated lignocellulosic waste as substrate under SSF

Biomass	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		
Pine needles (T ₁)	3.40* (0.17)** (6.42)***	2.20 (0.11) (4.58)	2.80 (0.14) (5.50)	20.10 (1.01) (37.52)	15.30 (0.77) (31.88)	17.70 (0.89) (34.90)	24.60 (1.23) (46.42)	16.70 (0.84) (34.79)	20.65 (1.03) (40.60)	48.10 (2.41) (90.75)	34.20 (1.71) (71.25)	41.15 (2.06) (81.00)	0.53	0.48	0.50	28.90
Pine wood (T ₂)	3.6 (0.18) (6.43)	2.40 (0.12) (4.70)	3.00 (0.15) (5.56)	30.60 (1.53) (54.64)	21.80 (1.09) (41.92)	26.20 (1.31) (48.28)	29.40 (1.47) (52.50)	24.50 (1.23) (47.12)	26.95 (1.35) (49.81)	63.60 (3.18) (113.57)	48.70 (2.44) (93.65)	56.15 (2.81) (103.61)	0.56	0.52	0.54	23.43
Bamboo wood (T ₃)	3.50 (0.17) (6.60)	2.00 (0.10) (4.08)	2.75 (0.14) (5.34)	31.20 (1.56) (58.87)	23.60 (1.18) (48.16)	27.40 (1.37) (53.52)	33.90 (1.70) (63.96)	24.10 (1.21) (49.18)	29.00 (1.45) (56.57)	68.60 (3.43) (129.43)	49.70 (2.49) (101.43)	59.15 (2.96) (115.43)	0.53	0.49	0.51	27.55
Wheat straw (T ₄)	4.8 (0.24) (4.06)	3.4 (0.17) (4.35)	4.1 (0.21) (4.20)	48.00 (2.40) (40.67)	36 (1.80) (46.15)	42.00 (2.10) (43.41)	54.00 (2.70) (45.76)	40.00 (2.00) (51.28)	47.00 (2.35) (48.52)	106.40 (5.32) (90.16)	79.40 (3.97) (101.79)	92.90 (4.65) (95.97)	1.18	0.78	0.98	25.38
Rice straw (T ₅)	3.6 (0.18) (3.71)	3.0 (0.15) (4.34)	3.3 (0.17) (4.02)	36.00 (1.80) (37.11)	32.00 (1.60) (46.30)	34.00 (1.70) (41.70)	48.00 (2.40) (49.48)	38.00 (1.90) (55.07)	43.00 (2.15) (52.27)	87.60 (4.38) (90.30)	73.00 (3.65) (105.79)	80.30 (4.02) (98.04)	0.95	0.69	0.82	16.67
Mean	3.78 (0.16) (4.61)	2.6 (0.13) (4.41)		33.18 (1.66) (47.92)	25.74 (1.28) (42.20)		37.98 (1.90) (51.62)	28.66 (1.43) (47.48)		74.86 (3.75) (102.84)	57.00 (2.85) (94.78)		0.75	0.59		
CD _{T 0.05}	0.55 (0.012) (0.53)			0.83 (0.11) (1.23)			1.05 (0.26) (0.96)			1.72 (0.22) (2.24)			0.014			
CD _{B 0.05}	0.87 (0.019) (0.85)			1.32 (0.17) (1.95)			1.67 (0.41) (1.53)			2.73 (0.34) (3.55)			0.022			
CD _{TxB0.05}	1.18 (0.026) (1.14)			1.78 (0.24) (2.64)			2.26 (0.56) (2.06)			3.75 (0.50) (4.90)			0.030			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated x 100

Table 34. Xylanase production from *Rhizopus oryzae* W2 [JX 213812.1] using alkali pretreated lignocellulosic waste as substrate under SSF

Biomass	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		
Pine needles (T₁)	170.2* (8.51)** (321.13)***	143.4 (7.17) (298.75)	156.80 (7.84) (309.94)	0.53	0.48	0.50	15.75
Pine wood (T₂)	244.0 (12.2) (435.71)	202.2 (10.11) (396.47)	223.10 (11.15) (416.09)	0.56	0.52	0.54	17.13
Bamboo wood (T₃)	200.0 (10.0) (392.15)	166 (8.3) (338.77)	183.00 (9.15) (365.46)	0.53	0.49	0.51	17.00
Wheat straw (T₄)	362.5 (18.12) (307.20)	284.2 (14.21) (364.35)	323.35 (16.16) (335.77)	1.18	0.78	0.98	21.60
Rice straw (T₅)	304.2 (15.21) (313.60)	252.2 (12.61) (365.50)	278.20 (13.91) (339.55)	0.95	0.69	0.82	17.09
Mean	256.18 (12.80) (353.95)	209.6 (10.48) (352.76)		0.75	0.59		
CD _{T 0.05}	1.20 (1.12) (1.29)			0.014			
CD _{B 0.05}	1.90 (1.78) (2.04)			0.022			
CD _{TxB0.05}	2.58 (2.46) (2.76)			0.030			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = $\frac{\text{Untreated}-\text{Treated}}{\text{Treated}} \times 100$

Alkali pretreatment can result in sharp increase in saccharification with manifold yields. Alkaline pretreatment was shown to be more effective on agricultural residues than on wood materials (Taherzadeh and Karimi, 2008).

Similar findings have been quoted by Zhao *et al.* (2007) studied enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature. It was found that the enzymatic hydrolysis rate and efficiency can be significantly improved by the pretreatment. Compared with acid or oxidative reagents alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicelluloses and cellulose and avoiding fragmentation of hemicelluloses polymers. At low temperature chemicals used for pretreatment i.e. NaOH can remove lignin, disrupt the firm bonding between hemicellulose, cellulose, and lignin, and alter the structure of treated biomass it more accessible to hydrolysis enzymes. The xylanase production by a local soil isolate of *Trichoderma viride*, could be improved by using alkali treated straw as carbon source. Agricultural wastes contain a good amount of lignin, which protects the xylan to be attacked by hydrolytic enzymes. To make components of lignocellulosics more accessible, substrates were autoclaved with 10 ml of different concentrations of alkali (0.1, 0.5 and 1.0N) for 30 min (Goyal *et al.*, 2008). Raghavendra and Geeta (2007) reported that the agricultural crop residues such as paddy straw, wheat straw and sugarcane bagasse are abundantly available having rich source of sugars. In the present study, pretreatment process was developed for hydrolyzing paddy straw, wheat straw and sugarcane bagasse into fermentable sugars. The powdered substrates were delignified with 3 per cent NaOH and subjected for microbiological and commercial cellulase enzyme pretreatment. Among the fungal cultures *Trichoderma reesei* has yielded maximum reducing sugar of 22.30 mg/g in paddy straw, 25.56 mg/g in wheat straw and 28.56 mg/g in sugarcane bagasse. In case of commercial cellulase enzyme sugarcane bagasse has yielded maximum reducing sugar 136.86 mg/g after hydrolysis with 2.5 percent enzyme concentration at 45°C for 24 h of incubation.

Akhtar *et al.* (2001) produced cellulases by *Bacillus subtilis* used for saccharification of wheat straw, rice straw and bagasse. Wheat straw, rice straw

and bagasse were separately pretreated with (1-4% w/v) NaOH for 4 h. It has been seen that saccharification level has been increased to 33 in comparison to 4% saccharification of untreated wheat straw when the substrate was pretreated with 2% NaOH for 4h at room temperature. Sherief *et al.* (2010) investigated bioprocessing of lignocellulosic biomass for production of bioethanol using Thermotolerant *Aspergillus fumigatus* under Solid State Fermentation conditions. Alkali pretreatment was given to rice straw twenty grams of milled dried rice straw was suspended in 160 mL 1 % (w/v) NaOH and pretreated in an autoclave at 121°C for 1 h. The residues were collected and washed extensively with water until washings turned neutral. The residues were dried at 65°C for two days. Dried biomass is grinded to ensure more uniform particle size distribution.

Vaccarino *et al.* (1987) studied the effect of NaOH pretreatment on enzymatic digestibility of grape marc, and greatest degrading effects were obtained by pretreatment with 1% NaOH solution at 120°C. He *et al.* (2010) observed that the crystalline structure of corn stover was disrupted, and lignin was removed, while cellulose and hemicellulose were retained in corn stover by the pretreatment with 1% NaOH in 1 h. The reducing-sugar yield from the pretreated corn stovers increased from 20.2% to 46.7% when the NaOH concentration increased from 0% to 1%. The 1% NaOH pretreated corn stover had a holocellulose conversion of 55.1%.

4.3.2.3 Effect of Acid Pretreatment on Lignocellulosic Substrate

The different lignocellulosic substrate namely, pine needle (T₁), pine wood (T₂), bamboo (T₃), wheat straw (T₄) and rice straw (T₅) were treated with acid for assessing the activity of cellulase and xylanase enzymes. Data pertaining to cellulase titers viz., Fpase, CMCase and β -glucosidase and xylanase are presented in Table 35 to 38.

The significant difference in cellulase production was observed when different lignocellulosic substrate and pretreatment alone and in combination was used for the study in *T. longibrachearum* T2 (Table 35). The significantly maximum cellulase titers (76.40 U/gds) were noticed in wheat straw and

minimum (48.20 U/gds) in pine needle. Similarly, the significantly maximum cellulase production was observed in acid treated substrate (68.88 U/gds) in comparison to untreated substrate (55.28 U/gds). The combined study (TxB) showed that significantly most cellulase titers (81.8 U/gds) were estimated; when acid pretreated substrate of wheat straw was used as a substrate. The significantly least cellulase titers were observed in untreated substrate of pine needle (39.80 U/gds).

A cursory of data in Table 36 exhibited significantly highest xylanase titers (310.90 U/gds) were estimated in wheat straw, which was followed by rice straw (274.5 U/gds) and pine wood (258.75 U/gds). The significantly lowest xylanase titers were observed in pine needles (209.1 U/gds). Similarly, the significantly highest xylanase production was shown by acid pretreated substrate (263.06 U/gds) than untreated substrate (250.7 U/gds). Interaction study (TxB) revealed that significantly highest xylanase titers (319.60 U/gds) were estimated, when acid pretreated substrate of wheat straw was used as a substrate. The significantly lowest xylanase titers were observed in untreated substrate of pine needle (204.60 U/gds).

An appraisal of Table 37 revealed that the significant difference in cellulase production was observed when different lingocellulosic substrates were used for the study of *R. oryzae* W2. The significantly maximum cellulase titers (90.75 U/gds) were estimated in wheat straw, which was followed by rice straw (77.81 U/gds), bamboo (56.65 U/gds) and wood (53.95 U/gds). The significantly minimum cellulase titers (39.70 U/gds) were observed in pine needles. On the other hand, cellulase production was not influenced by acid pretreatment. The significantly maximum cellulase production was observed in untreated substrate (79.94 U/gds) than acid pretreated substrate (52.70 U/gds). Interaction study of different substrates (TxB) revealed that significantly maximum cellulase titers (106.4 U/gds) were recorded; when an untreated wheat straw was applied as a substrate. The significantly minimum cellulase titers were observed in acid pretreated substrate of pine needles (31.30 U/gds).

Table 35. Cellulase production from *Trichoderma longibracheatum* T2 [JX 213811.1] using acid pretreated lignocellulosic waste as substrate under SSF

Biomass	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
Pine needles (T ₁)	1.60* (0.08)** (3.40)***	2 (0.10) (4.16)	1.80 (0.09) (3.78)	14.20 (0.71) (30.21)	15.60 (0.78) (32.5)	14.90 (0.74) (31.35)	24.00 (1.20) (51.00)	39.00 (1.90) (81.25)	31.50 (1.57) (66.12)	39.80 (1.99) (84.68)	56.60 (2.83) (117.91)	48.20 (2.41) (101.3)	0.47	0.48	0.47	42.21
Pine wood (T ₂)	2.60 (0.13) (4.10)	3.20 (0.16) (5.00)	2.90 (0.14) (4.55)	16.40 (0.82) (26.45)	17.20 (0.86) (26.80)	16.80 (0.84) (26.61)	34.00 (1.70) (54.81)	48.00 (2.40) (75.00)	41.00 (2.05) (64.91)	53.00 (2.65) (85.48)	68.40 (3.42) (106.84)	60.70 (3.03) (96.16)	0.62	0.64	0.63	29.05
Bamboo wood (T ₃)	3.20 (0.16) (4.15)	3.60 (0.18) (4.55)	3.40 (0.17) (4.35)	19.60 (0.98) (25.44)	20.00 (1.00) (25.31)	19.80 (0.99) (25.37)	36.00 (1.80) (46.75)	42.00 (2.10) (53.16)	39.00 (1.95) (49.95)	58.80 (2.94) (76.36)	65.60 (3.28) (83.00)	62.20 (3.11) (79.68)	0.77	0.79	0.78	11.56
Wheat straw (T ₄)	7.00 (0.35) (6.66)	7.80 (0.39) (7.09)	7.40 (0.37) (6.87)	22.00 (1.10) (20.95)	28.00 (1.40) (25.45)	20.30 (1.25) (23.20)	42.00 (2.10) (40.00)	46.00 (2.300) (41.81)	44.50 (2.20) (40.90)	71.00 (3.55) (67.61)	81.800 (4.09) (74.36)	76.40 (3.82) (70.98)	1.05	1.10	1.07	15.21
Rice straw (T ₅)	5.20 (0.26) (5.97)	6.00 (0.30) (6.59)	5.43 (0.28) (6.28)	16.60 (0.83) (19.08)	24.00 (1.20) (26.37)	25.00 (1.01) (22.89)	32.00 (1.60) (36.78)	42.00 (2.10) (46.15)	37.50 (1.85) (41.46)	53.80 (2.69) (61.83)	72.00 (3.60) (79.12)	62.90 (3.14) (70.47)	0.87	0.91	0.89	33.82
Mean	3.92 (0.19) (4.85)	4.45 (0.22) (5.47)		17.76 (0.88) (24.43)	0.41 (1.10) (27.29)		33.60 (1.68) (45.87)	43.60 (2.17) (59.47)		55.28 (2.76) (75.19)	68.88 (3.44) (92.25)		0.75	0.78		
CD _{T 0.05}	0.27 (0.057) (0.34)			0.81 (0.195) (0.50)			0.13 (0.58) (0.59)			0.87 (0.78) (1.15)			0.034			
CD _{B 0.05}	0.60 (0.036) (0.85)			1.38 (0.123) (0.93)			1.97 (0.36) (1.7)			1.37 (0.49) (1.83)			0.53			
CD _{TxB0.05}	0.83 (0.080) (1.16)			1.86 (0.28) (1.30)			2.68 (0.80) (1.45)			1.83 (1.06) (2.50)			0.074			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Treated- untreated/untreated x100

Table 36. Xylanase production from *Trichoderma longibracheatum* T2 [JX 213811.1] using acid pretreated lignocellulosic waste as substrate under SSF

Biomass	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
Pine needles (T₁)	204.60* (10.23)** (435.31)***	213.60 (10.68) (435.91)	209.10 (10.45) (435.61)	0.47	0.48	0.475	11.73
Pine wood (T₂)	252.80 (12.64) (407.74)	264.70 (13.23) (381.56)	258.75 (12.39) (394.65)	0.62	0.64	0.63	3.67
Bamboo wood (T₃)	222.60 (11.13) (289.09)	237.80 (11.89) (297.25)	230.20 (11.51) (293.17)	0.77	0.79	0.78	8.71
Wheat straw (T₄)	302.20 (15.11) (289.71)	319.60 (15.98) (285.35)	310.90 (15.54) (287.5)	1.05	1.10	1.07	10.32
Rice straw (T₅)	269.40 (13.47) (309.65)	279.60 (13.98) (314.15)	274.50 (13.72) (311.90)	0.87	0.91	0.89	5.64
Mean	250.7 (12.51) (346.3)	263.06 (13.15) (342.84)		0.75	0.78		
CD_{T 0.05}	278.55 (1.61) (1.28)			0.034			
CD_{B 0.05}	440.42 (1.02) (1.94)			0.53			
CD_{TxB0.05}	597.2 (2.20) (2.64)			0.074			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = $\frac{\text{Treated} - \text{Untreated}}{\text{Untreated}} \times 100$

Table 37. Cellulase production from *Rhizopus oryzae* W2 [JX 213812.1] using acid pretreated lignocellulosic waste as substrate under SSF

Biomass	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1))	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		Untreated Biomass (B1)	Treated Biomass (B2)		
Pine needles (T ₁)	3.40** (0.17)** (6.42)**	2.00 (0.10) (3.92)	2.70 (0.14) (5.17)	20.10 (1.01) (37.92)	14.10 (0.71) (27.65)	17.10 (0.86) (32.79)	24.60 (1.23) (46.42)	15.20 (0.76) (29.80)	19.90 (1.00) (38.11)	48.10 (2.41) (90.75)	31.30 (1.57) (61.37)	39.70 (1.99) (76.06)	0.53	0.51	0.52	34.93
Pine wood (T ₂)	3.6 (0.18) (6.43)	2.2 (0.11) (4.00)	2.90 (0.15) (5.21)	30.60 (1.53) (54.64)	19.80 (0.99) (36.00)	25.20 (1.26) (45.32)	29.40 (1.47) (52.50)	22.30 (1.11) (40.55)	25.85 (1.29) (46.52)	63.60 (3.18) (113.57)	44.30 (2.22) (80.55)	53.95 (2.70) (97.06)	0.56	0.55	0.56	30.35
Bamboo wood (T ₃)	3.50 (0.18) (6.60)	2.00 (0.10) (3.92)	2.75 (0.14) (5.26)	31.20 (1.56) (58.87)	20.60 (1.03) (40.39)	25.90 (1.30) (49.63)	33.90 (1.70) (63.96)	22.10 (1.12) (43.33)	28.00 (1.40) (53.65)	68.60 (3.43) (129.43)	44.70 (2.24) (87.65)	56.65 (2.83) (108.54)	0.53	0.51	0.52	34.84
Wheat straw (T ₄)	4.8 (0.24) (4.07)	3.2 (0.16) (3.52)	4.00 (0.20) (3.79)	48.00 (2.40) (40.67)	33.99 (1.70) (37.35)	41.00 (2.05) (39.01)	54.00 (2.70) (45.76)	38.00 (1.90) (41.76)	46.00 (2.30) (43.76)	106.40 (5.32) (90.51)	75.19 (3.76) (82.63)	90.75 (4.55) (87.57)	1.18	0.91	0.91	29.33
Rice straw (T ₅)	3.6 (0.18) (3.79)	2.8 (0.14) (4.59)	3.20 (0.16) (4.19)	36.00 (1.80) (37.89)	30.00 (1.50) (49.18)	33.00 (1.65) (43.54)	48.00 (2.40) (49.48)	35.22 (1.76) (57.74)	41.61 (2.08) (54.13)	87.60 (4.38) (92.21)	68.02 (3.40) (111.51)	77.81 (3.89) (101.86)	0.95	0.61	0.78	22.35
Mean	3.78 (0.19) (5.46)	2.44 (0.12) (3.99)		33.18 (1.66) (46.00)	23.70 (1.18) (38.11)		37.98 (1.90) (51.83)	26.56 (1.33) (42.64)		74.94 (3.75) (103.30)	52.70 (2.64) (84.74)		0.75	0.56		
CD _{T 0.05}	0.20 (0.017) (0.26)			1.08 (0.095) (1.74)			1.19 (0.12) (1.79)			1.30 (0.82) (2.01)			0.021			
CD _{B 0.05}	0.32 (0.028) (0.41)			1.71 (0.15) (2.74)			1.88 (0.19) (2.86)			2.06 (0.27) (3.18)			0.033			
CD _{TxB0.05}	0.44 (0.040) (0.58)			2.32 (0.22) (3.72)			2.60 (0.26) (3.84)			2.80 (0.36) (4.34)			0.045			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated x 100

Data in Table 38 showed that significantly highest xylanase titers (335.55 U/gds) were estimated in wheat straw, which was followed by rice straw (283.20 U/gds), and by pine wood (232.00 U/gds). The significantly lowest xylanase titers were observed in pine needles (160.20 U/gds). Similarly, significantly highest xylanase production was observed in untreated substrate (256.18 U/gds) in comparison to acid pretreated substrate (223.80 U/gds). Interaction (TxB) revealed that significantly highest xylanase titers (362.50 U/gds) were recorded; when untreated substrate of wheat straw was used as a substrate. This was followed by acid pretreated substrate of wheat straw (308.60 U/gds), followed by untreated substrate of rice straw (304.20U/gds) and acid pretreated substrate of rice straw (262.20 U/gds).The significantly lowest xylanase titers were observed in acid pretreated substrate of pine needles (150.20 U/gds).

The action mode of dilute acid is to solubilize hemicellulose and remains lignin and cellulose intact so that the enzymatic digestibility of cellulose is enhanced.

In the related study by Dahoot and Noomrio (1996) the rate of β -glucosidase and CM-cellulase production by *Aspergillus fumigatus* in H_2SO_4 and HCl pretreated wheat straw mineral medium reached maximum. Ojumu *et al.* 2003 reported high cellulase activity from 3% pretreated saw dust, bagasse and corn cob as substrate, respectively. Studies by Delcampo *et al.* (2006) and Karimi *et al.* (2006) have established that 0.5% H_2SO_4 is optimal for treatment of wastes from vegetables and rice straw, respectively.

To promote carboxymethyl cellulase production, Iqbal *et al.* (2010) evaluated the effect of several kinetic parameters and one such parameter is pretreatment. Lignocellulosic wheat straw (10g) was carefully pretreated with 1 to 5% HCl in an Erlenmeyer flask (250 ml) kept at room temperature up to 2 h, autoclaved at 121°C and 15 lb/inch² pressure for 15min to ensure proficient deprivation of lignocellulosic contents of wheat straw to get optimum cellulase production. It was observed that pretreatment cause the removal of lignin and

Table 38. Xylanase production from *Rhizopus oryzae* W2 [JX 213812.1] using acid pretreated lignocellulosic waste as substrate under SSF

Biomass	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated Biomass (B ₂)		Untreated biomass (B ₁)	Treated Biomass (B ₂)		
Pine needles (T₁)	170.2* (8.51)** (321.13)***	150.2 (7.51) (306.53)	160.20 (8.01) (474.39)	0.53	0.51	0.52	11.75
Pine wood (T₂)	244.0 (12.2) (435.71)	220.0 (11.00) (415.01)	232.00 (11.60) (425.36)	0.56	0.55	0.56	9.84
Bamboo wood (T₃)	200.0 (10.0) (392.15)	178.0 (8.90) (349.01)	189.00 (9.45) (370.58)	0.53	0.51	0.52	11.00
Wheat straw (T₄)	362.5 (18.12) (307.20)	308.6 (15.43) (385.76)	335.55 (16.78) (346.48)	1.18	0.91	0.91	14.87
Rice straw (T₅)	304.2 (15.21) (313.60)	262.2 (13.11) (364.16)	283.20 (14.16) (338.88)	0.95	0.61	0.78	13.81
Mean	256.18 (12.80) (353.95)	223.8 (11.19) (428.32)		0.75	0.562		
CD _{T 0.05}	1.20 (0.86) (1.29)			0.021			
CD _{B 0.05}	1.99 (1.37) (2.64)			0.033			
CD _{TxB 0.05}	2.70 (1.90) (3.58)			0.045			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = $\frac{\text{Untreated}-\text{Treated}}{\text{Treated}} \times 100$

hemicelluloses successfully, while at the same time pretreatment also decreased the crystallinity of cellulose that improves the porosity characteristic of substrate. A significant difference in enzyme activity was observed when pretreated substrate was inoculated with freshly prepared inoculum of *Trichoderma harzianum* with the change in acid concentration and became maximum $175 \pm 2.78 \mu\text{M}/\text{mL}/\text{min}$ at 2% while, further increase in concentration did not enhance the enzyme activity.

Zheng *et al.* (2006) reported that saline crops were evaluated for their potential to be used as feedstock for fermentable sugar production via dilute acid pretreatment and enzymatic hydrolysis. The saline crops included two woods, Athel (*Tamarixaphylla L*) and Eucalyptus (*Eucalyptus camaldulensis*), and two grasses, Jose Tall Wheat grass (*Agropyronelongatum*) (JTW) and Creeping Wild Rye (*Leymus triticoides*) (CWR). Each of the biomass materials was first treated with dilute sulfuric acid under selected conditions (acid concentration, temperature and time) and then treated with enzymes (cellulases and β -glucosidase). The chemical composition (cellulose, hemicellulose and lignin contents) of each biomass material and the yields of total and different types of sugars after the acid and enzyme treatments were determined. The results showed that among the saline crops evaluated, the two grasses had the highest glucose yield (87% cellulose conversion) and fastest reaction rate during the enzyme

The rice straw was pretreated with dilute-acid hydrolysis by Karimi *et al.* (2006). The SSF experiments were carried out aerobically and anaerobically at 38°C; 50 g/l dry matter (DM) solid substrate concentration and 15 or 30 filter paper unit (FPU)/g DM of a commercial cellulase. The experiments were ended after 7 days, while an average of 2–3 days were usually enough to achieve the maximum ethanol yield. All the strains were able to produce ethanol from the pretreated rice straw with an overall yield of 40–74% of the maximum theoretical SSF yield, based on the glucan available in the solid substrate. *R. oryzae* had the best ethanol yield as 74% from rice straw followed by *M. indicus* with an overall yield of 68% with 15 FPU/g DM of cellulase.

4.3.2.4 Effect of Steam Pretreatment on Lingocellulosic Substrate

The different lingocellulosic substrate namely, pine needle (T₁), pine wood (T₂), bamboo (T₃), wheat straw (T₄) and rice straw (T₅) were treated with steam for assessing the activity of cellulase and xylanase enzymes. Data pertaining to cellulase titers viz., Fpase, CMCase and β -glucosidase and xylanase are presented in Table 39 to 42.

A perusal of Table 39 showed significant differences in cellulase production was observed when different lingocellulosic substrate and steam pretreatment was used for the study in *T. longibracheatum* T2. The significantly maximum cellulase titers (72.80 U/gds) were estimated in wheat straw and minimum (41.10 U/gds) in pine needle. Similarly, significantly maximum cellulase production was observed in steam treated substrate (59.16 U/gds) followed by untreated substrate (55.28 U/gds). The Interaction study of different substrate (Tx B) revealed that significantly maximum cellulase titers (74.6 U/gds) were estimated; when steam pretreated substrate of wheat straw was used as a substrate. The significantly minimum cellulase titers were observed in untreated substrate of pine needle (39.80 U/gds).

The scrutiny of data in Table 40 revealed significantly highest xylanase titers (318.90 U/gds) were estimated in wheat straw, which was followed by rice straw (278.0 U/gds) and bamboo wood (232.3 U/gds). The significantly lowest xylanase titers were observed in pine needles (216.6 U/gds). Similarly, significantly highest xylanase production was observed in steam treated substrate (263.2 U/gds) and lowest in untreated substrate (250.7 U/gds). Interaction study of different substrate (Tx B) revealed that significantly highest xylanase titers (335.60 U/gds) were estimated; when steam pretreated substrate of wheat straw was used as a substrate. The significantly lowest xylanase titers were observed in untreated substrate of pine needle (204.60 U/gds).

An appraisal of Table 41 revealed that the significant difference in cellulase production was observed when different lingocellulosic substrate was used for the study in *R. oryzae* W2. The significantly maximum cellulase titers (101.89 U/gds) were noticed in wheat straw and minimum (44.57 U/gds) in pine

Table 39. Cellulase production from *Trichoderma longibracheatum* T2 [JX 213811.1] using steam pretreated lignocellulosic waste as substrate under SSF

Biomass	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
Pine needles (T ₁)	1.60* (0.08)** (3.4)***	1.80 (0.09) (3.67)	1.70 (0.08) (3.53)	14.20 (0.71) (30.21)	14.60 (0.73) (29.79)	14.40 (0.72) (30.00)	24.00 (1.20) (51.00)	26.00 (1.30) (53.00)	25.00 (1.25) (52.00)	39.80 (1.99) (84.68)	42.40 (2.12) (86.50)	41.10 (2.05) (85.59)	0.47	0.49	0.48	6.5
Pine wood (T ₂)	2.60 (0.13) (4.10)	2.80 (0.14) (4.37)	2.70 (0.13) (4.23)	16.40 (0.82) (26.45)	16.80 (0.84) (26.25)	6.60 (0.83) (26.35)	34.00 (1.70) (54.81)	38.00 (1.90) (59.37)	36.00 (1.80) (57.10)	53.00 (2.65) (85.48)	57.60 (2.88) (90.00)	55.30 (2.76) (87.74)	0.62	0.64	0.63	8.67
Bamboo wood (T ₃)	3.20 (0.16) (4.15)	3.40 (0.17) (4.00)	3.30 (0.16) (4.07)	19.60 (0.98) (25.44)	20.00 (1.00) (25.00)	19.80 (0.99) (25.22)	36.00 (1.80) (46.75)	40.00 (2.00) (50.00)	38.00 (1.90) (48.37)	58.80 (2.94) (76.36)	63.20 (3.16) (79.00)	61.00 (3.05) (77.68)	0.77	0.80	0.78	7.48
Wheat straw (T ₄)	7.00 (0.35) (6.66)	7.40 (0.37) (6.66)	7.20 (0.36) (6.66)	22.00 (1.10) (20.95)	24.00 (1.20) (21.42)	23.00 (1.15) (18.00)	42.00 (2.10) (40.00)	44.00 (2.20) (39.28)	43.00 (2.15) (39.64)	71.00 (3.55) (67.61)	74.60 (3.16) (66.60)	72.80 (3.64) (67.10)	1.05	1.12	1.08	5.0
Rice straw (T ₅)	5.20 (0.26) (5.97)	5.20 (0.26) (5.30)	5.00 (0.26) (5.63)	16.60 (0.83) (19.08)	17.20 (0.86) (19.32)	16.90 (0.84) (19.20)	32.00 (1.60) (36.78)	36.00 (1.80) (40.44)	34.00 (1.70) (38.61)	53.80 (2.69) (61.83)	58.00 (3.73) (65.16)	55.90 (2.79) (63.16)	0.87	0.89	0.88	7.89
Mean	3.92 (0.19) (4.85)	4.12 (0.20) (4.64)		17.76 (0.88) (24.43)	18.52 (0.92) (24.36)		33.60 (1.68) (45.87)	36.80 (1.84) (48.42)		55.28 (2.76) (75.19)	59.16 (2.95) (77.32)		0.75	0.78		
CD _{T 0.05}	0.27 (0.036) (0.34)			0.81 (0.099) (0.50)			0.13 (0.44) (0.59)			0.86 (0.86) (1.15)			0.025			
CD _{B 0.05}	0.56 (0.022) (1.21)			1.53 (0.062) (2.4)			3.27 (0.28) (1.76)			2.0 (0.54) (1.70)			0.040			
CD _{TxB0.05}	0.78 (0.050) (1.64)			2.04 (0.136) (3.32)			4.44 (0.62) (2.38)			2.80 (1.20) (2.30)			0.054			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Treated- Untreated/Untreated x100

Table 40. Xylanase production from *Trichoderma longibracheatum* T2 [JX 213811.1] using steam pretreated lignocellulosic waste as substrate under SSF

Biomass	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
Pine needles (T₁)	204.60* (10.23)** (435.31)***	228.60 (11.43) (476.25)	216.60 (10.83) (455.70)	0.47	0.48	0.48	4.39
Pine wood (T₂)	252.80 (12.64) (407.74)	262.10 (13.10) (409.53)	257.45 (12.87) (408.63)	0.62	0.64	0.63	4.70
Bamboo wood (T₃)	222.60 (11.13) (289.09)	242.00 (12.10) (306.32)	232.30 (11.61) (297.70)	0.77	0.79	0.78	6.82
Wheat straw (T₄)	302.20 (15.11) (289.71)	335.60 (16.78) (305.00)	318.90 (15.94) (297.35)	1.05	1.10	1.08	5.06
Rice straw (T₅)	269.40 (13.47) (309.65)	286.60 (14.33) (312.74)	278.00 (13.90) (311.19)	0.87	0.91	0.88	3.78
Mean	250.70 (12.51) (346.30)	263.20 (13.54) (342.80)		0.78	0.78	0.75	4.39
CD _{T 0.05}	278.55 (2.12) (1.28)			0.025			
CD _{B 0.05}	1.97 (1.34) (2.01)			0.040			
CD _{TxB0.05}	2.66 (2.90) (2.72)			0.054			

* U/gds = μmoles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μmoles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Treated- Untreated/Untreated x100

Table 41. Cellulase production from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste as substrate under SSF

Biomass	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated Biomass (B ₁)	Treated Biomass (B ₂)		Mild steam treated Biomass	Treated Biomass (B ₂)		Mild steam pretreated Biomass	Treated Biomass (B ₂)		Untreated Biomass (B ₁)	Treated Biomass (B ₂)		Untreated Biomass (B ₁)	Treated Biomass (B ₂)		
Pine needles (T ₁)	3.40* (0.17)** (6.42)***	2.60* (0.13) (5.00)**	3.00 (0.15) (5.13)	20.10 (1.01) (37.92)	17.22 (0.86) (33.12)	18.66 (0.93) (35.52)	24.60 (1.23) (46.42)	21.22 (1.06) (40.81)	22.91 (1.15) (43.61)	48.10 (2.41) (90.75)	41.04 (2.05) (78.92)	44.57 (2.23) (84.84)	0.53	0.52	0.53	14.68
Pine wood (T ₂)	3.60 (0.18) (6.43)	2.80 (0.14) (5.90)	3.20 (0.16) (5.35)	30.60 (1.53) (54.64)	26.45 (1.32) (48.09)	28.53 (1.43) (51.37)	29.40 (1.47) (52.50)	26.11 (1.31) (47.47)	27.75 (1.39) (49.99)	63.60 (3.18) (113.57)	55.36 (2.77) (100.65)	59.48 (2.97) (107.11)	0.56	0.55	0.56	12.96
Bamboo wood (T ₃)	3.50 (0.17) (6.60)	2.00 (0.10) (4.00)	2.75 (0.14) (4.15)	31.20 (1.56) (58.87)	27.00 (1.35) (54.00)	29.10 (1.46) (56.43)	33.90 (1.70) (63.96)	30.00 (1.50) (60.00)	31.95 (1.60) (61.98)	68.60 (3.43) (129.43)	59.00 (2.95) (118.00)	63.80 (3.19) (123.72)	0.53	0.50	0.52	13.99
Wheat straw (T ₄)	4.8 (0.24) (4.06)	4.4 (0.22) (3.79)	4.60 (0.23) (3.92)	48.00 (2.40) (40.68)	43.45 (2.17) (37.46)	45.73 (2.29) (39.07)	54.00 (2.70) (45.76)	49.12 (2.46) (42.34)	51.56 (2.58) (44.05)	106.40 (5.32) (90.16)	96.97 (4.85) (83.59)	101.89 (5.09) (87.05)	1.18	1.16	1.17	8.86
Rice straw (T ₅)	3.60 (0.18) (3.79)	3.40 (0.17) (3.58)	3.50 (0.18) (3.68)	36.00 (1.80) (37.89)	32.11 (1.61) (33.80)	34.06 (1.70) (35.85)	48.00 (2.40) (50.53)	43.11 (2.16) (45.38)	45.55 (2.28) (47.95)	87.60 (4.38) (90.30)	78.62 (3.93) (82.76)	83.11 (4.16) (87.48)	0.95	0.95	0.95	10.25
Mean	3.78 (0.19) (5.46)	3.04 (0.15) (4.29)		33.18 (1.66) (46.00)	41.29 (1.46) (31.88)		37.98 (1.90) (51.83)	33.91 (1.70) (47.20)		74.94 (3.75) (103.06)	66.20 (3.31) (92.79)		0.75	0.736		
CD _{T 0.05}	0.18 (0.023) (0.25)			1.87 (0.16) (2.29)			4.28 (0.20) (1.73)			2.17 (0.27) (25.03)			0.24			
CD _{B 0.05}	0.29 (0.036) (0.40)			2.02 (0.26) (2.62)			6.77 (0.32) (2.73)			3.43 (0.42) (39.58)			0.38			
CD _{TxB0.05}	0.38 (0.050) (0.58)			2.74 (0.36) (4.98)			9.18 (0.45) (3.80)			4.70 (0.60) (53.68)			0.51			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated x 100

needles. On the other hand, significantly maximum cellulase production was recorded in untreated substrate (74.94 U/gds) and minimum in steam pretreated substrate (66.20 U/gds). Interaction study of different substrates (TxB) revealed that significantly maximum cellulase titers (106.4 U/gds) were estimated, when a untreated substrate of wheat straw was used as a substrate. This was followed by steam pretreated substrate of wheat straw (96.97 U/gds), untreated substrate of rice straw (87.6 U/gds) and steam treated substrate of rice straw (78.62 U/gds). The significantly minimum cellulase titers were observed in steam pretreated substrate of pine needles (41.04 U/gds).

The scrutiny of data in Table 42 depicted significantly highest xylanase titers (323.35 U/gds) were estimated in wheat straw, which was followed by rice straw (278.20 U/gds), followed by pine wood (223.10 U/gds). The significantly lowest xylanase titers were observed in pine needles (156.80 U/gds). On the other hand, significantly highest xylanase production was observed in untreated substrate (256.18 U/gds) than alkali pretreated substrate (209.60 U/gds). Interaction study of different substrate (TxB) revealed that significantly highest xylanase titers (362.50 U/gds) were estimated; when untreated substrate of wheat straw was used as a substrate. This was succeeded by steam pretreated substrate of wheat straw (345.00 U/gds), mild pretreated substrate of rice straw (304.20U/gds) and steam pretreated substrate of rice straw (281.0 U/gds). The significantly lowest xylanase titers were observed in steam pretreated substrate of pine needle (160.1 U/gds).

Steam pretreatment involves heating of biomass at high temperature pressure followed by mechanical disruption of the pretreated material either by violent discharge into a collecting vessel (explosion) or by mild blending after bleeding the steam pressure down to atmosphere (No explosion). In the present study no explosion technique has been used. The high pressure steam radically modified the plant cell wall structure yielding partially hydrolyzed hemicelluloses initially.

This type of steam explosion is also named autohydrolysis as in this steam water is only used. It is one of only very limiting number of cost effective

Table 42. Xylanase production from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste as substrate under SSF

Biomass	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated Biomass (B ₁)	Treated Biomass (B ₂)		Untreated Biomass (B ₁)	Treated Biomass (B ₂)		
Pine needles (T₁)	170.2 (8.51) (321.13)	160.1 (8.01) (298.75)	165.15 (8.26) (309.94)	0.53	0.52	0.53	5.93
Pine wood (T₂)	244.0 (12.2) (435.71)	225.6 (11.28) (396.47)	234.80 (11.74) (316.09)	0.56	0.55	0.56	7.54
Bamboo wood (T₃)	200.0 (10.0) (392.15)	180.3 (9.02) (338.77)	190.15 (9.51) (365.46)	0.53	0.50	0.52	9.85
Wheat straw (T₄)	362.5 (18.12) (307.20)	345.0 (17.25) (364.35)	353.75 (17.68) (335.77)	1.18	1.16	1.17	4.83
Rice straw (T₅)	304.2 (15.21) (313.60)	281.0 (14.50) (365.50)	292.60 (14.63) (339.55)	0.95	0.95	0.95	7.63
Mean	256.18 (12.80) (353.95)	209.6 (11.92) (352.76)		0.75	0.73		
CD _{T 0.05}	1.20 (0.86) (1.29)			0.24			
CD _{B 0.05}	2.48 (1.37)			0.38			
CD _{TxB0.05}	3.28 (1.90)			0.51			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = $\frac{\text{Untreated}-\text{Treated}}{\text{Treated}} \times 100$

pretreatment technologies that have been advanced to commercialized application. In this study, lower temperature i.e. 121°C and 1.5 lb pressure for 60 minutes has been found very effective to increase the accessibility of biomass inturn increasing the enzyme yield especially by *R. oryzae* W2 as compared to steam treatment i.e. 160 to 270°C as recommended in literature (Zheng *et al.*, 2009; Sun and Chang 2002; Viola *et al.* 2009;). Thus it can be proved more economical method for cellulase and xylanase production from *R. oryzae* W2. The action mode of uncatalyzed steam is similar to that of acid-aid chemical pretreatment except that during the steam treatment the biomass is hydrolyzed rapidly by circulating steam in the presence of much less moisture existing in the environment thus resulting in much more concentration of sugars from biomass in comparison. The major physicochemical changes of biomass during steam treatment are attributed to hemicelluloses solubilization and lignin transformation (Zheng *et al.* 2009; Kumar *et al.*, 2009;). These changes improve the digestibility of biomass consequently increasing the enzyme production by biodegrading microorganisms.

In steam pretreatment, that means a good compromise would be to balance the pretreatment severity so as to solubilize the hemicellulose component without degradation of the component sugars, thus leaving them in a fermentable form. Steam pretreatment combines both physical and chemical elements, causing the rupture of the wood cell wall structure, hydrolysis, and solubilization of the biomass components (Bura 2009). A locally isolated strain of *Trichoderma harzianum* for production of xylanase (EC 3.2.1.8) using lignocellulosic substrates for solid-state fermentation was revealed by Sanghvi *et al.* (2010).

Bura *et al.* (2009) altered the xylan content of corn stover and poplar using SO₂-catalyzed steam pretreatment to determine the effect on subsequent hydrolysis by commercial cellulase preparations supplemented with or without xylanases. Steam pretreated solids with xylan contents ranging from 1 to 19% (w/w) were produced. Higher xylan contents and improved hemicellulose recoveries were obtained with solids pretreated at lower severities or without SO₂-addition prior to pretreatment.

Ballesteros *et al.* (2002) reported hemicellulose and cellulose recovery and effectiveness of enzymatic hydrolysis of the cellulosic residue is presented for steam-explosion pretreatment of an agriculture residue (*Brassica carinata*) using different particle sizes. The parameters tested were: particle size (2/5, 5/8 and 8/12 mm), temperature (190 and 210 °C), and residence time (4 and 8 min). The compositional analysis of filtrate and water insoluble fibre after pretreatment and enzymatic digestibility data are presented. Larger steam-exploded particle (8/12 mm) results in higher cellulose and enzymic digestibilities.

Ohgren *et al.* (2007) reported that near theoretical glucose yield (96-104%) from acid-catalysed steam pretreated corn stover can be obtained if xylanases are used to supplement cellulases during hydrolysis. Xylanases hydrolyse residual hemicellulose, thereby improving the access of enzymes to cellulose. Under these conditions, xylose yields reached 70-74%. When pretreatment severity was reduced by using auto-catalysis instead of acid-catalysed steam pretreatment, xylose yields were increased to 80-86%. Partial delignification of pretreated material was also evaluated as a way to increase the overall sugar yield.

Fundamentally physicochemical pretreatments of substrate employed can be divided into physical and chemical methods but there is a strong interdependence of these processes. There is no perfect pretreatment method to be employed to lignocellulose material and remaining bottlenecks include generation of inhibitory chemicals, high particle load, high energy input and efficient separation of soluble sugars from solid residues. Specific pretreatment conditions are required for individual feedstocks and mechanistic model can help in the rational design of such processes. Therefore, it is especially important to optimize lignocellulose pretreatment methods because these are key steps in overall enzyme production. Basically pretreatment method render complex cellulose and hemicelluloses more amenable to enzymolysis by disrupting their complex structure, crystallinity, lignin seal and smaller pore size. Particle size of the substrate is related to substrate characterization and system capacity to interchange with microbial growth and heat and mass transfer during SSF process

(Krishna, 2005). Smaller particle size could provide larger surface area for microbial action and it is also advantageous for heat transfer and exchange of oxygen and carbon dioxide between air and solid surface. However, too small particles may result in substrate agglomeration which may interfere with microbial respiration and thus result in poor growth (Pandey *et al.*, 2000; Sindhu *et al.*, 2011). By commuting the biomass by physical pretreatments like milling (1.5 mm)/crushing/steam treatments were given to forestry and agricultural waste have resulted in enlarged surface proportionality to its volume thus making it more accessible for chemicals used in the present study i.e. alkali; NaOH and acid; H₂SO₄ to hydrolyze the substrate in turn producing more enzyme. However, milling process is energy-intensive, but satisfactory results obtained in the pretreatments designed in the present study rendered milling a necessary step for enzyme (Galbe and Zacchi 2007; Taherzadeh and Karimi 2008;) production from lignocellulosic biomass. Milling is considered an important physical pretreatment and is being utilized to enhance the hydrolyzability of lignocellulosic materials. It results in reduction of substrate particle size consequently increasing surface area, a decrease in cellulose crystallinity and its degree of polymerization (Taherzadeh and Karimi 2008; Zeng *et al.*, 2007, Sidiras and Koukios, 1989; Fan *et al.*, 1980). However, agricultural residue i.e. wheat straw, rice straw and pine needles being soft in nature were simply crushed with hand (1-10 cm) to obtain desirable results making it an energy saving modified methods.

The best pretreatment options are those which combine elements of both physical and chemical methods (Zhao *et al.*, 2007; Jin and Chen 2006; Vaccarino *et al.*, 1987; Azzam 1987). Milling of biomass followed by physical/chemical pretreatment i.e. steam, acid and alkali given to forestry as well as agriculture residue have resulted in higher titers of cellulase and xylanase in all the seven experiment carried out in the present study (Table No.31-42) hence has proved the efficiency of physicochemical pretreatment for enhanced synthesis of these enzymes.

The different types of inhibitor produced during pretreatment process in turn lowering down enzyme production have not studied separately but it has

been proved from the literature that the formation of furfural, hydroxyl methyl furfural, levulinic acid, formic acid, acetic acid, coumaric acid and phenols is very high in softwood and hardwood materials as compare to agriculture residue, thus, resulting in higher enzyme titers in case of agricultural residue. In woody biomass, lignin content is very high which during pretreatment may result in production of a cocktail of inhibitors like acids, alcohols and aldehydes leading to the suppression of enzyme synthesis. In softwoods, hemicelluloses sugar backbone is mannose with glucose and galactose side chains, while in hardwoods the backbone is xylan with side chains of arabinose and glucuronic acids (Persson *et al.*, 2006; Lavarack *et al.*, 2002; Emmel *et al.*, 2003). Additionally in hardwood species some of the xylose units are acetylated which during pretreatment give rise to high levels of acetic acid along with others inhibitors and thus inhibits subsequent enzyme production.

Among three different pretreatments of lignocellulosic biomass i.e. acid, alkali and steam used in the present experiment reflect the basic need of pretreatment for conversion of lignocellulosic biomass from its native form which is recalcitrant to cellulase and xylanase enzyme system into a form for which cellulose and hemicellulose hydrolysis is much more effective.

Among five substrates taken for the enzyme production study, wheat straw followed by rice straw has been rated the best substrates for cellulase and xylanase production by *R. oryzae* W2 and *T. longibrachaeum* T2 under SSF. As far as treatment is concerned, alkali treatment has emerged as the most suitable method to enhance the accessibility of biomass for enzymolysis by *T. longibrachaeum* T2 while untreated lignocellulosic biomass has been adjudged the best for cellulase and xylanase production from *R. oryzae* W2.

Overall, it has been observed that lignocellulosic forest waste can act as a suitable substrate for cellulase and xylanase production, though; different physico-chemical/biological pretreatments are prerequisite to enhance the yield of enzyme. As shown in Fig 41, 42, 43 and 44 pretreated agricultural and forest waste has resulted in more titers of cellulase and xylanase as compared to

untreated ones from *T. longibracheatum* T2. Various pretreatments given to biomass in the present study have responded differently to softwood and hardwood residues depending upon their chemical composition. Maximum enzyme activities in all the residues were shown after alkali pretreatment from *T. longibracheatum* T2. As far as cellulase and xylanase production profile is concerned, maximum cellulase and xylanase activity was observed in alkali pretreated wheat straw reaching upto 93.20 U/gds and 356.60 U/gds, respectively. Maximum percent increase in cellulase activity was observed in alkali pretreated rice straw whereas, xylanase activity was observed in alkali pretreated bamboo wood. *R. oryzae* W2 had responded best for untreated biomass for both cellulase and xylanase production. In this case highest cellulase and xylanase enzyme activity was found in untreated wheat straw reaching upto 106.40 U/gds and 362.50 U/gds. Maximum percent increase in cellulase was found in acid pretreated pine needles, while percent increase in xylanase was found in alkali pretreated wheat straw as shown in (Fig. 45, 46, 47 and 48). Therefore statistically cellulase and xylanase production in untreated and pretreated biomass was significantly different from each other.

Agricultural and forest wastes are believed to be as one of the abundant resource of sugars although limited work has been reported on them because of their complex structure for biodegradation. Therefore, various pretreatment technologies have been devised to increase its hydrolysis rate using hyper cellulase and xylanase producing *T. longibracheatum* T2 and *R. oryzae* W2 with an aim to reduce size, degree of polymerization, to preserve cellulose and hemicellulose fractions, to increase their structural porosity, to limit the formation of inhibitors and to turn it cost effective (Hu *et al.*, 2008). These screening variables which vary from biomass to biomass play a vital role to assess the effect of pretreatment on individual biomass. The relationship among structural and compositional factors reflects the complexity of lignocellulosic biomass matrix. The variability of these characteristics explains the change of enzymatic digestibility among different biomass (Zheng *et al.*, 2009). Thus each pretreatment acts specifically for agricultural wastes and wood to wood of forest species. It strongly proves that cellulase and xylanase production from enzymatic

hydrolysis of agricultural and forest biomass is substrate and pretreatment specific.

Overall, from Table No 31, 32, 35, 36, 39 and 40 for cellulase and xylanase production from *T. longibracheatum* T2 and using different physicochemical pretreated biomass a significant effect in terms of enzyme titers has been observed. As far as enzyme production from *T. longibracheatum* T2 is concerned (Table No. 31 and 32) alkali pretreatment has been found best except pine needles for cellulase followed by other treatments for enhancing the production of both the enzymes over untreated for forestry and agriculture residue. It proves that pretreatment of lignocellulosic biomass for increasing the enzyme production from potential cellulolytic/xylanolytic microorganisms by modifying the structure of biomass and increasing the accessibility of cellulose and hemicellulose degrading fungi to enhance the enzyme yield. In case of *R. oryzae* W2 for enzyme production from untreated and pretreated biomass, an interesting trend has been observed i.e. apparent higher cellulase and xylanase yield has been attained in untreated biomass over other pretreated biomass. The probable reason for that can be *R. oryzae* W2 might be capable of producing lignin degrading enzymes in addition to cellulase and xylanase. In that case degradation of untreated biomass can be rendered at par with biological pretreatment imposed by *R. oryzae* W2. Therefore a synergetic effect of lignase/laccase breaking down the protective shield of lignin around cellulose and hemicellulose would have resulted in exposed hexoses and pentoses without release of series of inhibitors otherwise being produced during different pretreatment. In addition, the crushing/milling of the biomass has also been done to attain smaller chip size (physical pretreatment) which also would have help for rapid growth of *R. oryzae* W2. From the results (Table No. 33, 34, 37, 38, 41 and 42), it can be concluded that freely available cellulose, intact fraction of hemicellulose as well as minimization of inhibitors would have facilitated the higher enzyme production from untreated (biologically pretreated) biomass by *R. oryzae* W2. The findings so obtained in the present study are highly encouraging ecofriendly approach improving the economics of the process. The detailed study in this field would be required to understand this mechanism more precisely.



Plate 8. Extracellular cellulase and xylanase production using untreated and pretreated wheat straw residue by *Trichoderma longibrachaeum* T2 under SSF A) control (untreated wheat straw; B) steam pretreated wheat straw; C) acid pretreated wheat straw and D) alkali pretreated wheat straw



Plate 9. Extracellular cellulase and xylanase production using untreated and pretreated rice straw residue by *Trichoderma longibrachaeum* T2 under SSF A) control (untreated rice straw; B) alkali pretreated rice straw; C) steam pretreated rice straw and D) acid pretreated rice straw



Plate 10. Extracellular cellulase and xylanase production using untreated and pretreated pine needles residue by *Trichoderma longibrachiatum* T2 under SSF A) control (untreated pine needles; B) alkali pretreated pine needles; C) steam pretreated pine needles and D) acid pretreated pine needles



Fig 11. Extracellular cellulase and xylanase production using untreated and pretreated pine wood residue by *Rhizopus oryzae* W2 under SSF A) control (Untreated pine wood; B) alkali pretreated pine wood; C) steam pretreated pine wood and D) acid pretreated pine wood



Plate 12. Extracellular cellulase and xylanase production using untreated and pretreated bamboo wood residue by *Trichoderma longibrachaeum* T2 under SSF A) control (untreated bamboo wood ; B) alkali pretreated bamboo wood; C) steam pretreated bamboo wood and D) acid pretreated bamboo wood



Plate 13. Extracellular cellulase and xylanase production using untreated and pretreated pine wood residue by *Rhizopus oryzae* W2 under SSF A) control (Untreated pine wood); B) acid pretreated pine wood; C) steam pretreated pine wood and D) alkali pretreated pine wood



Plate 14. Extracellular cellulase and xylanase production using untreated and pretreated rice straw residue by *Rhizopus oryzae* W2 under SSF A) control (untreated rice straw; B) acid pretreated rice straw; C) alkali pretreated rice straw and D) steam pretreated rice straw



Plate 15 Extracellular cellulase and xylanase production using untreated and pretreated pine needles residue by *Rhizopus oryzae* W2 under SSF A) control (untreated pine needles; B) acid pretreated pine needles; C) steam pretreated pine needles and C) alkali pretreated pine needles



Plate 16. Extracellular cellulase and xylanase production using untreated and pretreated bamboo wood residue by *Rhizopus oryzae* W2 under SSF A) control (untreated bamboo wood ; B) steam pretreated bamboo wood ; C) acid pretreated bamboo wood and C) alkali pretreated bamboo wood

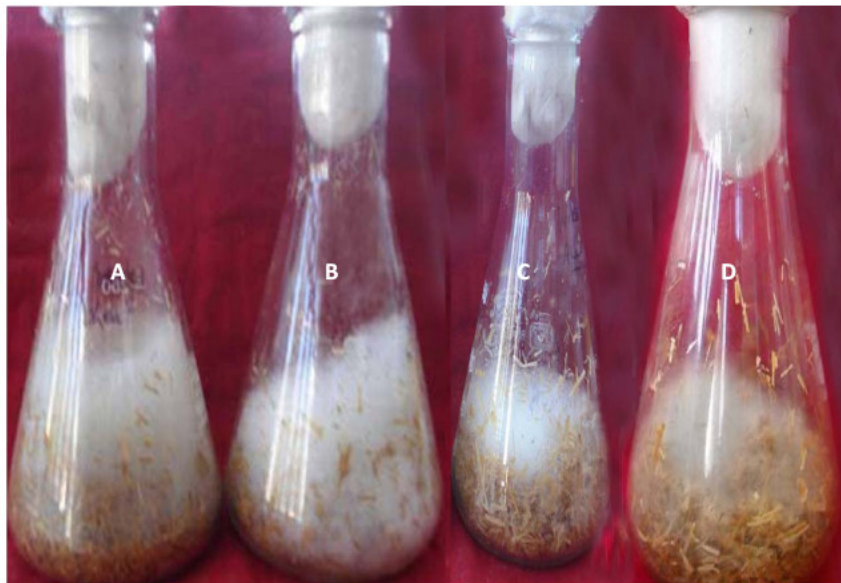
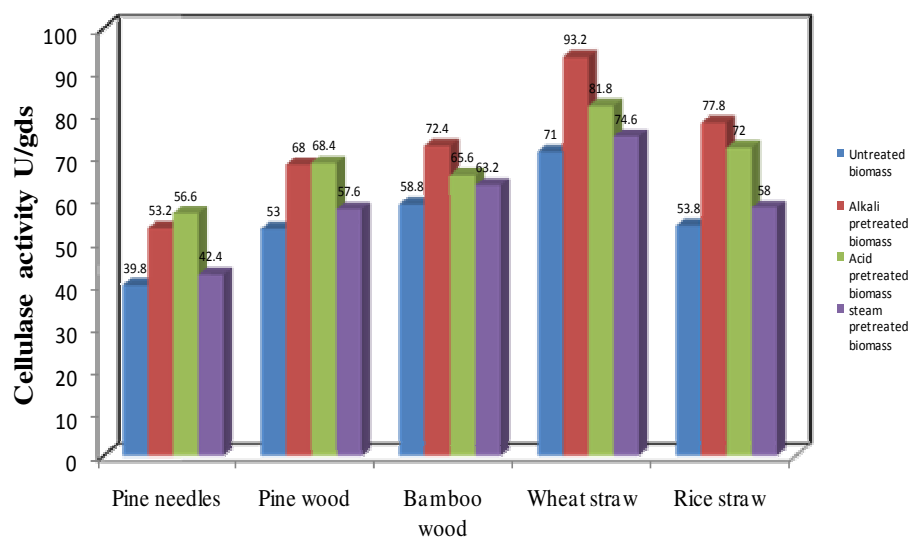


Plate 17. Extracellular cellulase and xylanase production using untreated and pretreated wheat straw residue by *Rhizopus oryzae* W2 under SSF A) control (untreated wheat straw; B) alkali pretreated wheat straw; C) acid pretreated wheat straw and D) steam pretreated wheat straw



Pretreated Lignocellulosic biomass

Fig 41. Effect of different pretreatment on lignocellulosic biomass for cellulase production from *T. longibracheatum* T2



Pretreated Lignocellulosic biomass

Fig 42. Effect of different pretreatment on lignocellulosic biomass for xylanase production from *T. longibracheatum* T2

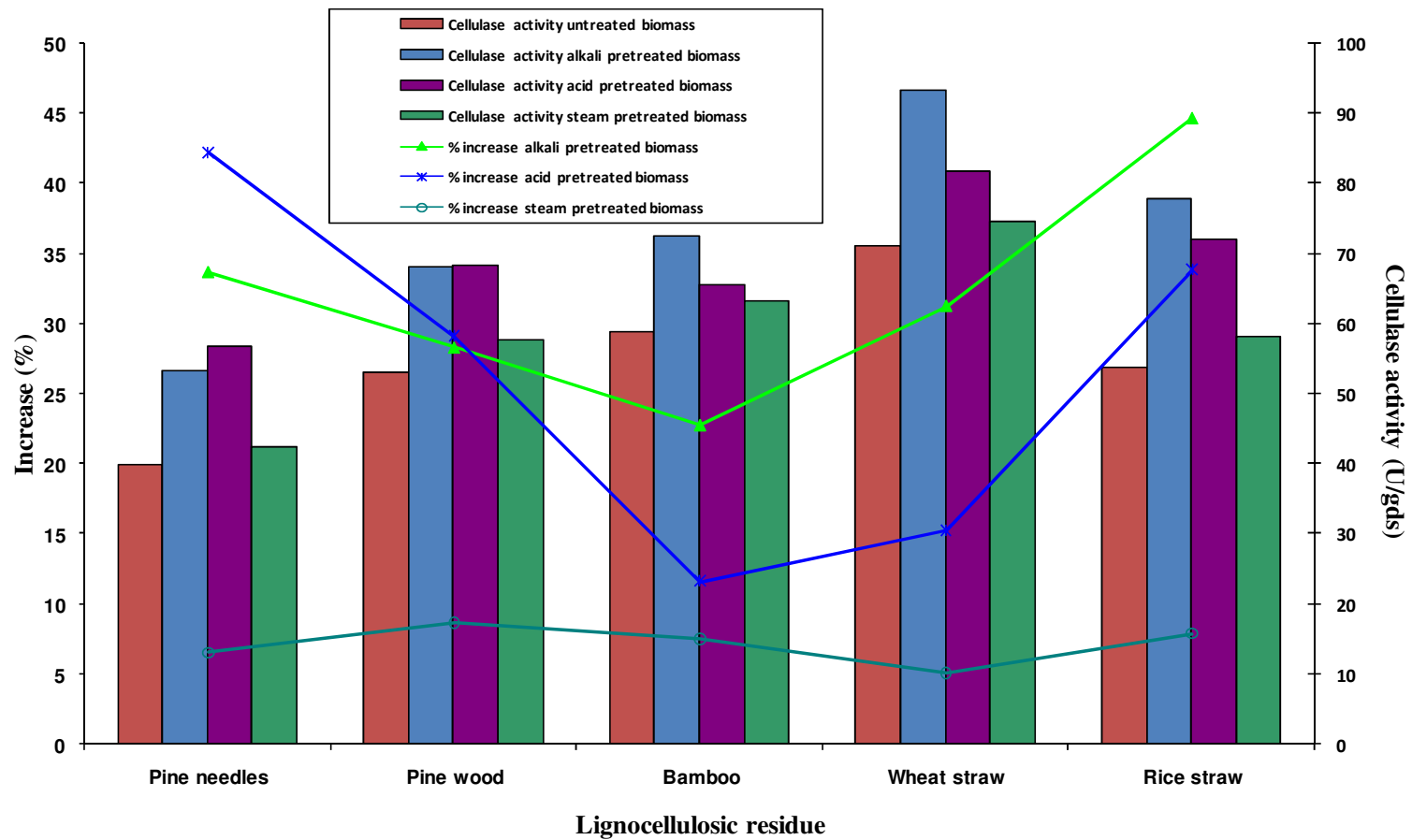


Fig 43. Comparison of percent increase in cellulase activity of pretreated over untreated lignocellulosic residue by *T. longibracheatum* T2

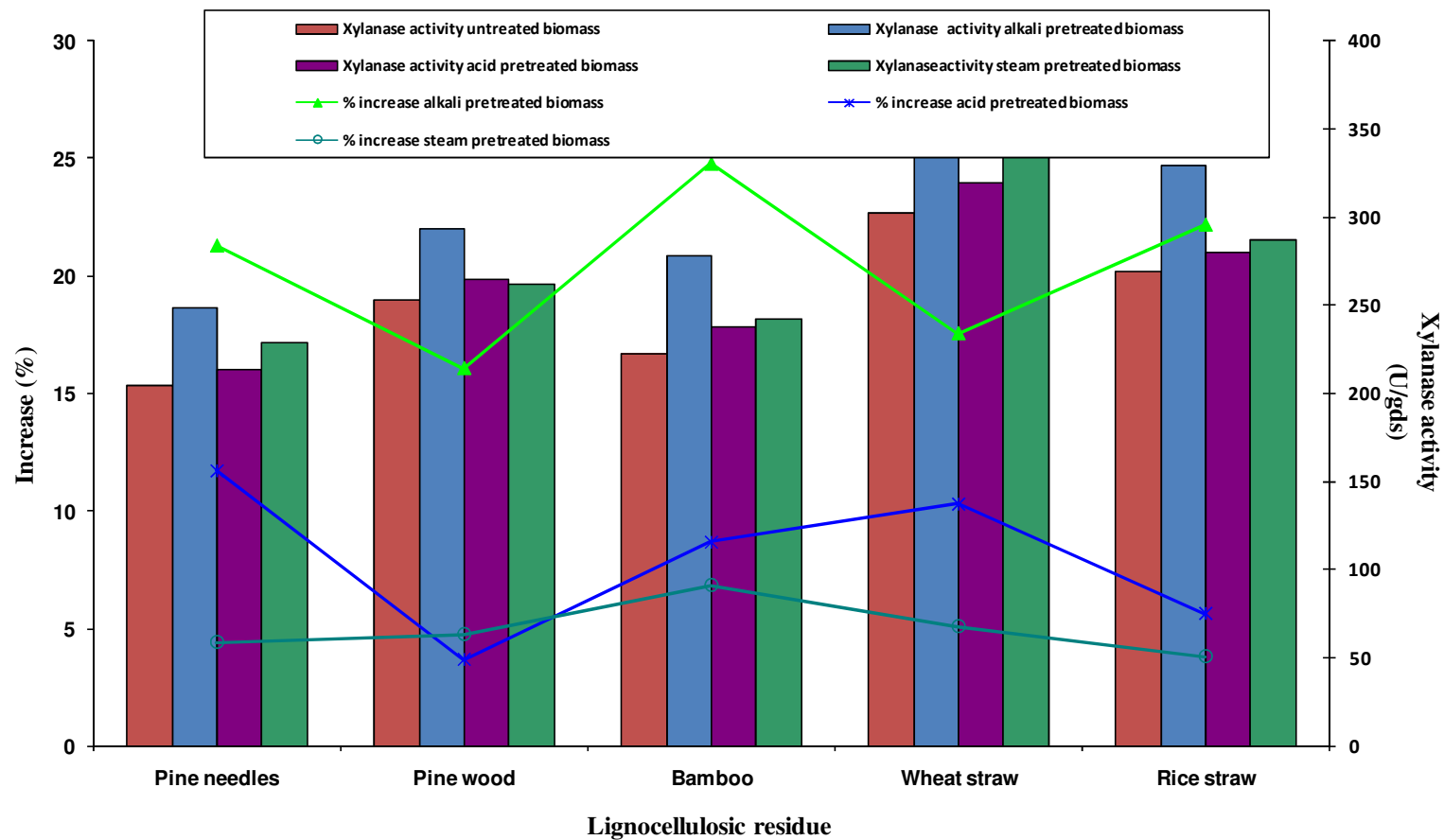


Fig 44. Comparison of percent increase in xylanase activity of pretreated over untreated lignocellulosic residue by *T. longibrachetum* T2

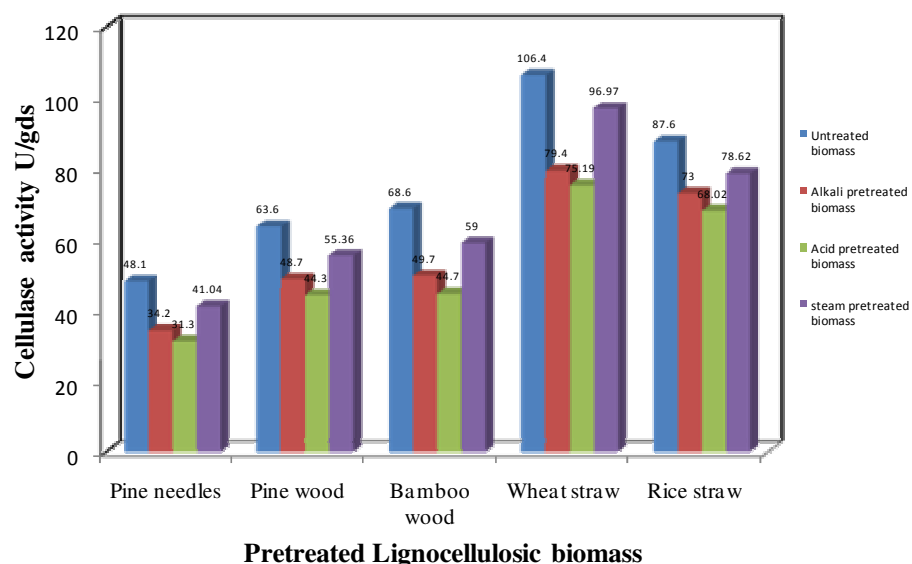


Fig 45. Effect of different pretreatment on lignocellulosic biomass for cellulase production from *R. oryzae* W2

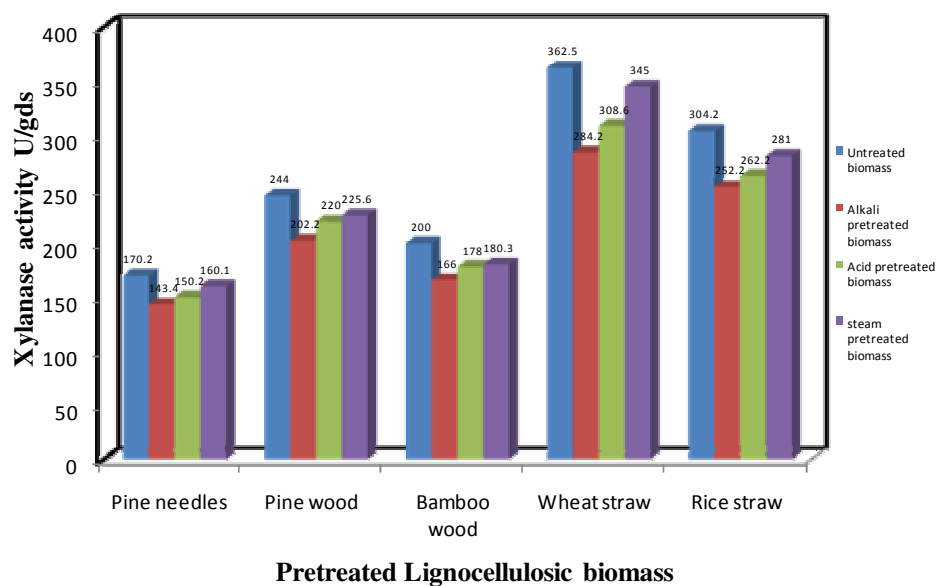


Fig 46. Effect of different pretreatment on lignocellulosic biomass for xylanase production from *R. oryzae* W2

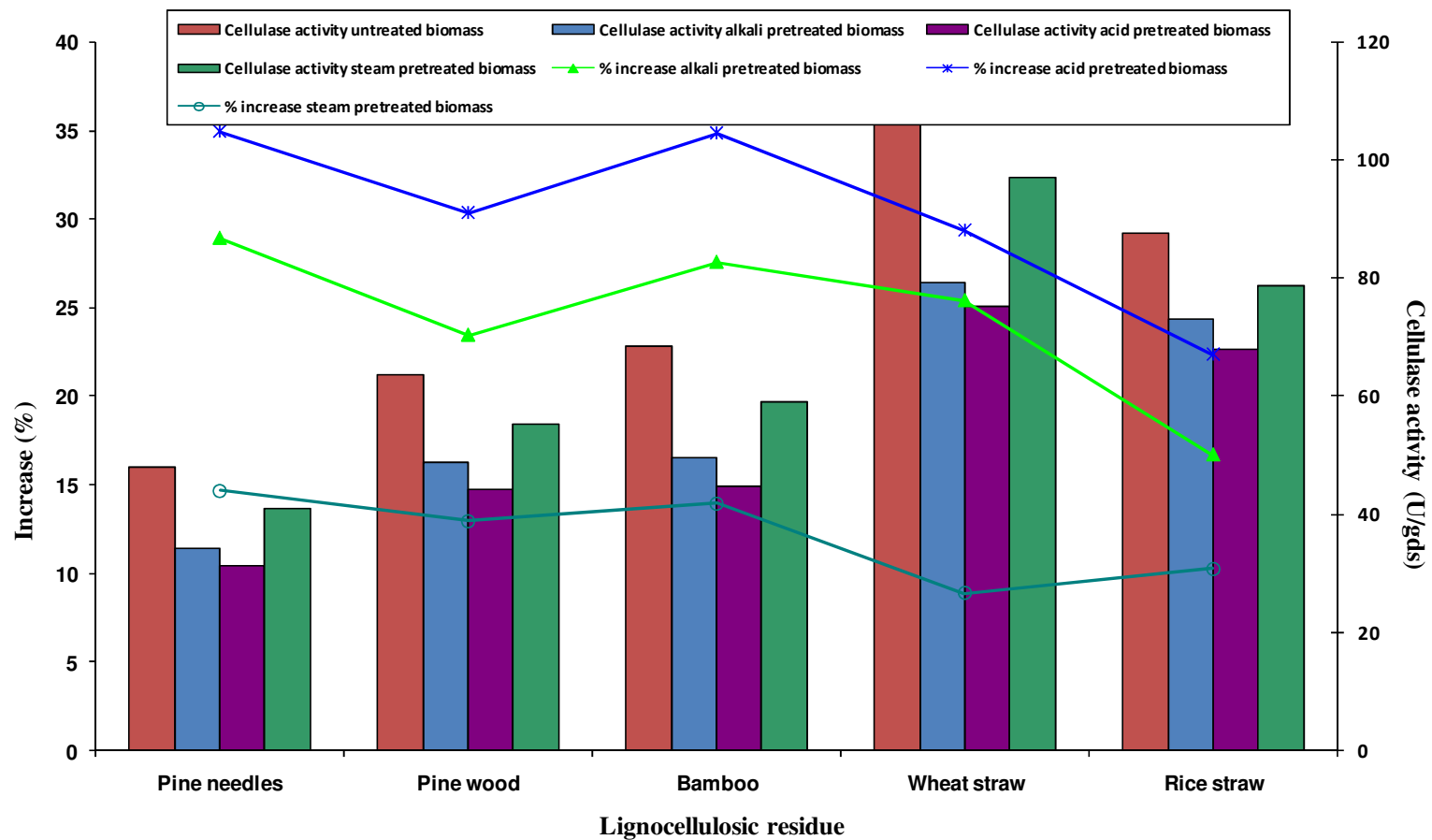


Fig 47. Comparison of percent increase in cellulase activity of untreated over pretreated lignocellulosic residue by *R. oryzae* W2

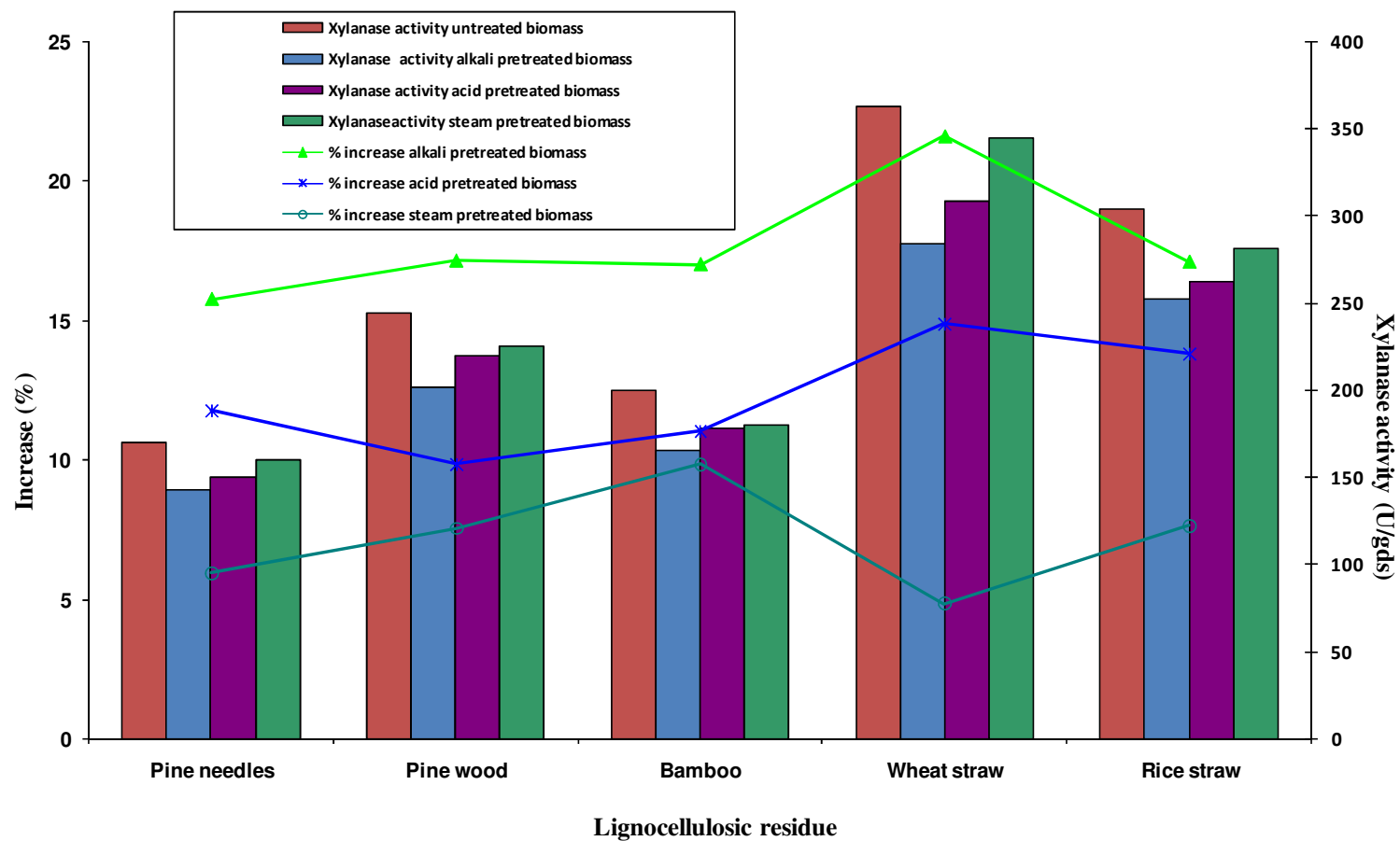


Fig 48. Comparison of percent increase in xylanase activity of untreated over pretreated lignocellulosic residue by *R. oryzae* W2

4.3.2.5 Effect of pH

The different pH of moistening agent namely, 3.5 (T₁), 4.0 (T₂), 4.5 (T₃), 5.0 (T₄), 5.5 (T₅), 6.0 (T₆) and 6.5 (T₇) were used for assessing the activity of cellulase and xylanase enzymes. Data pertaining to cellulase titers viz., Fpase, CMCase and β -glucosidase and xylanase are presented in Table 43 to 46.

A cursory of data in Table 43 revealed that the significant difference in cellulase production was observed when alkali treated and untreated agricultural lignocellulosic substrate wheat straw was used for study in *T. longibrachaeatum* T2 at varying pH of moistening agent. The significantly maximum cellulase titers (88.40 U/gds) were estimated at pH 5.0 (T₄), which was followed by pH 4.5 (78.40 U/gds), pH 5.5 (73.40 U/gds) and pH 4.0 (55.70 U/gds) as shown in Fig 49. The significantly minimum cellulase titers (28.90 U/gds) were observed at pH 3.5. Similarly, the significantly maximum cellulase production was observed in alkali treated substrate (67.71 U/gds) and minimum in untreated substrate (49.80 U/gds). Interaction study revealed that significantly maximum cellulase titers (103.8 U/gds) were estimated at pH 5.0 (T₄) and minimum in untreated substrate of wheat straw at pH 3.5 (30.40 U/gds).

Similarly, Table 44 showed that significantly highest xylanase titers (355.20 U/gds) were estimated at pH 5.0, which was followed by pH 4.5 (311.55 U/gds) and pH 5.5 (297.80 U/gds). The significantly lowest xylanase titers were observed at pH 6.5 (128.80 U/gds). Similarly, significantly highest xylanase production was observed in alkali treated substrate (278.93 U/gds) than untreated substrate (232.86 U/gds) as depicted in Fig 50. Interaction study (TXB) revealed that significantly highest xylanase titers (405.0 U/gds) were estimated at pH 5.0 (T₄), when alkali pretreated substrate of wheat straw was used as a substrate and lowest in untreated substrate at pH 6.5 (117.6 U/gds).

A perusal of Table 45 and Fig 51 depicted significant difference in cellulase production was observed when alkali treated and untreated agricultural lignocellulosic substrate wheat straw was used for study in *R. oryzae* W2 at varying pH of moistening agent. The significantly maximum cellulase titers

Table 43. Cellulase production at different pH from *Trichoderma longibracheatum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

pH	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
3.5 (T ₁)	1.20** (0.06)** (2.50)***	2.20 (0.11) (5.36)	1.70 (0.08) (3.93)	10.20 (0.51) (21.25)	12.00 (0.60) (29.26)	11.10 (0.55) (25.26)	16.00 (0.80) (33.33)	16.20 (0.81) (39.51)	16.10 (0.80) (46.42)	27.40 (1.37) (57.08)	30.40 (1.52) (74.14)	28.90 (1.44) (65.41)	0.48	0.41	0.45	10.9
4.0 (T ₂)	4.60 (0.23) (6.38)	5.60 (0.28) (7.08)	5.10 (0.25) (6.73)	15.20 (0.76) (21.10)	22.00 (1.10) (27.84)	18.60 (0.93) (24.47)	28.00 (1.40) (38.88)	36.00 (1.80) (45.56)	32.00 (1.60) (42.22)	47.80 (2.39) (66.38)	63.60 (3.18) (80.50)	55.70 (2.78) (73.44)	0.72	0.79	0.76	33.05
4.5 (T ₃)	6.40 (0.32) (7.03)	8.40 (0.42) (7.50)	7.40 (0.37) (7.26)	20.00 (1.00) (21.97)	32.00 (1.60) (28.57)	26.00 (1.30) (25.27)	38.00 (1.90) (41.75)	52.00 (2.60) (46.42)	45.00 (2.25) (44.08)	64.40 (3.22) (70.76)	92.40 (4.62) (82.50)	78.40 (3.92) (76.63)	0.91	1.12	1.02	28.00
5.0 (T ₄)	7.00 (0.35) (7.00)	9.80 (0.49) (8.16)	8.40 (0.42) (7.58)	22.00 (1.10) (22.00)	36.00 (1.80) (30.00)	23.10 (1.45) (24.62)	44.00 (2.20) (44.00)	58.00 (2.90) (48.33)	51.00 (2.55) (46.16)	73.00 (3.65) (73.00)	103.80 (5.19) (86.50)	88.40 (4.42) (79.75)	1.0	1.2	1.10	42.19
5.5 (T ₅)	6.20 (0.31) (7.12)	8.40 (0.42) (8.57)	7.30 (0.36) (7.84)	16.20 (0.81) (18.62)	30.00 (1.50) (30.61)	15.80 (1.55) (23.85)	36.00 (1.80) (41.37)	50.00 (2.50) (51.02)	43.00 (2.15) (46.19)	58.40 (2.92) (67.12)	88.40 (4.42) (90.20)	73.40 (3.67) (78.66)	0.87	0.98	0.93	51.36
6.0 (T ₆)	4.80 (0.24) (7.05)	6.20 (0.31) (9.53)	5.50 (0.27) (8.29)	13.60 (0.68) (20.00)	18.00 (0.90) (27.69)	29.00 (0.79) (26.00)	28.00 (1.40) (41.37)	36.00 (1.80) (55.38)	32.00 (1.60) (48.37)	46.40 (2.32) (68.23)	60.20 (3.01) (92.61)	53.30 (2.66) (80.42)	0.68	0.65	0.67	29.74
6.5 (T ₇)	3.60 (0.18) (15.65)	3.60 (0.18) (8.18)	3.60 (0.18) (11.91)	11.80 (0.59) (51.30)	15.00 (0.75) (34.09)	13.40 (0.67) (42.70)	15.80 (0.79) (68.69)	16.60 (0.83) (37.72)	16.20 (0.81) (53.20)	31.20 (1.56) (135.65)	35.20 (1.76) (80.00)	33.20 (1.66) (107.82)	0.23	0.44	0.34	12.82
Mean	4.83 (0.24) (7.53)	6.31 (0.31) (7.77)		15.57 (0.77) (25.18)	23.57 (1.17) (29.72)		29.40 (1.47) (44.80)	37.83 (1.89) (46.28)		49.80 (2.49) (76.89)	67.71 (3.38) (83.78)		0.70	0.80		
CD _{T 0.05}	0.38 (0.028) (0.62)			1.07 (0.26) (0.90)			1.09 (0.31) (1.02)			1.02 (0.89) (1.95)			0.044			
CD _{B 0.05}	0.71 (0.015) (1.16)			2.01 (0.14) (1.68)			2.00 (0.166) (0.19)			1.92 (0.47) (3.6)			0.082			
CD _{TxB0.05}	0.98 (0.040) (1.60)			2.78 (0.36) (2.32)			1.82 (0.44) (2.64)			2.64 (1.24) (5.04)			0.114			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Treated- Untreated/Untreated x100

Table 44. Xylanase production at different pH from *Trichoderma longibracheatum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

pH	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
3.5 (T ₁)	178.00* (8.90)** (370.83)***	193.80 (9.70) (472.68)	185.90 (9.30) (421.76)	0.48	0.41	0.45	8.87
4.0 (T ₂)	257.40 (12.87) (357.50)	266.80 (13.30) (337.72)	262.10 (13.00) (341.65)	0.72	0.79	0.76	3.65
4.5 (T ₃)	285.20 (14.26) (313.40)	337.90 (16.90) (301.69)	311.55 (15.58) (307.55)	0.91	1.12	1.02	18.47
5.0 (T ₄)	305.40 (15.27) (305.40)	405.00 (20.30) (337.50)	355.20 (17.78) (321.45)	1.0	1.2	1.10	32.69
5.5 (T ₅)	266.60 (13.33) (306.43)	329.00 (16.50) (335.71)	297.80 (14.91) (321.07)	0.87	0.98	0.93	23.45
6.0 (T ₆)	219.80 (10.99) (323.23)	280.00 (14.00) (430.76)	249.90 (12.49) (377.0)	0.68	0.65	0.67	27.38
6.5 (T ₇)	117.60 (5.88) (245)	140.00 (7.00) (318.18)	128.80 (6.44) (281.59)	0.23	0.44	0.34	19.04
Mean	232.86 (11.64) (317.40)	278.93 (13.95) (362.03)		0.70	0.80		
CD _{T 0.05}	1.10 (1.61) (29.66)			0.044			
CD _{B 0.05}	2.07 (0.86) (55.49)			0.082			
CD _{TxB0.05}	2.86 (2.24) (76.64)			0.114			

* U/gds = μ moles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μ moles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = $\frac{\text{Treated} - \text{Untreated}}{\text{Untreated}} \times 100$

Table 45. Cellulase production at different pH from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw as substrate under SSF

pH	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated Biomass (B ₁)	Treated Biomass (B ₂)		Untreated Biomass (B ₁)	Treated Biomass (B ₂)		Untreated Biomass (B ₁)	Treated Biomass (B ₂)		Untreated Biomass (B ₁)	Treated Biomass (B ₂)		Untreated Biomass (B ₁)	Treated Biomass (B ₂)		
3.5 (T ₁)	1.40* (0.07)** (5.80)***	1.10 (0.06) (5.50)	1.25 (0.065) (5.65)	7.75 (0.39) (32.29)	6.80 (0.34) (34.00)	7.28 (0.365) (33.14)	10.60 (0.53) (44.16)	9.50 (0.48) (47.50)	10.05 (0.50) (45.83)	19.75 (0.99) (82.29)	17.40 (0.87) (87.00)	18.58 (0.93) (84.64)	0.24	0.20	0.22	11.90
4.0 (T ₂)	2.80 (0.14) (5.70)	2.40 (0.12) (5.22)	2.60 (0.13) (5.46)	17.20 (0.86) (35.10)	16.00 (0.80) (34.78)	16.60 (0.83) (34.94)	24.00 (1.20) (57.50)	23.00 (1.15) (50.00)	23.50 (1.17) (53.75)	44.00 (2.20) (89.70)	41.40 (2.07) (90.00)	42.70 (2.13) (89.85)	0.49	0.46	0.48	5.91
4.5 (T ₃)	4.00 (0.20) (4.80)	3.50 (0.18) (4.38)	3.75 (0.19) (4.59)	26.00 (1.30) (31.70)	24.60 (1.23) (30.75)	25.30 (1.26) (31.22)	47.20 (2.36) (57.50)	46.00 (2.30) (57.50)	46.60 (2.33) (57.50)	77.20 (3.86) (94.10)	74.10 (3.71) (92.63)	75.65 (3.78) (93.37)	0.82	0.80	0.81	4.02
5.0 (T ₄)	5.20 (0.26) (4.29)	4.20 (0.21) (3.62)	4.70 (0.235) (3.95)	52.00 (2.60) (42.90)	48.00 (2.40) (41.38)	50.00 (2.50) (42.14)	57.00 (2.85) (47.01)	55.00 (2.75) (47.41)	56.00 (2.80) (47.21)	114.2 (5.71) (94.38)	107.20 (5.36) (92.41)	110.70 (5.53) (93.40)	1.21	1.16	1.19	6.13
5.5 (T ₅)	4.20 (0.21) (4.71)	3.70 (0.19) (4.35)	3.95 (0.20) (4.54)	36.00 (1.80) (40.44)	34.00 (1.70) (40.00)	35.00 (1.75) (40.22)	42.20 (2.11) (47.41)	41.80 (2.09) (49.18)	42.00 (2.10) (48.30)	82.40 (4.12) (92.58)	79.50 (3.98) (93.53)	80.95 (4.05) (93.06)	0.89	0.85	0.87	3.52
6.0 (T ₆)	3.20 (0.16) (5.00)	2.80 (0.14) (4.67)	3.00 (0.15) (4.83)	22.00 (1.10) (34.92)	19.80 (0.99) (33.00)	20.90 (1.0) (33.96)	29.00 (1.45) (46.03)	27.60 (1.38) (46.00)	28.30 (1.41) (46.02)	54.20 (2.71) (86.03)	50.20 (2.51) (83.67)	52.20 (2.61) (84.85)	0.63	0.60	0.62	7.38
6.5 (T ₇)	1.60 (0.08) (3.9)	1.30 (0.07) (3.33)	1.45 (0.075) (3.61)	12.00 (0.60) (29.26)	9.70 (0.48) (24.87)	10.85 (0.54) (27.06)	15.80 (0.79) (38.53)	14.40 (0.72) (36.92)	15.10 (0.75) (37.73)	25.40 (1.27) (65.13)	19.4 (0.97) (47.31)	22.40 (1.12) (56.22)	0.41	0.39	0.40	23.62
Mean	3.20 (0.16) (4.59)	2.71 (0.13) (4.43)		24.70 (1.23) (35.23)	22.7 (1.13) (34.11)		32.25 (1.61) (48.30)	31.04 (1.55) (47.79)		58.74 (2.93) (83.77)	56.46 (2.82) (86.34)		0.67	0.64		
CD _{T 0.05}	0.43 (0.019) (0.41)			0.72 (0.34) (0.64)			1.87 (0.38) (0.71)			0.64 (0.56) (0.62)			0.024			
CD _{B 0.05}	0.81 (0.035) (0.78)			1.34 (0.64) (1.20)			3.50 (0.71) (1.32)			1.20 (1.04) (1.16)			0.045			
CD _{TxB0.05}	1.10 (0.050) (1.08)			1.83 (0.90) (1.66)			4.84 (1.00) (1.82)			1.66 (1.48) (1.60)			0.062			

* U/gds = μ moles of reducing sugars released / min /g of biomass

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated X 100

Table 46. Xylanase production at different pH from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw as substrate under SSF

pH	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
3.5 (T ₁)	86.00* (4.30)** (358.33)**	79.00 (3.95) (395.00)	82.50 (4.12) (376.67)	0.24	0.20	0.22	8.14
4.0 (T ₂)	226.00 (11.30) (461.22)	218.00 (10.90) (473.91)	222.00 (11.10) (467.57)	0.49	0.46	0.48	3.54
4.5 (T ₃)	344.80 (17.24) (420.49)	334.00 (16.70) (417.50)	339.40 (16.97) (419.00)	0.82	0.80	0.81	3.13
5.0 (T ₄)	395.60 (19.78) (326.94)	387.60 (19.38) (334.14)	391.60 (19.58) (330.54)	1.21	1.16	1.19	2.02
5.5 (T ₅)	284.60 (14.23) (319.78)	274.00 (13.70) (322.35)	279.30 (13.96) (321.07)	0.89	0.85	0.87	3.72
6.0 (T ₆)	211.20 (10.56) (335.24)	202.00 (10.10) (336.67)	206.60 (10.33) (335.96)	0.63	0.60	0.62	4.36
6.5 (T ₇)	124.20 (6.21) (302.93)	118.00 (5.90) (302.56)	121.10 (6.05) (302.75)	0.41	0.39	0.40	4.99
Mean	238.91 (11.94) (360.70)	230.37 (11.51) (368.88)		0.67	0.64		
CD _{T 0.05}	1.24 (1.56) (2931.6)			0.024			
CD _{B 0.05}	2.33 (4.89) (5484.4)			0.045			
CD _{TxB0.05}	2.22 (3.78) (6972)			0.062			

* U/gds = μmoles of reducing sugars released / min /g of biomass

** IU/ml = μmoles of reducing sugars released/min/ml of enzyme

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated x 100

(110.70 U/gds) were estimated at pH 5.0 and minimum (18.58 U/gds) at pH 3.5. as shown in Fig. 51. Similarly, significantly maximum cellulase production was observed in untreated wheat straw (58.74 U/gds) than steam pretreated wheat straw (56.46 U/gds). Interaction study revealed that significantly maximum cellulase titers (114.20 U/gds) (T_4) were noticed in untreated substrate of wheat straw at pH 5.0 and minimum in steam pretreated wheat straw at pH 3.5 (17.40 U/gds).

The scrutiny of data in Table 46 revealed that highest xylanase titers (391.60 U/gds) were recorded at pH 5.0 and lowest at pH 3.5 (82.50 U/gds). Similarly, significantly highest xylanase production was observed in untreated substrate (238.91 U/gds) than steam pretreated substrate (230.37 U/gds). Interaction study revealed that significantly highest xylanase titers (395.60 U/gds) were estimated at pH 5.0, when untreated substrate of wheat straw was used as a substrate as depicted in Fig 52. The significantly lowest xylanase titers were observed in steam treated wheat straw (79.00 U/gds).

It is clear from the pH (Table No. 43 to 46), pH of the medium (vogel) affects the production of cellulase and xylanase from pretreated and native biomass by *T. longibracheatum* T2 and *R. oryzae* W2 under SSF. The enzyme maintained their stability over pH range of 4.5-5.5 but had maximum activity at pH 5.0. The pH 5.0 has been found best for both the potential fungi for hydrolytic enzyme production. Grag and Neelkantan (1981) found that optimum pH for fungal cellulase and protein production varies from species to species.

The pH value of the fermentation medium alongwith substrate for cellulase production can be considered as crucial for the enzyme production. There are remarkable changes which occur in the pH during SSF. These are mainly for the production of acids due to incomplete oxidation of the substrate or uptake of ammonium ions, which will cause the pH to fall, while the release of ammonia by deamination of nitrogen complex will increase the pH. In SSF pH can not be monitored so it is very difficult to control the pH. So, it is desirable to use the microorganisms which can grow over a wide range of pH and which have

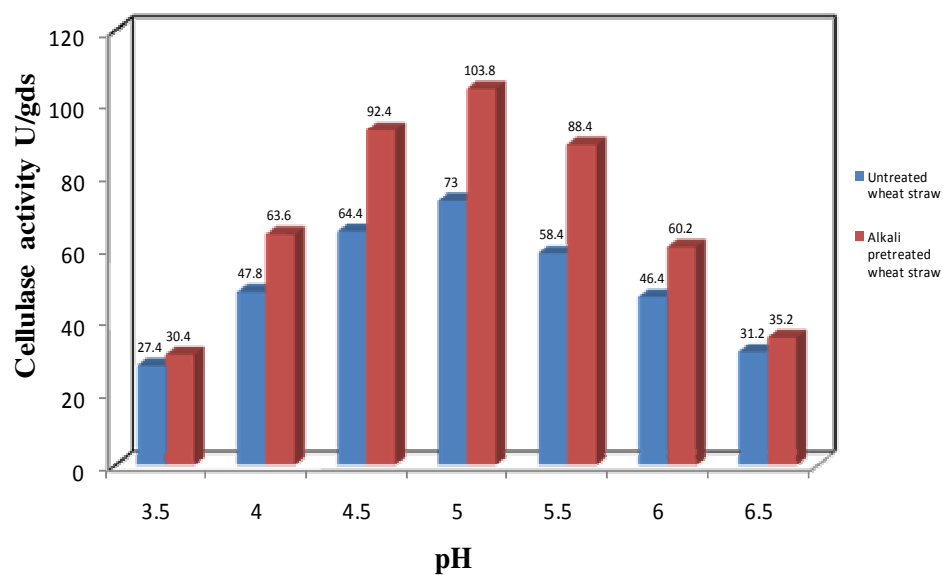


Fig 49. Effect of pH on cellulase production in alkali pretreated wheat straw from *T. longibracheatum* T2

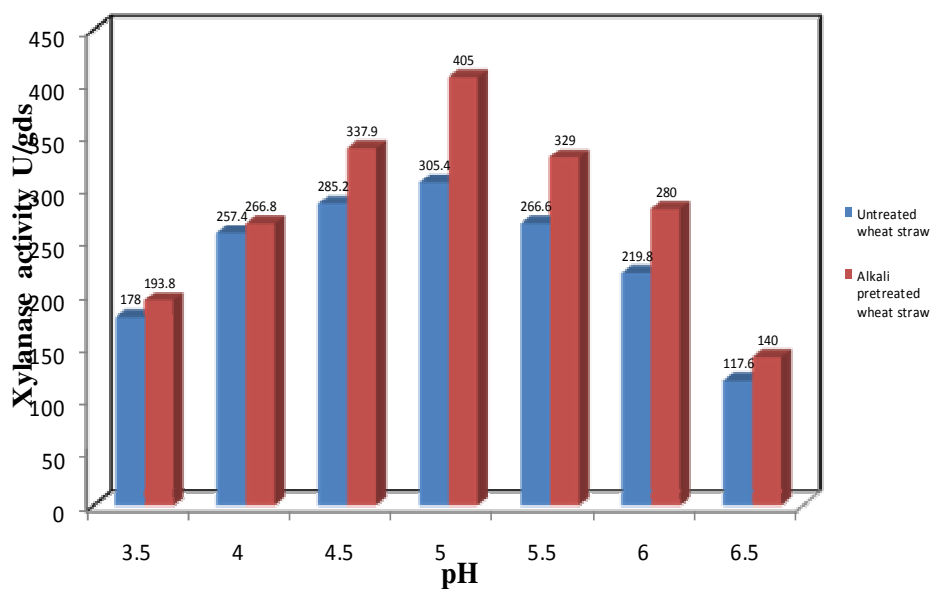


Fig 50. Effect of pH on xylanase production in alkali pretreated wheat straw from *T. longibracheatum* T2

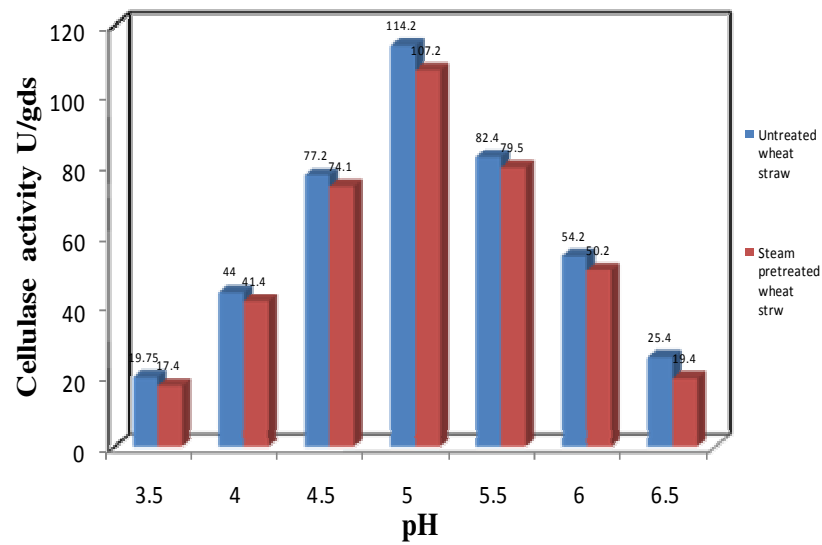


Fig 51. Effect of pH on cellulase production in steam pretreated wheat straw from *R. oryzae* W2

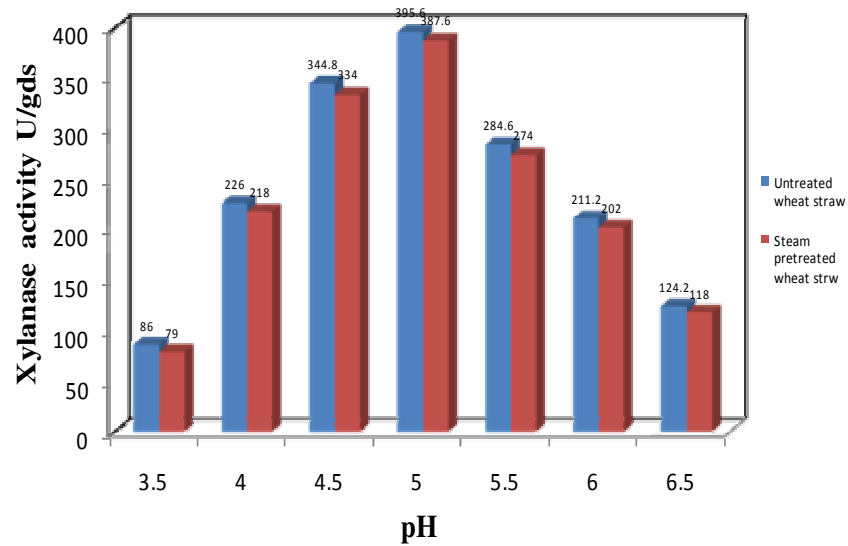


Fig 52. Effect of pH on xylanase production in steam pretreated wheat straw from *R. oryzae* W2

broad optima. By using different ratios of ammonium salts in the substrate, pH control in both natural and model SSF system can be obtained (Manpreet *et al.*, 2005).

Similar studies has reported by Maurya *et al.* (2012) on cellulase production have been carried out using the fungal strain *Trichoderma reesei* NCIM 992 by using three different lignocellulosic materials i.e. rice straw, sugarcane baggase and wheat bran under solid state fermentation. The effect of basic fermentation parameters (pH, temperature, and moisture content, particle size of substrate and moistening agent) on enzyme production was evaluated. Among three agricultural waste maximum cellulase production was observed in wheat bran as substrate. The optimal conditions for cellulase production for wheat bran were found to be: initial moisture content-70%, initial medium pH-5.0, temperature 30°C, moistening agents (MSS) and particle size of substrate (500 @m).

Pandit and Maheswari (2012) recorded the optimum culture conditions for the production of cellulase by *Pleurotus sajor-caju* were 10 days of incubation time at 25°C and pH 5.0 when sugarcane pressmud as substrate was pretreated with H₂O₂ + NaOH (2%) under solid state fermentation.

Umsza-Guez *et al.* (2011) optimized xylanase production by *A. awamori* under solid state fermentation conditions on tomato pomace optimum conditions for the determination of the enzyme activity was characterized. The xylanase activity shows an optimum pH of 5 and an optimum temperature of 50°C. The enzyme is activated by Mg²⁺, but strongly inhibited by Hg²⁺ and Cu²⁺. The enzymatic activity remains quite high if the extract is preserved in a range of pH from 3 to 10 and a temperature between 30°C to 40°C

Abo-State *et al.* (2010) observed that under solid state fermentation of wheat straw revealed that the optimum pH values for the three cellulases (CMcase, Fpase and Avicelase) and soluble protein production by *T. viride* as standard strain, *A. terreus* and *A. flavus* ranging were ranging from 3.5 to 5.0. Tap water was the best moistening agent in producing CMCase, Fpase and

Avicelase *A. terreus* MAM-F23, but phosphate buffer was the best moistening agent for CMCase and Fpase by *T. viride* and *A. flavus* MAM-F35.

Acharya *et al.* (2008) studied the different factors relevant for improvement of enzymatic hydrolysis of saw dust by using *Aspergillus niger*. Different cultural conditions were examined to assess their effect in optimizing enzyme production. Alkaline pretreated (2N NaOH) saw dust at 9.6% concentration gave 0.1813 IU/mL cellulase activity. Optimum pH for cellulase production was between 4.0 and 4.5. Submerged fermentation at 120 rpm at 28°C gave higher yields of cellulase compared to static condition. Several other parameters like inoculum size, time duration, nitrogen source and its concentration were also optimized for the cellulase production by using saw dust as substrate.

4.3.2.5 Effect of Temperature

The different incubation temperature namely, 20°C (T₁), 25°C (T₂), 30°C (T₃), 35°C (T₄) and 40°C (T₅) were used for assessing the activity of cellulase and xylanase enzymes. Data pertaining to cellulase titers viz., Fpase, CMCase and β -glucosidase and xylanase are presented in Table 47 to 50.

An appraisal of Table 47 revealed that the significant difference in cellulase production was observed when different temperatures were used for study in *T. longibrachearum* T2. The significantly maximum cellulase titers (103.5 U/gds) were estimated at temperature 30°C (T₃), which was followed by 25°C (87.4 U/gds), 35°C (82.00 U/gds) and 20°C (62.40 U/gds). The significantly minimum cellulase titers (24.4 U/gds) were observed at temperature 40°C as shown in Fig. 53. Similarly, significantly maximum cellulase production was observed in alkali treated wheat straw (86.28 U/gds) than untreated wheat straw (57.60 U/gds). Interaction study revealed that significantly maximum cellulase titers (126.20 U/gds) were estimated at temperature 30°C (T₃) of treated wheat straw and minimum cellulase titers were observed in untreated wheat straw at 40°C (22.6 U/gds).

Table 47. Cellulase production at different temperature from *Trichoderma longibracheatum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

Temperature °C	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
20 (T ₁)	4.60* (0.23)** (5.60)***	6.20 (0.31) (6.39)	5.40 (0.27) (5.90)	20.00 (1.00) (24.39)	28.00 (1.40) (28.86)	24.00 (1.20) (26.60)	26.00 (1.30) (31.70)	40.00 (2.00) (41.23)	33.00 (1.65) (36.50)	50.60 (2.53) (61.70)	74.20 (3.71) (76.49)	62.40 (3.12) (69.10)	0.82	0.97	0.895	46.64
25 (T ₂)	7.00 (0.35) (7.00)	9.80 (0.49) (8.16)	8.40 (0.42) (7.50)	22.00 (1.10) (22.00)	36.00 (1.80) (30.00)	29.00 (1.45) (26.00)	42.00 (2.10) (42.00)	58.00 (2.90) (48.33)	42.00 (2.50) (45.20)	71.00 (3.55) (71.00)	103.80 (5.19) (86.50)	87.40 (4.37) (78.80)	1.00	1.20	1.10	46.19
30 (T ₃)	7.80 (0.39) (6.78)	12.20 (0.61) (9.31)	10.00 (0.50) (8.00)	27.00 (1.35) (23.47)	48.00 (2.40) (36.64)	37.5 (1.87) (30.10)	46.00 (2.30) (40.00)	66.00 (3.30) (50.38)	46.00 (2.80) (45.20)	80.80 (4.04) (70.26)	126.20 (6.31) (96.33)	103.50 (5.17) (83.30)	1.15	1.31	1.23	56.60
35 (T ₄)	5.00 (0.25) (5.10)	7.00 (0.35) (7.00)	6.00 (0.30) (6.00)	20.00 (1.00) (20.40)	38.00 (1.90) (38.00)	29.00 (1.45) (29.20)	38.00 (1.90) (38.77)	56.00 (2.80) (56.00)	41.00 (2.35) (47.40)	63.00 (3.15) (64.28)	101.00 (5.05) (101.00)	82.00 (4.10) (82.60)	0.98	1.00	0.99	60.13
40 (T ₅)	1.00 (0.05) (3.22)	1.80 (0.09) (5.14)	1.40 (0.07) (4.10)	9.00 (0.45) (29.03)	10.00 (0.50) (28.57)	9.50 (0.47) (28.80)	12.60 (0.63) (40.64)	14.40 (0.72) (41.14)	20.20 (0.67) (40.90)	22.60 (1.13) (72.90)	26.2 (1.31) (74.85)	24.40 (1.22) (73.90)	0.31	0.35	0.33	15.92
Mean	5.08 (0.25) (5.50)	7.40 (0.37) (7.20)		19.60 (0.98) (23.90)	32.00 (1.60) (32.40)		26.00 (1.64) (38.62)	46.88 (2.34) (47.41)		57.60 (2.88) (68.02)	86.28 (4.31) (87.03)		0.85	0.96		
CD _{T 0.05}	0.62 (0.088) (0.69)			1.18 (0.22) (0.93)			0.85 (4.11) (0.73)			0.88 (0.81) (0.79)			0.064			
CD _{B 0.05}	0.99 (0.055) (1.09)			1.86 (0.140) (1.48)			1.34 (2.60) (1.16)			1.40 (0.51) (1.24)			0.12			
CD _{TxB0.05}	1.34 (0.120) (1.48)			2.52 (0.32) (2.00)			1.82 (5.60) (1.56)			1.90 (1.12) (1.68)			0.138			

* U/gds = μmoles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μmoles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Treated- Untreated/Untreated x100

Table 48. Xylanase production at different temperature from *Trichoderma longibracheatum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

Temp. °C	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
20 (T ₁)	211.20* (10.56)** (257.56)***	249.00 (12.45) (256.70)	230.10 (11.50) (257.13)	0.82	0.97	0.895	17.89
25 (T ₂)	305.40 (15.27) (305.40)	405.00 (20.25) (337.5)	355.20 (17.76) (321.45)	1.00	1.20	1.10	32.61
30 (T ₃)	379.80 (18.99) (330.26)	508.60 (25.43) (388.24)	444.20 (22.21) (359.25)	1.15	1.31	1.23	33.91
35 (T ₄)	191.20 (9.56) (195.10)	262.00 (13.10) (262.00)	226.60 (11.33) (228.55)	0.98	1.00	0.99	37.02
40 (T ₅)	104.60 (5.23) (337.41)	122.60 (6.13) (350.28)	113.60 (5.68) (343.84)	0.31	0.35	0.33	17.27
Mean	238.44 (11.92) (285.14)	309.44 (15.47) (318.94)		0.852	0.966		
CD _{T 0.05}	1.70 (1.33) (1.04)			0.064			
CD _{B 0.05}	2.70 (0.84) (1.65)			0.12			
CD _{TxB0.05}	0.36 (1.82) (2.24)			0.138			

* U/gds = μmoles of reducing sugars released / min / g of biomass

** IU/ml = Value in parentheses depict μmoles of reducing sugars released / min / ml of enzyme

***Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Treated- Untreated/Untreated x100

Table 49. Cellulase production at different temperature from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw as substrate under SSF

Temp. °C	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
20 (T ₁)	2.80* (0.14)** (5.40)***	2.60 (0.13) (5.42)	2.70 (0.135) (5.41)	46.00 (2.30) (90.19)	43.00 (2.15) (89.58)	44.50 (2.22) (89.89)	48.00 (2.40) (117.0)	46.80 (2.34) (97.50)	47.40 (2.37) (107.25)	96.80 (4.84) (189.80)	92.40 (4.62) (192.50)	94.60 (4.73) (191.15)	0.51	0.48	0.50	4.55
25 (T ₂)	5.20 (0.26) (4.29)	4.80 (0.24) (4.32)	5.00 (0.25) (4.31)	52.00 (2.60) (42.9)	51.00 (2.55) (45.95)	51.50 (2.57) (44.43)	57.00 (2.70) (47.01)	54.00 (2.70) (51.44)	55.50 (2.77) (49.23)	114.20 (5.71) (94.38)	109.90 (5.49) (101.71)	112.55 (5.68) (98.05)	1.21	1.11	1.16	3.77
30 (T ₃)	6.4 (0.32) (4.92)	5.90 (0.30) (4.72)	6.15 (0.31) (4.82)	60.00 (3.00) (46.15)	58.00 (2.90) (46.40)	59.00 (2.95) (46.28)	65.00 (3.25) (50)	63.00 (3.15) (50.40)	64.00 (3.20) (50.20)	131.41 (6.57) (101.0)	126.90 (6.35) (101.52)	129.15 (6.46) (101.2)	1.30	1.25	1.28	3.43
35 (T ₄)	2.20 (0.11) (5.11)	1.90 (0.10) (4.52)	2.05 (0.105) (4.82)	24.00 (1.20) (55.8)	23.00 (1.15) (54.76)	23.50 (1.17) (55.28)	28.00 (1.4) (65.1)	27.00 (1.35) (64.29)	27.50 (1.37) (64.70)	54.20 (2.71) (126.0)	51.90 (2.60) (123.57)	53.05 (2.65) (124.79)	0.43	0.42	0.43	4.24
40 (T ₅)	0.40 (0.02) (4.00)	0.30 (0.02) (3.33)	0.35 (0.02) (3.67)	0.80 (0.04) (8.00)	0.70 (0.04) (7.78)	0.75 (0.04) (7.89)	0.10 (0.01) (1.0)	0.08 (0.01) (0.89)	0.09 (0.01) (0.95)	1.30 (0.07) (13.00)	1.08 (0.05) (12.00)	1.19 (0.06) (12.50)	0.10	0.09	0.10	16.92
Mean	3.40 (0.17) (4.74)	3.10 (0.15) (4.46)		36.56 (1.82) (48.61)	35.14 (1.75) (48.89)		39.62 (1.98) (56.02)	38.79 (1.94) (52.90)		79.58 (3.98) (104.83)	77.03 (3.85) (106.26)		0.71	0.67		
CD _{T 0.05}	1.19 (0.020) (0.42)			1.18 (0.64) (0.73)			0.83 (0.59) (0.60)			0.76 (0.68) (0.61)			0.046			
CD _{B 0.05}	0.30 (0.033) (0.67)			1.87 (1.01) (1.15)			1.32 (0.93) (0.95)			1.20 (1.07) (0.96)			0.073			
CD _{TxB0.05}	2.18 (0.046) (0.90)			2.54 (1.36) (1.56)			1.78 (1.30) (1.28)			1.62 (1.46) (1.30)			0.098			

* U/gds = μmoles of reducing sugars released / min /g of biomass

** IU/ml = μmoles of reducing sugars released/min/ml of enzyme

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated x 100

Table 50. Xylanase production at different temperature from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw as substrate under SSF

Temp. °C	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
20 (T ₁)	184.00* (9.20)** (360.78)***	176.00 (8.80) (366.67)	180.00 (9.00) (363.73)	0.48	0.51	0.50	4.35
25 (T ₂)	395.60 (19.78) (326.94)	374.00 (18.70) (336.94)	384.80 (19.24) (331.94)	1.11	1.21	1.16	5.46
30 (T ₃)	490.00 (24.50) (376.92)	460.00 (23.00) (368.00)	475.00 (23.75) (372.46)	1.25	1.30	1.28	6.12
35 (T ₄)	224.00 (11.20) (520.93)	202.00 (10.00) (480.95)	213.00 (10.65) (500.94)	0.42	0.43	0.43	9.82
40 (T ₅)	62.00 (3.10) (620.00)	46.00 (2.30) (511.11)	54.00 (2.70) (565.56)	0.09	0.10	0.10	25.81
Mean	271.12 (13.55) (441.11)	251.60 (12.58) (412.73)		0.67	0.71		
CD _{T 0.05}	3.63 (1.08) (0.93)			0.073			
CD _{B 0.05}	5.75 (1.71) (1.47)			0.046			
CD _{TxB0.05}	7.78 (2.36) (2.00)			0.098			

* U/gds = μmoles of reducing sugars released / min /g of biomass

** IU/ml = μmoles of reducing sugars released/min/ml of enzyme

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated x 100

The scrutiny of data in Table 48 depicted that significantly highest xylanase titers (444.20 U/gds) were estimated at 30°C (T₃) and minimum at 40°C (113.6 U/gds). Similarly, significantly highest xylanase production was observed in alkali treated wheat straw substrate (309.44 U/gds) than untreated wheat straw substrate (238.44 U/gds). Interaction study revealed that significantly highest xylanase titers (508.6 U/gds) were estimated at 30°C (T₃), when alkali pretreated substrate of wheat straw was used as a substrate as shown in Fig 54. The significantly lowest xylanase titers were observed in untreated substrate at 40°C (104.6 U/gds).

A perusal of Table 49 revealed that significant difference in cellulase production was observed when pretreated agricultural lignocellulosic substrate wheat straw was used for study in *R. oryzae* W2 at different temperature. The significantly highest cellulase titers (129.15 U/gds) were estimated at 30°C (T₃), which was followed by 25°C (112.55 U/gds) and lowest (1.19 U/gds) at 40°C. Similarly, the significantly maximum cellulase production was observed in untreated wheat straw (79.58 U/gds) than steam pretreated wheat straw (77.03 U/gds). Interaction study revealed that significantly maximum cellulase titers (131.41 U/gds) were estimated at 30°C (T₃), when an untreated agricultural waste of wheat straw was used as a substrate and minimum in steam pretreated wheat straw at 40°C (1.08 U/gds) as depicted in Fig 55.

The scrutiny of data in Table 50 revealed that significantly highest xylanase titers (475.0 U/gds) were observed at 30°C and lowest was observed at 40°C (54.0 U/gds). Similarly, significantly highest xylanase production was observed in untreated substrate (271.12 U/gds) than steam pretreated substrate (251.60 U/gds). Interaction study revealed that significantly highest xylanase titers (490.00 U/gds) were estimated at 30°C, when untreated substrate of wheat straw was used as a substrate as shown in Fig. 56. The significantly lowest xylanase titers were observed in steam treated wheat straw at incubation temperature 40°C (46.0 U/gds).

Like pH, temperature is one of the most important parameter essential for the success of SSF reaction. Microorganisms grow slowly at a temperature below

or above the normal growth temperature because of reduced rate of cellular production. If the growth temperature is too high but not lethal, these may be premature induction target protein expression. Incubation temperature is a most important factors among the all the physical variables affecting the SSF performance because both cell growth and production of enzymes are usually sensitive to temperature (Cardona and Sanchez, 2007).

Study presented (Table No. 47 to 50) reflects the mesophilic nature of both the enzyme producing fungi showing 30°C as optimum temperature for cellulase and xylanase enzyme synthesis. Maximum enzyme at this temperature seems to be favourable for their growth and their is direct correlation between growth and production of enzyme. For cellulase and xylanase production by *T. longibrachetum* T2 and *R. oryzae* W2 at 30°C was found to be most effective. Enzyme production started to decline after further increase in temperature. This temperature seems a cardinal factor affecting the amount of enzyme and rate of growth of organism and the increase in temperature has the general effect of increase in enzyme activity (20 to 30°C) but the enzyme begins to suffer thermal inactivation at higher temperature (30 to 40°C). When the temperature of the medium was changed on either side of optimum range (30°C), a decline in enzyme activities were observed. The temperature usually employed for in SSF is in the range of 25-35°C and it depends upon the growth kinetics of the organisms employed (Sindhu *et al.*, 2011).

Microbial growth in SSF generates significant amount of metabolic heat. It has been reported that 100-300 kJ of heat per kg of cell mass is generated in a SSF Process. Establishment of temperature gradient and localized overheating of the substrate occurs because of the insufficient removal of heat from the substrate. Heat transfer problem in SSF includes temperature gradients that may cause belated microbial activity, dehydration of the medium and undesirable metabolic deviations. Heterogenous materials make heat removal difficult due to low transfer coefficient and low thermal conductivity (Manpreet *et al.*, 2005). Temperature can rise rapidly, because there is little water to absorb the heat or in other words the specific heat capacity of the fermenting mass is much lower than

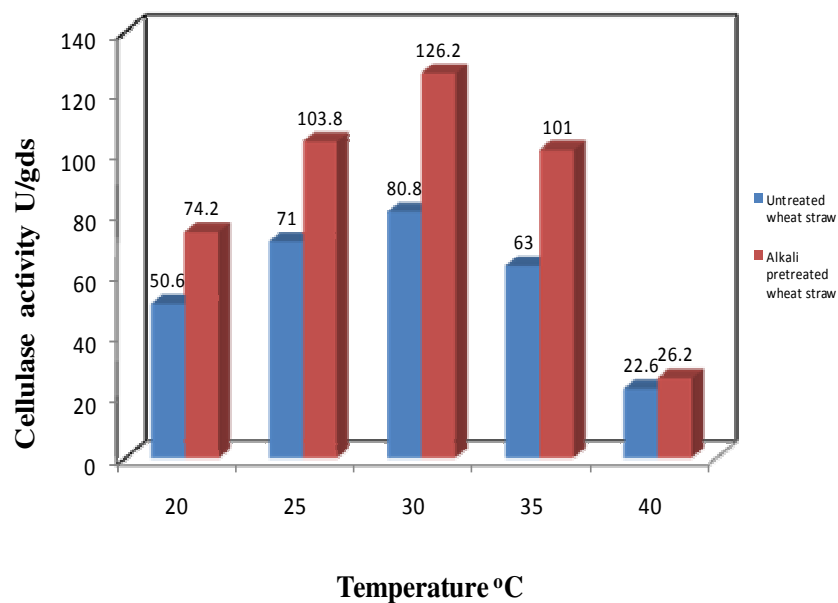


Fig 53. Effect of temperature on cellulase production in alkali pretreated wheat straw from *T. longibracheatum* T2

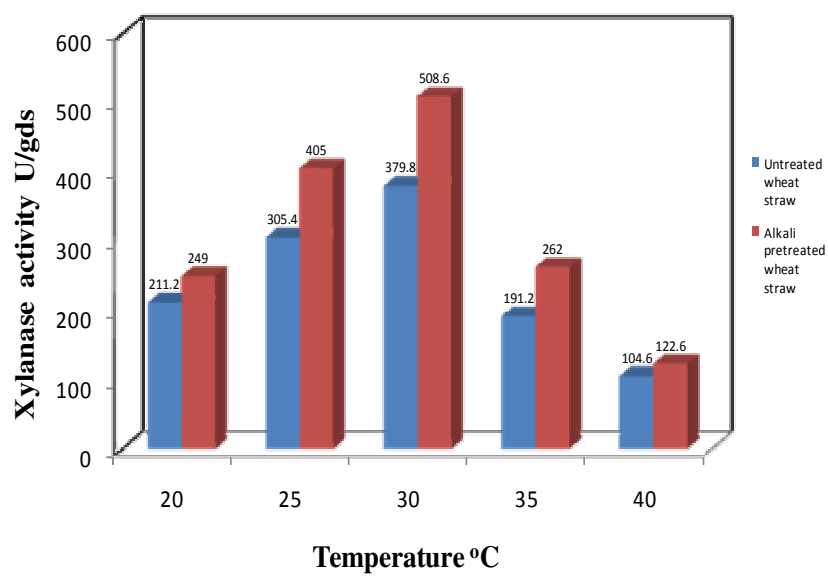


Fig 54. Effect of temperature on xylanase production in alkali pretreated wheat straw from *T. longibracheatum* T2

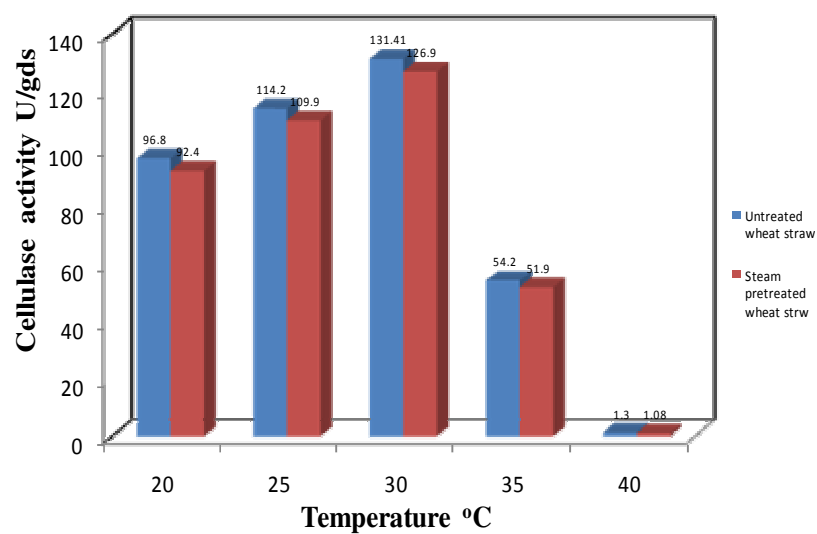


Fig 55. Effect of temperature on cellulase production in steam pretreated wheat straw from *R. oryzae* T2

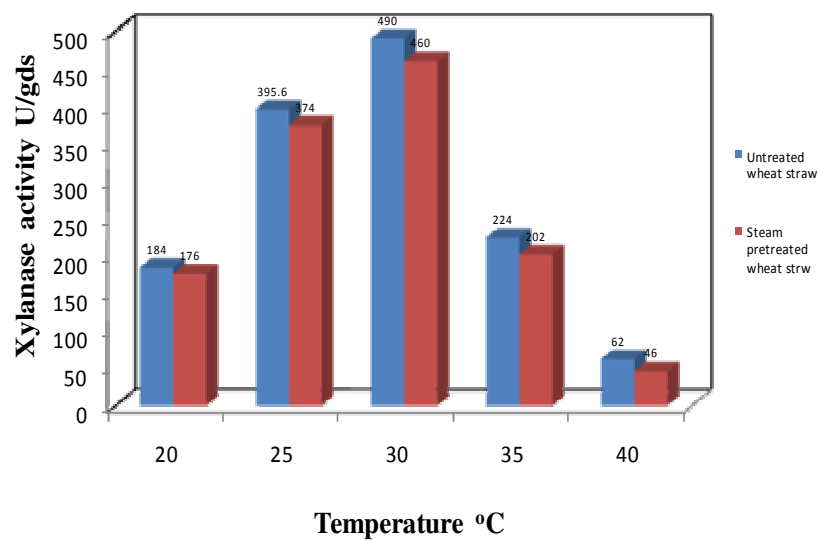


Fig 56. Effect of temperature on xylanase production in steam pretreated wheat straw from *R. oryzae* T2

that of water. Therefore, heat generated must be dissipated immediately as most of the microorganisms used in SSF are mesophilic, having optimal temperature for growth between 20 and 40°C and maximum growth below 50°C.

Similar finding has been reported by Mrudula and Murugammal (2011) recorded the optimum conditions for the maximum biosynthesis for cellulase by *A. niger* were at pH 6 and temperature 30°C at 72 hour of incubation time. The CMcase and Fpase activities recorded in SSF were 8.89 and 3.56 U/g dry mycelial bran.

Malik *et al.* (2010) described the optimization of process parameters for the production of cellulases by *Trichoderma viride* using submerged fermentation technique. The fermentation experiments were carried out in shake flasks using pretreated bagasse. Maximum production of cellulases (CMCase 1.57 U/ml/min, FPase 0.921 U/ml/min) was observed after a fermentation period of 72h at an incubation temperature of 30°C. Initial pH of the culture medium was also optimized and a pH of 5.5 was found to support maximum growth and enzyme production (CMCase 1.66 U/ml/min and FPase 0.932 U/ml/min) by *T. viride*. Different inorganic nitrogen sources were evaluated for the production of cellulases and Ammonium sulphate was found to be the best.

Fatma and Fadel (2010) reported the production of cellulase by *Trichoderma reesei* F-418 cultivated on alkali treated rice straw using solid state fermentation (SSF) technique. The high cellulase activity was obtained when the fungus was cultivated on substrate with about 75 % (v/w) moisture, pH 4.8 for 5 days incubation at 28±2°C, as it gave 16.2 IU/g substrate. The obtained cellulase of 1.2 IU/ ml culture filtrate was applied for saccharification (5% w/v) of alkali treated rice straw, in 0.1M citrate buffer pH 4.8 in shaker water bath of 100 rpm. Sugary solution of 1.07 % glucose was achieved after 16 h. The sugary solution was concentrated to give 10% (w/v) glucose.

Sanghvi *et al.* (2010) showed that maximum xylanase production was observed in a wheat straw/rice straw ratio of 1:1, with a particle size of 0.45–0.5 mm by *T. harzanium*. Media supplementation with xylose as a carbon source in a

ratio 1:5 (carbon source/substrate) gave maximum activity (157 IU/ml). Nitrogen supplementations from yeast extract produced xylanase activity of 141 IU/ml. Optimum xylanase production was observed at 30°C and pH 5.0. Xylanase production was enhanced in the presence of Ca^{2+} and Zn^{2+} ions.

Haq *et al.* (2006) studied different agricultural residue such as wheat bran, wheat straw, rice bran, and rice husk and soybean meal for cellulase production from *T. harzianum*. Among them wheat bran was found as best substrate for cellulase production. The production of enzyme was significantly improved when wheat bran was moistened with mineral salts CMC solution (1:1 ratio) at 28°C and pH 6.5.

4.3.2.6 Effect of Moisture Ratio

The different moisture ratio namely, 1:3.0 (T_1), 1:3.5 (T_2), 1:4.0 (T_3), 1:4.5 (T_4), 1:5.0 (T_5), 1:5.5 (T_6), 1:6.0 (T_7) and 1:6.5 (T_8) were used for assessing the activity of cellulase and xylanase enzymes. Data pertaining to cellulase titers viz., Fpase, CMCase and β -glucosidase and xylanase are presented in Table 51 to 54.

A perusal of Table 51 revealed that the significant difference in cellulase production was observed when *T. longibrachiatum* T2 was used for study at different moisture ratio. The significantly maximum cellulase titers (114.45 U/gds) were estimated at moisture ratio of 1:4.5 (T_4), which was followed by 1:4.0 (T_3) (105.30 U/gds), 1:5.0 (T_6) (103.50U/gds) and 1:3.5 (83.50 U/gds). The significantly minimum cellulase titers (19.50 U/gds) were observed at moisture ratio of 1:6.5. Similarly, significantly maximum cellulase production was observed in alkali treated wheat straw (90.71 U/gds) than untreated wheat straw (60.20 U/gds) as shown in Fig 57. Interaction study revealed that significantly maximum cellulase titers (139.90 U/gds) were estimated at moisture ratio of 1:4.5 30°C (T_4) and minimum in untreated wheat straw at moisture ratio of 1:6.5 (19 U/gds).

The scrutiny of data in Table 52 showed that significantly highest xylanase titers (491.35 U/gds) were estimated at 1:4.0 (T_4), which was followed

Table 51. Cellulase production at different moisture ratio from *Trichoderma longibracheatum*T2 [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

Moisture Ratio	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
1:3:0 (T ₁)	4.80 (0.24) (9.79)	5.80 (0.29) (7.73)	5.30 (0.26) (8.76)	16.00 (0.80) (32.65)	28.00 (1.40) (37.33)	22.00 (1.10) (34.99)	24.00 (1.20) (48.97)	42.00 (2.10) (56.00)	33.00 (1.65) (52.49)	44.80 (2.24) (91.42)	75.80 (3.79) (101.06)	60.30 (3.01) (96.24)	0.49	0.75	0.62	69.19
1:3:5 (T ₂)	6.40 (0.32) (8.64)	9.60 (0.48) (8.64)	8.00 (0.40) (8.64)	22.00 (1.10) (29.72)	38.00 (1.90) (34.23)	30.00 (1.15) (31.97)	37.00 (1.85) (50.00)	54.00 (2.70) (48.64)	45.50 (2.27) (49.32)	65.40 (3.27) (88.37)	101.60 (5.08) (91.53)	83.50 (4.17) (89.95)	0.74	1.11	0.93	55.35
1:4:0 (T ₃)	8.20 (0.41) (10.00)	12.40 (0.62) (9.32)	10.30 (0.51) (9.66)	26.00 (1.30) (31.70)	50.00 (2.50) (37.59)	38.00 (1.90) (34.64)	46.00 (2.30) (56.09)	68.00 (3.40) (51.12)	57.00 (2.85) (53.61)	80.20 (4.01) (97.80)	130.40 (6.52) (98.04)	105.30 (5.26) (97.92)	0.82	1.33	1.08	62.59
1:4:5 (T ₄)	9.00 (0.45) (7.43)	13.90 (0.70) (10.00)	11.45 (0.57) (8.71)	28.00 (1.40) (23.14)	54.00 (2.70) (38.84)	41.00 (2.05) (30.99)	52.00 (2.60) (42.97)	72.00 (3.60) (51.79)	62.00 (3.10) (47.38)	89.00 (4.45) (73.55)	139.90 (6.99) (100.64)	114.45 (5.72) (87.10)	1.21	1.39	1.30	57.19
1:5:0 (T ₅)	7.80 (0.39) (6.78)	12.20 (0.61) (9.31)	10.00 (0.50) (8.04)	26.00 (1.30) (22.60)	48.00 (2.40) (36.64)	37.00 (1.85) (29.62)	47.00 (2.35) (40.86)	66.00 (3.30) (50.38)	56.50 (2.82) (45.62)	80.80 (4.04) (70.26)	126.20 (6.31) (96.33)	103.50 (5.17) (83.30)	1.15	1.31	1.23	56.18
1:5:5 (T ₆)	5.20 (0.26) (5.59)	7.20 (0.36) (7.20)	6.20 (0.31) (6.39)	20.00 (1.00) (21.50)	36.00 (1.80) (36.00)	28.00 (1.40) (28.75)	42.00 (2.10) (45.16)	50.00 (2.50) (50.00)	46.00 (2.30) (47.58)	67.20 (3.36) (72.25)	93.20 (4.66) (93.20)	80.20 (4.01) (82.73)	0.93	1.00	0.97	38.69
1:6:0 (T ₇)	3.60 (0.18) (8.37)	3.40 (0.17) (7.39)	3.50 (0.17) (7.88)	15.20 (0.76) (35.34)	19.60 (0.98) (42.60)	17.40 (0.87) (38.97)	16.40 (0.82) (38.13)	15.60 (0.78) (33.91)	16.00 (0.80) (36.02)	35.20 (1.76) (81.86)	38.60 (1.93) (83.91)	36.90 (1.84) (82.89)	0.43	0.46	0.45	9.65
1:6:5 (T ₈)	1.40 (0.07) (7.36)	1.80 (0.09) (8.57)	1.60 (0.08) (7.96)	9.20 (0.46) (48.42)	9.60 (0.48) (45.71)	9.40 (0.47) (47.06)	8.40 (0.48) (44.21)	8.60 (0.43) (40.95)	8.50 (0.42) (42.58)	19.00 (0.95) (100.00)	20.00 (1.00) (95.23)	19.50 (0.97) (97.62)	0.19	0.21	0.20	5.26
Mean	5.80 (0.29) (7.99)	8.2875 (0.41) (8.52)		20.3 (1.01) (30.63)	35.40 (1.77) (38.61)		34.10 (1.70) (45.80)	47.03 (2.35) (47.85)		60.20 (3.01) (84.44)	90.71 (4.53) (94.99)		0.75	0.945		
CD _{T 0.05}	0.26 (0.037) (0.44)			1.08 (0.19) (0.56)			0.99 (0.38) (0.69)			0.58 (0.99) (0.74)			0.020			
CD _{B 0.05}	0.52 (0.018) (0.88)			2.17 (0.098) (1.13)			1.99 (0.193) (1.38)			1.17 (0.48) (1.48)			0.041			
CD _{TxB0.05}	0.72 (0.052) (1.22)			3.00 (0.26) (1.56)			2.76 (0.54) (1.96)			1.62 (1.36) (2.06)			0.058			

* U/gds = μ moles of reducing sugars released / min /g of biomass

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated x 100

Table 52. Xylanase production at different moisture ratio from *Trichoderma longibracheatum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

Moisture ratio	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
1:3.0 (T ₁)	224.60 (11.23) (458.37)	304.60 (15.23) (406.13)	264.60 (13.23) (432.25)	0.49	0.75	0.62	35.62
1:3.5 (T ₂)	266.80 (13.34) (360.54)	353.00 (17.65) (318.02)	309.90 (15.49) (339.28)	0.74	1.11	0.93	32.31
1:4.0 (T ₃)	311.20 (15.56) (379.51)	437.20 (21.86) (328.72)	374.20 (18.71) (354.11)	0.82	1.33	1.08	40.49
1:4.5 (T ₄)	405.50 (20.28) (335.12)	577.20 (28.86) (415.25)	491.35 (24.57) (375.18)	1.21	1.39	1.30	42.34
1:5.0 (T ₅)	379.80 (18.99) (330.26)	508.60 (25.43) (388.24)	444.20 (22.21) (359.25)	1.15	1.31	1.23	33.91
1:5.5 (T ₆)	253.40 (12.67) (272.47)	344.60 (17.23) (344.60)	299.00 (14.95) (308.53)	0.93	1.0	0.97	35.99
1:6.0 (T ₇)	179.60 (8.98) (417.67)	283.20 (14.16) (615.65)	231.40 (11.57) (516.66)	0.43	0.46	0.45	57.68
1:6.5 (T ₈)	64.20 (3.21) (337.89)	84.80 (4.24) (403.81)	74.50 (3.72) (370.85)	0.19	0.21	0.20	32.09
Mean	260.63 (13.03) (361.47)	361.65 (18.08) (402.55)		0.74	0.94		
CD _{T 0.05}	0.94 (0.64) (25.74)			0.020			
CD _{B 0.05}	1.88 (0.82) (51.48)			0.041			
CD _{TxB0.05}	2.32 (2.30) (71.98)			0.058			

* U/gds = μ moles of reducing sugars released / min /g of biomass

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

% increase = Untreated-Treated/Treated X 100

1:5.0 (444.20 U/gds) and 1:4.0 (374.20 U/gds). The significantly lowest xylanase titers were observed at 1:6.5 (74.50 U/gds). Similarly, significantly highest xylanase production was observed in alkali treated wheat straw substrate (361.65 U/gds) than untreated wheat straw substrate (260.63 U/gds) as depicted in Fig 58. Interaction study (TxB) revealed that significantly highest xylanase titers (577.20 U/gds) were estimated at 1:4.5 (T₃), when alkali pretreated substrate of wheat straw was used as a substrate and significantly lowest xylanase titers were observed in untreated substrate at 1:6.5 (64.20 U/gds).

An overview of Table 53 revealed that the significant difference in cellulase production was observed when *R. oryzae* W2 at different moisture ratio was used for study. The significantly highest cellulase titers (146.40 U/gds) were estimated at 1:4.5 (T₄), which was followed 1:5.0 (129.21 U/gds), at 1:4.0 (107.25 U/gds) and at 1:5.5 (97.05 U/gds). The significantly lowest cellulase titers (3.52 U/gds) were observed at moisture ratio of 1:6.5. Similarly, significantly maximum cellulase production was observed in untreated wheat straw (80.98 U/gds) than steam pretreated wheat straw (78.69 U/gds) as shown in Fig 59. Interaction study (TxB) revealed that significantly maximum cellulase titers (151.0 U/gds) were estimated at 1:4.5 (T₄), when an untreated agricultural waste of wheat straw was used as a substrate. The significantly minimum cellulase titers were observed in steam pretreated wheat straw at 1:6.5 (3.40 U/gds).

An appraisal of data in Table 54 revealed that significantly highest xylanase titers (548.0 U/gds) were observed at 1:4.5, which was followed by 1:5.0 (472.0 U/gds) and 1:4.0 (427.00 U/gds). The significantly lowest xylanase titers were observed at 1:6.5 (130.0 U/gds). Similarly, significantly highest xylanase production was observed in untreated substrate (364.75 U/gds) than steam pretreated substrate (336.25 U/gds) as shown in Fig 60. Interaction study (TxB) revealed that significantly highest xylanase titers (564.0 U/gds) were estimated at 1:4.5 (T₄), when an untreated substrate of wheat straw was used as a substrate. The significantly lowest xylanase titers were observed in steam treated wheat straw at 1:6.5 (122.0 U/gds).

Table 53. Cellulase production at different moisture ratio from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw as substrate under SSF

Moisture ratio	Cellulase															
	Fpase		Mean	CMCase		Mean	β -glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
1:3.0 (T ₁)	1.8* (0.09)** (4.28)***	1.70 (0.08) (4.25)	1.75 (0.085) (4.27)	22 (1.10) (52.38)	20.50 (1.03) (51.25)	21.25 (1.06) (51.82)	20.00 (1.00) (47.61)	17.80 (0.89) (44.50)	18.90 (0.94) (46.06)	43.80 (2.19) (104.28)	40.0 (2.09) (100.00)	41.90 (2.14) (102.14)	0.42 	0.40 	0.41 	8.68
1:3.5 (T ₂)	3.00 (0.15) (3.61)	1.60 (0.09) (2.05)	2.3 (0.11) (2.83)	34 (1.70) (40.96)	31.30 (1.57) (40.13)	32.65 (1.63) (40.55)	38.00 (1.9) (45.78)	35.90 (1.80) (46.03)	36.95 (1.85) (45.91)	75.0 (3.75) (90.36)	68.80 (3.44) (88.21)	71.90 (3.59) (89.28)	0.83 	0.78 	0.81 	8.27
1:4.0 (T ₃)	4.2 (0.21) (3.47)	3.80 (0.19) (3.80)	4.00 (0.20) (3.64)	50 (2.50) (41.32)	46.80 (2.34) (46.80)	48.40 (2.42) (44.06)	56.00 (2.80) (46.20)	53.70 (2.69) (53.70)	54.85 (2.74) (49.95)	110.2 (5.51) (91.07)	104.3 (5.22) (104.30)	107.25 (5.36) (97.68)	1.21 	1.00 	1.11 	5.35
1:4.5 (T ₄)	7.60 (0.38) (5.31)	6.20 (0.31) (4.84)	6.9 (0.34) (5.08)	69 (3.45) (48.25)	65.00 (3.25) (50.78)	67.00 (3.35) (49.52)	74.40 (3.72) (52.0)	70.60 (3.53) (55.16)	72.50 (3.62) (53.58)	151.0 (7.55) (105.59)	141.80 (7.09) (110.78)	146.40 (7.32) (108.18)	1.43 	1.28 	1.36 	6.09
1:5.0 (T ₅)	6.4 (0.32) (4.92)	6.10 (0.30) (5.55)	6.25 (0.31) (5.24)	60 (3.00) (46.15)	58.00 (2.90) (52.73)	41.50 (2.95) (49.44)	65.00 (3.25) (50.0)	62.90 (3.15) (57.18)	63.95 (3.20) (53.59)	131.41 (6.57) (101.0)	127.00 (6.35) (115.45)	129.21 (6.46) (108.22)	1.30 	1.10 	1.20 	3.36
1:5.5 (T ₆)	4.6 (0.23) (4.6)	4.20 (0.21) (5.25)	4.4 (0.22) (4.93)	42 (2.10) (42)	41.00 (2.05) (51.25)	59.00 (2.07) (46.63)	52.00 (2.60) (52.0)	50.30 (2.52) (62.88)	51.15 (2.56) (57.44)	98.6 (4.93) (98.6)	95.50 (4.78) (119.38)	97.05 (4.85) (108.99)	1.0 	0.80 	0.90 	3.14
1:6.0 (T ₇)	2.2 (0.11) (3.85)	2.00 (0.10) (3.70)	2.1 (0.105) (3.78)	22 (1.10) (38.59)	20.00 (1.00) (37.04)	21.00 (1.05) (37.82)	28.00 (1.40) (49.12)	26.70 (1.34) (49.44)	27.35 (1.37) (49.28)	52.2 (2.61) (91.57)	48.70 (2.44) (90.19)	50.45 (2.52) (90.88)	0.57 	0.54 	0.56 	6.70
1:6.5 (T ₈)	0.6 (0.03) (2.5)	0.45 (0.02) (2.14)	0.525 (0.025) (2.32)	1.6 (0.08) (6.66)	1.45 (0.07) (6.90)	1.53 (0.075) (6.78)	1.80 (0.10) (7.50)	1.50 (0.08) (7.14)	1.65 (0.08) (7.32)	3.64 (0.18) (15.16)	3.40 (0.17) (16.19)	3.52 (0.17) (15.67)	0.24 	0.21 	0.23 	6.59
Mean	3.80 (0.19) (4.0)	3.26 (0.16) (3.94)		37.58 (1.87) (39.54)	35.51 (1.77) (42.11)		41.90 (2.10) (43.78)	39.93 (2.00) (47.00)		83.23 (4.04) (81.85)	78.68 (3.93) (93.06)		0.88 	0.76 		
CD _{T 0.05}	0.36 (0.033) (0.31)			0.81 (0.41) (0.48)			0.49 (0.46) (0.44)			0.91 (0.55) (0.54)			0.057 			
CD _{B 0.05}	0.72 (0.067) (0.63)			1.62 (0.83) (0.96)			0.99 (0.93) (0.88)			1.83 (1.11) (1.09)			0.115 			
CD _{TxB0.05}	1.0 (0.10) (0.86)			2.24 (1.14) (1.36)			1.38 (1.30) (1.22)			2.54 (1.44) (1.52)			0.158 			

* U/gds = μ moles of reducing sugars released / min /g of biomass

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

% increase = Untreated-Treated/Treated X 100

Table 54. Xylanase production at different moisture ratio from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw as substrate under SSF

Moisture ratio	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
1:3.0 (T ₁)	262.00* (13.10)** (623.81)***	236.00 (11.8) (590.00)	249.00 (12.45) (606.91)	0.42	0.40	0.41	9.92
1:3.5 (T ₂)	346.00 (17.30) (416.87)	318.00 (15.9) (407.69)	332.00 (16.60) (412.28)	0.83	0.78	0.81	8.09
1:4.0 (T ₃)	450.00 (22.50) (371.90)	404.00 (20.20) (404.00)	427.00 (21.35) (387.95)	1.21	1.00	1.11	10.22
1:4.5 (T ₄)	564.00 (28.20) (394.41)	532.00 (26.60) (415.63)	548.00 (27.40) (402.05)	1.43	1.28	1.36	5.67
1:5.0 (T ₅)	490.00 (24.50) (490.00)	454.00 (22.70) (412.73)	472.00 (23.60) (451.37)	1.30	1.10	1.20	7.35
1:5.5 (T ₆)	426.00 (21.30) (327.69)	400.00 (20.00) (500.00)	413.00 (20.65) (413.85)	1.0	0.80	0.90	6.10
1:6.0 (T ₇)	242.00 (12.10) (424.56)	224.00 (11.20) (414.81)	233.00 (11.65) (419.69)	0.57	0.54	0.56	7.44
1:6.5 (T ₈)	138.00 (6.90) (575.00)	122.00 (6.10) (580.95)	130.00 (6.50) (577.98)	0.24	0.21	0.23	11.59
Mean	364.75 (18.23) (453.03)	336.25 (16.81) (465.73)		0.88	0.76		
CD _{T 0.05}	0.92 (1.69) (14.71)			0.057			
CD _{B 0.05}	1.85 (3.39) (29.42)			0.115			
CD _{TxB 0.05}	2.58 (4.75) (40.86)			0.158			

* U/gds = μ moles of reducing sugars released / min /g of biomass

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

% increase = $\frac{\text{Untreated-Treated}}{\text{Treated}} \times 100$

Table No. 51 to 54 showed that the ratio of initial moisture level (1:4.5) yielded the highest cellulase and xylanase production. This could be attributed to the faster growth of the organism, at this moisture content and the subsequent early initiation of enzyme production. Many investigators have reported similar effects of moisture content on this enzyme synthesis (Szendfy *et al.*, 2006; Bakri *et al.*, 2003; Xin and Geng, 2010). Therefore, importance of moisture level in solid state fermentation media and its influence on microbial growth and product synthesis may be attributed to the direct impact of moisture on the physical properties of solid substrate.

All microorganisms require a source of water to enable cellular functioning to occur effectively. They have a semi-permeable cell membrane, which permits water molecules to enter the cell through osmosis and come to equilibrium with the outer environment. The total water of any substance is the sum of the bound water (water of constitution), which is held in chemical union with other compounds by very strong forces, and free water, which is weakly bound. Free water is the most readily available for microbial growth and metabolism. Scott (1957) suggested that the water activity (a_w) would be the best to describe the water availability for microbial activity. Low moisture content is known as to decrease the metabolic and enzymatic activity, probably owing to reduced solubility of nutrients from the solid substrates. Low swelling and higher water tension (1:2) (Moo-Young *et al.*, 1983; Lonsane *et al.*, 1985). On the other hand higher moisture level can cause the reduction in enzyme yield due to steric hindrance to cell growth of enzyme producing strains resulted in the reduction of solid matrix porosity (interparticle space). Less porosity interferes with oxygen transfer and in turn influences the cell growth (Bakri *et al.*, 2003). As oxygen transfer affects both the growth and the metabolism of the fungi, the solid substrate should contain suitable amount of water to enhance mass transfer (Xin and Geng, 2010).

In a similar study a higher cellulase activity was recorded by Gao *et al.* (2008) using *A. terreus* M11 on corn stover having 80% moisture for enzyme activity up to 581 U/gds. Xin and Geng (2010) reported that optimal moisture

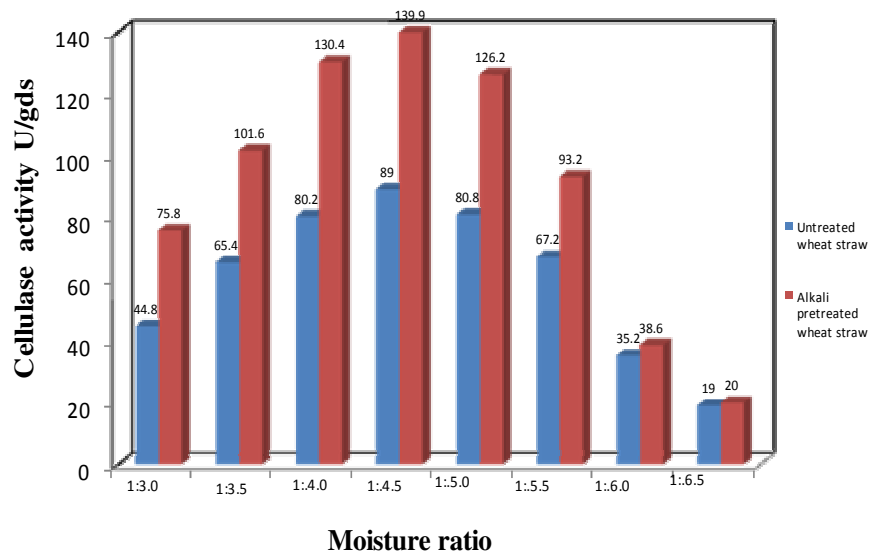


Fig 57. Effect of moisture ratio on cellulase production in alkali pretreated wheat straw from *T. longibracheatum* T2

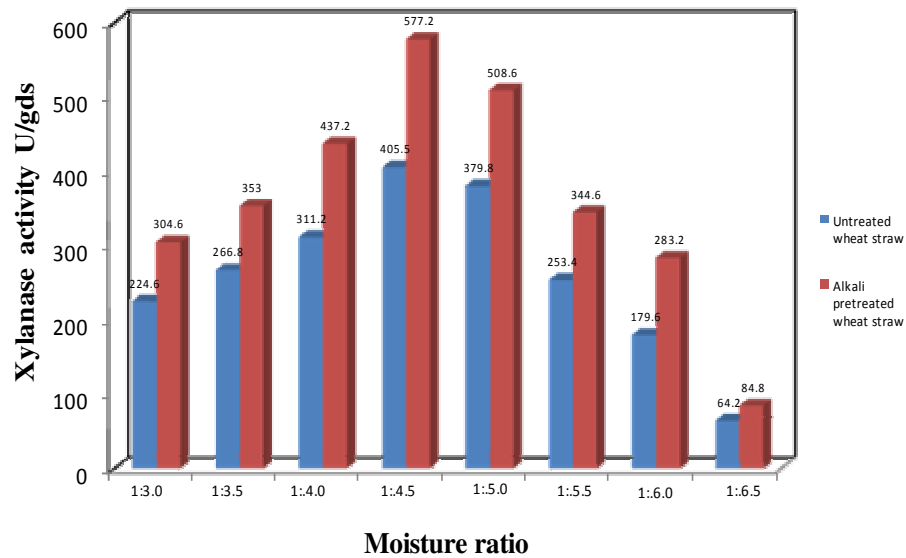


Fig 58. Effect of pH on xylanase production in alkali pretreated wheat straw from *T. longibracheatum* T2

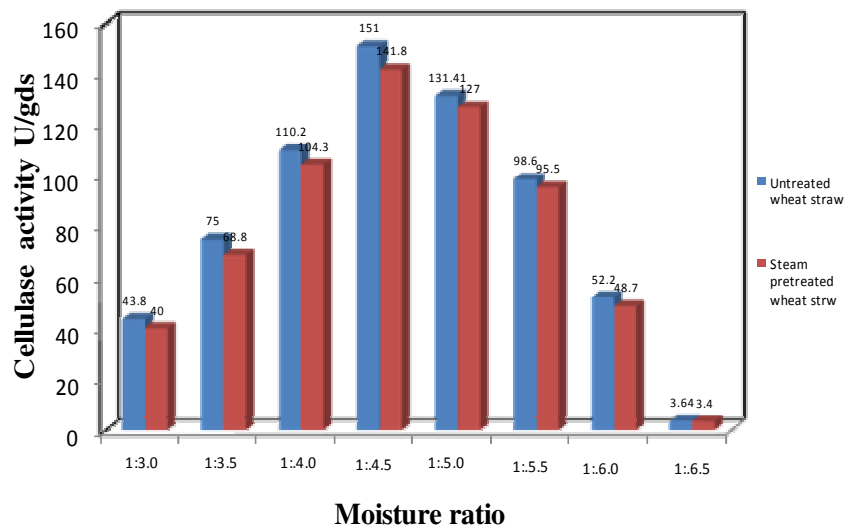


Fig 59. Effect of moisture ratio on cellulase production in steam pretreated wheat straw from *R. oryzae* W2

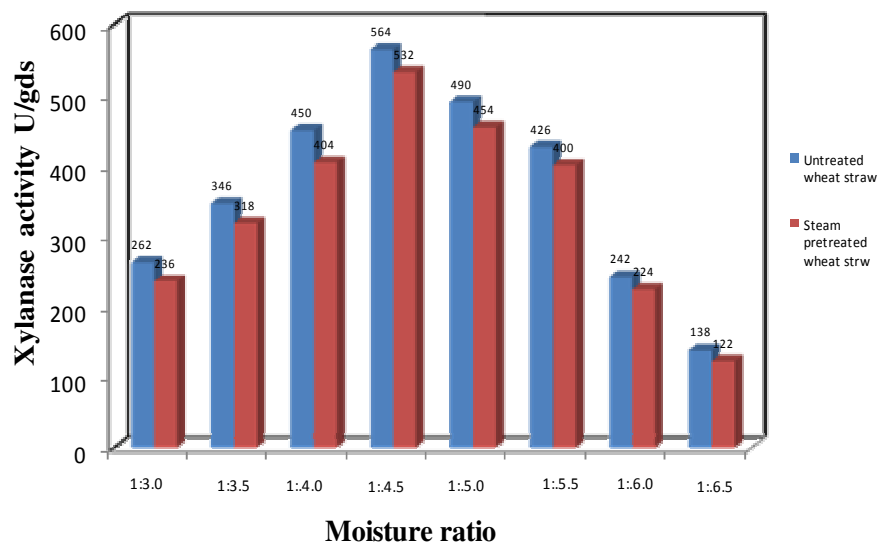


Fig 60. Effect of moisture ratio on xylanase production in steam pretreated wheat straw from *R. oryzae* W2

content in the solid substrate for Fpase and CMcase appears to be 80%. However, further increase in moisture content influenced the Fpase and CMcase enzymes negatively. On the other hand, higher moisture contents (85-87.5%) were necessarily for xylanase, β -xylosidase and β -glucosidase production.

Pal and Khanum (2010) affirmed the effects of solid substrates, initial moisture content, moistening medium, temperature and incubation time on xylanase production by *A. niger* DFR-5 was studied and the highest activity (2596 IU/g dry substrate (gds)) was achieved in medium that contained wheat bran (WB) and soybean cake (SBC) at a ratio of 70:30, was moistened to 70% with MSS-2 mineral salt solution, and incubated for 6 days at 40°C. Water at 37°C was suitable for efficient recovery of enzyme from moldy WB-SBC medium. The extraction parameters for xylanase were optimized with respect to minimum volume of extractant using a central composite rotational design (CCRD). The maximum recovery of xylanase (4465 $\pm$ 52 IU/gds) with 92.5% desirability was obtained employing water (10 ml/gds) as extractant at 200 rpm for 60 min.

Singhania *et al.* (2010) reported that maximum cellulase production obtained was 154.58 U/gds when pretreated sugarcane bagasse was used as a substrate by *T. reesei* NRRL 11460. The optimal conditions using for cellulase production using sugarcane bagasse were found to be initial moisture content 66%, initial pH 7.0, at incubation temperature 28°C and incubation time of 72h. Szendfy *et al.* (2006) affirmed that highest xylanase yield was obtained from *A. oryzae* NRRL1808 at 83% to 80% initial moisture content on eucalyptus (2168 IU/gdm) and bagasse pulp (1250 IU/gdm), respectively.

Sherief *et al.* (2010) the effect of cultural conditions including; incubation period, initial pH, incubation temperature, moisture level, different nitrogen sources, different lignocelluloses as carbon source and different ratios of mixed rice straw and wheat bran (9:1-1:9) by *A. fumigatus* were evaluated. The fungus expressed high enzyme production after 4.0 days incubation at moisture level 75%, initial pH 5-6, at 40°C in presence of NaNO₃ as an inorganic nitrogen

source. The recorded activities were 14.71, 8.51, 0.93, 0.68 and 42.7 IU/g for CMCase, β -glucosidase, exoglucanase, endoglucanase and xylanase, respectively.

Bakri *et al.* (2003) reported optimal initial moisture content for xylanase production was 83% by *P. canescens* 10-10c under solid state fermentation. Among different agricultural wastes tested i.e. wheat bran, untreated wheat straw, treated wheat straw, beet pulp and soja meal, and untreated wheat straw gave the highest xylanase production. The addition of 0.4g of xylan or easily metabolizable sugar such as glucose and xylose at a concentration of 2% to wheat straw enhanced xylanase production.

4.3.2.7 Effect of Incubation Time

The different incubation time namely, 72h (T₁), 96h (T₂), 120h (T₃), 144h (T₄), 168h (T₅), 192h (T₆), 216h (T₇) and 240h (T₈) were used for assessing the activity of cellulase and xylanase enzymes. Data pertaining to cellulase titers viz., Fpase, CMCase and β -glucosidase and xylanase are presented in Table 55 to 58.

An overview of Table 55 revealed that significant difference in cellulase production was observed when different incubation time was used for the study in *T. longibracheatum* T2. The significantly maximum cellulase titers (135.8 U/gds) were estimated in wheat straw at 168 h, which was followed by 192 h (122.4 U/gds) and 216h (116.2 U/gds). The significantly minimum cellulase titers (22.90 U/gds) were observed 72h of incubation time. Similarly, significantly maximum cellulase production was observed in alkali treated substrate (103.76 U/gds) than untreated substrate (68.69 U/gds). Interaction study (TxB) revealed that significantly maximum cellulase titers (173.20 U/gds) were estimated at incubation time of 168 h, when pretreated substrate of wheat straw was used as shown in Fig 61. The significantly maximum cellulase titers were observed in untreated wheat straw at incubation time 72h (22.80 U/gds).

The scrutiny of data in Table 56 revealed that significantly highest xylanase titers (582.90 U/gds) was recorded in alkali treated wheat straw at incubation time of 168h (T₅), which was followed by 192h (T₆) (523.50 U/gds)

Table 55. Cellulase production at different incubation time from *Trichoderma longibracheatum* T2 [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

Day	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
3 rd	1.60 (0.08) (5.16)	2.40 (0.12) (5.85)	2.00 (0.10) (5.50)	7.40 (0.37) (23.87)	8.60 (0.43) (20.98)	8.00 (0.40) (22.43)	13.80 (0.69) (44.52)	12.00 (0.60) (29.27)	12.90 (0.64) (36.90)	22.80 (1.14) (73.55)	23.00 (1.15) (56.10)	22.90 (1.14) (64.83)	0.31	0.41	0.36	0.88
4 th	3.60 (0.18) (6.43)	4.80 (0.24) (6.96)	4.20 (0.21) (6.69)	12.90 (0.65) (23.04)	17.20 (0.86) (24.93)	15.05 (0.75) (23.99)	24.00 (1.20) (42.86)	32.00 (1.60) (46.38)	28.00 (1.40) (44.62)	40.50 (2.03) (72.32)	54.00 (2.70) (78.26)	47.25 (2.36) (75.29)	0.56	0.69	0.63	33.33
5 th	6.40 (0.32) (6.88)	8.20 (0.41) (7.39)	7.30 (0.36) (7.135)	17.80 (0.89) (19.14)	28.00 (1.40) (25.23)	22.90 (1.14) (22.19)	36.00 (1.80) (38.71)	54.00 (2.70) (48.65)	45.00 (2.25) (43.68)	60.20 (3.01) (64.73)	90.20 (4.51) (81.26)	75.20 (3.76) (73.00)	0.93	1.11	1.02	49.83
6 th	8.30 (0.42) (7.55)	12.60 (0.63) (9.62)	10.45 (0.52) (8.58)	22.00 (1.10) (20.00)	46.00 (2.30) (35.11)	34.00 (1.70) (27.56)	52.00 (2.60) (47.27)	72.00 (3.60) (54.96)	62.00 (3.10) (51.12)	82.30 (4.12) (74.82)	112.60 (5.63) (85.95)	97.45 (4.87) (80.39)	1.10	1.31	1.21	36.82
7 th	9.40 (0.47) (7.01)	16.20 (0.81) (11.33)	12.80 (0.64) (9.17)	32.00 (1.60) (23.88)	70.00 (3.50) (48.95)	51.00 (2.55) (36.42)	57.00 (2.85) (42.54)	87.00 (4.35) (60.84)	72.00 (3.60) (51.69)	98.40 (4.92) (73.43)	173.20 (8.66) (121.12)	135.80 (6.79) (97.28)	1.34	1.43	1.39	76.02
8 th	9.10 (0.46) (6.85)	14.70 (0.74) (10.43)	11.90 (0.60) (8.64)	30.00 (1.50) (23.62)	58.00 (2.90) (41.13)	44.00 (2.20) (32.18)	54.00 (2.70) (40.94)	79.00 (3.95) (56.03)	66.50 (3.32) (48.49)	93.10 (4.66) (71.42)	151.70 (7.59) (107.59)	122.40 (6.12) (89.51)	1.27	1.41	1.34	67.25
9 th	8.20 (0.41) (6.78)	14.20 (0.71) (9.93)	11.20 (0.56) (8.36)	29.00 (1.45) (23.14)	55.00 (2.75) (38.85)	42.00 (2.10) (31.00)	53.00 (2.65) (41.32)	73.00 (3.65) (51.80)	63.00 (3.15) (46.56)	90.20 (4.51) (71.24)	142.20 (7.11) (85.47)	116.20 (5.81) (78.36)	1.21	1.39	1.30	37.82
10 th	8.00 (0.40) (6.61)	13.90 (0.70) (10.00)	10.45 (0.57) (8.30)	28.0 (1.40) (23.14)	54.0 (2.70) (38.84)	41.0 (2.00) (30.99)	52.00 (2.60) (42.97)	72.0 (3.60) (51.79)	62.00 (3.10) (47.38)	89.00 (4.45) (72.72)	139.90 (7.00) (100.64)	113.45 (5.72) (87.10)	1.21	1.39	1.30	57.19
Mean	6.575 (0.34) (6.67)	10.4125 (0.54) (8.86)		21.76 (1.12) (22.74)	40.48 (2.01) (34.04)		40.35 (2.13) (42.27)	58.88 (3.13) (50.46)		68.69 (3.60) (71.66)	103.76 (5.54) (88.76)		0.96	1.11		
CD _{T 0.05}	0.33 (0.091) (0.41)			0.96 (0.32) (0.74)			0.72 (0.89) (42.69)			0.80 (1.06) (0.54)			0.034			
CD _{B 0.05}	0.67 (0.045) (0.82)			1.93 (0.161) (1.41)			1.44 (0.44) (85.39)			1.61 (0.53) (1.09)			0.069			
CD _{TxB0.05}	0.92 (0.128) (1.14)			2.68 (0.58) (2.06)			1.40 (1.26) (118.58)			2.24 (1.18) (1.50)			0.096			

* U/gds = μ moles of reducing sugars released / min /g of biomass

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

% increase = Untreated-Treated/Treated X 100

Table 56. Xylanase production at different incubation time from *Trichoderma longibracheatum* [JX 213811.1] using alkali pretreated lignocellulosic waste wheat straw as substrate under SSF

Day	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
3 rd	96.00 (4.8) (309.68)	124.60 (6.23) (303.90)	110.30 (5.51) (306.79)	0.31	0.41	0.36	29.79
4 th	226.80 (11.34) (405.00)	277.20 (13.83) (401.74)	252.00 (12.60) (403.37)	0.56	0.69	0.63	22.22
5 th	311.20 (15.56) (334.62)	414.40 (20.72) (373.33)	362.80 (18.14) (353.98)	0.93	1.11	1.02	33.16
6 th	403.40 (20.17) (366.73)	555.20 (27.76) (423.82)	479.30 (23.96) (395.28)	1.10	1.31	1.21	37.63
7 th	496.80 (24.84) (370.75)	669.00 (33.45) (467.83)	582.90 (29.14) (419.29)	1.34	1.43	1.39	34.66
8 th	442.40 (22.12) (348.35)	604.60 (30.23) (428.79)	523.50 (26.17) (388.57)	1.27	1.41	1.34	36.66
9 th	405.40 (20.17) (335.04)	577.20 (28.86) (415.25)	491.30 (24.56) (375.15)	1.21	1.39	1.30	42.38
10 th	322.20 (16.11) (339.16)	424.80 (21.24) (375.93)	373.50 (18.67) (357.55)	0.95	1.13	1.04	31.84
Mean	338.03 (16.90) (351.17)	455.88 (22.79) (398.82)		0.96	1.11		
CD _{T 0.05}	1.15 (1.71) (1.10)			0.034			
CD _{B 0.05}	2.30 (0.85) (2.20)			0.069			
CD _{TxB0.05}	2.18 (2.40) (3.04)			0.096			

* U/gds = μ moles of reducing sugars released / min /g of biomass

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

% increase = $\frac{\text{Untreated-Treated}}{\text{Treated}} \times 100$

and 216 h (491.30 U/gds). The significantly lowest xylanase titers were observed in 72 h (110.30 U/gds). Similarly, significantly highest xylanase production was observed in alkali treated substrate (455.88 U/gds) than untreated substrate (338.0 U/gds) as depicted in Fig 62. Interaction study (TxB) revealed that significantly highest xylanase titers (669.00 U/gds) was estimated at 168h of incubation time, when alkali pretreated substrate of wheat straw was used as a substrate. The significantly lowest xylanase titers were observed in untreated substrate at incubation time of 72h (96.0 U/gds).

An appraisal of Table 57 revealed that the significant difference in cellulase production was observed when different incubation time was used for study in *R. oryzae* W2. The significantly highest cellulase titers (176.80 U/gds) were estimated at 120h (T₃), which was followed by 144h (158.1 U/gds), and at 168h (149.35 U/gds). The significantly lowest cellulase titers (34.15 U/gds) were observed at incubation time of 240h. Similarly, significantly maximum cellulase production was observed in untreated wheat straw (109.56 U/gds) than steam pretreated wheat straw (107.26 U/gds) as. Interaction study (TxB) revealed that significantly maximum cellulase titers (179.4 U/gds) were estimated at 120h (T₃), when an untreated agricultural waste of wheat straw was used as a substrate as shown in Fig 63. The significantly minimum cellulase titers were observed in steam pretreated wheat straw at 240h of incubation time (32.30 U/gds).

The scrutiny of data in Table 58 revealed that significantly highest xylanase titers (705.55 U/gds) were observed at 120h (T₃) of incubation time, which was followed by 144h (621.35 U/gds), and 168h (T₅) (561.00 U/gds). The significantly lowest xylanase titers were observed at 72h of incubation time (182.95 U/gds). Similarly, the significantly highest xylanase production was observed in untreated substrate (412.98 U/gds) than steam pretreated substrate (407.84 U/gds). Interaction study (TxB) revealed that significantly highest xylanase titers (709.0 U/gds) were estimated at 120h (T₃), when untreated substrate of wheat straw was used as a substrate. The significantly lowest xylanase titers were observed in steam treated wheat straw at 72 h (T₁) of incubation temperature (180.90 U/gds) as shown in Fig 64.

Table 57. Cellulase production at different incubation time from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw as substrate under SSF

Day	Cellulase															
	Fpase		Mean	CMCase		Mean	β-glucosidase		Mean	Total cellulase		Mean	Protein (mg/ml)		Mean	% increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
3 rd	2.60* (0.13)** (4.12)***	2.20 (0.11) (3.73)	2.40 (0.12) (3.93)	44.00 (2.20) (10.67)	42.10 (2.11) (71.36)	43.05 (2.15) (41.02)	43.50 (2.17) (69.04)	39.00 (1.95) (66.10)	41.25 (2.06) (67.57)	90.10 (4.50) (143.00)	83.30 (4.17) (141.19)	86.70 (4.33) (142.09)	0.63	0.59	0.61	7.55
4 th	6.20 (0.31) (4.88)	5.90 (0.30) (4.92)	6.05 (0.30) (4.90)	46.00 (2.30) (36.22)	44.60 (2.23) (37.17)	45.30 (2.26) (36.70)	50.00 (2.50) (39.37)	47.80 (2.39) (38.81)	48.90 (2.44) (39.09)	102.20 (5.11) (80.47)	98.30 (4.92) (81.92)	100.25 (5.01) (81.20)	1.27	1.20	1.24	3.82
5 th	11.40 (0.57) (7.0)	10.90 (0.55) (7.08)	11.15 (0.56) (7.04)	80.00 (4.00) (49.68)	77.60 (3.88) (50.39)	78.80 (3.94) (50.04)	88.00 (4.40) (54.6)	85.70 (4.29) (55.65)	86.85 (4.34) (55.13)	179.40 (8.97) (111.4)	174.20 (8.71) (113.12)	176.80 (8.84) (112.26)	1.61	1.54	1.58	2.90
6 th	9.50 (0.48) (6.25)	9.10 (0.46) (6.36)	9.30 (0.47) (6.31)	74.00 (3.70) (48.68)	70.50 (3.53) (49.30)	72.25 (3.61) (48.99)	77.60 (3.88) (51.0)	75.10 (3.76) (52.52)	76.35 (3.82) (51.76)	161.5 (8.08) (106.25)	154.70 (7.74) (108.18)	158.10 (7.91) (107.22)	1.52	1.43	1.48	4.21
7 th	7.60 (0.38) (5.30)	7.30 (0.37) (5.41)	7.45 (0.37) (5.36)	69.00 (3.45) (48.25)	67.50 (3.38) (50.00)	68.25 (3.41) (49.13)	74.40 (3.72) (52.0)	72.90 (3.65) (54.00)	73.65 (3.68) (53.00)	151.00 (7.55) (105.59)	147.70 (7.39) (109.41)	149.35 (7.47) (107.50)	1.43	1.35	1.39	2.19
8 th	4.60 (0.23) (3.60)	4.20 (0.21) (3.82)	4.40 (0.22) (3.71)	56.00 (2.80) (44.44)	53.90 (2.70) (49.00)	54.95 (2.75) (46.72)	46.00 (2.30) (36.05)	44.80 (2.24) (40.73)	45.40 (2.27) (38.39)	106.6 (5.33) (84.6)	102.90 (5.15) (93.55)	104.75 (5.24) (89.08)	1.26	1.10	1.18	3.47
9 th	3.20 (0.16) (3.90)	2.80 (0.14) (3.68)	3.00 (0.15) (3.79)	36.00 (1.80) (44.44)	33.80 (1.69) (44.47)	34.90 (1.74) (44.46)	30.00 (1.50) (37.0)	28.10 (1.41) (36.97)	29.05 (1.45) (36.99)	69.20 (3.46) (85.43)	64.70 (3.24) (85.13)	66.95 (3.35) (85.28)	0.81	0.76	0.79	6.50
10 th	2.00 (0.10) (3.27)	1.60 (0.08) (2.76)	1.80 (0.09) (3.02)	16.00 (0.80) (26.22)	14.40 (0.72) (24.83)	15.20 (0.76) (25.23)	18.00 (0.90) (29.50)	16.30 (0.82) (28.10)	17.15 (0.86) (28.80)	36.00 (1.80) (59.0)	32.30 (1.62) (55.69)	34.15 (1.71) (57.35)	0.61	0.58	0.60	10.28
Mean	5.89 (0.29) (4.79)	5.50 (0.27) (4.72)		52.63 (2.63) (38.58)	50.55 (2.53) (47.06)		63.25 (2.67) (46.07)	51.21 (2.56) (46.61)		112.00 (5.60) (96.96)	107.26 (5.36) (98.52)					
CD _{T 0.05}	0.40 (0.046) (0.33)			0.70 (0.53) (1.57)			2.14 (0.53) (0.96)			2.47 (0.53) (0.60)			0.20			
CD _{B 0.05}	0.81 (0.090) (0.67)			1.41 (1.07) (3.14)			4.28 (1.06) (1.93)			4.94 (1.06) (1.21)			0.41			
CD _{TxB0.05}	1.12 (0.13) (0.94)			1.96 (1.56) (4.36)			5.94 (1.52) (2.70)			6.86 (1.52) (1.68)			0.60			

* U/gds = μ moles of reducing sugars released / min /g of biomass

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

% increase = Untreated-Treated/Treated X 100

Table 58. Xylanase production at different incubation time from *Rhizopus oryzae* W2 [JX 213812.1] using steam pretreated lignocellulosic waste wheat straw waste as substrate under SSF

Day	Xylanase		Mean	Protein (mg/ml)		Mean	% Increase
	Untreated biomass (B ₁)	Treated biomass (B ₂)		Untreated biomass (B ₁)	Treated biomass (B ₂)		
3 rd	185.00* (9.25)** (293.60)***	180.90 (9.05) (306.61)	182.95 (9.15) (300.11)	0.63	0.59	0.61	2.22
4 th	364.60 (18.23) (287.0)	360.70 (18.04) (300.58)	362.65 (18.13) (293.79)	1.27	1.20	1.24	1.07
5 th	709.00 (35.45) (440.37)	702.10 (35.11) (455.91)	705.55 (25.28) (448.14)	1.61	1.54	1.58	0.97
6 th	623.4 (31.17) (410.13)	619.30 (30.97) (433.08)	621.35 (31.07) (421.61)	1.52	1.43	1.48	0.66
7 th	564.00 (28.20) (394.4)	558.20 (27.91) (415.70)	561.10 (28.05) (405.05)	1.43	1.35	1.39	1.03
8 th	427.6 (21.28) (339.36)	420.50 (21.03) (385.91)	424.05 (21.20) (362.64)	1.26	1.10	1.18	1.66
9 th	282.6 (14.13) (348.88)	276.40 (13.82) (366.32)	279.50 (13.97) (357.60)	0.81	0.76	0.79	2.19
10 th	147.60 (7.38) (241.96)	144.60 (7.23) (249.31)	146.10 (7.30) (245.61)	0.61	0.58	0.60	2.03
Mean	412.98 (20.64) (412.98)	407.84 (20.39) (364.18)		1.1425	1.07		
CD _{T 0.05}	2.00 (1.03) (0.84)			0.20			
CD _{B 0.05}	4.00 (2.07) (1.69)			0.41			
CD _{TxB0.05}	5.56 (2.90) (2.34)			0.60			

* U/gds = μ moles of reducing sugars released / min /g of biomass

*** Value in parentheses depict specific activity i.e. enzyme activity/g of protein

** IU/ml = μ moles of reducing sugars released/min/ml of enzyme

% increase = $\frac{\text{Untreated-Treated}}{\text{Treated}} \times 100$

Incubation time is one of the most important factors among all the physical variables affecting the SSF performance. Varying incubation time from 3 to 10 days for the production of cellulase and xylanase enzyme was investigated showing maximum cellulase and xylanase production at 5th and 7th day by *R. oryzae* W2 and *T. longibrachetum* T2, respectively. The decrease in enzyme activity upon prolonged incubation may be due to irreversible adsorption of enzyme to substrate or due to feed back inhibition/denaturation of enzymes, resulted from the variation of pH and lesser cellular metabolism with ageing during fermentation or this could be due to loss of moisture (Xin and Geng , 2010; Liu *et al.*, 2007). Singh *et al.* (2009) reported that the decrease of cellulase enzyme activity may be due to the accumulation of cellobiose.

Omojasola *et al.* (2008) reported maximum cellulase production from pineapple waste as a substrate from *T. longibrachetum*, *A. niger* and *S. cerevisiae*, *T. longibrachetum* produced the highest amount of glucose among the cultures tested (0.92mg/0.5ml) from pineapple pulp at pH 4.5 and temperature of 45°C on day 7 of fermentation. The highest amount of glucose produced by *A. niger* was also from pineapple pulp (0.63mg/0.5ml) at pH 3.5 and temperature of 40°C on day 5 of fermentation. The highest amount of glucose produced by *S. cerevisiae* was from pineapple pulp (0.54mg/0.5ml) at pH 4.5 and temperature of 45°C on day 5 of fermentation.

Bhatt *et al.* (2012) reported maximum xylanase (1530 IU/gds) production with substrate concentration (4/g), incubation period (5th day), moisture level (45%), pH 6 and temperature at 25°C by *Aspergillus flavus* FPDN1. Danial and Ambikapathy (2011) observed that Different cultural conditions were examined such as pH, temperature and incubation period to assess their effect in optimizing enzyme production. Alkaline pretreated (2N NaOH) leaf litters of *Artocarpus heterophylla*, *Mangifera indica* and *Tectona grandis* showed 1.371, 1.832 and 1.183 IU/mL cellulase activity, respectively. Optimum pH for cellulase production was 5, temperature 30°C and the incubation period 5th day.

Irshad *et al.* (2011) observed the production of xylanases by *H. lanuigosa* in solid state fermentation was carried out on different carbon and nitrogen

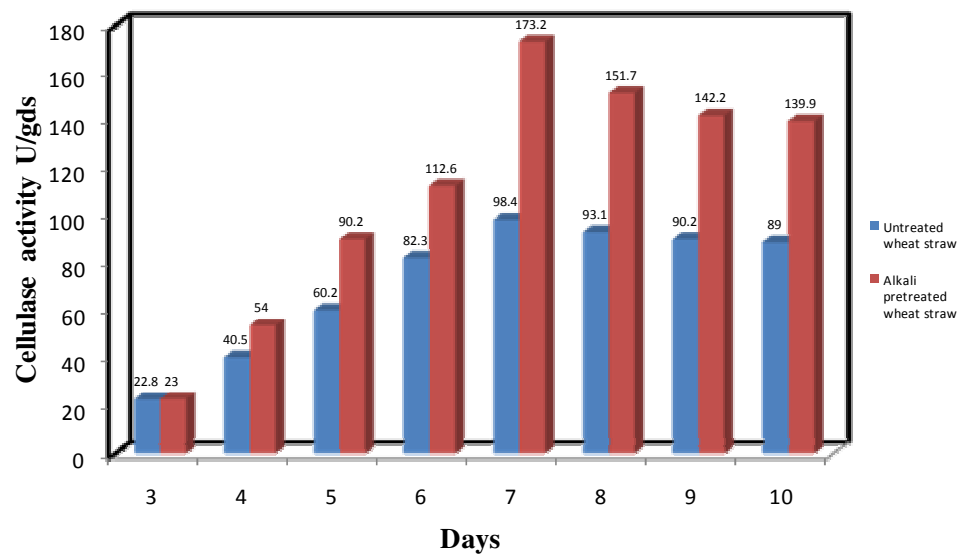


Fig 61. Effect of Incubation time on cellulase production in alkali pretreated wheat straw from *T. longibracheatum* T2

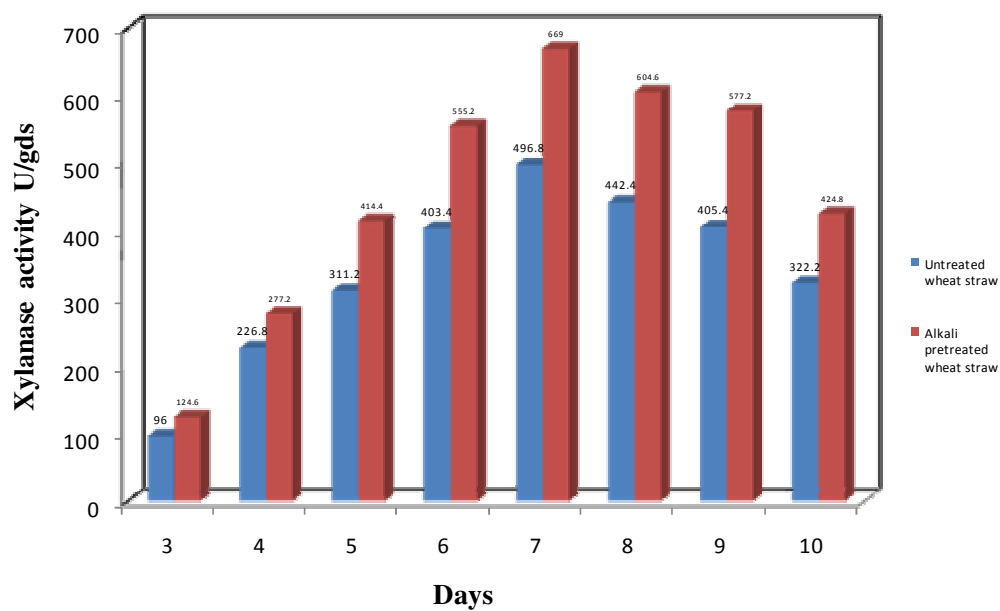


Fig 62. Effect of incubation on xylanase production in alkali pretreated wheat straw from *T. longibracheatum* T2

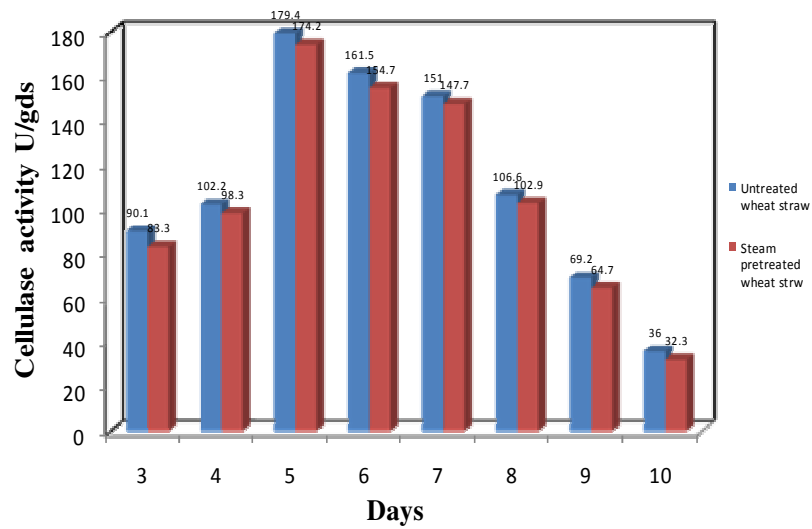


Fig 63. Effect of incubation time on cellulase production in steam pretreated wheat straw from *R. oryzae* W2

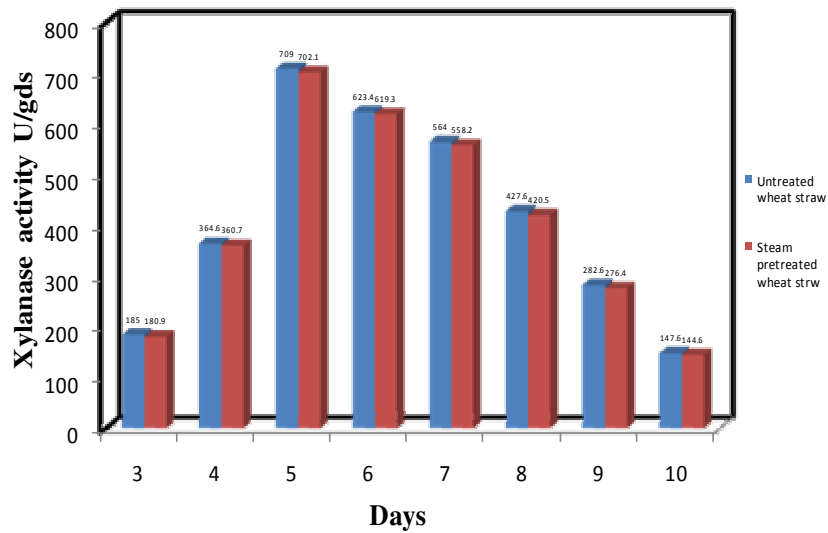


Fig 64. Effect of incubation time on xylanase production in steam pretreated wheat straw from *R. oryzae* W2

sources. Optimum temperature for xylanase and β -xylosidase production was 45°C while maximum productivity was on pH 5.5 to 6 of the fermentation medium by *H. lanuigosa* in solid culture screening from 15 different carbon sources (inducers). Xylanase and β -xylosidase accumulated extra cellular. Vogel's salt medium was found optimum to provide ionic strength. The productivities (IU/F/h) of xylanases from *H. lanuigosa* at pH 5.5 and 45°C in solid state fermentation using xylan was 35.5 IU/F/h and wheat bran 32.9 IU/F/h after 120 h. Similarly β -xylosidase showed maximum productivity 5.49 IU/F/h from xylan in solid state fermentation and 2.97 IU/F/h on wheat bran.

Park *et al.* (2002) reported that an inoculum size of 5×10^5 spores/g, initial moisture content of 65%, cultivation time of 5 days and 10 times concentration of basal medium containing 50 times concentration of corn steep liquor were optimum for xylanase production in SSF. Under the optimized conditions, the activity and productivity of xylanase obtained after 5 days of fermentation were 5,071 IU/g of rice straw and 14790 IU/L/h, respectively. The xylanase activity predicted by a polynomial model was 5,484 IU/g of rice straw. Highest xylanase activity (13230 U/gds) was obtained with the initial moisture contents of 83% by an extracellular xylanase producing thermophilic fungus *P. thermophila* J18 at 50°C on the lignocellulosic material when wheat straw was used as substrate.

Microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Enzyme production is closely controlled in microorganisms and therefore, to improve its productivity, these controls can be exploited and modified. Cellulase and xylanase yields appear to depend on a complex relationship involving a variety of factors like inoculum size (carbon source and cellulase quality), pH value, and temperature, presence of inducers, medium additives, aeration, and growth time. To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favourable for the microorganism for over-production of the desired metabolite. An elaborated investigation is therefore, required to establish the optimum conditions to scale up enzyme production in an individual fermentation process. The optimization of these

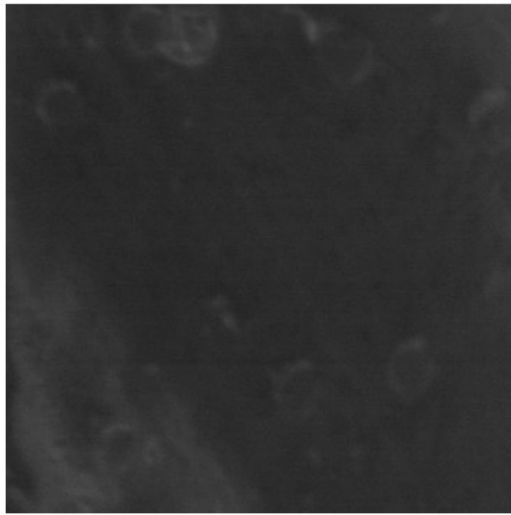
fermentation factors particularly physical and chemical parameters are of primary importance in the development of any fermentation process owing their impact on the yield practicability and economy of the process (Wenster-Botz, 2000). The optimal conditions derived for *T. longibracheatum* T2 and *R. oryzae* W2 for cellulase and xylanase production reveal their acidophilic and mesophilic nature. The significant increase after optimization extracellular cellulase and xylanase activity from *T. longibracheatum* T2 and *R. oryzae* W2 are the main highlight of solid state fermentation. The gradual increase in cellulase titers of *T. longibracheatum* ranging from 93.20 U/gds to 173.20 U/gds (63.65%) (Fig 65) and xylanase titers from 356.60 U/gds to 669.0 U/gds (95.96%) (Fig 66); is an outcome of optimizing the various bioprocess parameters i.e. pretreatment, substrate, pH, temperature, moisture ratio and incubation time. Similarly, the stepwise increase in cellulase titers of *R. oryzae* W2 ranging from 106.40 to 179.40 U/gds (107.38%) (Fig 67) and xylanase titers ranging from 362.50 to 709.0 U/gds (125.64%) (Fig 68) has been noticed.

4.3.2.8 Scanning Electron Microscopy of Pretreated and Untreated Wheat Straw

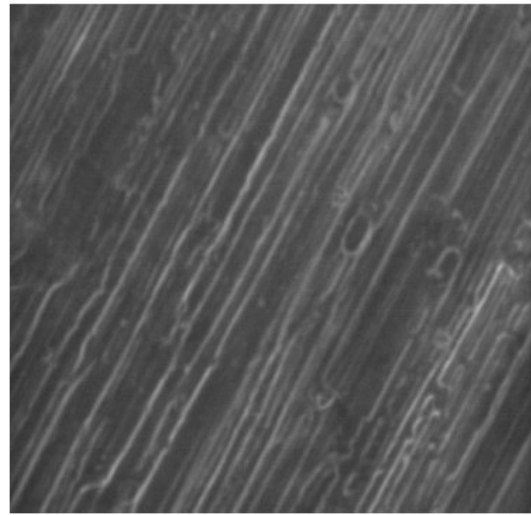
Most native celluloses are mixtures of cellulose fibres. Each natural fiber is essentially a composite in which rigid cellulose microfibrils are embedded in soft matrix composed of lignin and hemicelluloses. Hemicelluloses and to some extent pectin are primary components of the binding substances elementary bast fibres, while lignin plays the part of stabilizer. The degree of polymerization in hemicelluloses is lower than cellulose. Hydrogen bonds are formed between xylan and cellulose. Due to this linking effect of hemicelluloses, hemicelluloses degradation leads to disintegration of fibres into cellulose microfibrils leading into lower fibre bundle strength (Lian *et al.*, 2011)

4.3.2.8.1 Scanning Electron Microscopy of Wheat straw

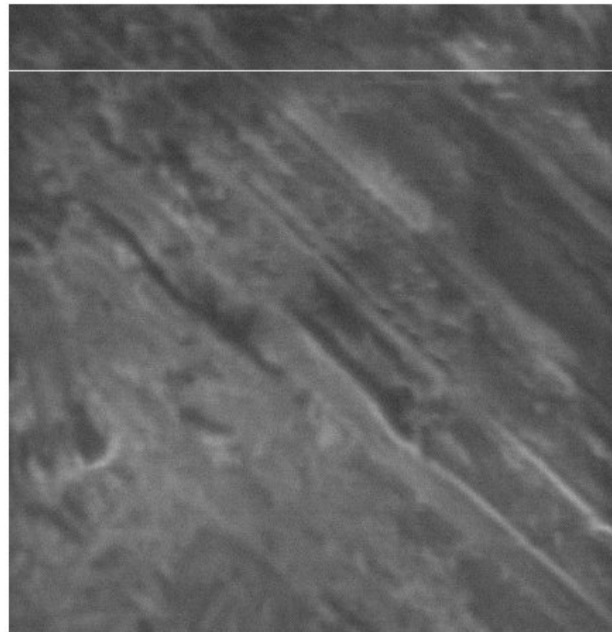
Plate 18 a, b and c revealed the SEM images of wheat straw chip in native, alkali pretreated and alkali pretreated + *T. longibracheatum* T2 (7 days growth). Native wheat straw chip showed compact structure (Plate 18a), alkali pretreated wheat straw showed more tiny holes and cracks probably due to



a) Native wheat straw

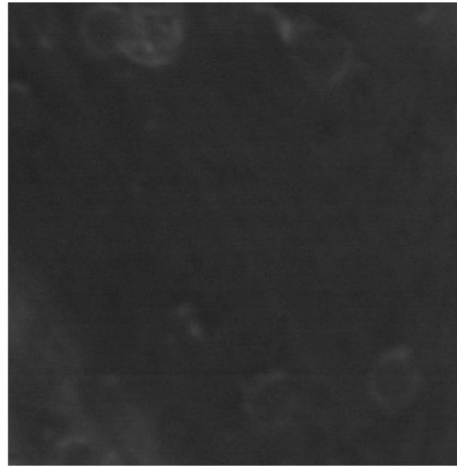


b) Alkali pretreated wheat straw (7 days)

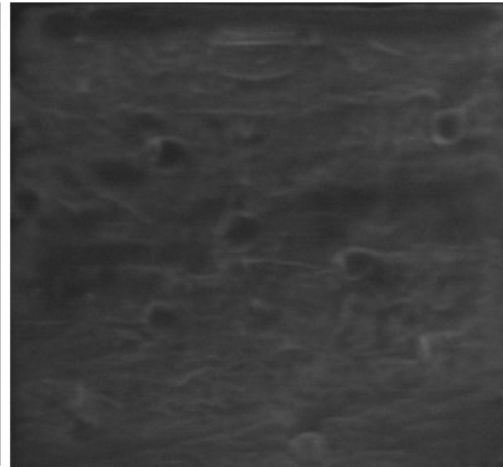


c) Alkali and *T. longibracheatum* treated wheat straw (7 days)

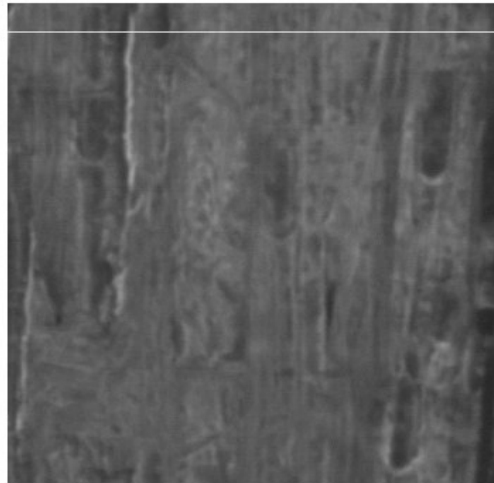
Plate 18. Scanning electron micrograph (SEM) of *T. longibracheatum* T2 of wheat straw



a) Native wheat straw



b) Untreated and *R. oryzae* treated wheat straw (5 days)



c) Steam and *R. oryzae* treated wheat straw (5 days)

Plate 19. Scanning electron micrograph (SEM) of *R. oryzae* W2 of wheat straw

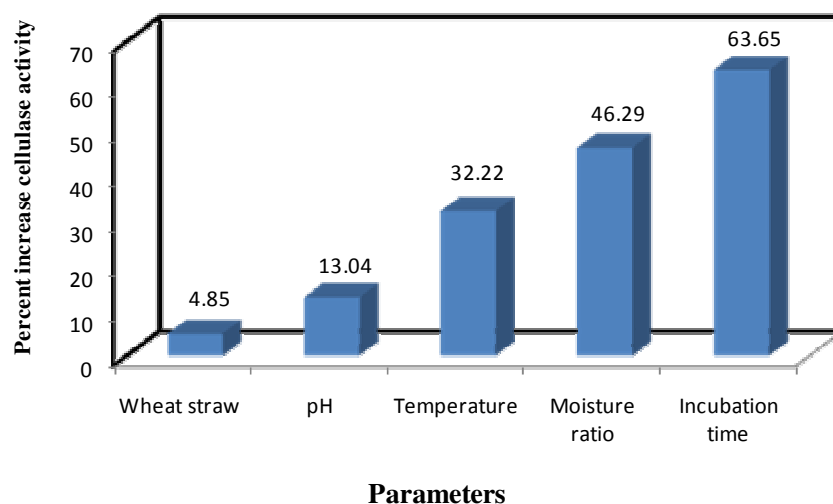


Fig 65. An overview of percent increase in cellulase activity from *T. longibracheatum* T2 in alkali pretreated wheat straw after optimization of different parameters under SSF

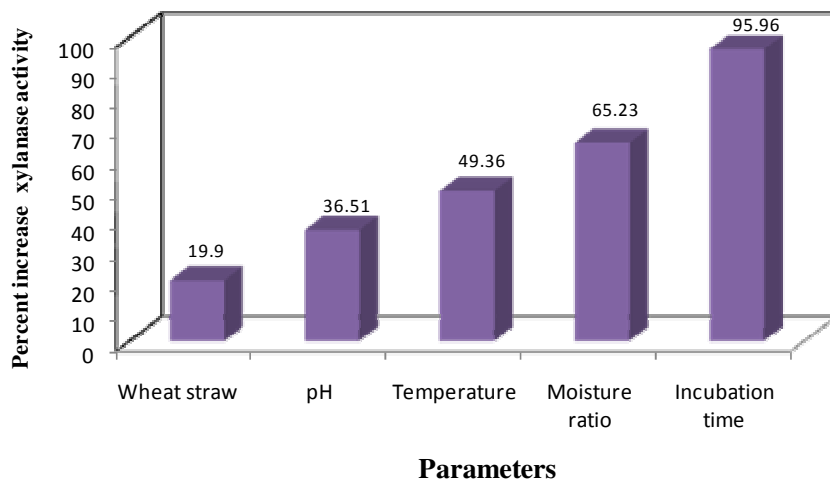


Fig 66. An overview of percent increase in xylanase activity from *T. longibracheatum* T2 in alkali pretreated wheat straw after optimization of different parameters under SSF

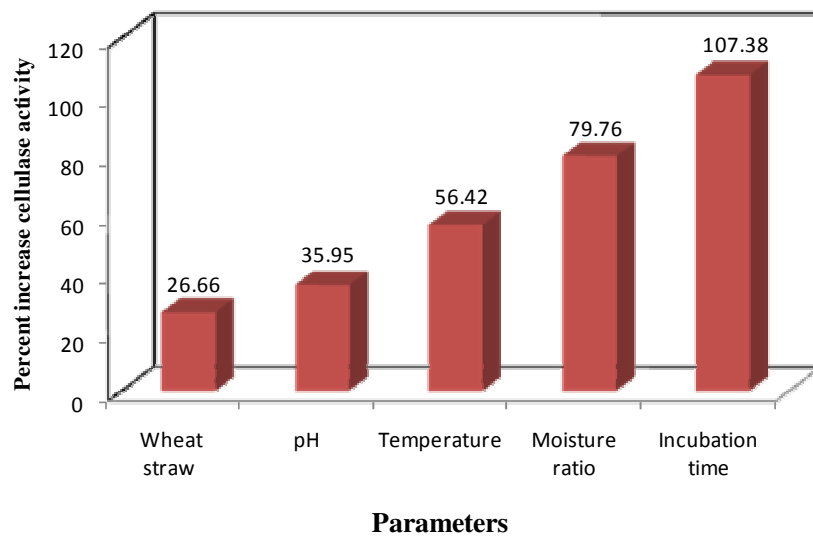


Fig 67. An overview of percent increase in cellulase activity *R. oryzae* W2 in untreated wheat straw after optimization of different parameters under SSF

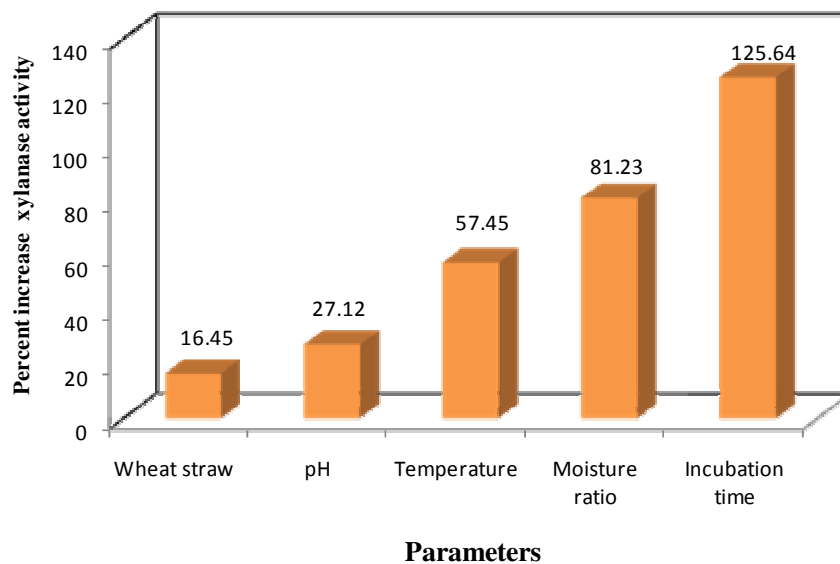


Fig 68. An overview of percent increase in cellulase activity from *R. oryzae* W2 in untreated wheat straw after optimization of different parameters under SSF

removal of lignin and some fraction of hemicellulose (Plate 18b) whereas fungus treated wheat straw had rough fibrous surface, tiny holes and cracks presumably owing to assimilation of cellulose/xylan from biomass.

Plate 19 a, b and c revealed the SEM images of wheat straw chip in native, untreated + *R. oryzae* W2 pretreated and steam pretreated + *R. oryzae* W2 (5 days growth). Native wheat straw chip showed compact structure (Plate 19a) whereas fungus treated wheat straw had rough fibrous surface, tiny holes and cracks presumably resulting from dissolution of lignin and consequently assimilating cellulose/xylan from biomass.

4.4 PURIFICATION AND CHARACTERIZATION OF XYLANASE FROM HYPER XYLANOLYTIC MICROORGANISMS

4.4.1 Purification and Characterization of Xylanase Enzyme from Bacterial Isolate

Enzyme xylanase was partially purified by two step strategy including lyophilization, ion exchange and gel exclusion column Chromatography.

4.4.1.1 *Bacillus amyloliquefaciens* SH8

4.4.1.1.1 Lyophilization: The xylanase containing cell free supernatant was concentrated for 6h in lyophilizer (Allied Frost, make). In the present study the direct method of concentration by lyophilization was opted because of high sensitivity of xylanase enzyme towards ammonium sulphate. Xylanase lost its complete activity during salt saturation method even at initial concentration of $\text{NH}_4(\text{SO}_4)_2$. Though, ammonium sulphate is a recommended method to stabilize protein but at times it may prove toxic to some enzymes probably depending upon the nature of the of protein and also due to the possible traces of metals which might be detrimental to the protein of interest (Scopes, 1982; Bollag and Edelstein, 1991).

4.4.1.1.2 Ion Exchange Chromatography

Lyophilized crude enzyme was subjected to ion exchange chromatography column DEAE cellulose. The column was packed with DEAE cellulose and the concentrated crude enzyme (2ml) was loaded on pre-

equilibrated gel filtration column with the help of Pasteur pipette. Phosphate buffer pH (6.9) was used as elution buffer. A total of 60 fractions with flow rate 6 min/3ml each were collected in chromatographic vials under cool environment. The optical density of each collected fraction was measured at 280 nm. The fractions of 0.1 M which showed highest enzyme activity by calorimetric method i.e. 5-14 (8.0, 11.0, 16.77, 24.11, 33.11, 41.0, 40.1, 30.0, 24.1, 14.21 IU/ml) were pooled as depicted in Fig 69 and concentrated further by lyophilization. The xylanase activity was checked in these active fractions. The enzyme activity of pooled fractions was checked by quantitative calorimetric estimation assays.

4.4.1.1.3 Gel Exclusion Column Chromatography

The void volume of sephadex G-100 column was calculated by loading blue dextran solution (2 mg/ml) on to the column. Fractions of 2 ml were collected and optical density (OD) was measured at 540 nm. The void volume was found to be 66 ml. The pooled post-DEAE-cellulose xylanase fraction was then further enriched by preparative sephadex G-100 gel filtration column Chromatography. The column was packed with sephadex G-100 and the concentrated crude enzyme (2 ml) was loaded on pre-equilibrated gel filtration column with the help of Pasteur pipette. 0.1 M Phosphate buffer pH (6.9) was used as elution buffer. A total of 60 fractions with flow rate 6min/3ml each were collected in chromatographic vials at room temperature (10 to 15°C). The optical density of each collected fraction was measured at 280 nm. The maximum enzyme activity was observed in fraction no 8 to 15 i.e. 18.0, 30.0 53.0, 5.1, 65.4, 48.0, 37.1, 28.0 IU) (Fig 70). The xylanase activity was checked in these active fractions. The fractions having positive xylanase activity were pooled together and were lyophilized. The enzyme activity of pooled fractions was checked by quantitative calorimetric estimation assays. The lyophilized enzyme preparation was stored at (-20°C) for further use.

4.4.1.1.4 SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) *Bacillus amyloliquefaciens* SH8

The purity and molecular weight of pooled fractions were determined by SDS-PAGE. 10 µl of pooled sample +5µl of loading dye were loaded in the wells of 12% of gel. The electrophoresis was run at 150V for 2h. After the gel was run,

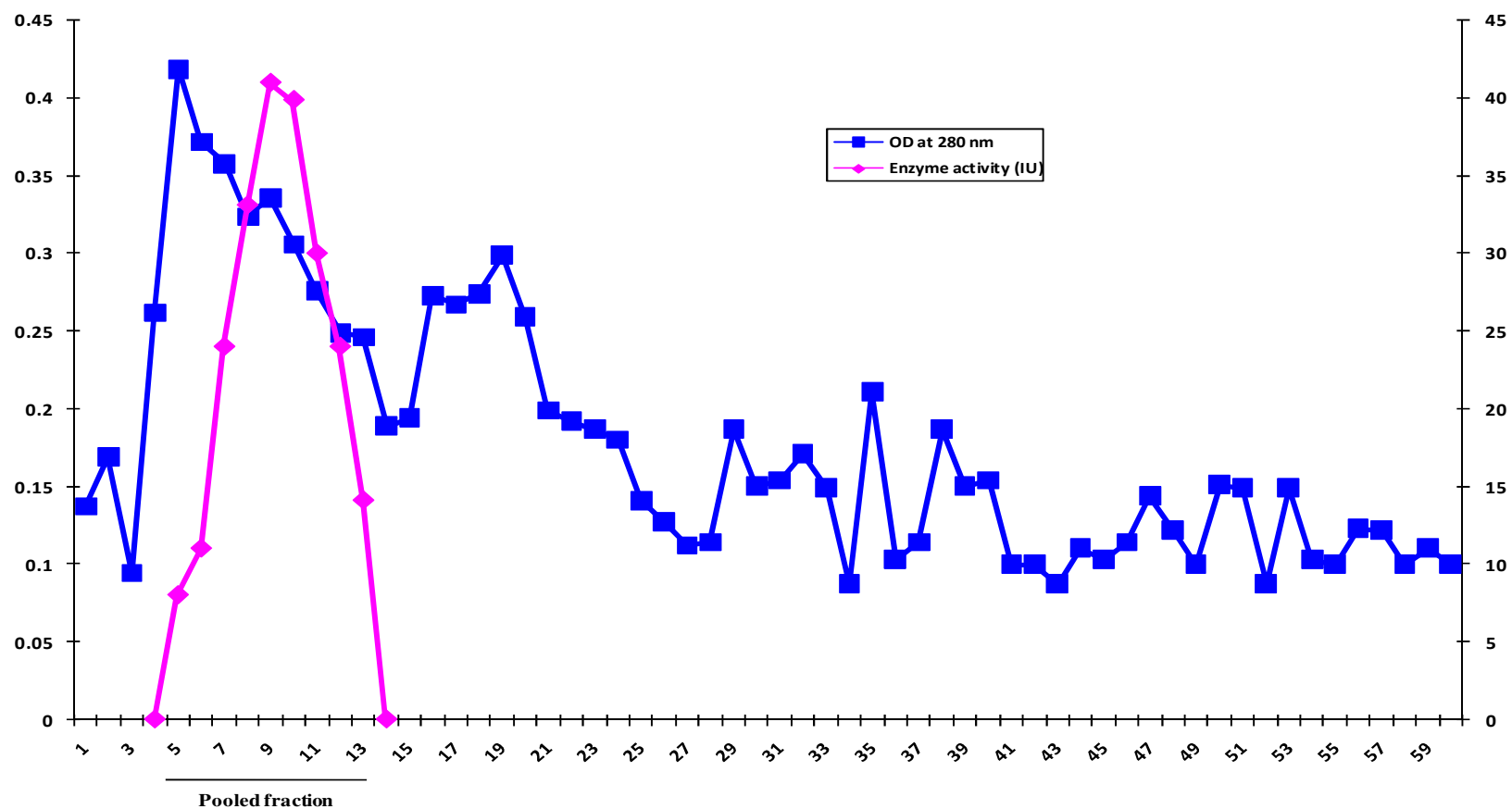


Fig 69. Ion exchange chromatography of extracellular xylanase of *Bacillus amyloliquefaciens* SH8 on DEAE cellulose

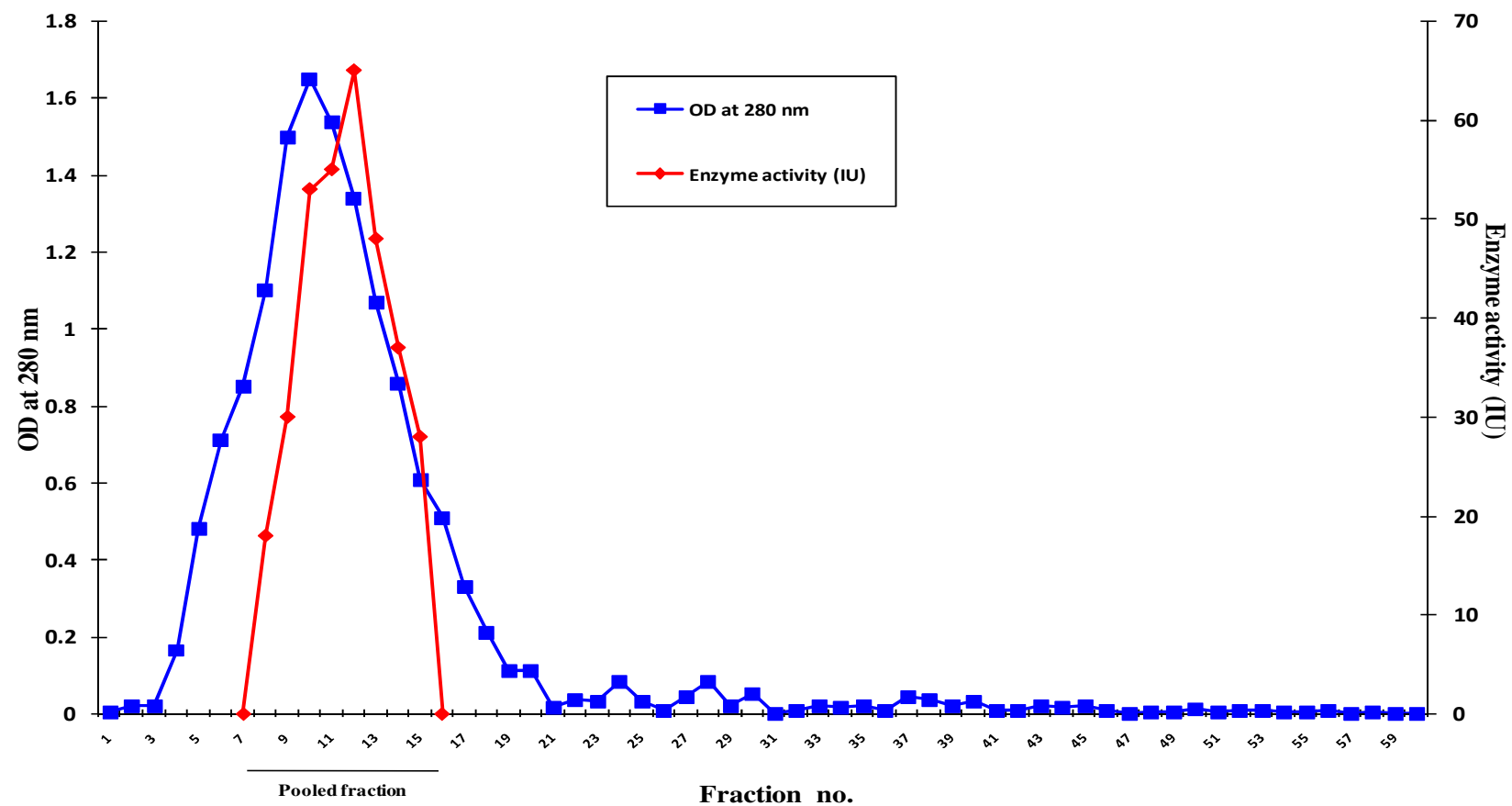


Fig. 70. Gel exclusion chromatography of xylanase active fraction (obtained after ion exchange) of *B. amyloliquefaciens* SH8 on sephadex *G-100*

the standard procedure of staining and destaining was followed. Molecular weight was determined by comparing the relative mobility of standard protein molecular weight marker of 14.3 kDa–97.4 kDa. Multiple bands ranging of 14 kDa to 97.4 kDa of partially purified xylanase enzyme of *Bacillus amyloliquefaciens* SH8 was observed (Plate 20). These bands may be different subunits of same enzyme on electrophoresis gel.

Table 59. Purification stages of xylanase produced by *B. amyloliquefaciens* SH8

Steps	Volume (ml)	Activity units IU/ml	Total* activity	Protein** mg/ml	Specific*** activity	Purification [♦] fold	Yield ^{♦♦} %
Crude	100	65.0	6500	1.20	54.16	1.0	100
Lyophilized	20	71.2	7120	1.31	54.35	1.0	100
Ion exchange	2	74.8	7480	0.83	90.12	1.65	63.35
Gel filtration	2	79.6	7960	0.30	265.3	4.88	22.90

* Total activity was determined by the multiplication of volume and activity

** Protein concentration was determined by Lowry's method

*** Specific activity is the activity unit/protein concentration

♦ Purification fold is increase in the specific activity

♦♦ Recovery % is remaining protein concentration as % of the initial protein concentration

Table 59 showed the purification stages of partially purified xylanase by *B. amyloliquefaciens* SH8. The final recovery in ion exchange Chromatography of partially purified xylanase was 1.65 fold with 63.35% yield and final specific activity of 90.12 was observed. The enzyme activity of partially purified xylanase was 4.88 fold with 22.90% yield and final specific activity of 265.3 in gel exclusion chromatography.

The results are in close conformity with those of Aygan and Arikan (2009) observed that a multifunctional endoxylanase producing *Bacillus* sp. was isolated from soil. Enzyme synthesis occurred at temperatures between 20°C and 60°C with an optimum 37°C. Analysis of the enzyme by SDS-PAGE revealed 4 active enzyme bands, which were estimated to be 66.5 kDa, 80.6 kDa, 95.5 kDa, and 108.4 kDa.

Sa -Pereira *et al.* (2002) a *Bacillus* sp. CCMI 966, characterized as *Bacillus subtilis*, has a duplication time of about 24 min. It produces at least two extracellular xylanases, Xyl I and Xyl II. The extracellular xylanase activity seems to be strongly correlated with the biomass growth profile. The Xyl II isoenzyme was purified by ammonium sulphate precipitation and anionic exchange Chromatography, with a purification factor of 8.3. The molecular weight of the isoenzyme was estimated by SDS-PAGE revealing that Xyl II is a multimeric enzyme with a catalytic subunit of about 20 kDa. Under non-denaturing conditions, a molecular weight of about 340 kDa was obtained by native PAGE gel and of 20 kDa by gel filtration chromatography. The enzyme showed an optimum pH and temperature of 6.0 at 60°C. Xyl II was stable at 40°C for 180 min at pH 6.0. The specificity of Xyl II for different substrates was evaluated. Xyl II presents a higher affinity towards OSX, with a K_m of 1.56 g l^{-1} and showed the ability to hydrolyze laminarin, with a K_m of 1.02 g l^{-1} .

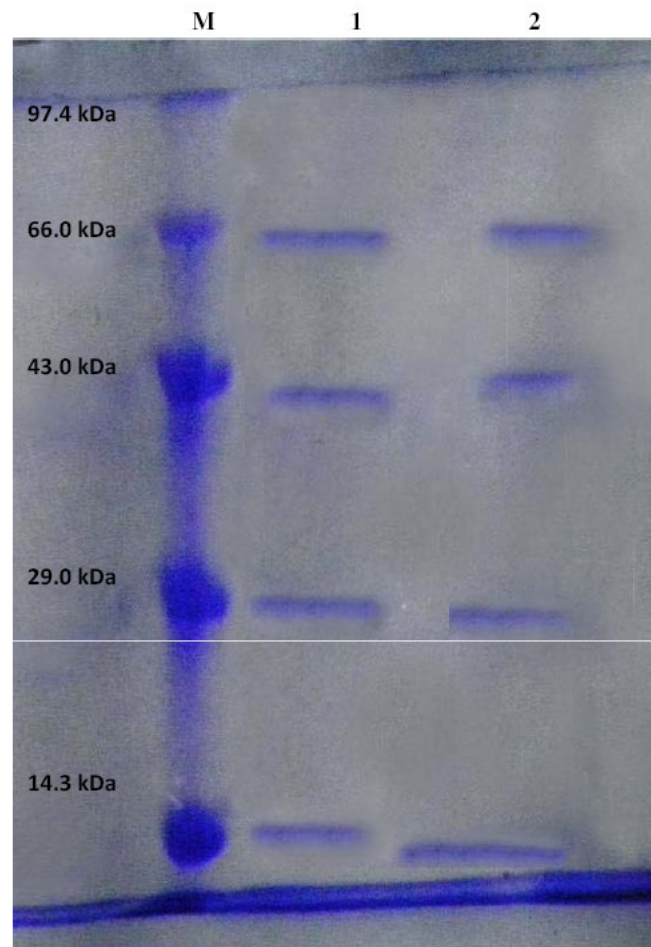
4.4.1.1.5 Characterization of Partially Purified Xylanase

After partial purification, the enzyme was characterized to find out the conditions at which it showed best performance. The detail of parameters is given below:

4.4.1.1.5.1 Effect of pH

The effect of various pH was determined in different buffers with the pH values ranging from (3, 4, 5....12) on the partially purified xylanase activity is depicted in Fig 71. Activity of the enzyme was found to be greatly affected by pH changes. The enzyme exhibited high activity in a range spanning 5.0 to 7.0. The highest enzyme activity was found at 6.0 pH (81.83 IU/ml). When the pH of the enzyme medium was change on either side of optimum range from acidic to neutral pH and alkaline pH, a decline in enzyme activity was observed.

Most enzymes have a characteristic pH at which their activity is maximal, above or below this pH, the activity declines. The change in enzyme activity with the change in pH showing sharp peak (optimum pH) is a trend obtained because the enzymes contains some amino acids which have side groups that are ionised at



Lane 1. = Partially purified xylanase of ion exchange chromatography
Lane 2. = Partially purified xylanase of gel chromatography
M = Marker

Plate 20. SDS PAGE of partially purified xylanase of *Bacillus amyloliquifaciens* SH8

a particular pH range. Some of these residues will be present at active sites of enzymes where virtually catalysis of chemical reaction occurs.

The results are in close conformity with those of Aygan and Arikan (2009) observed that a multifunctional endoxylanase producing *Bacillus* sp. was isolated from soil. Enzyme synthesis occurred at temperatures between 20°C and 60°C with an optimum 37°C. Analysis of the enzyme by SDS-PAGE revealed 4 active enzyme bands, which were estimated to be 66.5 kDa, 80.6 kDa, 95.5 kDa, and 108.4 kDa. The enzyme has a broad temperature range, between 20 and 90°C, with an optimum at 40°C; and maximum activity was at pH 6.0. The enzyme showed a gradual decrease in the remaining activity as the pre-incubation temperature increase.

The effect of pH on the activity of xylanase was determined in *Bacillus* sp. strain BP-7 by Lopez *et al.* (1998). The enzyme was most active at neutral pH, showing the highest activity at pH 6. However, the xylanase showed a good level of activity at alkaline pH, exhibiting 58% of its maximum activity at pH 8 and 26% at pH 9. Roy and Rowshanul (2009) reported that the SDS PAGE gave a single band at 32kDa. The optimum pH and temperature for the optimized enzyme was 40°C and 6.0, respectively by *Bacillus cereus* isolated from soil. Sa-Pereira *et al.* (2002) reported that enzyme showed an optimum pH and temperature of 6.0 at 60°C by *Bacillus subtilis*.

Kumar *et al.* (2010) reported that maximum production of xylanase from *Bacillus pumilis* MTCC 8964 observed in late log phase at pH 6.0 with at 40°C. High activity of xylanase was recorded with acetate buffer at pH 6.0 and 60°C (241.4 U/ml). Molecular mass of enzyme was estimated to 14.3 kDa with SDS PAGE after gel exclusion chromatography.

4.4.1.1.5.2 Effect of Temperature

The effect of various temperatures regime on the enzyme activity of partially purified xylanase was presented in Fig. 72 ranging from 30°C, 40°C.....100°C for 10 min. It was found that the enzyme exhibited maximum

activity at 90°C (99.89 IU/ml). When the temperature increased above 90°C the activity of the enzyme was affected negatively and gradually reduced. As the denaturation of the enzymatic protein occurs at elevated temperatures therefore, after certain level of temperature increase (above 90°C), the enzyme activity decreased rapidly. In nutshell, the enzyme was most efficient at 90°C as compared with other tested temperatures.

The highest enzyme activity at 90°C by *Bacillus amyloliquefaciens* SH8 proves its highly thermostable nature. Thermostability of the xylanase enzyme render its highly important industrial use. Mostly, the available commercial enzymes are heat sensitive in nature thus restricting their use for various industrial applications. Thermostable enzymes are highly sought after in the industries because of the reaction conditions pertained that are generally being pertained at elevated temperature as most of the enzymes are proteins and get denatured due to irreversible distortion of their three dimensional structure thus, resulting in decreased enzyme activity. On the other hand thermostable enzymes have unique protein in their structure turing them thermostable in nature.

Virtually, thermostable enzymes are occurs rarely in nature and thus are of immense value for biotechnological industries because this strain SH8 was isolated from the compost. So, therefore its thermostable nature can be correlated with its origin. Since in compost during composting the temperature shoots up to 70°C reflecting the possibilities of thriving the thermophiles in enriched compost. It has been reported in literature that thermostable microorganisms mostly yield thermostable enzymes.

The present results are in close harmony with the recent findings of Harris *et al.* (1997) reported a thermophilic xylanase from *Bacillus* strain D3 suitable for use as a bleach booster in the paper pulping industry has been identified and characterized. The enzyme is suited to the high temperature and alkaline conditions needed for using xylanases in the pulp industry. The xylanase is stable at 60°C and relatively stable at high temperatures, with a temperature optimum of 75°C. The pH optimum is 6, but the enzyme is active over a broad pH range.

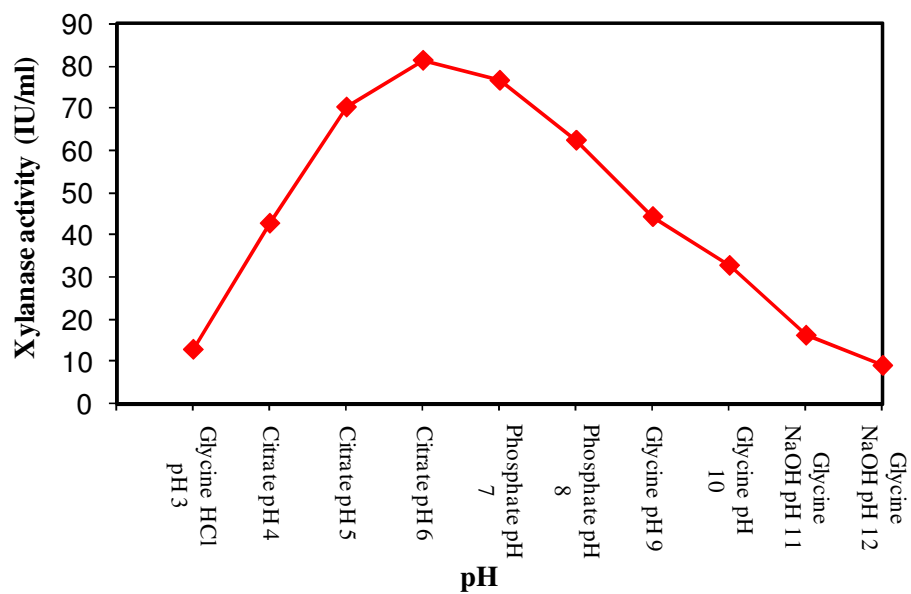


Fig 71. Effect of different pH on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens* SH8

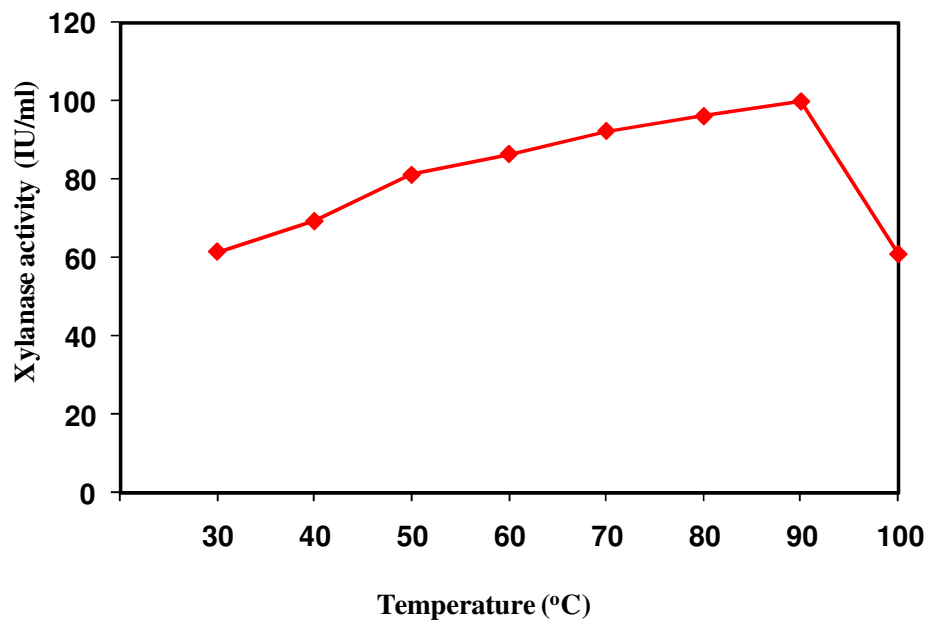


Fig 72. Effect of different temperature on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens* SH8

Srinivas *et al.* (2010) reported a highly thermostable alkaline xylanase was purified to homogeneity from culture supernatant of *Bacillus* sp. JB 99 using DEAE-Sephadex and Sephadex G-100 gel filtration with 25.7-fold increase in activity and 43.5% recovery. The molecular weight of the purified xylanase was found to be 20 kDa by SDS-PAGE and zymogram analysis. The enzyme was optimally active at 70°C, pH 8.0 and stable over pH range of 6.0-10.0. The relative activity at 9.0 and 10.0 were 90% and 85% of that of pH 8.0, respectively. The enzyme showed high thermal stability at 60°C with 95% of its activity after 5 h. The K_m and V_{max} of enzyme for oat spelt xylan were 4.8 mg/ml and 218.6 μ M/min/mg, respectively.

Cordeiro *et al.* (2002) showed that an aerobic, thermophilic, xylanolytic bacterium was isolated from local soil. The results of 16S rRNA sequence comparisons indicated that the isolate was closely related to *B. caldoxylolyticus* and *Bacillus* sp. strain AK1. These organisms exhibited 94% levels of ribosomal DNA sequence homology. Studies on the β -xylanase characterization from liquid cultures grown on beechwood xylan revealed that the enzyme retained 100% of activity for 2h at temperatures ranging from 30 to 50°C, while at 60, 70 and 100°C, 10%, 11% and 29% of the original activities were lost, respectively. The optimum pH of the enzyme was found to be between 6.5 and 7.0. After incubation of crude enzyme solution for 24h at 25°C and at pH 5.5 to 8.0, a decrease of about 12% of its original activity was observed.

4.4.1.1.5.3 Effect of Metal Ions

The results in Fig. 73 showed that Hg^{2+} was strong inhibitor of enzyme activity while Cu^{2+} , Na^+ and Mg^{2+} had a slight inhibition effect on enzyme activity. The inhibition of the enzyme was generally obtained with ions that reacted with sulfhydryl groups such as Hg^{2+} , suggesting that there was an important cysteine residue in or close to the active site of the enzyme. Inactivation of xylanase by Hg^{2+} has been reported by others authors also. K^+ ions were found inert and it do not affect the activity of this enzyme. However, stimulatory effects were obtained with Zn^{2+} , Ca^{2+} and Mn^{2+} (101.10, 103.80 and

102.45 IU/ml). The moderate to high increase on xylanase activities by these cation have also been reported in the literature.

Kiddinamoorthy *et al.* (2008) reported the purified enzyme had an apparent molecular weight of 42 kDa and showed optimum activity at 70°C and pH 7. The enzyme was stable at 60-80°C at pH 7 and pH 5–11 at 37°C. Metal ions Mn^{2+} and Co^{2+} increased activity by twofold, while Cu^{2+} and Fe^{2+} reduced activity by fivefold as compared to the control. At 60°C and pH 6, the K_m for oat-spelt xylan was 2.23 mg/ ml and V_{max} was 296.8 IU/ mg protein.

Chivero *et al.* (2001) purified endoxylanase from tow isolates of *Bacillus* sp. Endoxylanase activity of the for isolate SB9-a was inhibited by Hg^{2+} , Ag^+ and Mn^{2+} ions, while Fe^{3+} , K^+ , Ca^{2+} and Cu^{2+} ions stimulated xylanase activity.

4.4.1.1.5.4 Effect of Substrate Specificity

The substrate specificity by the partially purified xylanase for different substrates was depicted in Fig 74. The xylanase enzymes exhibited different specificity and activity towards various polysaccharide used here as substrates. Xylanase of *Bacillus amyloliquefaciens* SH8 strongly hydrolyzed Birch wood xylan (106.1 IU/ml) followed by oat spelt xylan (99.89 IU/ml). However, it could not hydrolyze cellulose, avicel and CMC. Thus, this enzyme is cellulase free proves the true nature of xylanase enzyme. The results are in concord with those of Srinivasan *et al.* (2010). The enzyme purified from *Cellulosimicrobium* sp. MTCC 10645 exhibited greater binding affinity exclusively for xylans but not for avicel, CMC, cellobiose, starch, or p-nitrophenyl xylopyranoside. Parachloromercuric benzoate and iodoacetamide were found stimulatory, while potassium permanganate, cysteine, and cystine markedly reduced the activity by Kambli and Jadhav (2012).

4.4.1.1.5.5 Effect of Substrate Concentration

The effect of various substrate concentrations was determined in values ranging from (0.2, 0.4.....2.0%) on the partially purified xylanase activity is depicted in Fig 75. Enzyme activity was found to be greatly affected by substrate

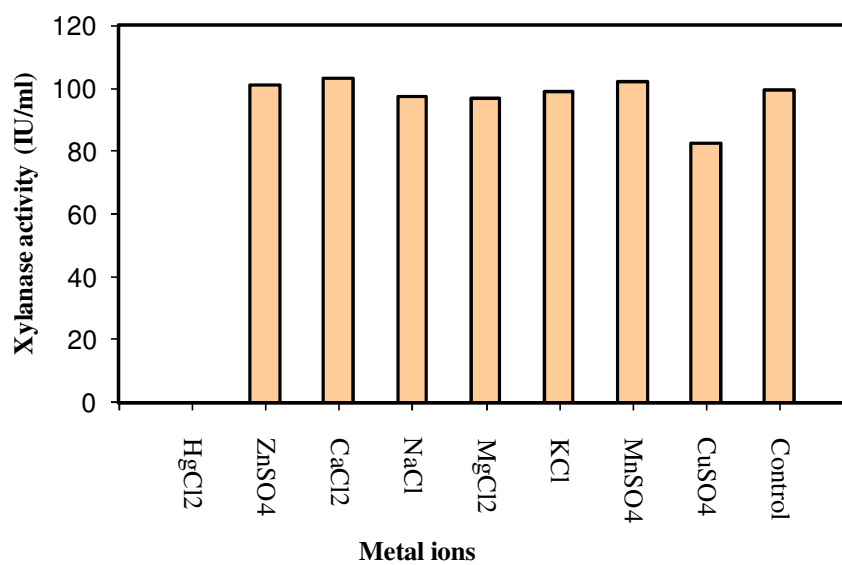


Fig 73. Effect of different Metal ions on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens* SH8

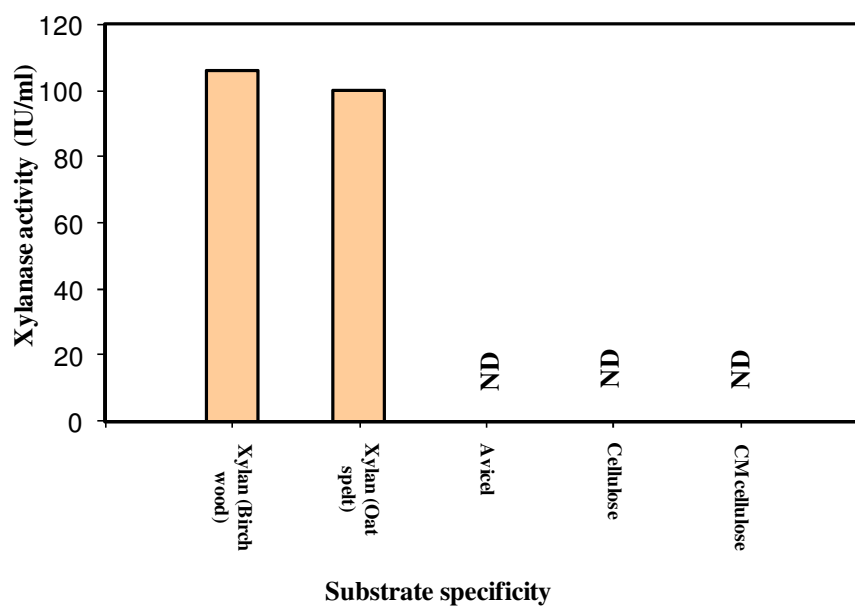


Fig 74. Effect of different Substrate on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens* SH8

concentration. The enzyme exhibited a gradual increase in enzyme activity from 0.2% to 1.8% (68.23 to 119.5 IU/ml) reaching a constant value to (119.5 IU/ml) at 1.8 and 2.0% of substrate concentration, respectively. The stability in enzyme activity at increasing concentration of reflects the attainment of saturation point of substrate furthermore leading to nil or negligible increase in enzyme activity. In general, increase in the substrate concentration gradually increases the velocity of the enzyme reaction within the limited range of substrate levels. A rectangular hyperbola is obtained when velocity is plotted against the substrate concentration. Three distinct phases of the reaction are observed as shown in figure. At low substrate concentration at phase A, the velocity of the reaction is directly proportional to the substrate level. In the second phase B, substrate concentration is not directly proportional to the enzyme activity. In the third and final phase C, the reaction is independent of the substrate concentration by Satyanarayana (2004).

4.4.1.1.5.6 Determination of Enzyme Kinetics

The kinetic parameters for hydrolysis of birch wood xylan were determined by plotting the initial velocities towards different concentrations of substrate as depicted in Fig. 76. The enzyme showed Michaelis-Menten behaviour, and the kinetic constant for birch wood xylan substrate was calculated. The kinetic parameters (K_m and V_{max}) were determined using Line weaver-Burk plot at 90°C (pH 6.0) with different concentrations of Birchwood xylan ranging from 2,4.....20 mg/ml. The K_m and V_{max} of partially purified xylanase were 5.83 mg/ml and 166.67 μ mol/mg/min. This indicates that the specificity of towards birch wood xylan substrate is high (K_m value is low).

The *Bacillus* sp. WTU-06 had K_m and V_{max} values of 3.45 mg ml⁻¹ and 387.3 μ mol min⁻¹ mg⁻¹, respectively. The deduced internal amino acid sequence of *Bacillus* sp. WTU -06 xylanase resembled the sequence of β -1,4-endoxylanase which is a member of glycoside hydrolase family 11 (Wang *et al.*, 2010). Monisha *et al.* (2009) reported that the xylanase had K_m of 4.0 mg/ml and V_{max} of 0.068X10⁻⁴ mM min⁻¹ mg⁻¹.

The one step purification and characterization of an extracellular cellulose free xylanase from a newly isolate alkalophilic and moderately thermophilic strain of *Bacillus subtilis* ASH was studied by Sanghi *et al.* (2010). Xylanase was purified to homogeneity by 10.5 fold with approximately 43 % recovery using ion exchange chromatography though CM-Sephadex C-50. The purified enzyme revealed a single band on SDS-Page gel with molecular mass of 23 kDa. It showed an optimum pH at 7.0 and was stable over the pH range 6.0-9.0. The purified enzyme xylanase did not lose any activity upto 45°C; however, it retained 80% and 51% of its activity after pre-incubation at 55°C and 60°C, respectively. The enzyme obeyed Michaelis-Menton kinetics towards birchwood xylan with apparent K_m 3.33 mg/ml and V_{max} 100 IU/m

4.4.1.2 *Bacillus tequilensis* SH0

4.4.1.2.1 Lyophilization:

Same as section.....4.4.1.1.1

4.4.1.2.2 Ion Exchange Chromatography

Lyophilized crude enzyme was subjected to ion exchange Chromatography column DEAE cellulose. The column was packed with DEAE cellulose and the concentrated crude enzyme (2ml) was loaded on pre-equilibrated gel filtration column with the help of Pasteur pipette. Phosphate buffer pH (6.9) was used as elution buffer. A total of 60 fractions with flow rate 6min/3ml each were collected in chromatographic vials under cool environment. The optical density of each collected fraction was measured at 280 nm. The fractions of 0.1 M showed highest enzyme activity by calorimetric method i.e. 6-12 (28.0, 44.1, 39.20, 32.11, 28.6 19.2 IU) were pooled and concentrated further by lyophilization as shown in Fig. 77. The xylanase activity was checked in these active fractions. The enzyme activity of pooled fractions was checked by quantitative calorimetric estimation assays.

4.4.1.2.3 Gel Exclusion Column Chromatography:

The void volume of sephadex G-100 column was calculated by loading blue dextran solution (2 mg/ml) on to the column. Fractions of 2ml were

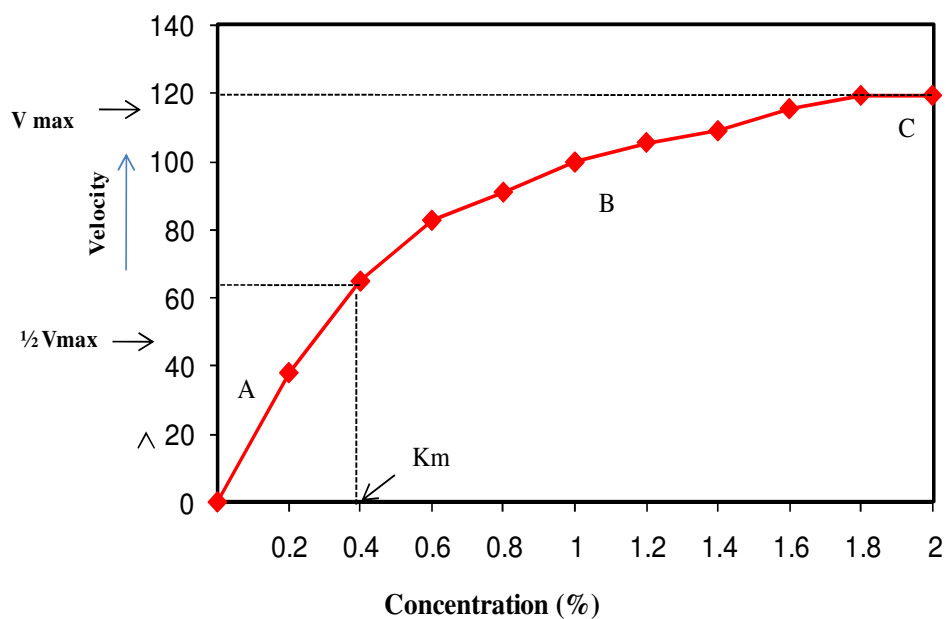


Fig 75. Effect of different concentration (%) of substrate on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquifaciens* SH8

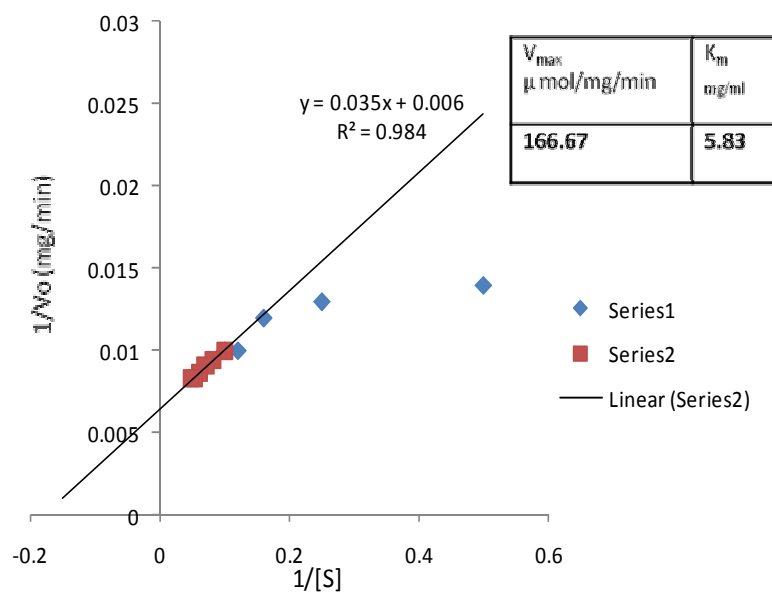


Fig 76. Lineweaver-Burk double reciprocal plot for the determination of K_m and V_{max} of partially purified xylanase from *Bacillus amyloliquifaciens* SH8

collected and optical density (OD) was measured at 540 nm. The void volume was found to be 66 ml. The pooled post-DEAE-cellulose xylanase fraction was then further enriched by preparative sephadex G-100 gel filtration column Chromatography. The column was packed with sephadex G-100 and the concentrated crude enzyme (2 ml) was loaded on pre-equilibrated gel filtration column with the help of Pasteur pipette. Phosphate buffer pH (6.9) was used as elution buffer. A total of 60 fractions with flow rate 6min/3ml each were collected in chromatographic vials under cool environment. The optical density of each collected fraction was measured at 280 nm. The maximum protein was observed in fraction no 10 to 17 i.e. 3.1, 34.2, 36.0, 57.89, 51.20, 42.0, 31.0, 13.0 IU (Fig. 78). The xylanase activity was checked in these active fractions. These fractions having positive xylanase activity were pooled together and were lyophilized. The enzyme activity of pooled fractions was checked by quantitative calorimetric estimation assays. The lyophilized enzyme preparation was stored at (-20°C) for further use.

4.4.1.2.4 SDS-PAGE (Sodium dodecyl sulphate - polyacrylamide gel electrophoresis) *Bacillus tequilensis* SH0

The purity and molecular weight of pooled fractions were determined by SDS-PAGE. 10 µl of pooled sample + 5µl of loading dye were loaded in the wells of 12% of gel. The electrophoresis was run at 150V for 2h. After the gel was run, the standard procedure of staining and destaining was followed. Molecular weight was determined by comparing the relative mobility of standard protein molecular weight marker of 14.3 kDa–97.4 kDa. Multiple bands ranging of 14 kDa to 97.4 kDa of partially purified xylanase enzyme of *Bacillus tequilensis* SH0 was observed (Plate 21). These bands may be different subunits of same enzyme on electrophoresis gel.

Table 60 showed the purification stages of partially purified xylanase by *Bacillus tequilensis* SH0. The final recovery in ion exchange chromatography of partially purified xylanase was 1.82 fold with 57.69% yield and final specific activity of 72.95 was observed. The enzyme activity of partially purified xylanase

was 5.30 fold with 21.53% yield and final specific activity of 211.53 in gel exclusion Chromatography.

Table 60. Purification stages of xylanase produced by *B. tequilensis* SH0

Steps	Volume (ml)	Activity units IU/ml	Total* activity	Protein** mg/ml	Specific*** activity	Purification [♦] fold	Yield ^{♦♦} %
Crude	100	41.00	4100	1.18	34.75	1.0	100
Lyophilized	20	49.56	4956	1.30	38.12	1.0	100
Ion exchange	2	54.71	5471	0.75	72.95	1.82	57.69
Gel filtration	2	59.35	5935	0.28	211.96	5.30	21.53

* Total activity was determined by the multiplication of volume and activity

** Protein concentration was determined by Lowry's method

*** Specific activity is the activity unit/protein concentration

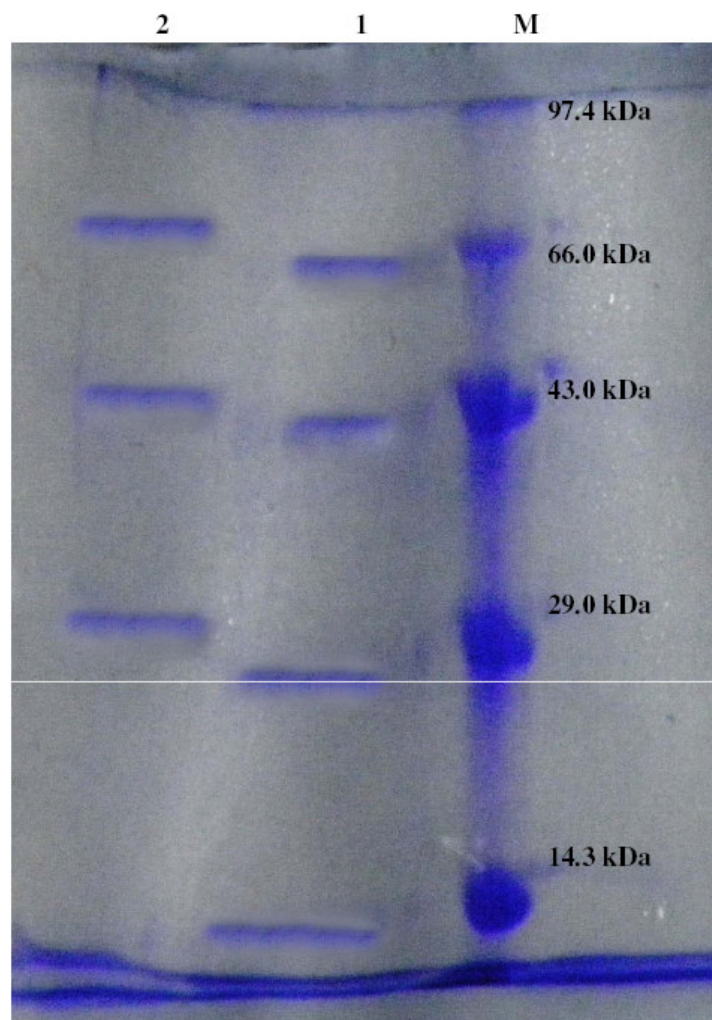
♦ Purification fold is increase in the specific activity

♦♦ Recovery % is remaining protein concentration as % of the initial protein concentration

Damiano *et al.* (2006) reported that the alkalophilic bacteria *Bacillus licheniformis* 772 produces significant quantities of thermostable cellulase-free xylanases. The crude xylanase was purified to apparent homogeneity by gel filtration (G-75) and ionic exchange chromatography (carboxymethyl sephadex, Q sepharose, and Mono Q), resulting in the isolation of two xylanases. The molecular masses of the enzymes were estimated to be 17 kDa (X-I) and 40 kDa (X-II), as determined by SDS-PAGE. The K_m and V_{max} values were 1.8 mg/mL and 7.05 U/mg protein (X-I), and 1.05 mg/mL and 9.1 U/mg protein (X-II). The xylanases demonstrated optimum activity at pH 7.0 and 8.0–10.0 for xylanase X-I and X-II, respectively, and retained more than 75% of hydrolytic activity up to pH 11.0. The purified enzymes were most active at 70 and 75°C for X-I and X-II, respectively, and, retained more than 90% of hydrolytic activity after 1h of heating at 50°C and 60°C for X-I and X-II, respectively.

4.4.1.2.5 Characterization of partially purified xylanase

After partial purification, the enzyme was characterized to find out the conditions at which it showed best performance. The detail of parameters is given below:



Lane 1. = Partially purified xylanase of ion exchange chromatography
Lane 2. = Partially purified xylanase of gel exchange chromatography
M = Marker

Plate 21. SDS PAGE of partially purified xylanase of *Bacillus tequilensis* SH0

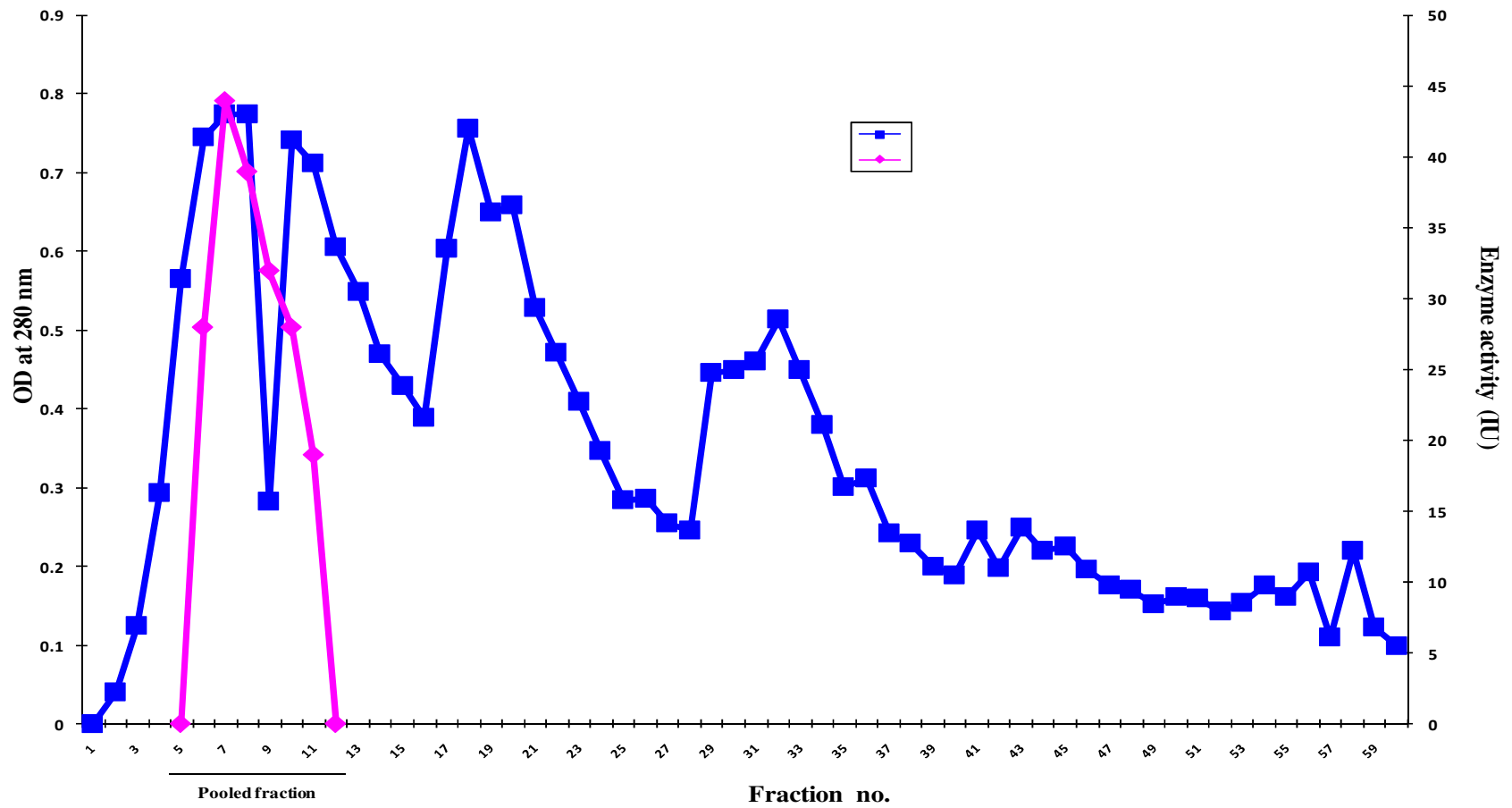


Fig. 77 Ion exchange chromatography of extracellular crude xylanase of *Bacillus tequilensis* SH0 on DEAE Cellulose

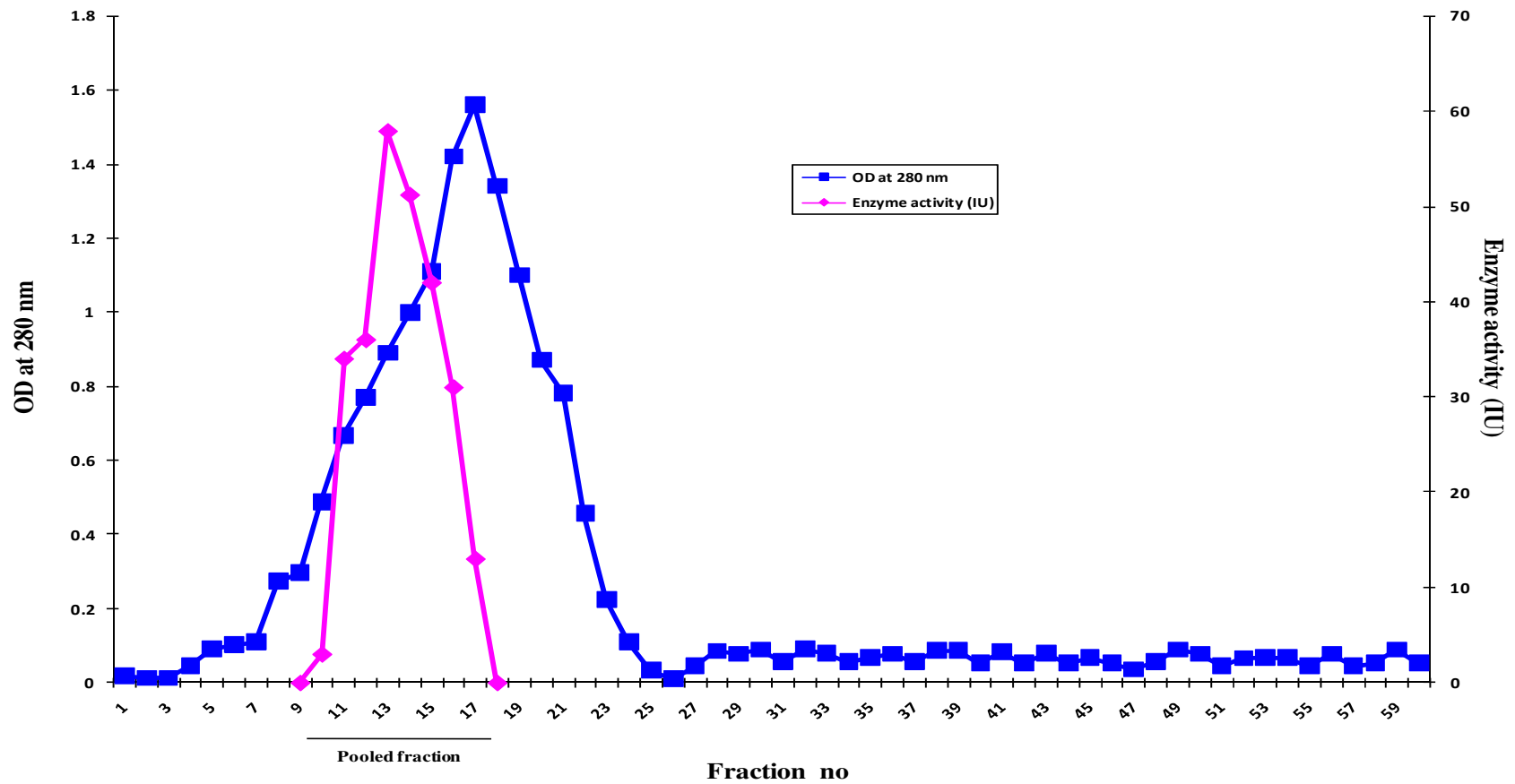


Fig 78. Gel exclusion chromatography of xylanase active fraction (obtained after ion exchange) of *B. tequilensis* SH0 on sephadex G-100

4.4.1.2.5.1 Effect of pH

The effect of various pH was determined in different buffers with the pH values ranging from (3, 4, 5.....12) on the partially purified xylanase activity is depicted in Fig. 79. Activity of the enzyme was found to be greatly affected by pH changes. The enzyme exhibited high activity in a range spanning 5.0 to 7.0. The highest enzyme activity was found at 6.0 pH (62.20 IU/ml). When the pH of the enzyme medium was change on either side of optimum range from acidic to neutral pH and alkaline pH, a decline in enzyme activity was observed.

Most enzymes have a characteristic pH at which their activity is maximal, above or below this pH, the activity declines. The change in enzyme activity with the change in pH showing sharp peak (optimum pH) is a trend obtained because the enzymes contains some amino acids which have side groups that are ionised at a particular pH range. Some of these residues will be present at active sites of enzymes where virtually catalysis of chemical reaction occurs.

Mittal *et al.* (2012) reported the xylanase was purified to homogeneity from crude extract of *Bacillus* sp. KS09 using ammonium sulphate fractioning and CM-Sephadex C-50. The final purification fold was 10.20 with a recovery of 36.18%. The enzyme was optimally active at 50°C, pH 7.0 and stable over a broad pH range of 6.0-11.0. The residual activity at 6.0-11.0 pH was 100% even upto 3 h of incubation. The enzyme showed 75, 70 and 60% thermal stability at 50, 55 and 60°C, respectively after 1h of incubation. The kinetic parameters (K_m 22.59 mM; V_{max} 76.93 IU/mL) were estimated using Lineweaver-Burk plot for purified xylanase.

Atanakhanokchai *et al.* (1999) reported that an alkaliphilic bacterium, *Bacillus* sp. strain K-1, produces extracellular xylanolytic enzymes such as xylanases, β -xylosidase, arabinofuranosidase, and acetyl esterase when grown in xylan medium. One of the extracellular xylanases that is stable in an alkaline state was purified to homogeneity by affinity adsorptiondesorption on insoluble xylan. The enzyme bound to insoluble xylan but not to crystalline cellulose. The molecular mass of the purified xylan-binding xylanase was estimated to be

approximately 23 kDa. The enzyme was stable at alkaline pH up to 12. The optimum temperature and optimum pH of the enzyme activity were 60°C and 5.5, respectively. Metal ions such as Fe^{2+} , Ca^{2+} , and Mg^{2+} greatly increased the xylanase activity, whereas Mn^{2+} strongly inhibited it.

4.4.1.2.5.2 Effect of Temperature

The effect of various temperatures regime on the enzyme activity of partially purified xylanase was presented in Fig. 80 ranging from 30°C, 40°C.....100°C for 10 min. It was found that the enzyme exhibited maximum activity at 90°C (74.90 IU/ml). When the temperature increased above 90°C the activity of the enzyme was affected negatively and gradually reduced. As the denaturation of the enzymatic protein occurs at elevated temperatures therefore, after certain level of temperature increase (above 90°C), the enzyme activity decreased rapidly. In nutshell, the enzyme was most efficient at 90°C as compared with other tested temperatures.

Enzymes are sensitive to high temperature. Enzymes are proteineous in nature having three dimensional structures which gets distorted at high temperature and consequently gets coagulated. The breakage of weak hydrogen bonds lead to unfolding of long protein chains and this reaction is irreversible showing permanent inactivation of enzyme with increased temperature beyond a certain point.

Enzyme properties of new thermostable xylanase from alkalophilic and thermophilic *Bacillus* sp. isolate DM-15 was reported by Ozcan *et al.* (2011). The enzyme had a broad temperature range between 40-90°C and the optimum activity was observed at 60°C. The relative enzyme activities were 77, 91, 96, 68 and 57% at 40, 50, 70, 80 and 90°C, respectively, whereas only 17% activity was retained at 100°C.

Purification and characterization of thermostable xylanase from thermophilic fungus *Rhizomucor mieher* (Cooney and R. Emerts) Schipper was reported by Fawzi (2010). The enzyme was purified 29.1 fold homogeneity by

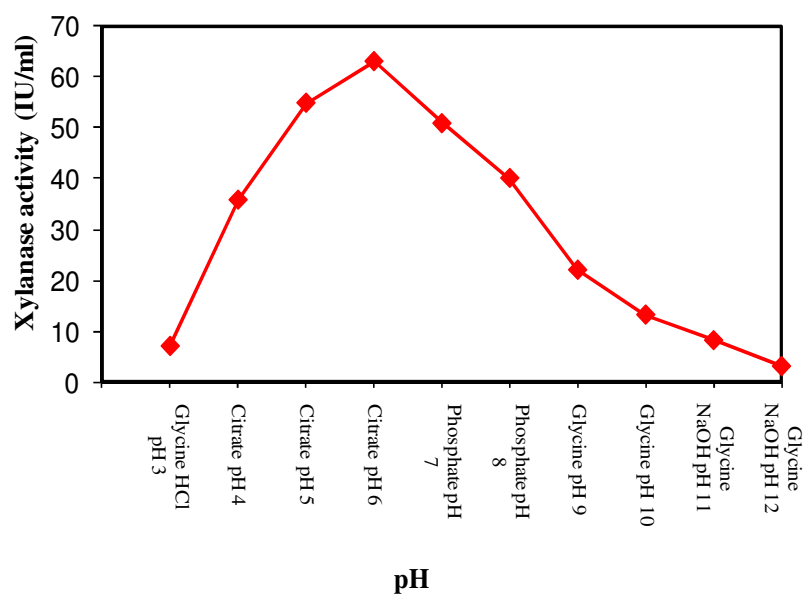


Fig 79. Effect of different pH on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0

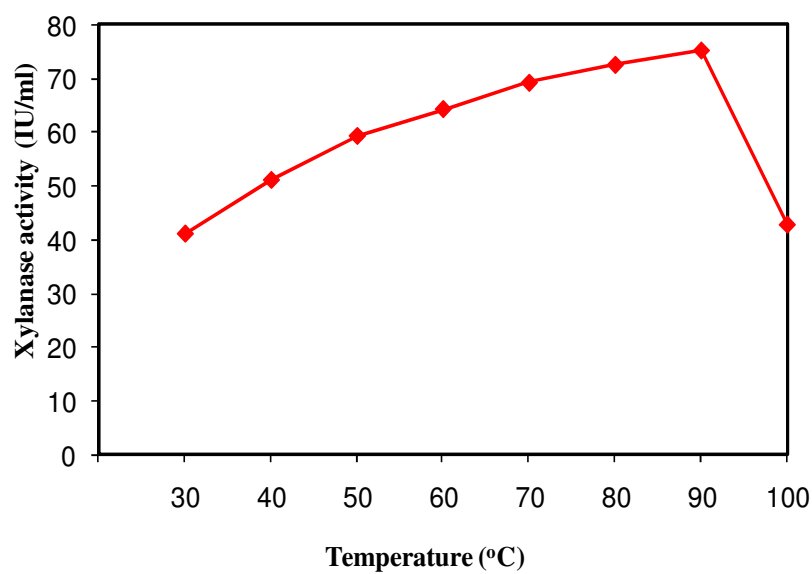


Fig 80. Effect of different temperature on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0

ammonium sulphate precipitation, sephadex G-100 gel filtration and diethylaminoethyl cellulose anion exchange chromatography. The enzyme was highly active with a range of pH from 5.0 to 6.5. The optimum temperature of the purified enzyme was the 70 and 75°C and half life of the xylan as at 90°C was 30 min. K_m and V_{max} values at 50°C of the purified enzyme were 0.055 mg/ml and 113.5 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively.

Ratanachomsri *et al.* (2006) observed that *Marasmius* sp. was chosen for further characterization because of its high xylanolytic activity at temperature as high as 90°C. The crude enzyme possessed high thermostability and pH stability. Purification of this xylanase was carried out using an anion exchanger followed by hydrophobic interaction chromatography, yielding the enzyme with >90% homogeneity. The molecular mass of the enzyme was approximately 40 kDa. The purified enzyme retained broad working pH range of 4-8 and optimal temperature of 90°C. When using xylan from birchwood as substrate, it exhibits K_m and V_{max} values of 2.6 ± 0.6 mg/ml and 428 ± 26 U/mg, respectively. Hiremath and Patil (2011) characterized xylanase from *Bacillus* sp. which showed optimum xylanolytic activity at pH more than 10 and at temperature 70°C.

The optimal pH and temperature for the xylanase activity were 3.0 and 80°C, respectively from brown rot fungus *Laetioporus sulphureus*. Although the highest activity was at 80°C, it showed highest thermostability at 60°C by Lee *et al.* (2009).

4.4.1.2.5.3 Effect of Metal Ions

The results shows that Hg^{2+} was strong inhibitor of enzyme activity while Cu^{2+} , Na^+ and Mg^{2+} had a slight inhibition effect on enzyme activity as depicted in Fig. 81. The inhibition of the enzyme was generally obtained with ions that reacted with sulfhydryl groups such as Hg^{2+} , suggesting that there was an important cysteine residue in or close to the active site of the enzyme. Inactivation of xylanase by Hg^{2+} has been reported by others authors also. K^+ ions were found inert and it do not affect the activity of this enzyme. However, stimulatory effects were obtained with Zn^{2+} , Ca^{2+} and Mn^{2+} (77.10, 78.0 and

76.88 IU/ml). The moderate to high increase on xylanase activities by these cation have also been reported in the literature.

Yin *et al.* (2010) depicted the purified xylanase activity, with an M_w of 19 kDa, had an optimal pH and temperature at 5.0 and 50°C, respectively, and was stable at pH 5.0-9.0 or 50°C. It was inhibited by Cu^{2+} , Fe^{3+} , Hg^{2+} , phenylmethyl sulfonyl fluoride (PMSF), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), W-ethylmaleimide (NEM), and leupeptin but activated by K^+ , Na^+ , Co^{2+} , Mg^{2+} , 3-mercaptopropanol (9-ME) and glutathione (GSH). The purified xylanase had high specificity to beechwood, birchwood, and oat spelt xylans.

4.4.1.2.5.4 Effect of Substrate Specificity

The substrate specificity by the partially purified xylanase for different substrates was depicted in Fig. 82. The xylanase enzymes exhibited different specificity and activity towards various polysaccharide used here as substrates. Xylanase of *Bacillus amyloliquefaciens* SH8 strongly hydrolyzed Birch wood xylan (82.10 IU/ml) followed by oat spelt xylan (76.10 IU/ml). However, it could not hydrolyze cellulose, avicel and CMC. Thus, this enzyme is cellulase free proves the true nature of xylanase enzyme. The results are in concord with those of Jones *et al.* (2012) reported that *Bacillus subtilis* SJ01 degraded xylan substrates (C1: 0.24 U/mg, C2: 0.14 U/mg Birchwood xylan) with higher efficiency than amorphous cellulose substrates (C1: 0.002 U/mg, C2: 0.01 U/mg carboxymethyl cellulose).

4.4.1.2.5.5 Effect of Substrate Concentration

The effect of various substrate concentrations was determined in values ranging from (0.2, 0.4.....2.0%) on the partially purified xylanase activity is depicted in Fig. 83. Enzyme activity was found to be greatly affected by substrate concentration. The enzyme exhibited a gradual increase in enzyme activity from 0.2% to 1.8% (39.12 to 96.87 IU/ml) reaching a constant value to (96.87 IU/ml) at 1.8 and 2.0% of substrate concentration, respectively. The stability in enzyme activity at increasing concentration of reflects the attainment of saturation point of substrate furthermore leading to nil increase in enzyme activity. In general,

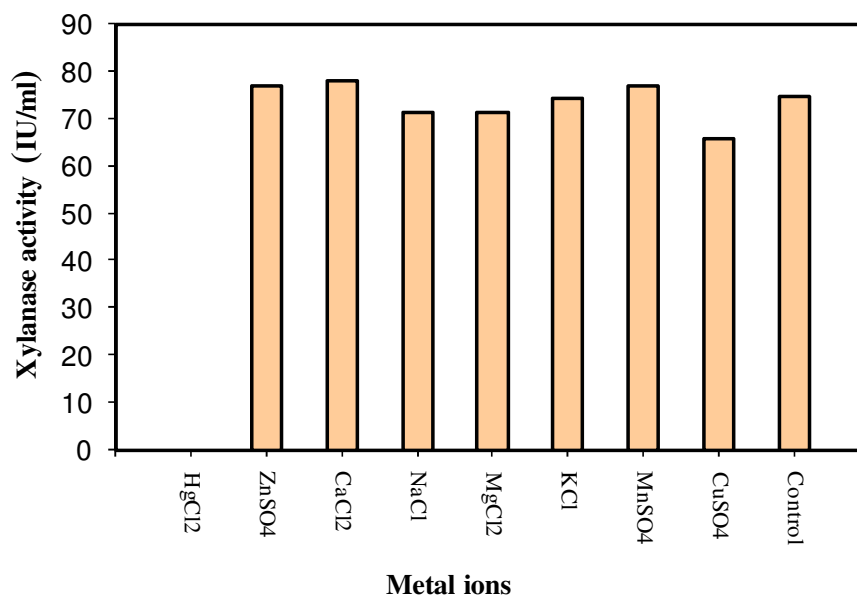


Fig 81. Effect of different Metal ions on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0

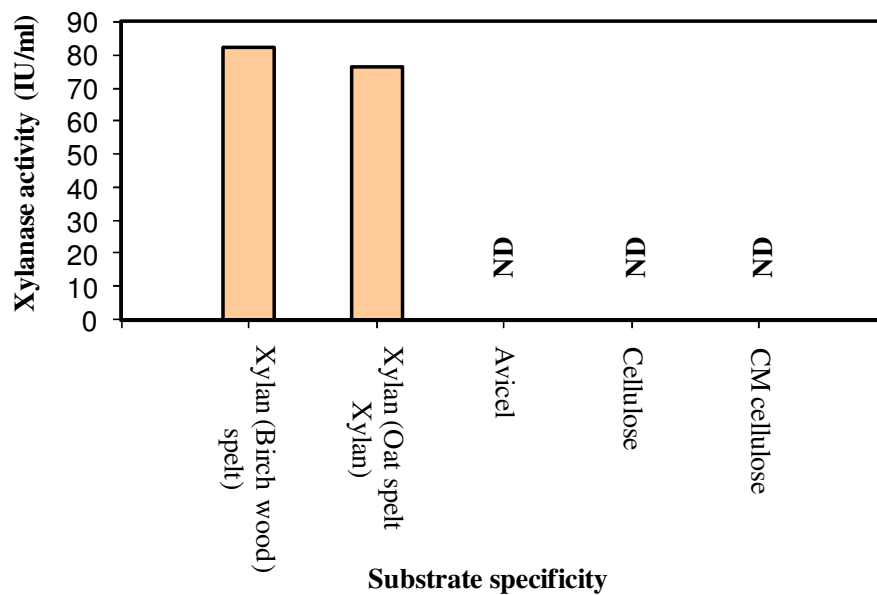


Fig 82. Effect of different substrate on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0

increase in the substrate concentration gradually increases the velocity of the enzyme reaction within the limited range of substrate levels. A rectangular hyperbola is obtained when velocity is plotted against the substrate concentration. Three distinct phases of the reaction are observed as shown in figure. At low substrate concentration at phase A, the velocity of the reaction is directly proportional to the substrate level. In the second phase B, substrate concentration is not directly proportional to the enzyme activity. In the third and final phase C, the reaction is independent of the substrate concentration by Satyanarayana (2004).

4.4.1.2.5.6 Determination of Enzyme Kinetics

The kinetic parameters of for hydrolysis of birch wood xylan were determined by plotting the initial velocities towards different concentrations of substrate as shown in Fig 84. The enzyme showed Michaelis-Menten behaviour, and the kinetic constant for birch wood xylan substrate was calculated. The kinetic parameters (K_m and V_{max}) were determined using Line weaver-Burk plot at 90°C (pH 6.0) with different concentrations of Birchwood xylan ranging from 2-20 mg/ml. The K_m and V_{max} of partially purified xylanase were 10.99 mg/ml and 166.67 μ mol/mg/min. This indicates that the specificity of towards birch wood xylan substrate is high (K_m value is low).

Heck *et al.* (2006) purified xylanase enzyme by cation exchange and gel filtration chromatography from *Bacillus circulans* BL53 and the molecular weight was 38 kDa by SDS-PAGE. Maximum pH and temperature for the maximum activity was obtained using a 2² factorial design over an expensive range of pH (5.0-8.0) and temperature (40-80°C). The K_m and V_{max} values were 9.9 mg/xylan/ml and 25.25 μ mol/min, respectively. The purified enzyme activity was stimulated by Co^{2+} , Mn^{2+} and protein disulphide reducing agents, but strongly inhibited by Hg^{2+} ions and SDS.

Mandal *et al.* (2011) an extracellular xylanase from the fermented broth of *Bacillus cereus* BSA1 was purified and characterized. The enzyme was purified to 3.43 fold though ammonium sulphate precipitation, DEAE cellulose

chromatography and followed by gel filtration through Sephadex-G-100 column. The molecular mass of the purified xylanase was about 33 kDa. The enzyme was an endoxylanase as it initially degraded xylan to xylooligomers. The purified enzyme showed optimum activity at 55°C and at pH 7.0 and remained reasonably stable in a wide range of pH (5.0-8.0) and temperature (40-65°C). The K_m and V_{max} values were found to be 8.2 mg/ml and 181.8 $\mu\text{mol}/(\text{min}/\text{mg})$, respectively. The enzyme had no apparent requirement of cofactors, and its activity was strongly inhibited by Cu^{2+} , Hg^{2+} . It was also a salt tolerant enzyme and stable up to 2.5 M of NaCl and retained its 85% activity at 3.0 M.

4.4.2 Purification and characterization of xylanase enzyme from fungal isolate

4.4.2.1 *Trichoderma longibrachiatum* T2

4.4.2.1.1 Lyophilization:

Same as in.....4.4.1.1.1

4.4.2.1.2 Ion Exchange Chromatography

Lyophilized crude enzyme was subjected to ion exchange Chromatography column DEAE cellulose. The column was packed with DEAE cellulose and the concentrated crude enzyme (2ml) was loaded on pre-equilibrated gel filtration column with the help of Pasteur pipette. Phosphate buffer pH (6.9) was used as elution buffer. A total of 60 fractions with flow rate 6min/3ml each were collected in chromatographic vials under cool environment. The optical density of each collected fraction was measured at 280 nm. The 0.2 M fractions of KCl showed highest enzyme activity by calorimetric method i.e. 13-19 (11.1, 23.0, 37.0, 41.20, 39.0, 24.0, 9.0, 0 IU) were pooled and concentrated further by lyophilization as shown in Fig. 85. The xylanase activity was checked in these active fractions. The enzyme activity of pooled fractions was checked by quantitative calorimetric estimation assays.

4.4.2.1.3 Gel Exclusion Column Chromatography

The void volume of sephadex G-100 column was calculated by loading blue dextran solution (2mg/ml) on to the column. Fractions of 2ml were collected

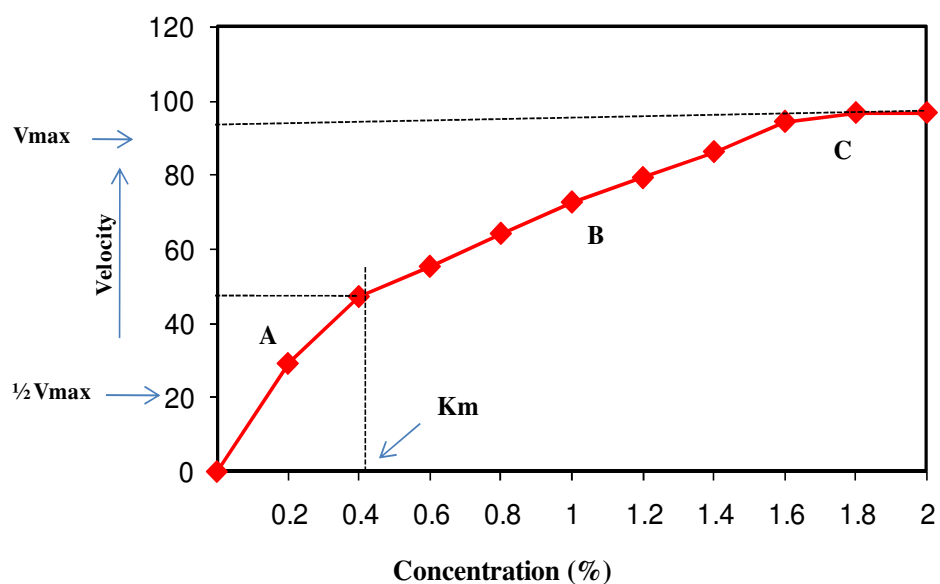


Fig 83. Effect of different Concentration (%) of substrate on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0

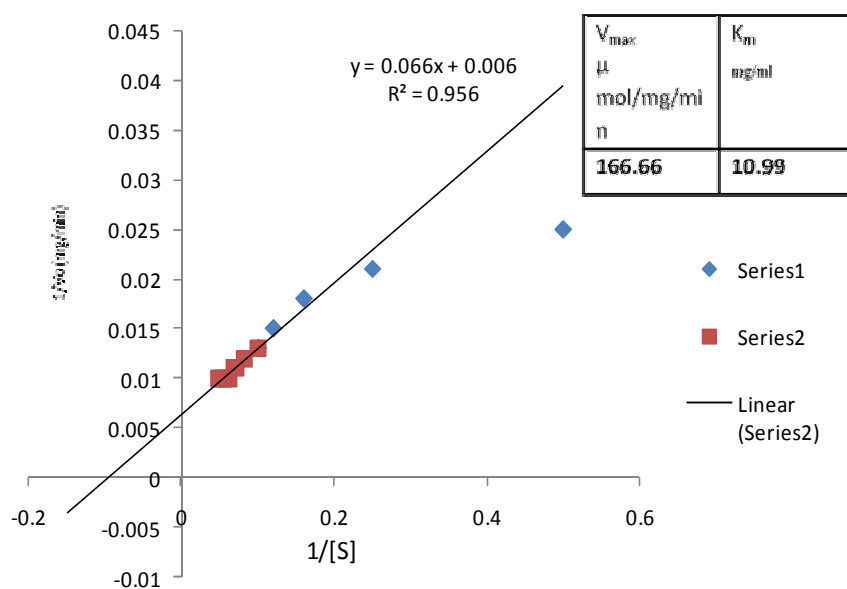


Fig 84 Lineweaver –Burk double reciprocal plot for the determination of K_m and V_{max} of partially purified xylanase from *Bacillus tequilensis* SH0

and optical density (OD) was measured at 540 nm. The void volume was found to be 66 ml. The pooled post-DEAE-cellulose xylanase fraction was then further enriched by preparative sephadex G-100 gel filtration column Chromatography. The column was packed with sephadex G-100 and the concentrated crude enzyme (2 ml) was loaded on pre-equilibrated gel filtration column with the help of Pasteur pipette. Phosphate buffer pH (6.9) was used as elution buffer. A total of 60 fractions with flow rate 6min/3ml each were collected in chromatographic vials under cool environment. The optical density of each collected fraction was measured at 280 nm. The maximum protein was observed in fraction no 8 to 16 i.e. 6.10, 11.0, 23.10, 46.20, 45.0, 33.1, 27.32, 18.0, 11.2 (Fig. 86). The xylanase activity was checked in these active fractions. These fractions having positive xylanase activity were pooled together and were lyophilized. The enzyme activity of pooled fractions was checked by quantitative calorimetric estimation assays. The lyophilized enzyme preparation was stored at (-20°C) for further use.

4.4.2.1.4 SDS–PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) *Trichoderma longibracheatum* T2

The purity and molecular weight of pooled fractions were determined by SDS-PAGE. 10µl of pooled sample + 5µl of loading dye were loaded in the wells of 10% of gel. The electrophoresis was run at 150V for 2h. After the gel was run, the standard procedure of staining and destaining was followed. Molecular weight was determined by comparing the relative mobility of standard protein molecular weight marker of 14.3 kDa–97.4 kDa. Heterogenous bands ranging of 14 kDa to 97.4 kDa of partially purified xylanase enzyme of *Trichoderma longibracheatum* T2 was observed (Plate 22). These bands may be different subunits of same enzyme on electrophoresis gel.

Table 61 showed the purification stages of partially purified xylanase by *Trichoderma longibracheatum*T2. The final recovery in ion exchange chromatography of partially purified xylanase was 1.82 fold with 61.93% yield and final specific activity of 46.58 was observed. The enzyme activity of partially purified xylanase was 5.23 fold with 23.87% yield and final specific activity of 133.89 in gel exclusion chromatography.

Table 61. Purification stages of xylanase produced by *T. longibracheatum* T2

Steps	Volume (ml)	Activity units IU/ml	Total* activity	Protein** mg/ml	Specific*** activity	Purification* fold	Yield** %
Crude	100	33.40	3340	1.43	23.36	1.0	100
Lyophilized	20	39.67	3967	1.55	25.59	1.0	100
Ion exchange	2	44.72	4472	0.96	46.58	1.82	61.93
Gel filtration	2	49.54	4954	0.37	133.89	5.23	23.87

* Total activity was determined by the multiplication of volume and activity

** Protein concentration was determined by Lowry's method

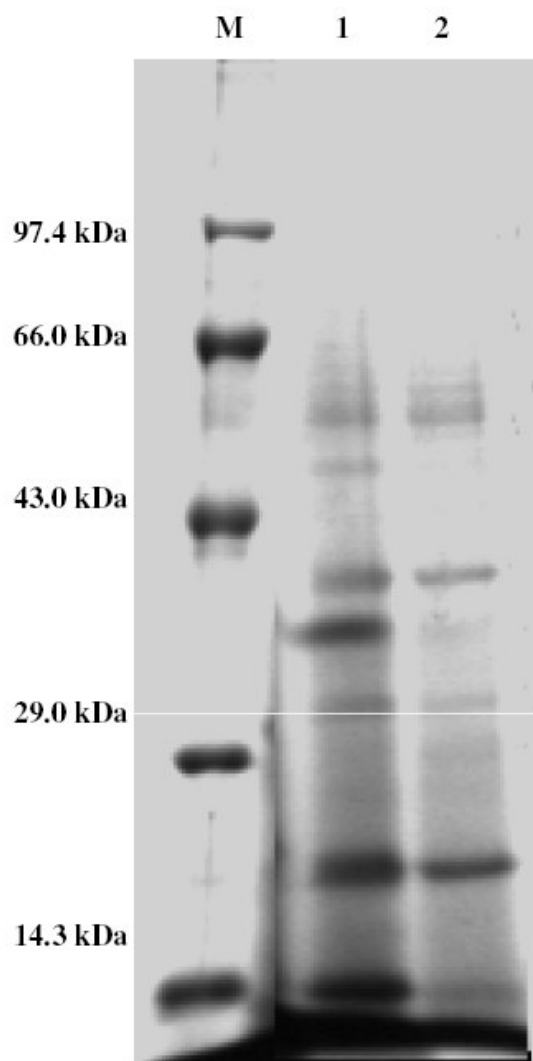
*** Specific activity is the activity unit/protein concentration

♦ Purification fold is increase in the specific activity

♦♦ Recovery % is remaining protein concentration as % of the initial protein concentration

Dobrev and Zhekova (2012) obtained an extracellular multiple form of endoxylanase was isolated from the xylanolytic complex of *Aspergillus niger* B03. The enzyme was purified to a homogenous form using ultrafiltration, anion exchange chromatography and gel filtration. It was a nonglycosylated protein with a molecular weight of 20,000 Da as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and 21,000 Da as determined by gel filtration. The optimal pH for the enzyme action was 5.0 and the optimal temperature was 55°C. Endoxylanase stability was significantly improved in the presence of glycerol and sorbitol. The enzyme activity was activated by Mn^{2+} and Co^{2+} , and it was inhibited by Ag^+ , Cu^{2+} , Fe^{3+} , Fe^{2+} , and Pb^{2+} . The substrate specificity and the product profile of the enzyme suggested that it was an endoxylanase. The enzyme showed a synergism with another endoxylanase from *A. niger* B03 in xylan hydrolysis.

Knob and Carmona (2008) purified two xylanases from the crude culture filtrate of *Penicillium sclerotium* to homogeneity by a rapid and efficient procedure, using ion exchange and molecular exclusion chromatography. Molecular masses estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were 23.9 and 33.1 kDa for xylanase 1 and 11, respectively. The native enzyme molecular masses of 23.8 and 30.8 kDa were estimated for xylanase 1 and 11, respectively by molecular exclusion chromatography. The K_m



Lane 1. = Partially purified xylanase of ion exchange chromatography
Lane 2. = Partially purified xylanase of gel chromatography
M = Marker

Plate 22. SDS PAGE of xylanase by *Trichoderma longibrachiatum* T2

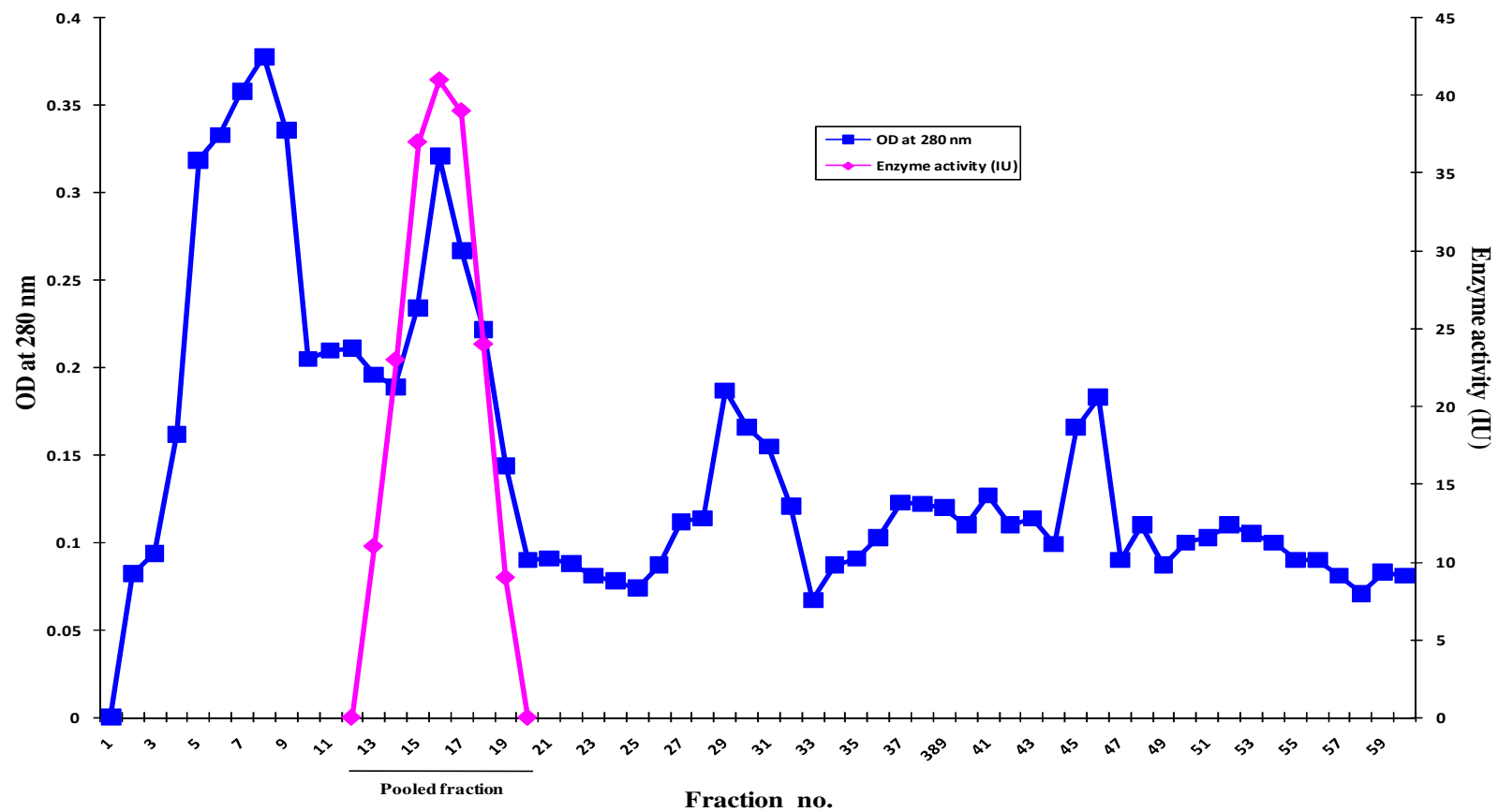


Fig 85. Ion exchange chromatography of extracellular xylanase of *Trichoderma longibracheatum* T2 on DEAE cellulose

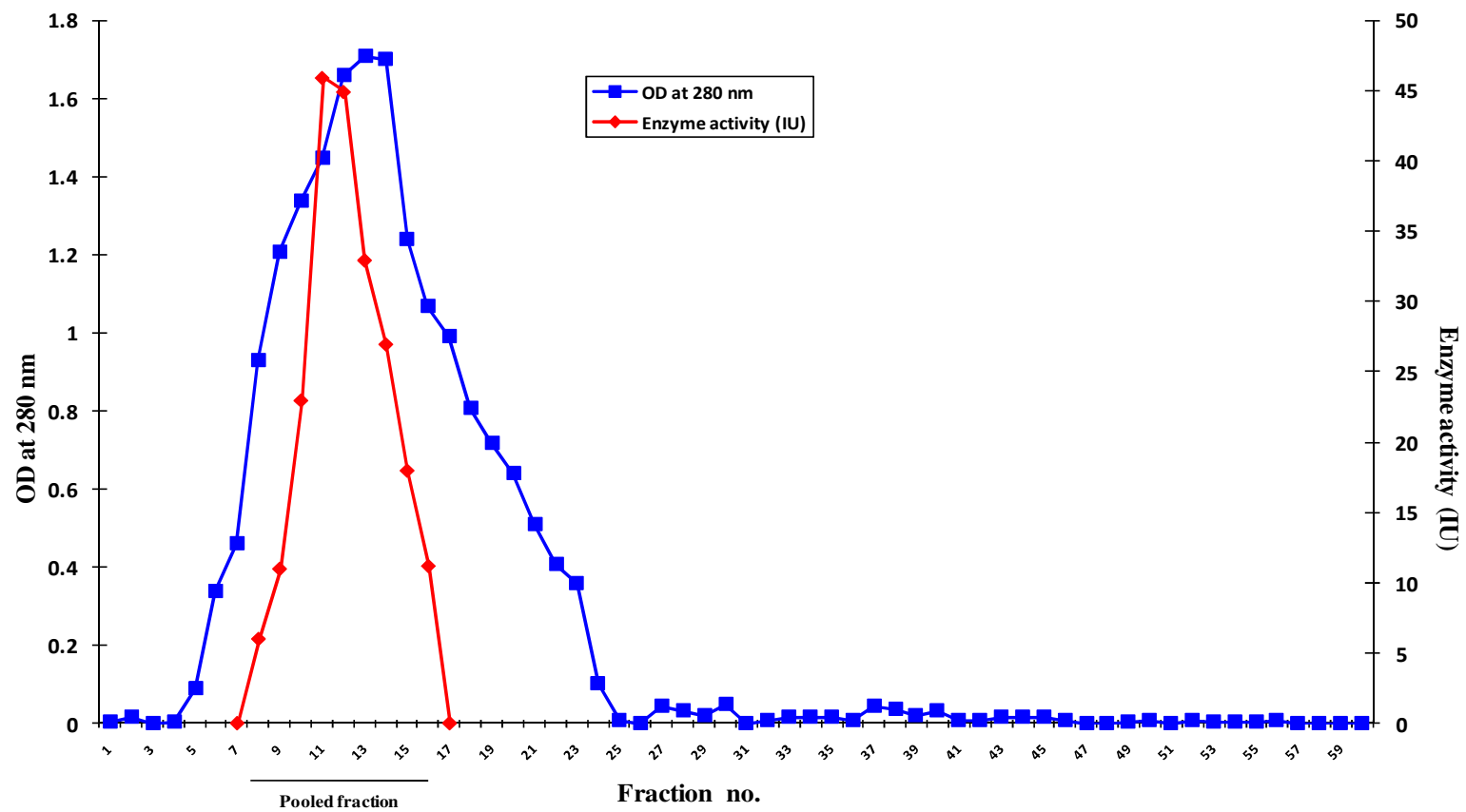


Fig 86. Gel exclusion chromatography of xylanase active fraction (obtained after ion exchange) of *Trichoderma longibrachetum* T2 on sephadex G-100

value of xylanase for birchwood xylan and for oat spelt xylan were 6.5 and 2.6 mg ml⁻¹, respectively whereas the K_m values of xylanase 11 for the substrates were 26.61 and 23.45 mg/ml.

4.4.2.1.5 Characterization of Partially Purified Xylanase

After partial purification, the enzyme was characterized to find out the conditions at which it showed best performance. The detail of parameters is given below:

4.4.2.1.5.1 Effect of pH

The effect of various pH was determined in different buffers with the pH values ranging from (3,4,5.....12) on the partially purified xylanase activity is depicted in Fig 87. Activity of the enzyme was found to be greatly affected by pH changes. The enzyme exhibited high activity in a range spanning 5.0 to 7.0. The highest enzyme activity was found at 6.0 pH (49.45 IU/ml). When the pH of the enzyme medium was change on either side of optimum range from acidic to neutral pH and alkaline pH, a decline in enzyme activity was observed.

The purified xylanase (specific activity 591 U/mg protein) was a monomeric glycoprotein with a molecular weight (MW) of 22400 as determined by SDS-PAGE by Saha (2002). The optimum pH and temperature for the action of the enzyme were at 5.0-5.5 and 55°C, respectively. The purified xylanase was fully stable at pH 5.0-7.5 and temperature up to 55°C.

Chidi *et al.* (2008) reported that maximum xylanase activity (35 U/ml) was observed after 96 h at 35°C and pH 6 in 1% oat spelt xylan from *A. terreus* UL 4209. The xylanase was purified to homogeneity by gel filtration on Sephacryl S-200. This enzyme was found to be a single subunit protein of 22 kDa showing optimal activity at 35°C and pH 6. The enzyme retained 95% activity at 35 - 40°C after 4h incubation at pH 6 and at 50°C the half-life was 5.8 h. The apparent K_m and V_{max} values were 3.57 mg/ml and 55.5 mol/min per mg protein, respectively.

Goulart *et al.* (2005) reported purified enzyme obtained from *Rhizopus stolonifer* after molecular exclusion chromatography in Sephacryl S-200H

showed optimum temperature at 45°C and pH optima at pH 6.0 and 9.0, respectively.

4.4.2.1.5.2 Effect of Temperature

The effect of various temperatures regime on the enzyme activity of partially purified xylanase was presented in Fig. 88 ranging from 30°C, 40°C, ...80°C for 10 min. It was found that the enzyme exhibited maximum activity at 60°C (57.98 IU/ml). When the temperature increased above 80°C the activity of the enzyme was affected negatively and gradually reduced. As the denaturation of the enzymatic protein occurs at elevated temperatures therefore, after certain level of temperature increase (above 80°C), the enzyme activity decreased rapidly. In nutshell, the enzyme was most efficient at 60°C as compared with other tested temperatures

Enzymes are sensitive to high temperature. Enzymes are proteineous in nature having three dimensional structure which gets distorted at high temperature and consequently gets coagulated. The breakage of weak hydrogen bonds lead to unfolding of long protein chains and this reaction is irreversible showing permanent inactivation of enzyme with increased temperature beyond a certain point.

The present results are in harmony with the recent findings of Coral *et al.* (2002) A thermostable xylanase was isolated from an *Aspergillus niger* wild type strain in a liquid Czapek Dox medium, containing oat spelts xylan as the sole carbon source. The molecular mass of the enzyme was estimated to be about 36 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimum pH for activity was found to be 7.5. The temperature optimum of the enzyme was found to be 60°C at pH 7.5. The enzyme remained stable up to 100°C, yet lost about 50% of its activity after 15 min at this temperature.

Wakiyaman *et al.* (2010) purified an extracellular endo-1,4-β- xylanase with specific activity of 566 U/mg from the culture filtrate of a filamentous fungus, *Aspergillus japonicas* strain MU-2, grown on oat spelt xylan. The purified enzyme showed a single band on SDS PAGE with an apparent m(r) of

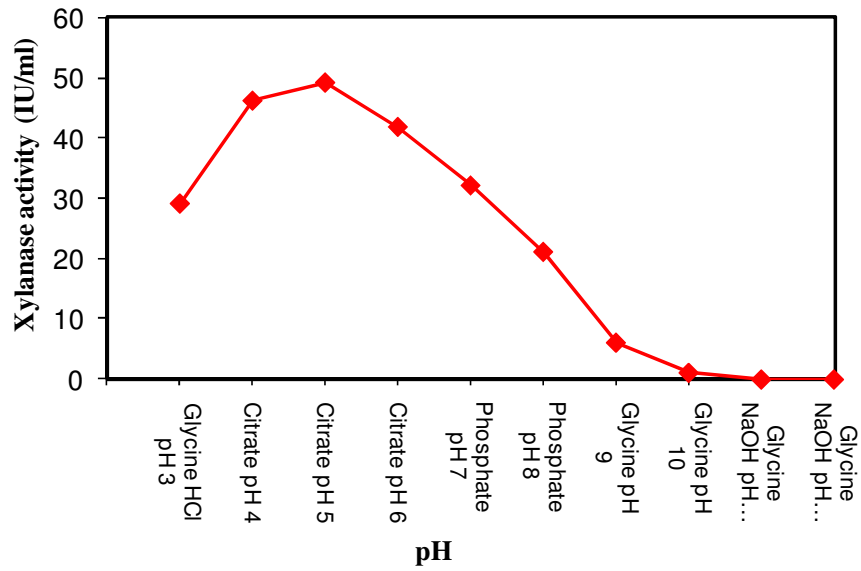


Fig 87. Effect of different pH on enzyme activity of partially purified xylanase produced by *Trichoderma longibracheatum* T2

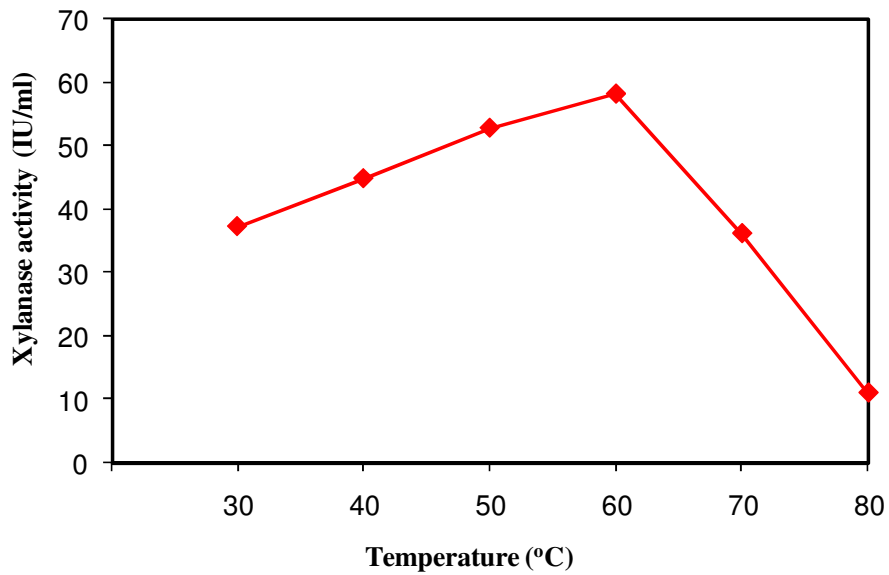


Fig 88. Effect of different temperature on enzyme activity of partially purified xylanase produced by *Trichoderma longibracheatum* T2

25.1 kDa. Xylanase activity was optimal at pH 5.0 and 60°C the xylanase gene (*xynA*) encoded a 42 residue prepropeptide and a 191 residue mature protein. The *xynA* protein showed the highest sequence identity of 69% to *Aspergillus niger*. *XynB* (Da 174549), which belongs to the glycoside hydrolase family 11.

4.4.2.1.5.3 Effect of Metal Ions

The results shows that Hg^{2+} was strong inhibitor of enzyme activity while Cu^{2+} , Na^{+} and Mg^{2+} had a slight inhibition effect on enzyme activity as depicted in Fig. 89. The inhibition of the enzyme was generally obtained with ions that reacted with sulfhydryl groups such as Hg^{2+} , suggesting that there was an important cysteine residue in or close to the active site of the enzyme. Inactivation of xylanase by Hg^{2+} has been reported by others authors also. K^{+} ions were found inert and it do not affect the activity of this enzyme. However, stimulatory effects were obtained with Zn^{2+} , Ca^{2+} and Mn^{2+} (61.33, 63.67 and 61.13 IU/ml). The moderate to high increase on xylanase activities by theses cation have also been reported in the literature.

Yang *et al.* (2010) reported that an extracellular xylanase from *Aspergillus niger* C3486 grown on a medium containing D-xylose was purified to homogeneity as indicated by disc acrylamide gel electrophoresis with an apparent molecular mass of about 25 kDa using DEAE-Sephadex A-50 and Sephadex G-100 column chromatography. The xylanase was purified 14.79 fold with 29.88% recovery. Optimal temperature and pH for activity was observed at 55°C and 5.5, respectively. The extracellular purified xylanase had K_m value and V_{max} of 0.104 mg/ml and $24.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. The metal ions Hg^{2+} and Cu^{2+} showed some inhibition effects, while Mg^{2+} and Cr^{3+} had small stimulating effects on the activity.

Sanghvi *et al.* (2010) reported that maximum xylanase production was observed in a wheat straw/rice straw ratio of 1:1, with a particle size of 0.45–0.5 mm. Media supplementation with xylose as a carbon source in a ratio 1:5 (carbon source/substrate) gave maximum activity (157 IU/ml). Nitrogen supplementations from yeast extract produced xylanase activity of 141 IU/ ml.

Optimum xylanase production was observed at 30°C and pH 5.0. Xylanase production was enhanced in the presence of Ca^{2+} and Zn^{2+} ions. Ammonium sulfate fractionation (20-80% saturation) of partially purified xylanase yielded 76.5% of the enzyme with a 3.53-fold purification. The molecular weight of xylanase was found to be ca. 29,000 Da by SDS-PAGE. These studies have shown that Ca^{2+} enhances xylanase activity, while Mn^{2+} and Zn^{2+} ions also had a positive effect on xylanase activity (Cesar and Mirsa 1996; Castro *et al.*, 1997).

Purification and characterization of thermostable xylanase from *Cereus pterogonous* plant species was reported by Vikramathithan *et al.* (2010). The enzyme was purified to homogeneity by ammonium sulfate (80%) fractionation ion exchange and size exclusion Chromatography. The enzyme showed a final specific activity of 216.2 U/g and the molecular mass of protein was 80 kDa. The optimum pH and temperature for xylanase activity were 5.0- and 80°C, respectively, with oat spelt xylan as a substrate the enzyme yielded a K_m value of 2.24 mg/ml and V_{max} of $5.8 \mu \text{mol min}^{-1} \text{mg}^{-1}$. In the presence of the metal ions (1 mM) Co^{2+} , Mn^{2+} , Ni^{2+} , Ca^{2+} and Fe^{3+} the activity of the enzyme increased, whereas strong inhibition of the enzyme activity was observed with the use of Hg^{2+} , Cd^{2+} , and Cu^{2+} while partial inhibition was noticed with Zn^{2+} and Mg^{2+} .

4.4.2.1.5.4 Effect of Substrate Specificity

The substrate specificity by the partially purified xylanase for different substrates was depicted in Fig. 90. The xylanase enzymes exhibited different specificity and activity towards various polysaccharide used here as substrates. Xylanase of *Bacillus amyloliquefaciens* SH8 strongly hydrolyzed Birch wood xylan (68.12 IU/ml) followed by oat spelt xylan (63.0 IU/ml). However, it could not hydrolyze cellulose, avicel and CMC. Thus, this enzyme is cellulase free proves the true nature of xylanase enzyme.

The results are in concord with those of Ratanachomsri *et al.* (2006) purified enzyme retained broad working pH range of 4-8 and optimal temperature of 90°C. When using xylan from birchwood as substrate, it exhibits K_m and V_{max} values of $2.6 \pm 0.6 \text{ mg/ml}$ and $428 \pm 26 \text{ U/mg}$, respectively. The enzyme rapidly

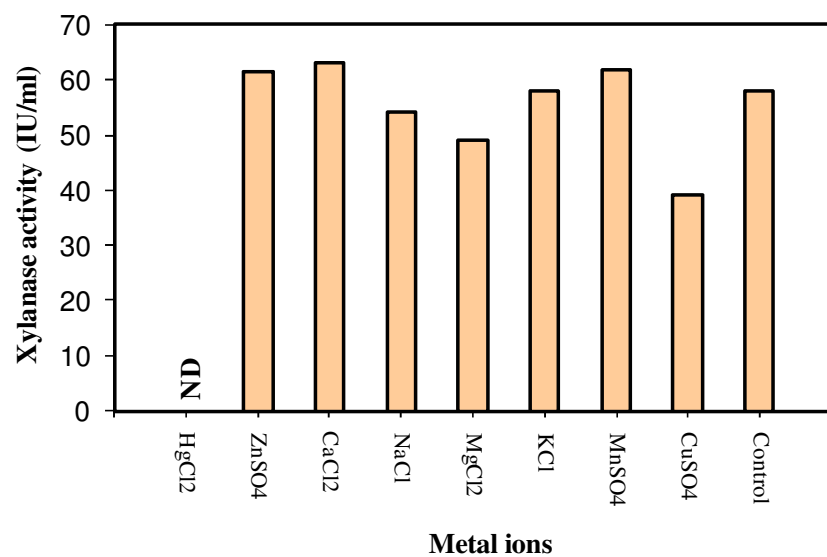


Fig 89. Effect of different metal ions on enzyme activity of partially purified xylanase produced by *Trichoderma longibrachaeum* T2

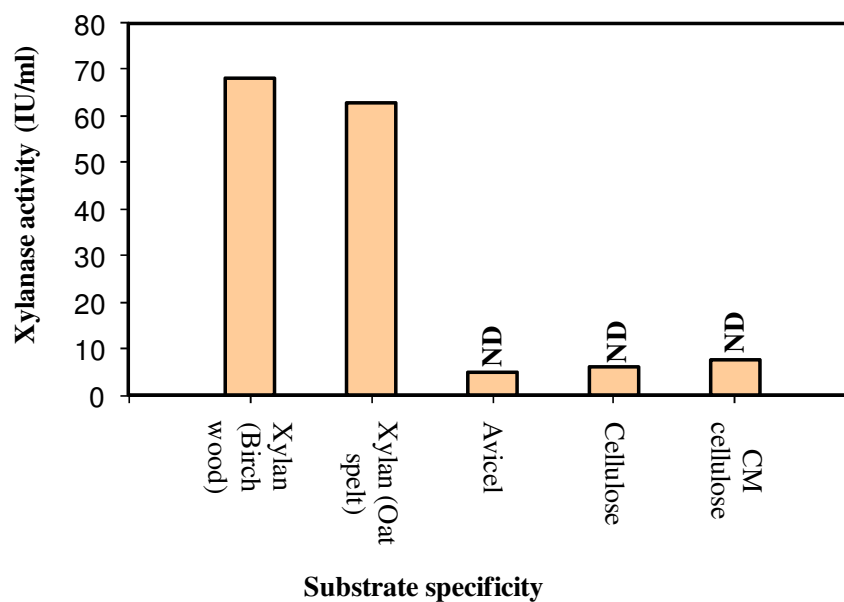


Fig 90. Effect of different substrate specificity on enzyme activity of partially purified xylanase produced by *Trichoderma longibrachaeum* T2

hydrolysed xylans from birchwood, beechwood and exhibited lower activity on xylan from wheat bran, or celluloses from carboxymethylcellulose and Avicel.

Soren *et al.* (2010) that a low molecular weight endo-xylanase (EC 3.2.1.8) was purified from an edible mushroom *Termitomyces clypeatus* grown in submerged medium with oat spelt xylan. Xylanase was purified to apparent homogeneity by ammonium sulfate fractionation and gel filtration Chromatography. Its molecular weight was determined by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 12 kDa. The enzyme was found to be most active at 50°C and pH 5.0, being most stable at pH 6.5. The K_m for oat spelt xylan was determined to be 10.4 mg/ml. The specificities of the enzyme was observed to be highly specific towards oat spelt xylan and was inhibited by mercuric chloride (HgCl_2), N-bromosuccinimide, and trans-1,2-diaminocyclohexane- $\text{N}',\text{N}',\text{N}',\text{N}'$ -tetraacetic acid strongly. The inhibitory action of N-bromosuccinimide on enzyme confirmed the presence of one tryptophan residue in its substrate-binding site.

4.4.2.1.5.5 Effect of Substrate Concentration

The effect of various substrate concentrations was determined in values ranging from (0.2, 0.4%.....2.0%) on the partially purified xylanase activity is depicted in Fig 91. Enzyme activity was found to be greatly affected by substrate concentration. The enzyme exhibited a gradual increase in enzyme activity from 0.2% to 1.8% (37.10 to 83.15 IU/ml) reaching a constant value to (83.15 IU/ml) at 1.8 and 2.0% of substrate concentration, respectively. The stability in enzyme activity at increasing concentration of reflects the attainment of saturation point of substrate furthermore leading to nil or negligible increase in enzyme activity. In general, increase in the substrate concentration gradually increases the velocity of the enzyme reaction within the limited range of substrate levels. A rectangular hyperbola is obtained when velocity is plotted against the substrate concentration. Three distinct phases of the reaction are observed as shown in figure. At low substrate concentration at phase A, the velocity of the reaction is directly proportional to the substrate level. In the second phase B, substrate concentration is not directly proportional to the enzyme activity. In the third and final phase C,

the reaction is independent of the substrate concentration by Satyanarayana (2004).

4.4.2.1.5.6 Determination of Enzyme Kinetics

The kinetic parameters of for hydrolysis of birch wood xylan were determined by plotting the initial velocities towards different concentrations of substrate as depicted in Fig. 92. The enzyme showed Michaelis-Menten behaviour, and the kinetic constant for birch wood xylan substrate was calculated. The kinetic parameters (K_m and V_{max}) were determined using Line weaver-Burk plot at 90°C (pH 6.0) with different concentrations of Birchwood xylan ranging from 2, 4,.....20 mg/ml. The K_m and V_{max} of partially purified xylanase were 1.55 mg/ml and 125.0 μ mol/mg/min. This indicates that the specificity of towards birch wood xylan substrate is high (K_m value is low).

Knob and Carmona (2008) purified two xylanases from the crude culture filtrate of *Penicillium sclerotium*. The K_m value of xylanase for birchwood xylan and for oat spelt xylan were 6.5 and 2.6 mg/ml, respectively whereas the K_m values of xylanase 11 for the substrates were 26.61 and 23.45 mg/ml.

Sohpal and Singh (2010) V_{max} and K_m obtained using Lineweaver-Burk plot method were 1.935 μ Mol/L min and 0.131 mol/L correspondingly. Enzyme activity was investigated with different buffers over wide range of pH (5.0-10.0) at room and optimum kinetic temperature indicates that pH 6.0 - 7.0 found highly suitable.

4.5 ENCODING OF CELLULASE AND XYLANASE GENE FROM CELLULOLYTIC AND XYLANOLYTIC MICROORGANISMS.

4.5.1 Encoding of Cellulase and Xylanase Gene from Bacteria

4.5.1.1 Encoding of Cellulase Gene by *Bacillus subtilis* BM-1

4.5.1.1.1 Isolation of Genomic DNA

Genomic DNA of isolates was isolated by using DNA purification kit (Banglore Genei, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The genomic DNA obtained from *B. subtilis* BM-1 isolate had a concentration of 40 ng/ μ l.

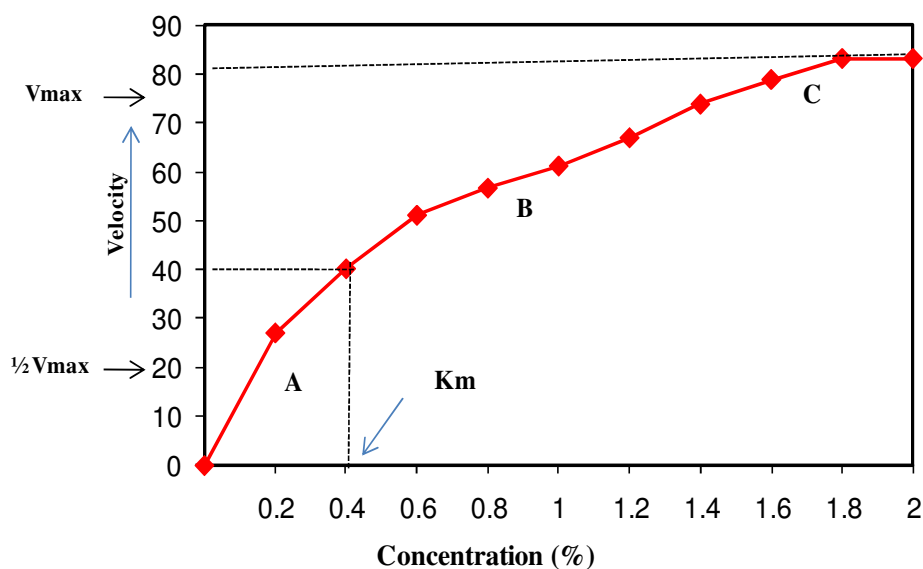


Fig 91. Effect of different concentration on Xylanase activity of *Trichoderma longibrachaeum* T2

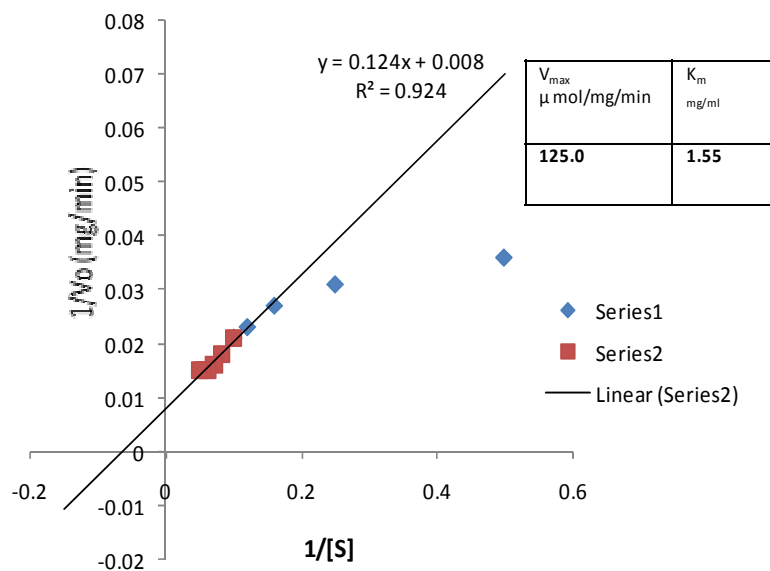


Fig 92. Lineweaver –Burk double reciprocal plot for the determination of K_m and V_{max} of partially purified xylanase from *Trichoderma longibrachaeum* T2

4.5.1.1.2 PCR Amplification

The primers were selected by taking into account nucleotide sequences of cellulase encoded genes of *Bacillus subtilis* BM-1 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. These sets of primers were used in PCR to amplify cellulase encoded gene. In total 2 set of primers were used to amplify cellulase producing gene. The amplification was achieved with one set of primer, i.e. (5'- GCGAAGGAGGAAAAGATCAG-3') (5'- 'ATAATCGCCGCTTTGTGC-3') of expected amplicon size 1432 bp using a gradient PCR reaction. Annealing temperature was varied in between 45 to 55°C to see the effect of temperature on the specific amplification. Plate 23 revealed that annealing temperature 55°C was best for the amplification of cellulase encoded gene of *Bacillus subtilis* BM-1 as a clear expected amplification product of ~1400 bp was obtained. Other sets of primers i.e. (5'- GCGAAGGAGGAAAAGATCAG-3') (5'- 'GGTTCGTGTTCCCCAAATCAG-3') that was used in PCR amplification was failed to amplify with cellulase mediated genes as nil amplification or a nonspecific amplification was observed. The amplified PCR product obtained after amplification was visualized using ethidium bromide on 2 % agarose gel (Plate 23). PCR product was purified and submitted for sequencing to Xcelaris, Ahmadabad, India.

4.5.1.1.3 Nucleotide Sequencing

Following sequences were obtained which were matched with existing gene sequences available on-line on <http://www.ncbi.nih.gov/blast>.

>BM1_60_S4766 sequence exported from BM1_60_S4766_022_F04.ab1

```
CCGGTTTGGTTATATTTTCGATTACGTTATTGACAATGGGCGGCATGCTGGCTTCGCCG
GCATCACCAGCAGGGACAAAACGCGAGTAGCCAAGAATGGCCAGCTTAGCATAAAAGGT
ACACAGCTCGTTAACCGAGACGGTAAAGCGGTACAGCTGAAGGGGATCAGTTCACACGGA
TTGCAATGGTATGGAGAATATGTCAATAAAGACAGCTTAAATGGCTGAGGGACGATTGG
GGTATCACCGTTTCCGTGCAGCGATGTATACGGCAGATGGCGGTTATATTGACAACCCG
TCCGTGAAAAATAAAGTAAAAGAAGCGGTTGAAGCGGCAAAAGAGCTTGGGATATATGTC
ATCATTGACTGGCATATCTTAAATGACGGTAACCCAAACCAAAATAAAGAGAAGGCAAAA
GAATTCTTCAAGGAAATGTCAAGTCTTTACGGAAACACGCCAAACGTCATTTATGAAATT
GCAAACGAACCAACGGTGATGTGAACTGGAAGCGTGATATTAAACCCTATGCGGAAGAA
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GTGATTTCCGTTATCCGCAAAAATGATCCAGACAACATCATCATTGTCTCGGAACCGGTACA
TGGAGCCAGGATGTGAATGATGCAGCCGATGACCAGCTAAAAGATGCAAACGTTATGTAC
GCACTTCATTTTTATGCCGGCACACACGGCCAGTTTTTACGGGATAAAGCAAACATATGCA
CTCAGCAAAGGAGCGCCTATTTTTGTGACAGAATGGGGGACAAGTGATGCTTCCGAAAT
GGCGGTGTATTCCTTGATCAATCGCGGGAATGGCTGAAATATCTCAATAGCAAGACCATT
AGCTGGGTGAACTGGAATCTTTCTGATAAGCAGAAATCATCCTCAGCTTTAAGGCCGGGG
GCATCTAAAACAGGCGGCTGGGCAGGTGTCGGATTTATCTGCTTCAAGAACATTCCGGTAG
AAGAAAACATTCTCGGCACCAAGATTTCGACGAAGGACATT

>BM_1_89_S4766 sequence exported from BM_1_89_S4766_018_H04s.eq

CATAAAGAGAAGGCAAAAGAATTTTTAAGGAAATGTCAAGTCTTTACGGAAACACGCCA
AACGTCATTTATGAAATTGCAAACGAACCAAACGGTGATGTGAACTGGAAGCGTGATATT
AAACCGTATGCGGAAGAAGTGATTTCCGTTATCCGCAAAAATGATCCAGACAACATCATC
ATTGTCTCGGAACCGGTACATGGAGCCAAGATGTGAATGATGCAGCCGATGATCAGCTAAAA
GATGCAAACGTCATGTACGCGCTTCATTTTTATGCCGGCACACACGGCCAATCTTTACGG
GATAAAGCAAACATGCACTCAGTAAAGGAGCGCCTATTTTCGTGACGGAATGGGGAAACA
AGCGACGCGTCTGGAAATGGCGGTGTATTCCTTGACCAGTCGCGGGAATGGCTGAATTAT
CTCGACAGCAAGAACATCAGCTGGGTGAACTGGAATCTTTCTGATAAGCAGGAATCATCC
TCAGCGTTAAAGCCGGGAGCATCTAAAACAGGCGGCTGGCCGCTTACAGATTAACTGCT
TCAGGAACATTCGTAAGAGAAAACATTCTCGGCAACAAAGATTCAACGAAAGAACGCCCT
GAAACGCCAGCACAAAGATAACCCCGCACAGGAAAACGGCATTCTGTACAATACAAAGCA
GGGGATGGGGGTGTGAACAGCAACCAAATCCGCCCCGAGCTTCACATAAAAAATAACGGC
AATGCGACGGTTGATTTAAAAGATGTCACTGCCCGTTACTGGTATAACGCGAAAAACAAG
GGCCAAAACCTTTGACTGTGACTACGCGCAGATTGGATGCGGCAATCTGACCCACAAATTT
GTGACGCTGCATAAACCTAAGCAAGGTGCAGATACCTATCTGGAACGGGTTTTAAACA
GGAACGCTGTCACCGGGAGCAAGCACAGGAATATTCAGCTTCGTCTTCAATGATGAC
TGGAGTAGTTATGCACAAAGCGGTGATTATTCCTTTTTTC

The obtained sequences were evaluated by entering into NCBI database and BLAST to search for previously reported sequences. The obtained sequence matched with previous reported gene sequence of *Bacillus subtilis* and it showed 97% to 98% homology with *Bacillus subtilis* (Table 62). Phylogenetic tree of *Bacillus subtilis* BM-1 with respect to other *Bacillus subtilis* bacteria as inferred by neighbor joining method has been presented in (Fig. 93). The obtained sequences were further evaluated for searching endoglucanase protein by translated nucleotide sequence by entering into NCBI database and xBLAST to search for previous reported cellulase protein sequences. The obtained gene sequences showed 98% protein homology with previous reported endo-beta-1, 4-glucanase proteins of *Bacillus subtilis* (Table 63). Thus the sequences proving the true nature of the genes responsible for cellulase production in *B. subtilis* SH8.

Table 62. Sequence alignment of cellulase gene from *Bacillus subtilis* BM-1 using BLAST

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
HM470252.1	<i>Bacillus subtilis</i> strain shu-3 endoglucanase (eglS) gene, complete cds	1714	1714	95%	0.0	98%

Table 63. Sequence alignment of cellulase protein from translated nucleotide of *Bacillus subtilis* BM-1 using xBLAST

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
ADP76528.1	endo-beta-1,4-glucanase, partial [<i>Bacillus subtilis</i>]	530	530	81%	0.0	98%

The genomic DNA of *Bacillus* sp. was isolated by Zaffer *et al.* (2011) and had been employed for the PCR amplification of cellulase gene. A PCR product of cellulase gene of 1650 bp was successfully amplified. This amplified gene was cloned using suitable vector and cellulase gene was confirmed finally.

4.5.1.2 Encoding of Xylanase Gene from *Bacillus amyloliquefaciens* SH8

4.5.1.2 Isolation of Genomic DNA

Genomic DNA of isolates was isolated by using DNA purification kit (Bangalore Genei, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The genomic DNA obtained from SH8 isolate had a concentration of 45 ng/ µl.

4.5.1.2.1 PCR Amplification

The primers were selected by taking into account nucleotide sequences of xylanase encoded genes of *Bacillus amyloliquefaciens* SH8 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. These setss of primer were used in PCR to amplify xylanase encoded gene. In total 2 set of primers were used to amplify xylanase producing gene. The amplification was achieved with both set of primers, i.e. (5'-CATGTGTATGTGCTTCCTCGTC-3') (5-

‘AATGTCCGGTATGGCTTCAG-3’) and (5’-GCAGTCATGTGTATGTGCTTCC-3’) (5-‘CTTCAGCCGTCCATTTTCG-3’) of expected amplicon size 718 bp and 709 bp using a gradient PCR reaction. Annealing temperature was varied in between 45 to 55⁰C to see the effect of temperature on the specific amplification. The annealing temperature of 55.0⁰C was best for the amplification of cellulase encoded gene *Bacillus amyloliquefaciens* SH8 as a clear expected amplification product of ~700 bp were obtained. The amplified PCR product obtained after amplification was visualized using ethidium bromide on 2% agarose gel (Plate 23). PCR product was purified and submitted for sequencing to Xcelaris, Ahmadabad, India.

4.5.1.2.2 Nucleotide Sequencing

Following sequences were obtained which were matched with existing gene sequences available on-line on <http://www.ncbi.nih.gov/blast.1>

```
>SH83_57_S4766 sequence exported from SH83_57_S4766_051_G07.ab1
GCACTGGATTGGTGTAAGGATGCAACAAACAACCCATTCTCAAGCCGCGCCAATCAC
GCCAAACGGGAAAACCATCATACAAAAGAGGAAAAACAAAAGGCGGAGCCCCGTTACTAT
ATCAATCCGAAAAGTTTTCTGTGGAATAATGGATGAACAGAAAGAGAAAAAACCGATC
GCATTGGTAACGATCGATGACGCGCCTGATCAGCATGCCGCTGAGATTGCGGACAACTG
CACGATCTCAATGCGTCAGCGATCTTTTTTGTGAACGGACATTTTTTGAAAAAGATGAA
AATAAGAAAGCGCTTAAGCATATTCATGAGTTAGGTTTTATGATCGGAAATCATACAATG
ACTCATCAAACTTAAAGATCTTTCCGAACAAAAACAACGGGAAGAAATCATTGGCGTC
AATAAAGCGGTTGAAAAGGTAATAGGAGAGAAGCCCGCATTTTTCAGGGCGCCTTTCGGA
AGCAATACAGACTACAGTGACAGACTTATAAAAAAAGAAAGGTATGGTGAAAATGAACTGG
ACCTTCGGGTATGATTGGGATCAGAAATATCAATCCAAAAAAGCGCTTACCGATATCACA
CTGAACAGTCCTTATCTGCGGGACGGGGCCAACATATTGATGCATGACCGGAAATGGACG
GCTGAAGCCTCCCCGGGACATTA
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>SH84_58_S4766 sequence exported from SH84_58_S4766_064_A08.ab1
TCCTTCCGCCCCCTCATTTTCGGTCATGCATCAATATGTTGGCCCCGTCCCGCAGATAAG
GACTGTTCAAGTGTGATATCGGTAAGCGCTTTTTTGGATTGATATTCTGATCCCAATCAT
ACCCGAAGGTCCAGTTCATTTTCACCATACCTCTTTTTTTATAAGTCTGTCACTGTAGT
CTGTATTGCTTCCGAAAGGCGCCCTGAAAAATGCGGGCTTCTCTCTATTACCTTTTCAA
CCGCTTTATTGACGCCAATGATTTCTCCCGTTGTTTTGTTTCGGAAAGATCTTTTAAGT
TTTGATGAGTCATTGTATGATTTCCGATCATAAACCTAACTCATGAATATGCTTAAGCG
CTTTCTATTTTCATCTTTTTTCAAAAAATGTCCGTTCAAAAAAAGATCGCTGACGCAT
TGAGATCGTGAGTTGTCCGCAATCTCAGCGGCATGCTGATCAGGCGCGTCATCGATCG
TTACCAATGCGATCGGTTTTTCTCTTCTGTTTCATCCATTTTTTCCACAGAAAACTTT
TCGGATTGATATAGTAACGGGGCTCCGCCTTTTGTTTTTCCTCTTTTGTATGATGGTTTT
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CCCGTTTGGCGTGATTGGCCGCGGCTTGAGAATGGGGTTTGTGTTGCATCTTTTCATT
TGATTACGGAGGCTTG

>SH83_87_S4766 sequence exported from SH83_87_S4766_049_H07.ab1

CGGCCATCGGACAGGCGGGTCATATGTTGGCGCCGTCCCGCAATAGGACTGTTTCAGTGTG
ATATCGGTAAGCGCTTTTTTGGATTGATATTTCTGATCCCAATCATACCCGAAGGTCCAG
TTCATTTTCACCATACTTCTTTTTTATAAGTCTGTCACTGTAGTCTGTATTGCTTCCG
AAAGGCGCCCTGAAAAATGCGGGCTTCTCTCCTATTACCTTTTCAACCGCTTTATTGACG
CCATGATTTCTTCCCGTTGTTTTGTTTCGGAAAGATCTTTAAGTTTTGATGAGTCATTG
TATGATTTCCGATCATAAAACCTAACTCATGAATATGCTTAAGCGCTTTCTTATTTTCAT
CTTTTTTCAAAAAATGTCCGTTACAAAAAAGATCGCTGACGCATTGAGATCGTGCAGTT
TGTCCGCAATCTCAGCGCATGCTGATCAGGCGCGTCATCGATCGTTACCAATGCGATCG
GTTTTTCTCTTTCTGTTTCATCCATTTTTTCCACAGAAAACTTTTCGGATTGATATAGT
AACGGGGCTCCGCCTTTTGTTCCTCTTTTGTATGATGGTTTTCCCGTTTGGCGTGAT
TGGCCGCGGCTTGAGAATGGGGTTTGTGTTTGCATCTTTTCATTGATTGGCAGCCGA
CGAGGAAGAAATATCACCATGAA

>SH84_88_S4766 sequence exported from SH84_88_S4766_062_B08.ab1

TGCAGTCTCAAGTTATGTGCTTCCTCGCCGGCTGCCAATCAAATGAAAAGATGCAAACAA
ACAAACCCATTCTCAAGCCGCGGCCAATCACGCCAAACGGGAAAACCATCATACAAAAG
AGGAAAAACAAAAGGCGGAGCCCCGTTACTATATCAATCCGAAAAGTTTTTCTGTGAAA
AAATGGATGAACAGAAAGAGAAAAAACCGATCGCATTGGTAACGATCGATGACGCGCCTG
ATCAGCATGCCGCTGAGATTGCGGACAACTGCACGATCTCAATGCGTCAGCGATCTTTT
TTGTGAACGGACATTTTTTGAAAAAAGATGAAAATAAGAAAGCGCTTAAGCATATTCATG
AGTTAGGTTTTATGATCGGAAATCATACAATGACTCATCAAACTTAAAAGATCTTTCCG
AACAAAAACAACGGGAAGAAATCATTGGCGTCAATAAAGCGGTTGAAAAGGTAATAGGAG
AGAAGCCCGCATTTTTCAGGGCGCCTTTCGGAAGCAATACAGACTACAGTGACAGACTTA
TAAAAAAGAAGGTATGGTGAAAATGAACTGGACCTTCGGGTATGATTGGGATCAGAAAT
ATCAATCCAAAAAGCGCTTACCGATATCACTGAACAGTCCTATCTCCGGATCGGCC
AATCTTCTGTCC

The obtained sequences were evaluated by entering into NCBI database and BLAST to search for previously reported sequences. The obtained sequence matched with previous reported gene sequence of *Bacillus amyloliquefaciens* and it showed 96% homology with *Bacillus amyloliquefaciens* (Table 64). Phylogenetic tree of *Bacillus amyloliquefaciens* SH8 with respect to *Bacillus amyloliquefaciens* bacteria as inferred by neighbor joining method has been presented in (Fig. 94). The obtained sequences were further evaluated for searching xylanase protein by translated nucleotide sequence by entering into NCBI database and xBLAST to search for previous reported xylanase protein sequences. The obtained gene sequences showed 97% protein homology with

previous reported endo-1,4-beta-xylanase D protein of *Bacillus amyloliquefaciens* Y2 and polysaccharide deacetylase protein of *Bacillus amyloliquefaciens* sub sp. *plantarum* (Table 70). Showing a strong correlation between xylanase gene of *B. amyloliquefaciens* SH8 and consequently related protein translated from nucleotide sequence from this strain.

Table 64. Sequence alignment of xylanase gene from *Bacillus amyloliquefaciens* SH8 using BLAST

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
CP000560.1	<i>Bacillus amyloliquefaciens</i> FZB42, complete genome	1098	1098	97%	0.0	96%

Table 65. Sequence alignment of xylanase protein from translated nucleotide by *Bacillus subtilis* BM-1 using xBLAST

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
YP_006328295.1	endo-1,4-beta-xylanase D [<i>Bacillus amyloliquefaciens</i> Y2] >gblAFJ61570.1 endo-1,4-beta-xylanase D [<i>Bacillus amyloliquefaciens</i> Y2]	423	423	94%	3e-147	97%
EKE47070.1	polysaccharide deacetylase [<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> M27]	435	435	94%	2e-152	97%

Similar, work to sequence glucanase enzyme and xylanase enzyme was from *B. subtilis* has been reported by Lu *et al.* (2006). Sequencing of xylanase gene from different *Bacillus* sp. and further express them successfully in clones has been cited in literature to improve either enzyme production or to construct a clone of desirable characteristics for various applications in relevant industries. Jalal *et al.* (2009) used specific primers of xylanase gene of *B. subtilis* strain R5 gene resulted in the amplification of 0.65 kbp DNA. The PCR amplified DNA fragment was ligated into cloning vector and then *E. coli* cells were transformed.

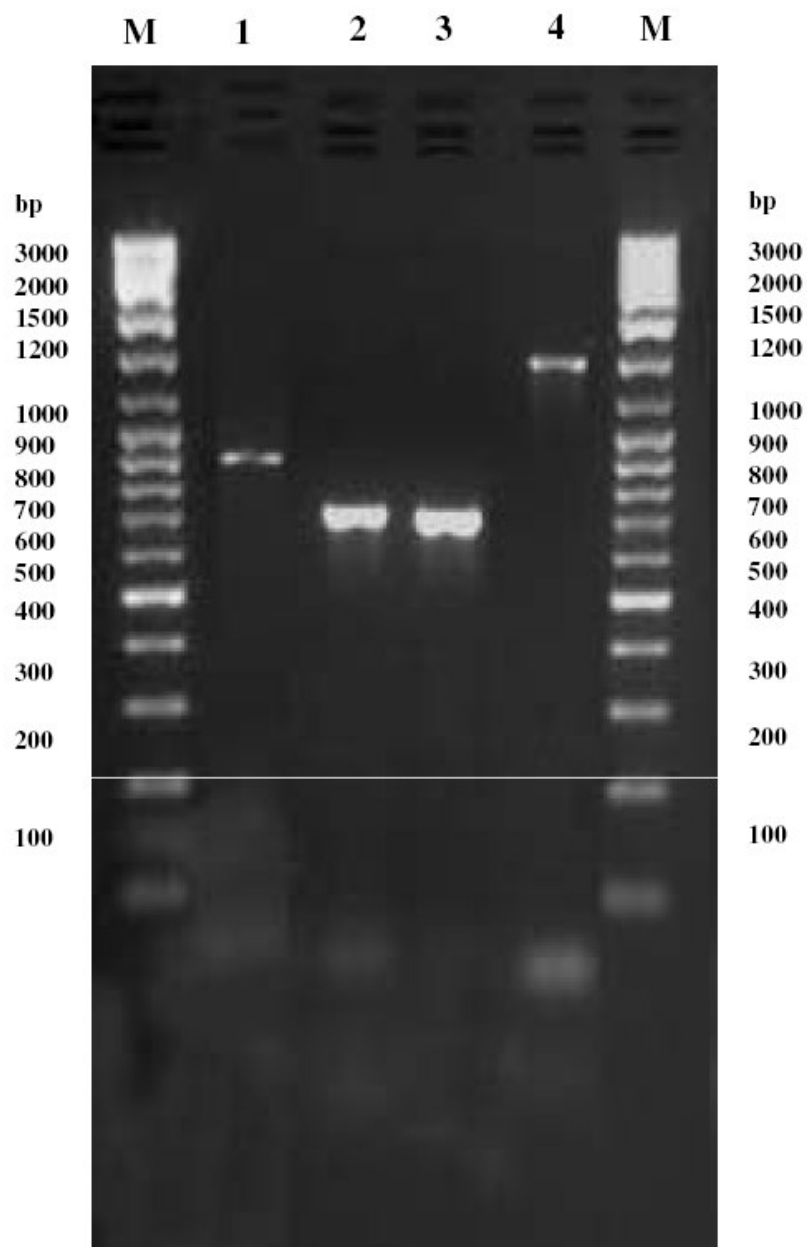


Plate 23 PCR amplification of chromosomal DNA

Lane 1. = *Rhizopus oryzae* W2
 Lane 2. = *Bacillus amyloliquifacins* SH8
 Lane 3. = *Bacillus amyloliquifacins* SH8
 Lane 4. = *Bacillus Subtilis* BM-1
 M = Marker

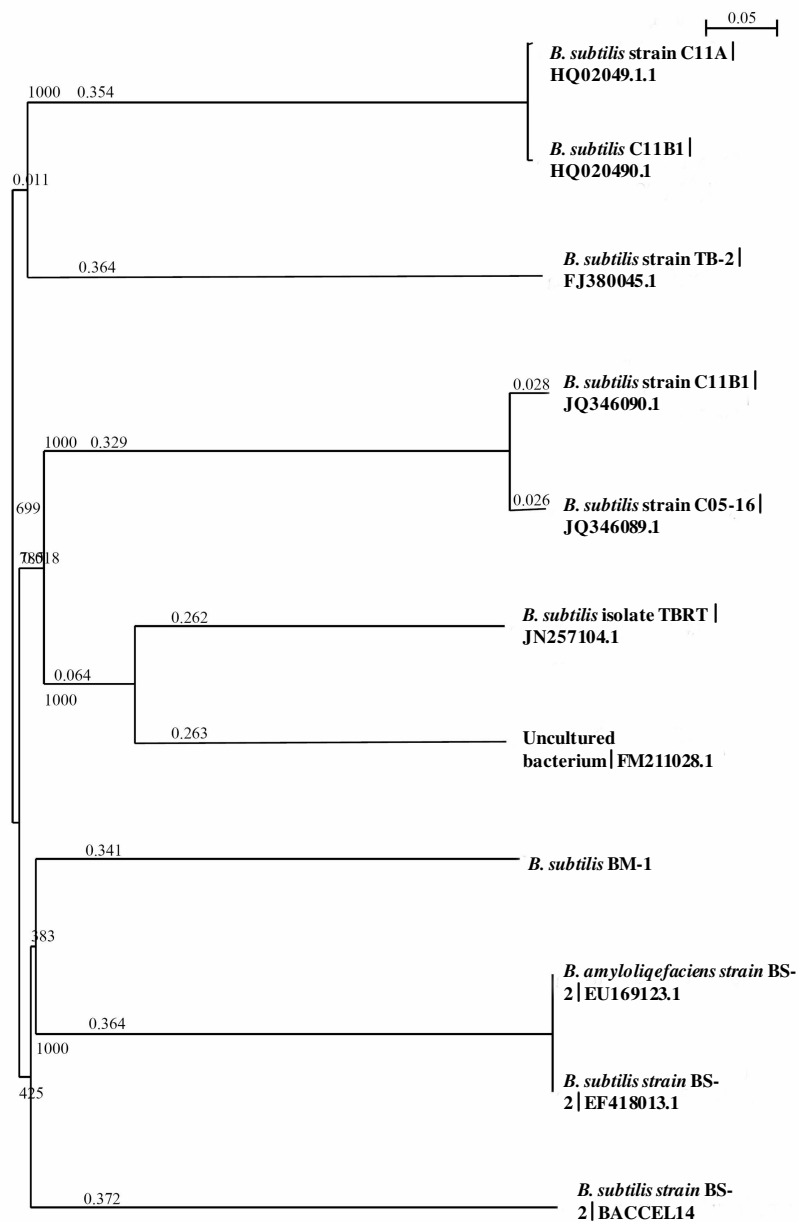


Fig 93. Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *B. subtilis* among the genus *Bacillus* based on a distance matrix analysis of endoglucanase gene sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

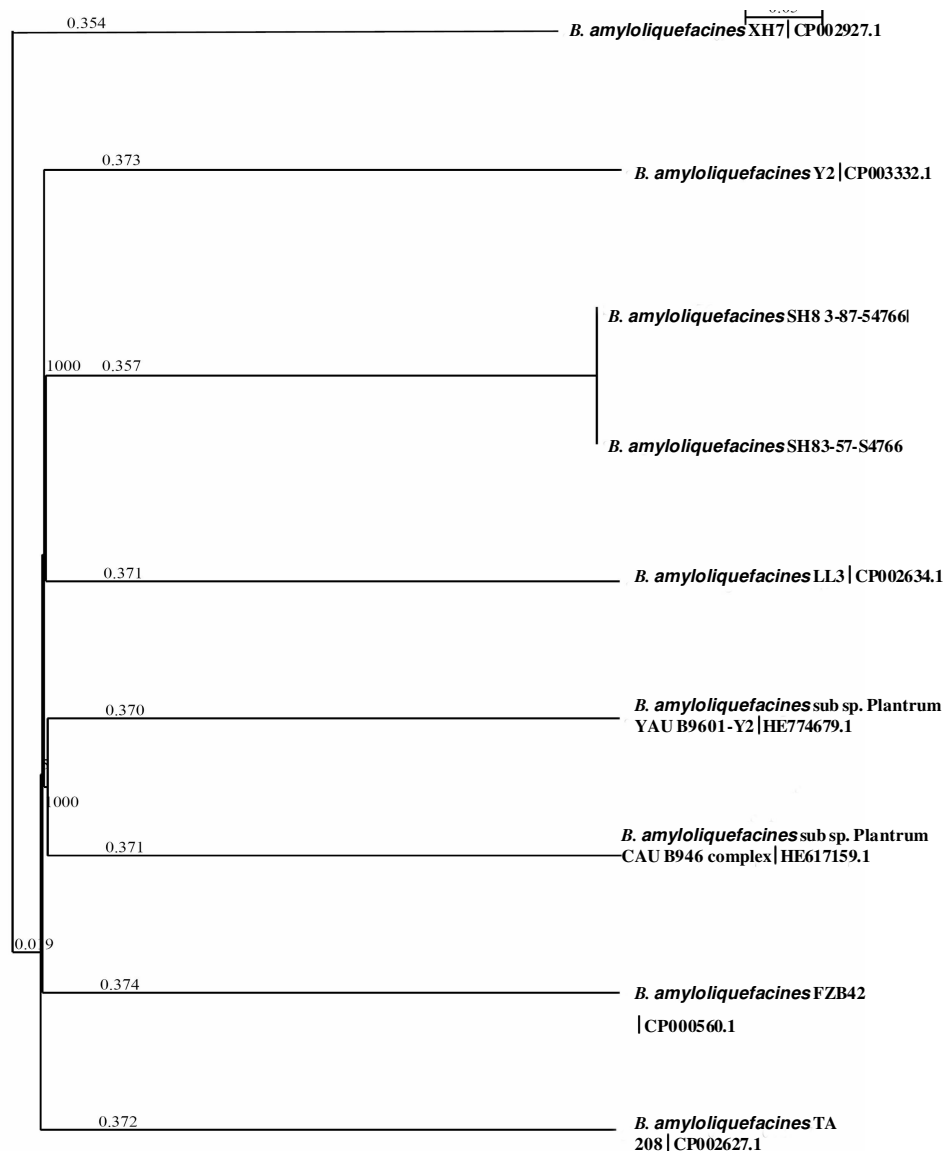


Fig 94. Neighbor-joining tree constructed in Clustal X showing phylogenetic relationship of *B. amyloliquefacines* SH8 among the genus *Bacillus* based on a distance matrix analysis of *xyn* gene sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

4.5.1.3 Encoding of Xylanase Gene from *Bacillus tequilensis* SH0

4.5.1.3.1 Isolation of Genomic DNA

Genomic DNA of isolates was isolated by using DNA purification kit (Banglore Genei, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The genomic DNA obtained from SH0 isolate had a concentration of 22 ng/ µl.

4.5.1.3.2 PCR Amplification

The primers were selected by taking into account nucleotide sequences of xylanase encoded genes of *Bacillus tequilensis* SH0 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma-Genosys. These sets of primers were used in PCR to amplify xylanase encoded gene. In total 2 set of primers were used to amplify xylanase producing gene. The multiple amplification was achieved with both set of primers, i.e. (5'-GGGGAAGGCTGGCTTATATC-3') (5'-ATGGTGTGCCTTCACTTCC-3') and (5'-AGGGCAACCTCATGTCAAAG-3') (5'-GTTAGCGGCAGTTCTTCGAG-3') of expected amplicon size 509 bp and 2020 bp using a gradient PCR reaction. Annealing temperature was varied in between 45 to 55°C to see the effect of temperature on the specific amplification (Plate 24). No specific amplification was observed in both the set of primers for xylanase gene.

4.5.2 Encoding of cellulase and xylanase gene from fungi

4.5.2.1 Encoding of Cellulase Gene from *R. oryzae* W2

4.5.2.1.1 Isolation of Genomic DNA

Genomic DNA of isolates was isolated by using DNA purification kit (Quaigen, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The genomic DNA obtained from *R. oryzae* W2 isolate had a concentration of 150 ng/µl.

4.5.2.1.2 PCR Amplification

The primers were selected by taking into account nucleotide sequences of cellulase encoded genes of *R. oryzae* W2 available after searching in the Gene

Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. These sets of primers were used in PCR to amplify cellulase encoded gene. In total 2 set of primers were used to amplify cellulase producing gene. The amplification was achieved with one set of primer, i.e. (5'- ACCAACTTGACCGAGCTCAC-3') (5'- TTCTGTATCCCGGGTCTGAG-3') of expected amplicon size 654 bp using a gradient PCR reaction. Annealing temperature was varied in between 45 to 55⁰C to see the effect of temperature on the specific amplification. Plate revealed that annealing temperature 45.7⁰C was found best for the amplification of cellulase encoded gene of *R. oryzae* W2 as a clear expected amplification product of ~700 bp was obtained. Other set of primers i.e. (5'- CCGAGAGCTTGGACCTACTG -3') (5'- CAGCTCGCCCTTTCTGTATC-3') that was used in PCR amplification was failed to amplify with cellulase mediated genes as nil amplification or a nonspecific amplification was observed. The amplified PCR product obtained after amplification was visualized using ethidium bromide on 2% agarose gel (Plate 23 and 25). PCR product was purified and submitted for sequencing to Xcelaris, Ahmadabad, India.

4.5.2.1.3 Nucleotide Sequencing

Following sequences were obtained which were matched with existing sequences available on-line on <http://www.ncbi.nih.gov/blast>.

>W2_63_S4766 sequence exported from W2_63_S4766_020_G04.ab1

```
GAGAATATCCTTTTCATCATTGTTCTCGGAGCCTCGGTGGATCTCCTGCAAGGACGCGGA
CAACTTCTATCGGTGCCCCCTCGGTGCGGTATTGCAGACAAAGGACCGAGGCGACTCAAC
TTTAATTGAGTCCCTGCATCCACTCATGCCTTTTTTCGCCGGATCTTGGGCTCCACCCATG
CTATTGATATCTCTCGGTGAGAGATTCCGACCGGATCTGACCCCGGTGATCTGGGCCC
ATCCGGGCGAGCTCTATAACTGACCTCCGCACTAGGCTAACGCACTGAACGTAAGTACTGGGAC
CGAATCCAAGTATGTATGTTGTCTTGATACTCTCTTTGGACCTCCATCGCCTTCTGCTA
GTCTTGATCTATCCATAACATTCCTCCGGCTGGCCTCAAGCACCTCCCTGTGATACTGC
CGGATGCTCTCGACCGATAAATCTCCGGTATGATCGGTGTGTTGCAACACCCGGTGAGAG
AGGTTGGATCGAACCCTGGACTCAGTTGTCTCTCAGATCCATCAAACCTGGCTCTGCAGT
ACCGATCGACTTAAGCACTGATCGATGGTCTCCACTCCCGTGCAAGAGATCCCCCAGTAT
CGAGGATCCCCTCTAATTGCTGTTGCTGACCGACCGTGAGCTCGGAAAATTTTGAAA
```

>W-2_64_S4766 sequence exported from W-2_64_S4766_047_A05.ab1

```
GCCCATTTTCCCAGAGCCTCGTTATTTCATTGCCCAATTCTTGCCGAGAGGGATCTGTTG
TGTCAGCATTGTCAAGTTGGTAGAGGGTGGTGTAAAGCGAAAATCCTACCATGGTCTGTTT
```

CTATTACCATTGCCATTTTATCATTTCCAGGACGATCAACTGTCTTCATCCACCAACTTA
CAGTCAACGTGTGAGCTCCCTCAAGTTGGTTGTTGAAGACAGTAGACCGTCTGGGAACT
GTTAAAACGGCCATGTTTCCCTCCACAGACCACATAAAGAACTTTGCCCATCCTATTTC
CTATATTTATCCCTCCACCTCATAT

The obtained sequences were evaluated by entering into NCBI database and BLAST to search for previously reported sequences. The obtained sequence matched with previous reported gene sequence and it showed 93% homology with *Aspergillus flavus*, *Nocardia brasiliensis* and *Faecalibacterium prausnitzii* (Table 66). Phylogenetic tree of *R. oryzae* W2 with respect to other fungus as inferred by neighbor joining method have been presented in (Fig 95). The obtained sequences were further evaluated for cellulase protein by translated nucleotide sequence by entering into NCBI database and xBLAST to search for previous reported cellulase protein sequences. The obtained gene sequences showed 58% protein homology with previous reported hypothetical protein of *Rhizopus deleamar* RA (Table 67).

A gene (cbh2) encoding cellobiohydrolase II was isolated from the fungus *Talaromyces emersonii* by rapid amplification of cDNA ends techniques and the equivalent genomic sequence was subsequently cloned by Murray *et al.* (2003).

Table 66. Sequence alignment of cellulase gene from *R. oryzae* W2 using BLAST

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
XM 002379043.1	<i>Aspergillus flavus</i> NRRL3357 hypothetical protein, mRNA	42.8	42.8	4%	3.5	93%
CP003876.1	<i>Nocardia brasiliensis</i> ATCC 700358, complete genome	42.8	42.8	4%	3.5	93%
FP929045.1	<i>Faecalibacterium prausnitzii</i> L2/6 draft genome	42.8	42.8	4%	3.5	93%

Table 67. Sequence alignment of cellulase protein from translated nucleotide by *R. oryzae* W2 using xBLAST

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
EIE81297.1	hypothetical protein RO3G_06002 [Rhizopus deleamar RA 99-880]	84.3	226	93%	2e-15	58%

4.5.2.2 Encoding of cellulase Gene from *T. longibracheatum* T2

4.5.2.2.1 Isolation of Genomic DNA

Genomic DNA of isolates was isolated by using DNA purification kit (Quaigen, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The genomic DNA obtained from T2 isolate had a concentration of 300 ng/ µl.

4.5.2.2.2 Isolation of Endoglucanase Gene I from *Trichoderma longibracheatum* T2

The primers were selected by taking into account nucleotide sequences of endoglucanase gene I encoded genes of *Trichoderma longibracheatum* T2 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. These sets of primers were used in PCR to amplify endoglucanase gene I encoded gene. In total 2 set of primers were used to amplify endoglucanase gene I producing gene. The multiple amplification was achieved with both set of primers, i.e. (5'-TCAGCTACACTGCCGTTGAC-3') (5-'GGCATTGCGAGTAGTCTGTG-3') and (5'-CATCTCAAACGCCTCCATC-3') (5-'AGGCATTGCGAGTAGTCTGTG-3') of expected amplicon size 1522 bp and 1553 bp using a gradient PCR reaction. Annealing temperature was varied in between 45 to 55°C to observe the effect of temperature on the specific amplification (Plate 26). Non specific amplification was observed in both the set of primers for endoglucanase gene I.

4.5.2.2.3 Isolation of Cellobiohydrolase II Gene from *Trichoderma longibrachatum* T2

The primers were selected by taking into account nucleotide sequences of Cellobiohydrolase II gene encoded genes of *Trichoderma longibracheatum* T2 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. These sets of primers were used in PCR to amplify Cellobiohydrolase II gene encoded gene. In total 2 set of primers were used to amplify Cellobiohydrolase II producing gene.

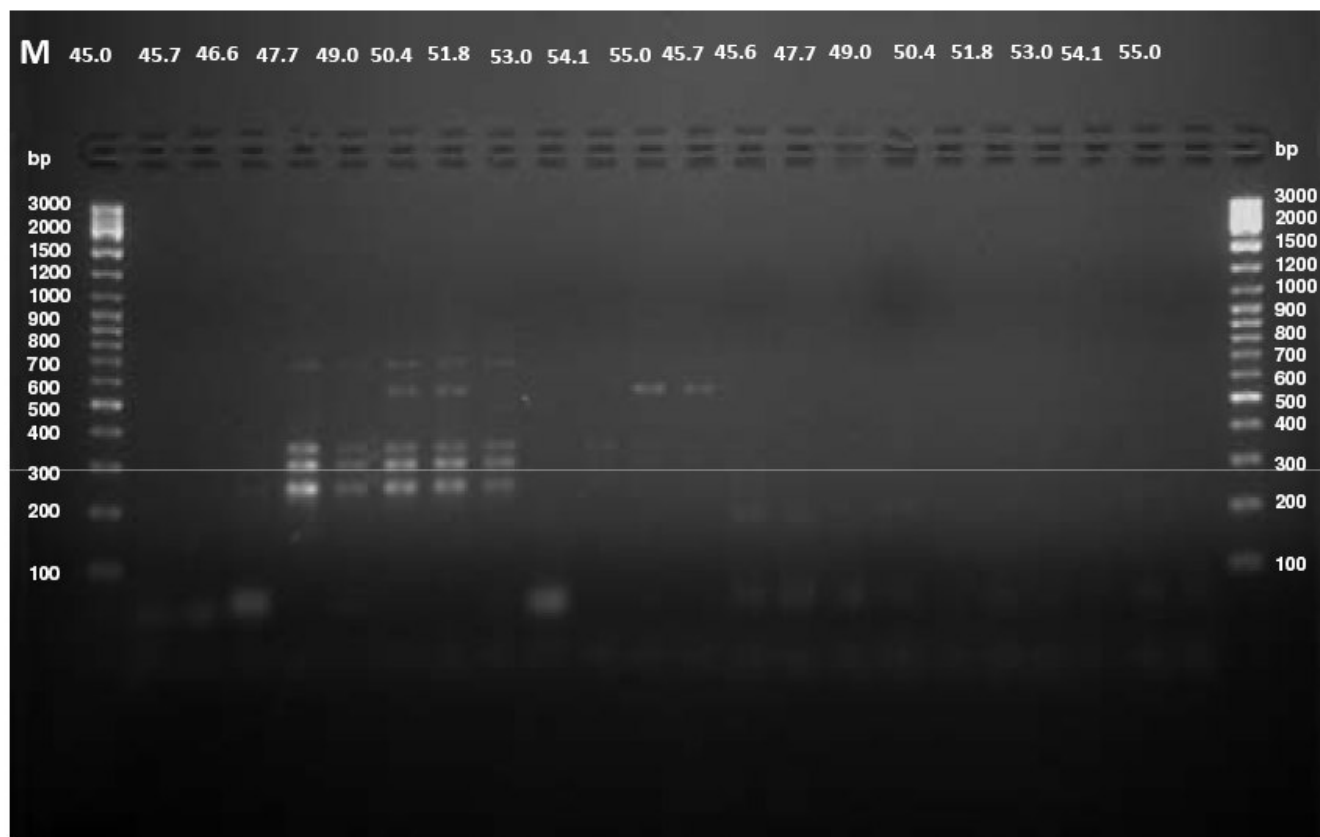


Plate 24. PCR amplification gene encoding of xylanase gene of chromosomal DNA of *Bacillus tequilensis* SH0 at different annealing temperatures

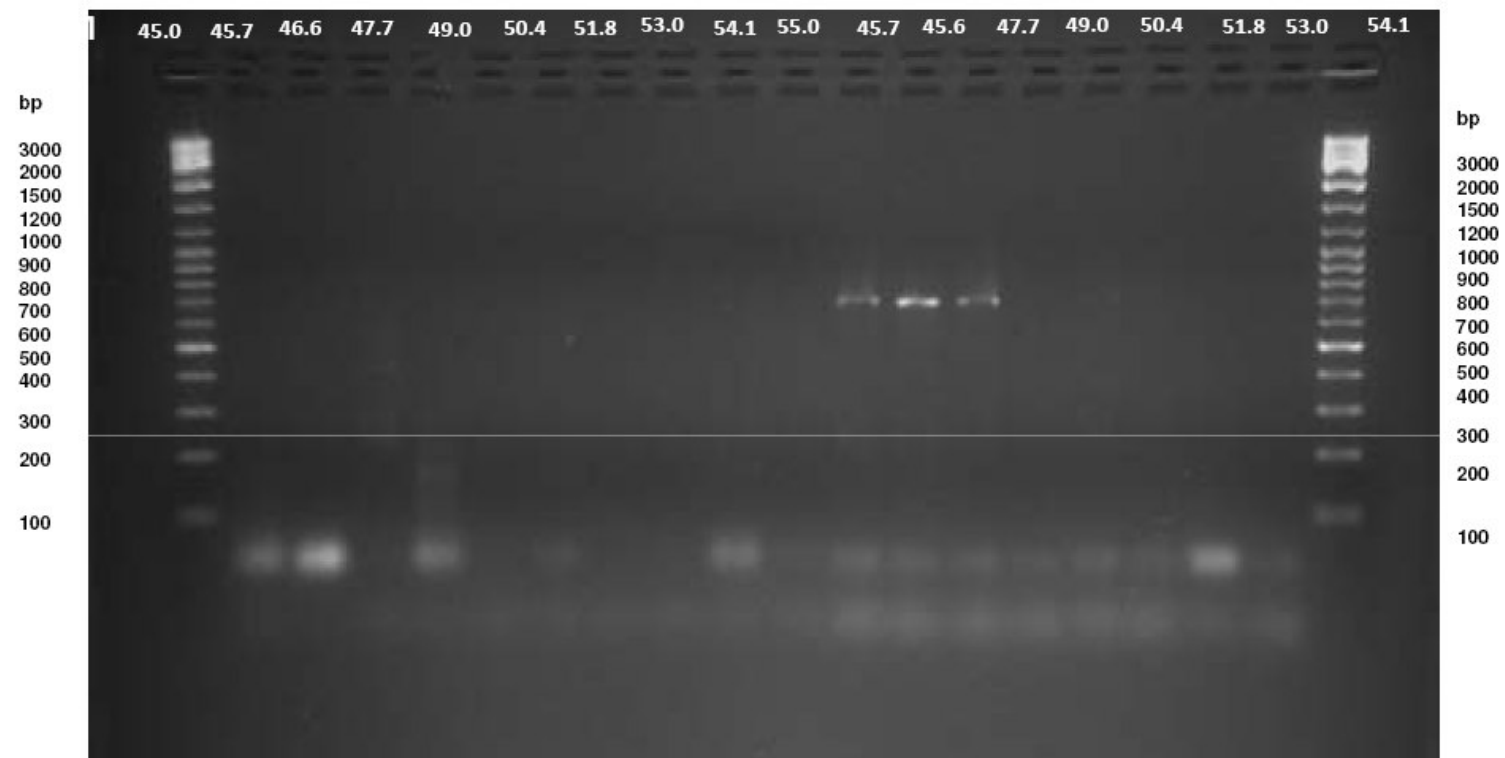


Plate 25. PCR amplification of gene encoding of cellulase gene chromosomal DNA of *Rhizopus oryzae* W2 at different annealing temperatures

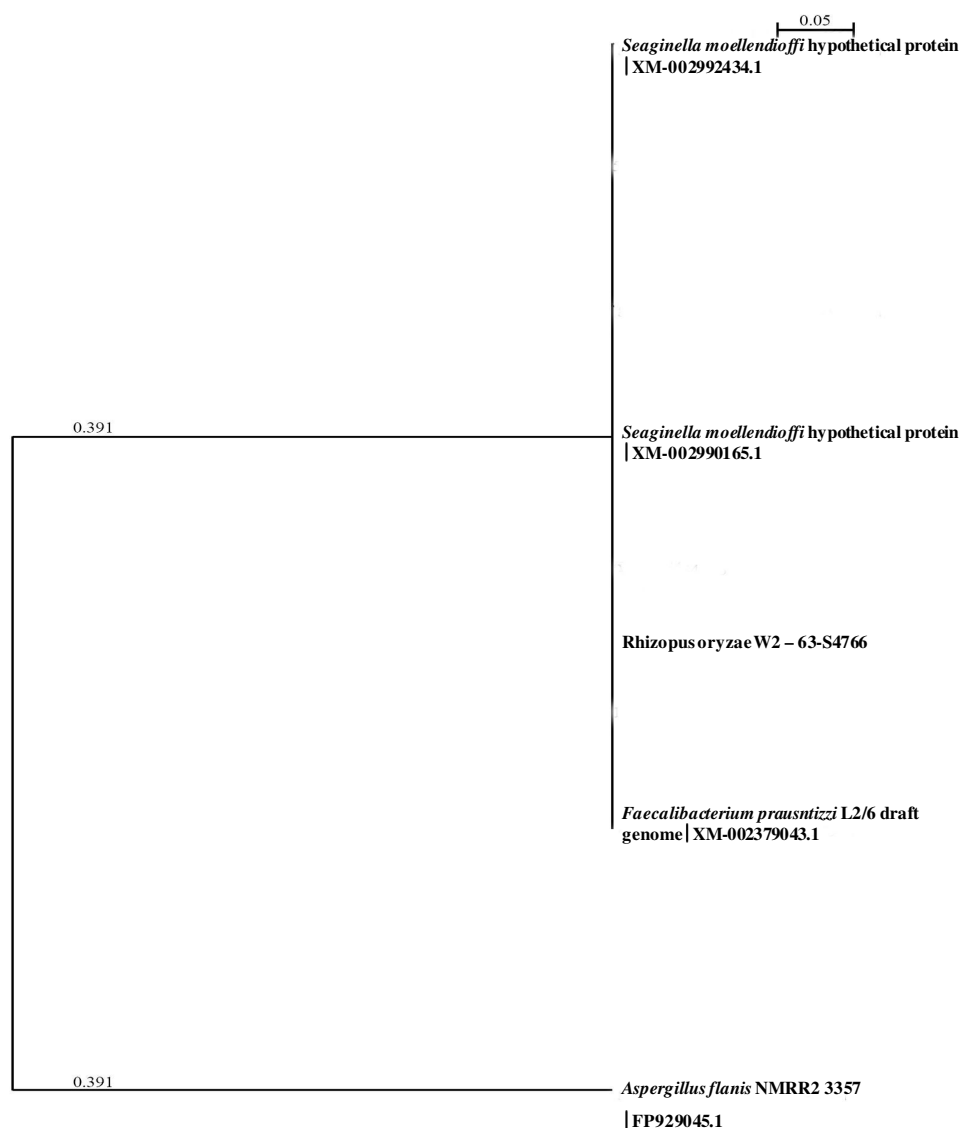


Fig 95. Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *R. oryzae* W2 among the genus *Rhizopus* based on a distance matrix analysis of endoglucanase gene sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

The multiple amplification was achieved with both set of primers, i.e. (5'-ATTCTCACCACGCTGGCTAC-3') (5'-TACAGGAACGATGGGTTTGC-3') and (5'-GCTGCACAAAGTAGGCTTGG-3') (5'-ATTGTCGGCATTCTCACCAC-3') of expected amplicon size 1571 bp and 1540 bp using a gradient PCR reaction. Plate 27 showed that annealing temperature was varied in between 45 to 55°C to see the effect of temperature on the specific amplification. Non specific amplification was observed in both the set of primers for Cellobiohydrolase II gene.

4.5.2.2.4 Isolation of β -glucosidase Gene from *Trichoderma longibrachaeum* T2

The primers were selected by taking into account nucleotide sequences of β -glucosidase gene encoded genes of *Trichoderma longibrachaeum* T2 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. These sets of primer were used in PCR to amplify β -glucosidase gene encoded gene. In total 2 set of primers were used to amplify β -glucosidase producing gene. The multiple amplification was achieved with both set of primers, i.e. (5'-TCAAGCTCTTTGCGCTCTTC-3') (5'-GCCCAAGGACTTTCAATGG-3') and (5'-AACGCTTCTGCCCATTTCTC-3') (5'-GACTTTCAATGGGGGTTTGC-3') of expected amplicon size 1400 bp and 1414 bp using a gradient PCR reaction. Plate 28 showed annealing temperature was varied in between 45 to 55°C to see the effect of temperature on the specific amplification. Non specific amplification was observed in both the set of primers for β -glucosidase gene.

4.5.2.2.3 Encoding of Xylanase Gene from *Trichoderma longibrachaeum* T2

The primers were selected by taking into account nucleotide sequences of xylanase gene encoded genes of *Trichoderma longibrachaeum* T2 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. These sets of primer were used in PCR to amplify xylanase gene encoded gene. In total 2 set of primers were used to amplify xylanase producing gene. The multiple amplification was achieved with both set of primers, i.e. (5'-GACAGCAAGCTCAACTGCATAG-

3') (5-'GGTAGTTCATCTGCCCAAGG-3') and (5'-CAGCAAGCTCAACT GCATAG-3') (5-'GCATTGAAGTGGTTCTGCAC-3') of expected amplicon size 747 bp and 704 bp using a gradient PCR reaction. Plate 29 showed annealing temperatures were varied in between 45 to 55°C to see the effect of temperature on the specific amplification. Non specific amplification was observed in both the set of primers for xylanase gene.

4.6 SCALE UP OF XYLANASE ENZYME OF *B. amyloliquefaciens* SH8

4.6.1 Scale up and Optimization of Xylanase Production by *B. amyloliquefaciens* SH8 in a Stirred Tank Bioreactor

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully the selected micro organisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution. Due to the development of large-scale fermentation technologies, the production of microbial enzymes accounts for a significant proportion of the biotechnology industry total output. Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic metres.

Xylanase enzyme is one among commercially important hydrolytic enzymes. These are used in biofuel production, paper and pulp industry, food industry, etc. Therefore large scale production of xylanase enzyme system is required. The intensive work is going on cellulase free xylanase production worldwide but low product yield, feedback inhibition and expensive xylan act as bottleneck in xylanase for large scale production. Since *B. amyloliquefaciens* SH8 has short fermentation time and additionally, being thermophilic in nature it has been selected for scale up process as thermophilic microorganisms generally synthesize heat stable enzyme.

4.6.1.1 Effect of Inoculum Age on Xylanase Production by *B. amyloliquefaciens* SH8 in a Stirred tank Bioreactor

The inoculum age has a pronounced effect on the xylanase production. The Inoculum age plays an important role in the production of xylanase

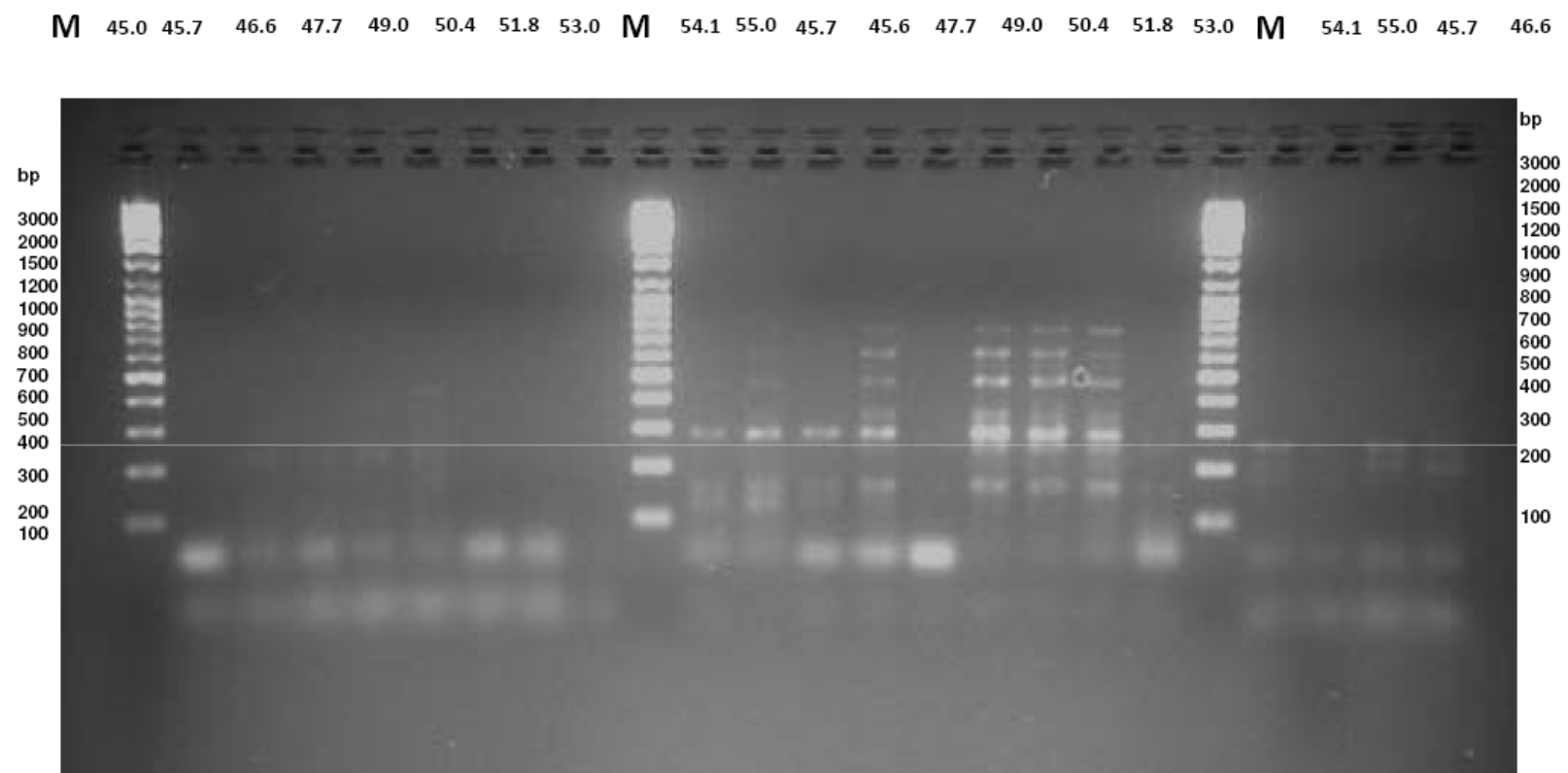


Plate 26. PCR amplification of gene encoding of endoglucanase gene1 chromosomal DNA of *Trichoderma longibrachiatum* T2 at different annealing temperatures

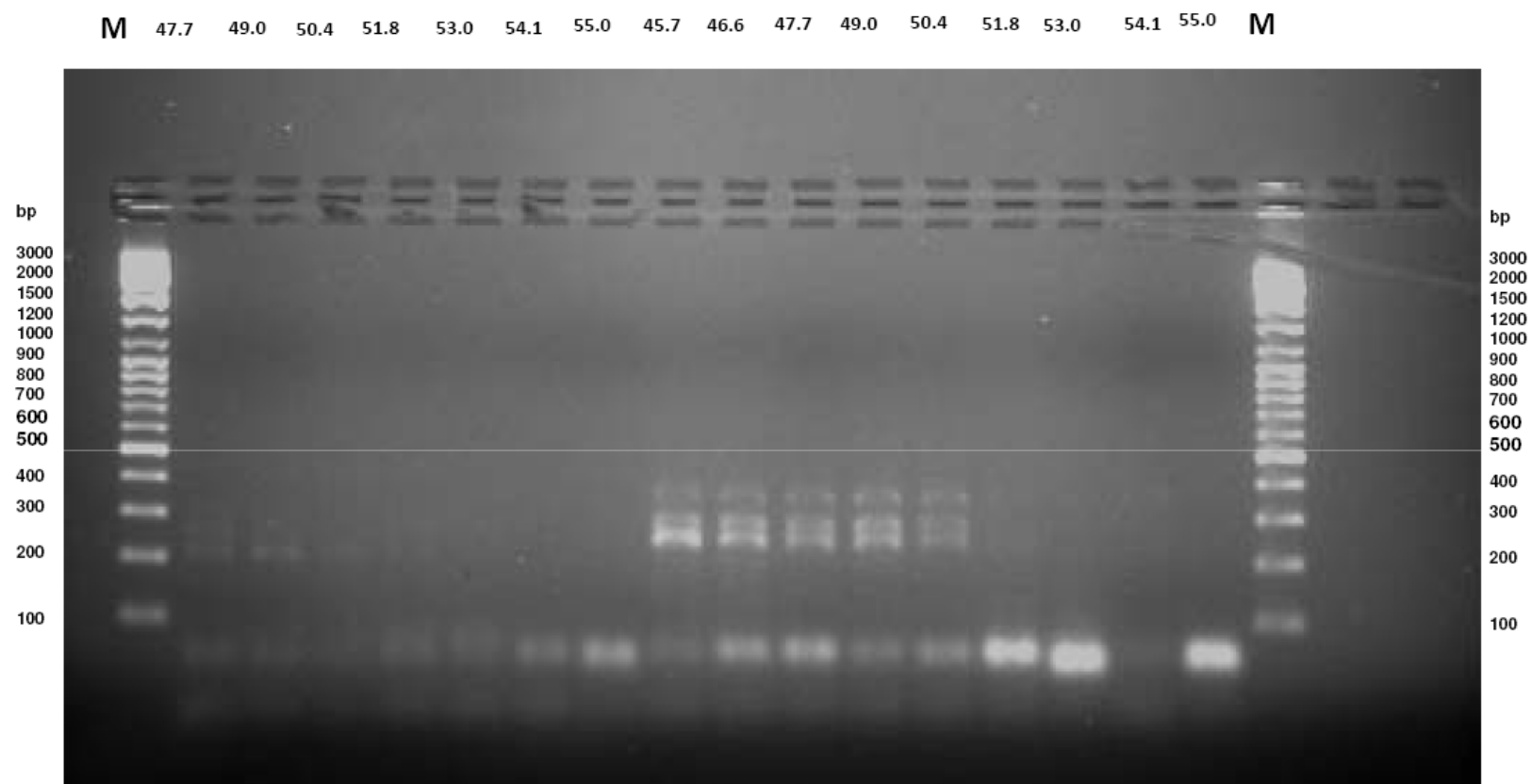


Plate 27. PCR amplification of gene encoding of cellobiohydrolase gene1 chromosomal DNA of *Trichoderma longibrachiatum* T2 at different annealing temperatures .

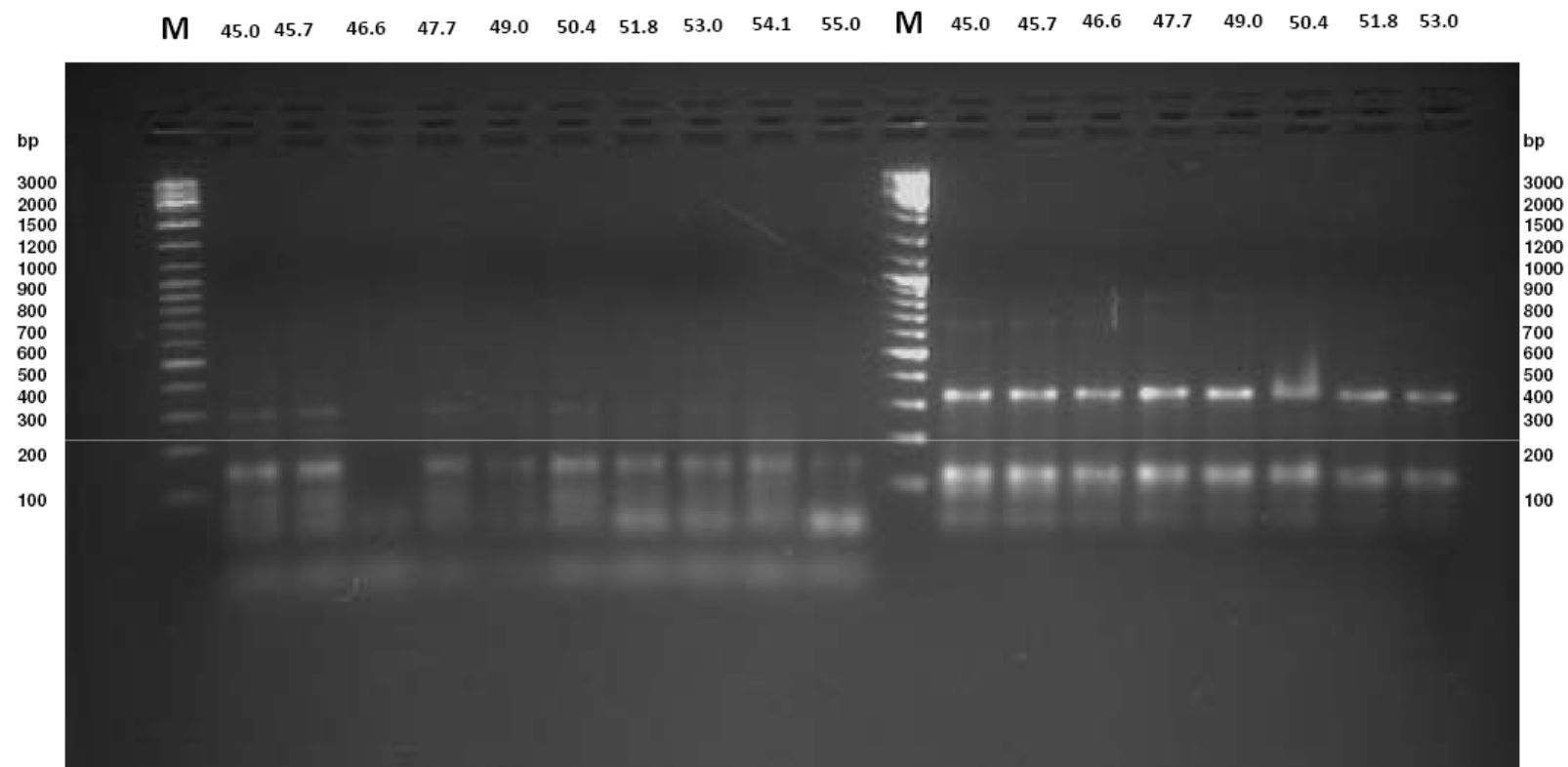


Plate 28 . PCR amplification of gene encoding of β -glucosidase gene1 chromosomal DNA of *Trichoderma longibrachiatum* T2 at different annealing temperatures

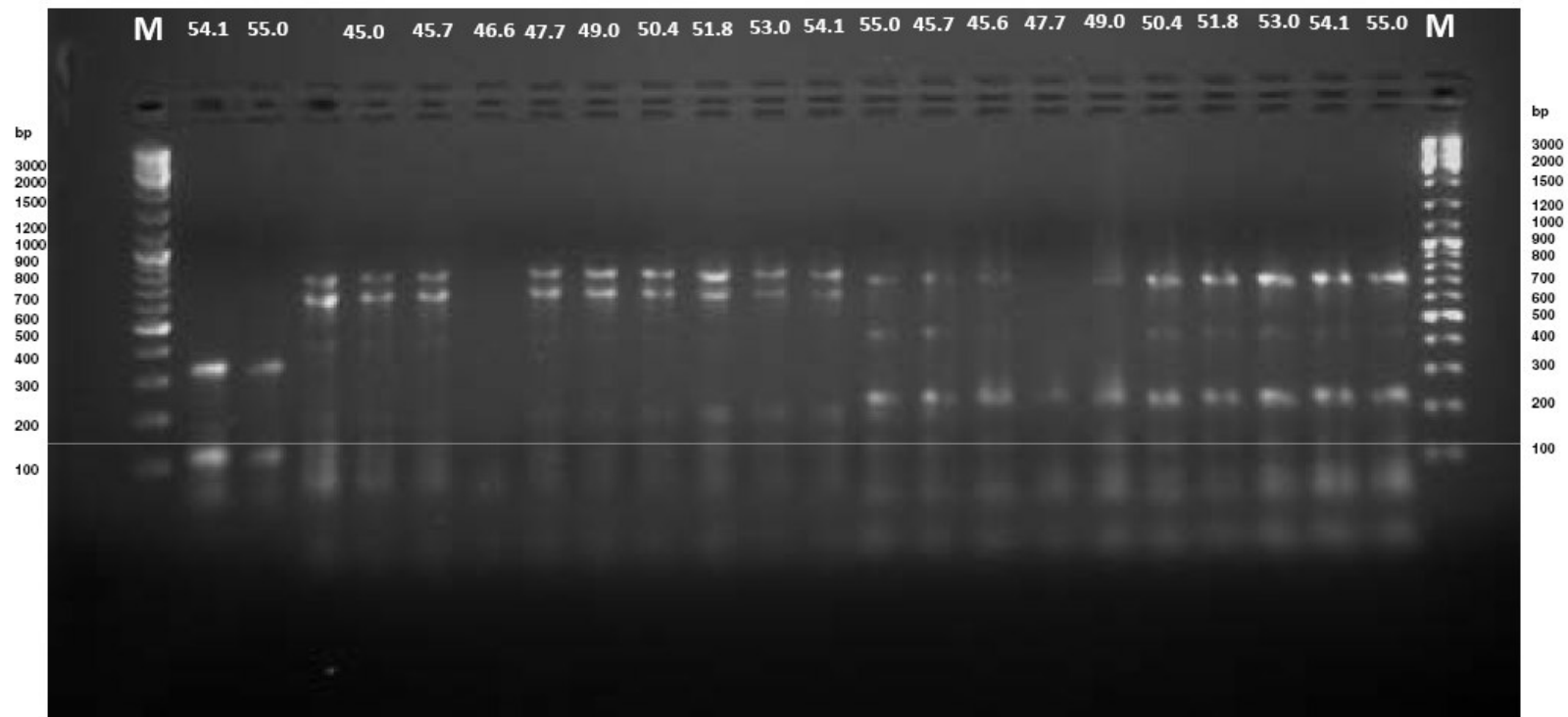


Plate 29. PCR amplification of gene encoding for xylanase gene chromosomal DNA of *Trichoderma longibrachiatum* T2 at different annealing temperatures

production. Inoculum age showed to be an important parameter to ensure dynamic growth in a stirred tank bioreactor as cell are highly active and energetic phase for xylanase production (Kapoor *et al.*, 2008). Inoculum age is particularly important in the sporulating bacteria because the sporulation induced at the end of the logarithmic phase and the use of an inoculum containing high percentage of spores would result a long log phase in subsequent fermentation. The younger culture of *B. amyloliquefaciens* SH8, being nonsporulating, entered the growth phase very soon, while the cultures older than 4h produced spores which took longer time to germinate, and therefore, the enzyme production was low. For each of three inoculums ages evaluated, sampling was performed at regular intervals for growth, protein and enzyme analyses. The different inoculum ages i.e. 2, 4 and 6h were used for assessing the activity of xylanase enzymes in stirred tank bioreactor by *B. amyloliquefaciens* SH8. A graphical representation of the data obtained for each of three bioreactor experiments are presented in Fig 96 to 99. The highest xylanase titers were observed at an inoculum age of 4h.

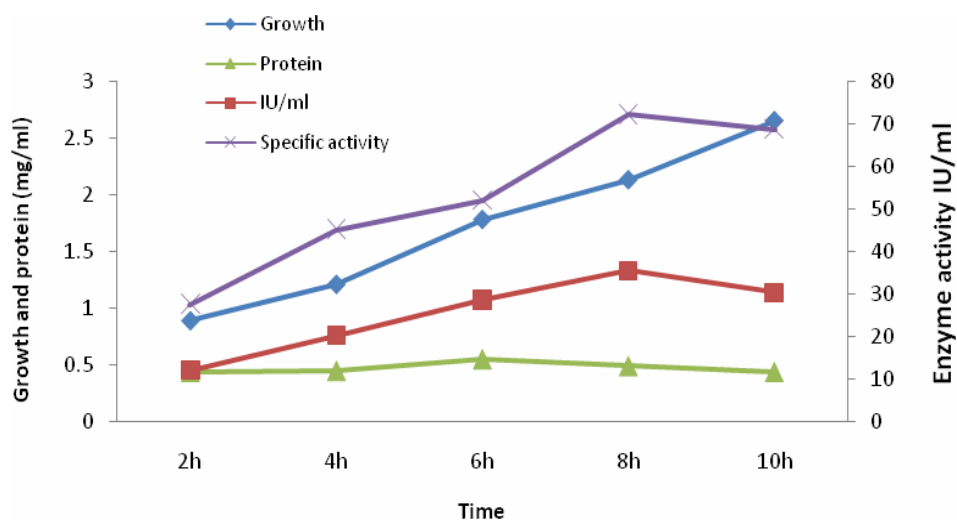


Fig. 96 Xylanase production in bioreactor at 2h inoculum age

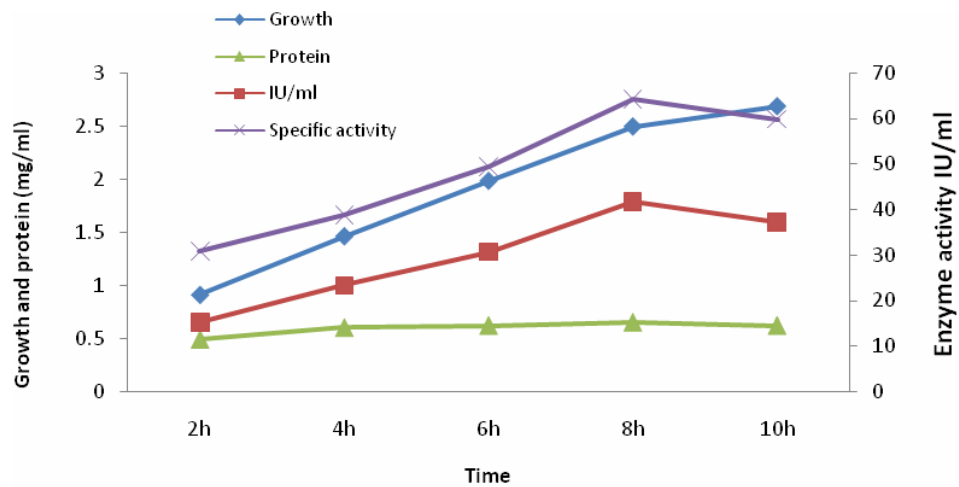


Fig. 97. Xylanase production in bioreactor at 4h inoculum age

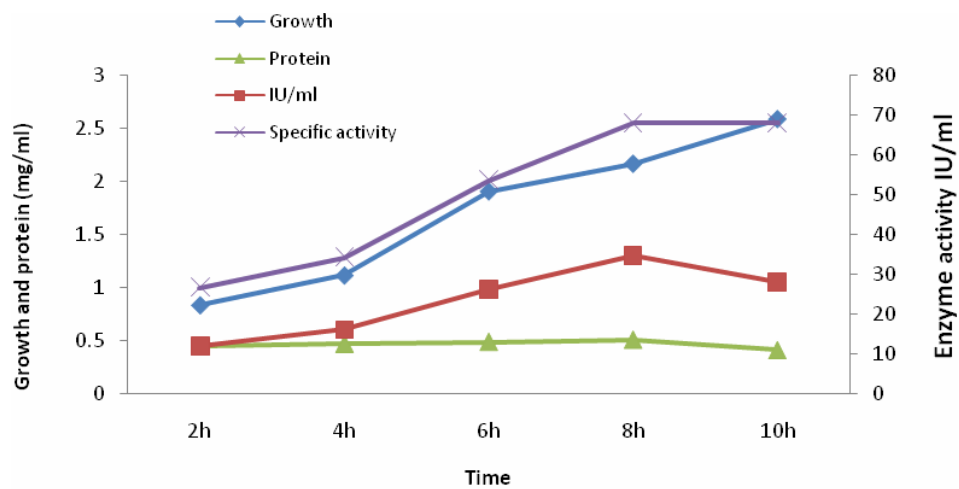


Fig. 98. Xylanase production in bioreactor at 6h inoculum age

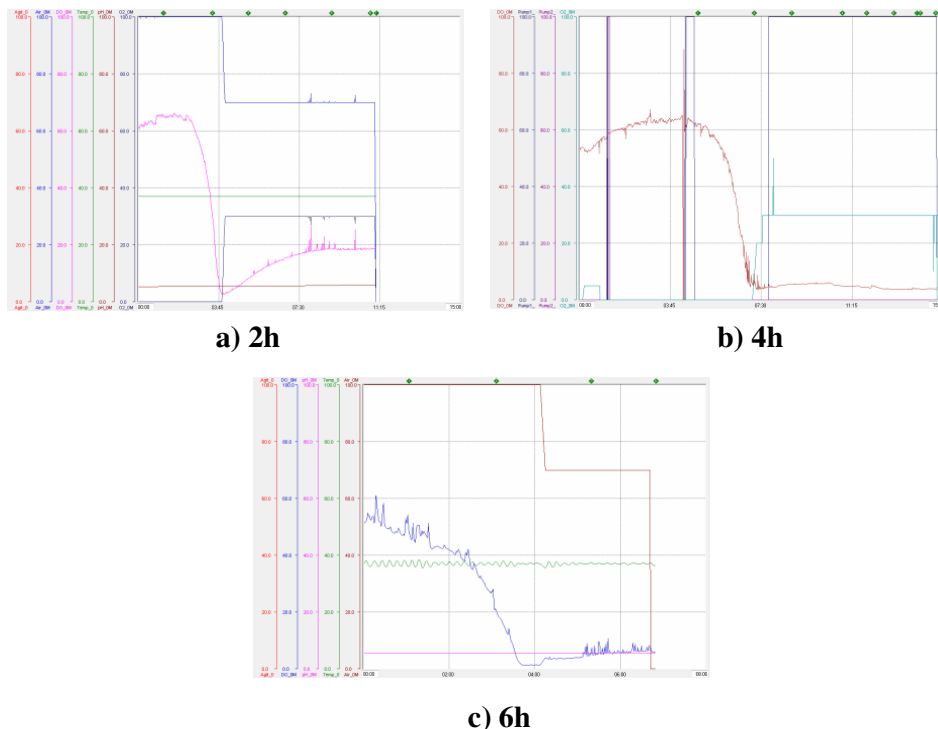


Fig. 99. Physiological behaviour of *Bacillus amyloliquefaciens* SH8 in a stirred tank bioreactor

In inoculum age of 4h a maximum xylanase titers of (41.75 IU/ml) were attained after 8h of fermentation time as shown in (Fig. 97), which was followed by 10h (37.11 IU/ml) and 6h of fermentation time (30.64 IU/ml). The minimum xylanase production was observed at 2h of fermentation time (15.11 IU/ml). There was a lag phase of approximately of 2h before xylanase synthesis commenced, and biomass production was begun approximately after 2h while Fig. 96 and 98 exhibited lesser xylanase production at inoculum age of 2h and 6h from *Bacillus amyloliquefaciens* SH8 as compared to inoculum age of 4h, respectively.

4.6.1.2 Effect of Inoculum Size on Xylanase Production by *B. amyloliquefaciens* SH8 in a Stirred Tank Bioreactor

For each of three inoculum sizes evaluated, sampling was performed at regular intervals for growth, protein and enzyme analyses. The different inoculums sizes namely 5%, 10% and 15% were used for assessing the activity of xylanase enzymes in stirred tank bioreactor by *B. amyloliquefaciens* SH8. A

graphical representation of the data obtained for each of three bioreactor experiment is presented in Fig. 100 to 103. The highest xylanase titers were observed at an inoculum size of 10%.

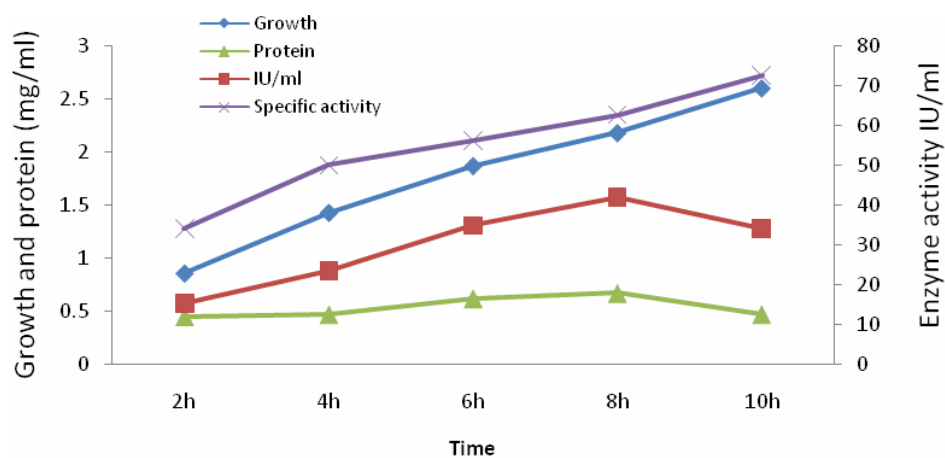


Fig. 100. Xylanase production in bioreactor at 5% inoculum size

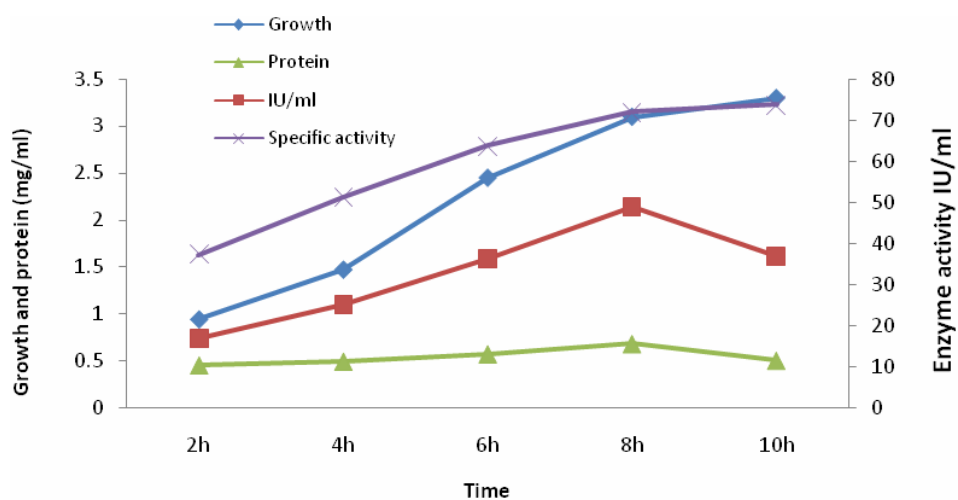


Fig. 101. Xylanase production in bioreactor at 10% inoculum size

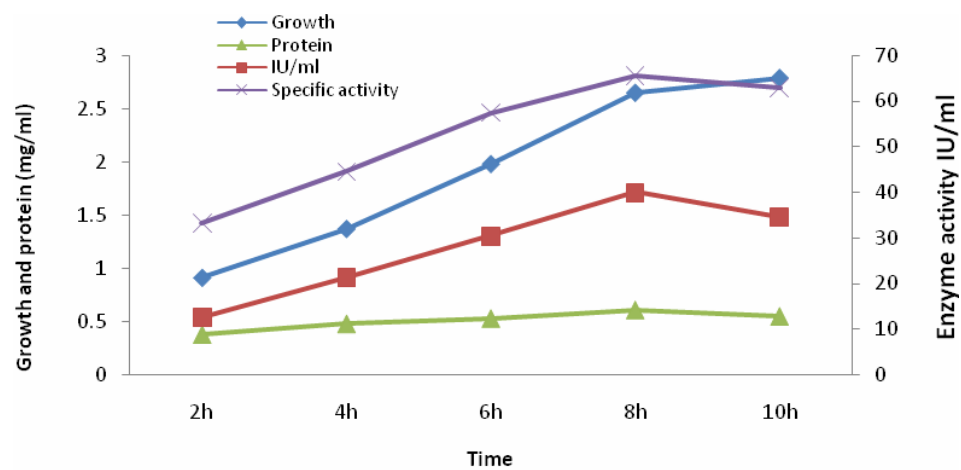


Fig. 102. Xylanase production in bioreactor at 15% inoculum size

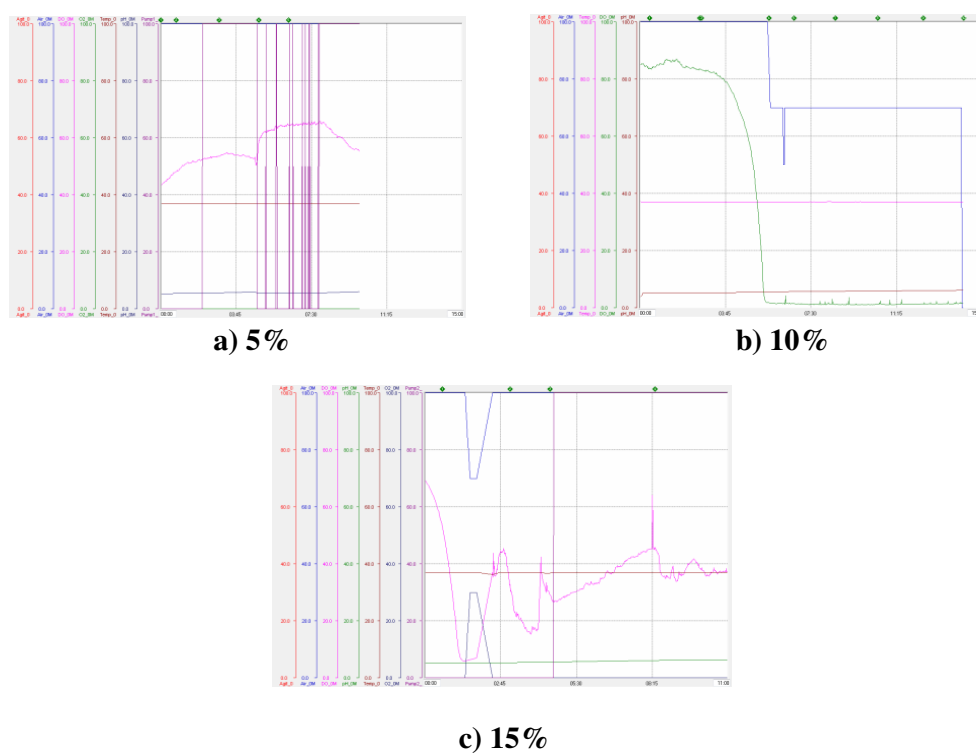


Fig. 103. Physiological behaviour of *Bacillus amyloliquefaciens* SH8 in a stirred tank bioreactor

The inoculum size of 10% showed a maximum xylanase titers of (48.91 IU/ml) after 8h of fermentation time as shown in (Fig 101), which was followed by 10h (36.86 IU/ml) and 6h (36.29 IU/ml). The minimum xylanase titers were observed at 2h of fermentation time (16.76 IU/ml). There was a lag phase of approximately 2h before xylanase synthesis commenced, and biomass production was begun approximately after 2h. In case of 5 and 15% inoculum size, xylanase produced was much less i.e. (19.0 to 42.0 IU/ml), respectively (Fig. 100 and 102).

The larger volume of stirred tank bioreactor also specifies the need of a higher inoculum concentration for optimum xylanase production. An optimum inoculum size of 10% (v/v; 9×10^6 CFU/ml) produced the maximum xylanase activity. Cell growth also was highest at the inoculums size of 10%. Inoculum size influences the substrate utilization in the medium resulting in the higher cell concentration at a time, and consequently more production of the enzyme. However, at higher inoculum size enzyme activity was ceased due to depletion of oxygen on account of high cell concentration and competition among bacterial cells for nutrients.

4.6.1.3 Effect of Aeration Rate on Xylanase Production by *B. amyloliquefaciens* SH8 in Stirred Tank Bioreactor

For each of three aeration rates evaluated, sampling was performed at regular intervals for growth, protein and enzyme analyses. The different aeration rates namely 0.5, 1.0 and 1.5vvm were used for assessing the activity of xylanase enzymes in stirred tank bioreactor by *B. amyloliquefaciens* SH8. A graphical representation of the data obtained for each of three bioreactor experiment is presented in Fig. 104 to 107. The highest xylanase titers were observed at aeration rates of 1.0 vvm.

The different aeration rate had been studied and it was found that cell growth and xylanase production from this aerobic isolate occurred only when air was supplied continuously to the bioreactor. The aeration rate in the stirred tank bioreactor is important for the growth of the bacteria and also increases the

oxygen exchange rate and helps in proper dissolution of O₂ in medium (Ibrahim, 1994).

Techapun *et al.* (2003) reported that aeration is essential for maintaining the metabolic activities of aerobic organisms. Nigam *et al.* (2012) studied that aeration also brings about mixing of the bioreactor contents thereby increasing the efficiency of bioreaction. The dissolved oxygen concentration in a suspension of respiring microorganisms depends on the rate of oxygen transfer from the gas phase to the liquid phase, rate at which oxygen diffuses to the microorganism and its consumption by the microorganism. During aerobic fermentation, the transfer of oxygen occurs from air bubble into the medium and ultimately to the site of reaction within the cell. Thus, the oxygen transfer from air bubbles, though the liquid medium, to the microbial cells has great importance. More the oxygen transfer, more is the oxygen availability for the organism. Oxygen transfer can be increased by increasing the aeration rate. The effect of aeration on the xylanase production in the bioreactor was evaluated by sparging air to the reactor at different rates (0.5, 1.0 and 1.5 vvm) and the results on the biomass accumulation and activity are mentioned in Fig. 104 to 107.

Aeration at 1.0 vvm produced the highest enzyme activity as shown in Fig while, xylanase production decreased at other aeration rates. The cell growth also was highest at 1.0 vvm. Aeration less than 1.0 vvm might not provide enough oxygen for cell growth. Whereas, an aeration rate greater than 1.0 vvm might have exceeded the oxygen need proving it toxic/lethal for cell growth and thus resulted in lower enzyme activity.

Maximum xylanase titers of (56.80 IU/ml) were attained after 8h of fermentation time at aeration rate of 1.0 vvm as shown in (Fig. 105), which was followed by 10h (37.86 IU/ml) and 6h (36.29 IU/ml). The minimum xylanase titers were observed at 2h of fermentation time (16.76 IU/ml). There was a lag phase of approximately of 2h before xylanase synthesis commenced, and biomass production had begun approximately after 2h while aeration rate of 0.5 vvm and 1.5 vvm showed lesser xylanase production as depicted in Fig 104 (19.0 to 35.60 IU/ml) and Fig. 106 (11.90 to 41.99 IU/ml).

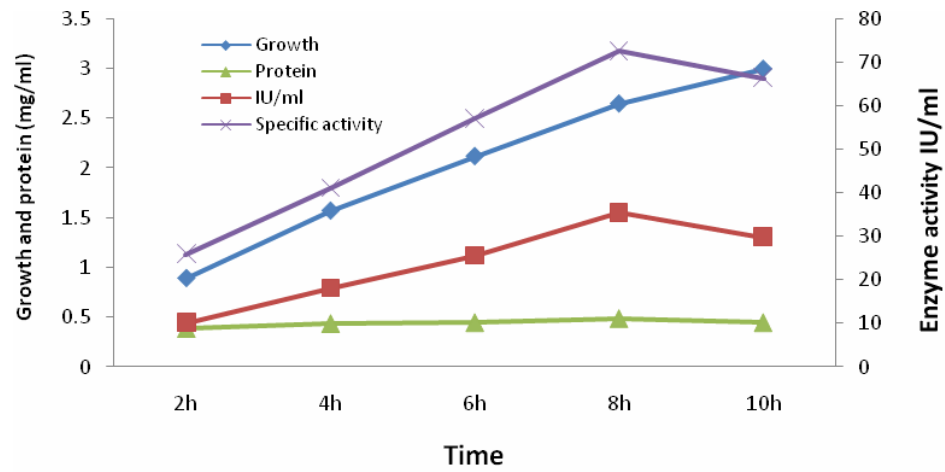


Fig. 104. Xylanase production in bioreactor at aeration rate of 0.5vvm

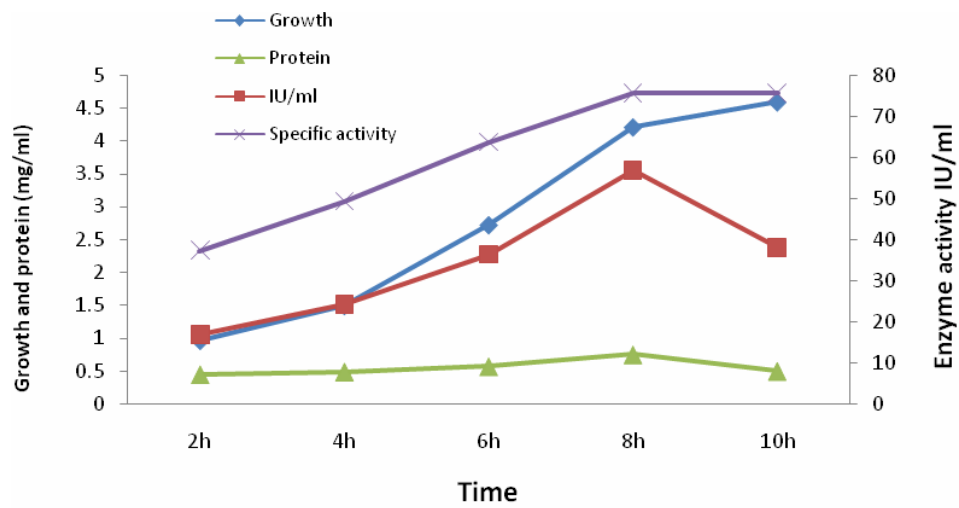


Fig. 105. Xylanase production in bioreactor at aeration rate of 1.0vvm

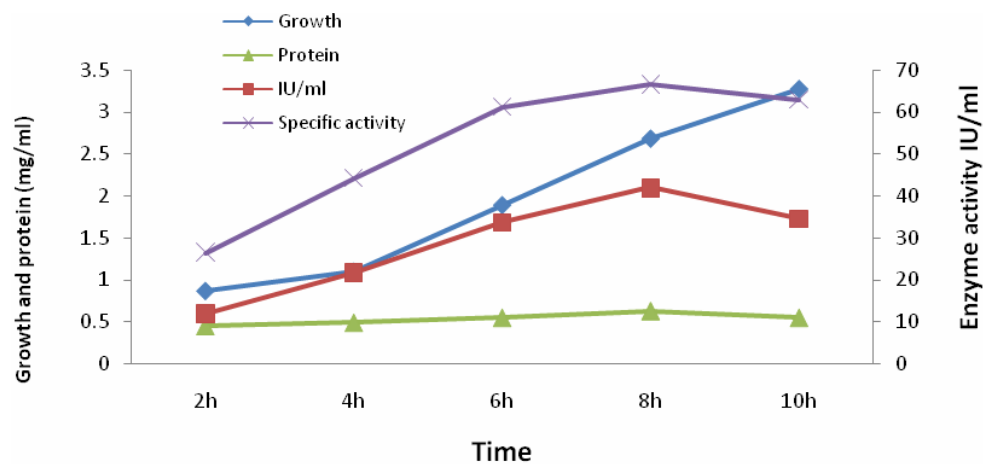


Fig. 106. Xylanase production in bioreactor at aeration rate of 1.5vvm

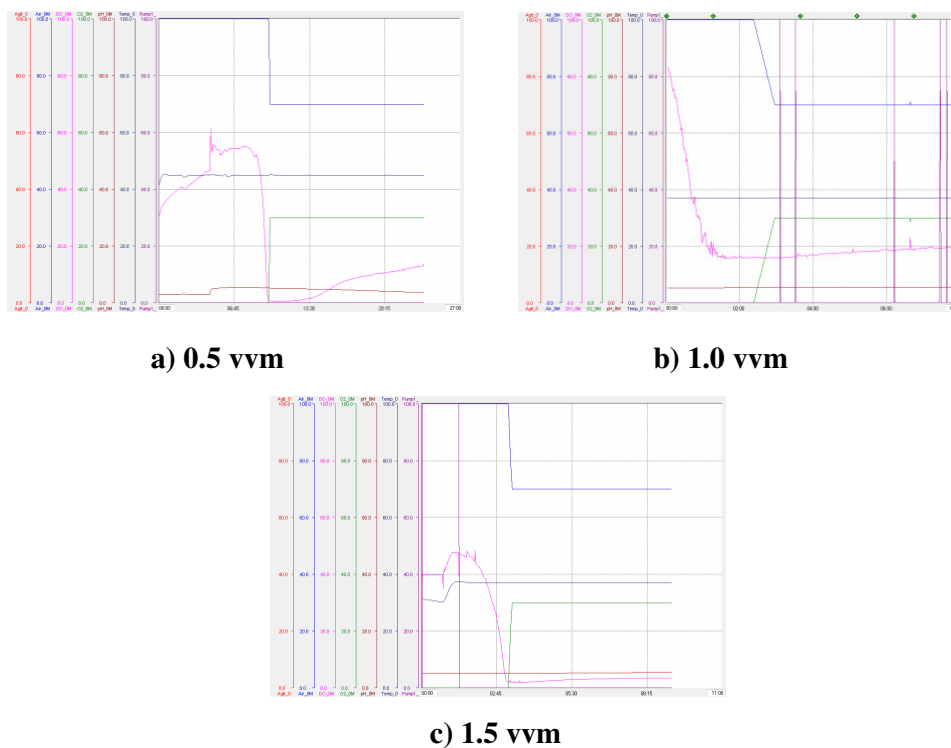


Fig. 107. Physiological behaviour of *Bacillus amyloliquefaciens* SH8 in a stirred tank bioreactor

4.6.1.4 Effect of Agitation on Xylanase Production by *B. amyloliquefaciens* SH8 in Stirred Tank Bioreactor

Agitation rate of the impellers in a stirred tank bioreactor plays an important role in the production of xylanase enzyme. Agitation showed an important parameter to ensure nutrient availability in a growth medium. An agitation rate of 200 rpm produced highest xylanase activity with the highest cell growth. Higher agitation may create conditions of higher nutrient availability and carbon sources to microorganisms by Nahas (1988).

Agitation brings about proper mixing of production broth as well as increases the oxygen transfer and thus has a tremendous effect on the productivity of the system. The optimization of agitation for maximum production of any culture needs to be carefully monitored, as it has both beneficial as well as deleterious effects (rupture of cells and thus less growth, change in the cell morphology, foam production at high agitation etc). At lower speed i.e. 100 rpm, cell biomass as well as enzyme activity was found to be lower than the activity observed at 200 rpm. The low activity at lower agitation speed could be attributed to the dearth of oxygen being experienced by the organism due to the insufficient mixing. The organism when allowed to grow at higher speed i.e. 300 rpm showed less biomass accumulation and decreased in activity of enzyme. The cells were observed under microscope at the end of the run and the disruption was found to be more as compared to that at lower agitation rate. Although, the organism grew faster at higher agitation, it got sheared more easily which adversely affected the enzyme synthesis. The enzyme activity at 200 rpm was marginally higher and obtained earlier. Hence, an agitation speed of 200 rpm is chosen as the optimal agitation speed for maximum synthesis of enzyme by Nigam *et al.* (2012).

An agitation rates of 200 rpm showed that maximum xylanase titers of (67.80 IU/ml) were attained after 8h of fermentation time as shown in (Fig. 109), which was followed by 10h (37.86 IU/ml) and (36.29 IU/ml). The minimum xylanase titers were observed at 2h of fermentation time (16.76 IU/ml). There

was a lag phase of approximately of 2h before xylanase synthesis commenced, and biomass production was begun approximately after 2h. In contrast, agitation rate lesser or higher than 200 rpm has resulted in drastic decrease in xylanase units i.e. 42.34 IU/ml and 52.10 IU/ml @ 100 and 300 rpm, respectively (Fig. 108 and 109).

The optimal temperature for *B. amyloliquefaciens* SH8 in stirred tank bioreactor for active enzyme production was 45°C. A few studies are available in the literature about commercial xylanase production from thermophilic microbial sources. Maximum yield of xylanase obtained in very short fermentation time i.e. 8h is another desirable trait for its commercial use. High xylanase titers (67.80 IU/ml) have been obtained in the reactor by using inexpensive residue wheat bran thus replacing expensive xylan source (oat spelt xylan, birch wood xylan, larch wood xylan and beach wood xylan) finally designating it a cost effective process. The above mentioned features are the main highlights of xylanase production from *B. amyloliquefaciens* SH8 that recommend its commercial scale production strongly with optimized production technology and low media cost scale up process as devised in the present study.

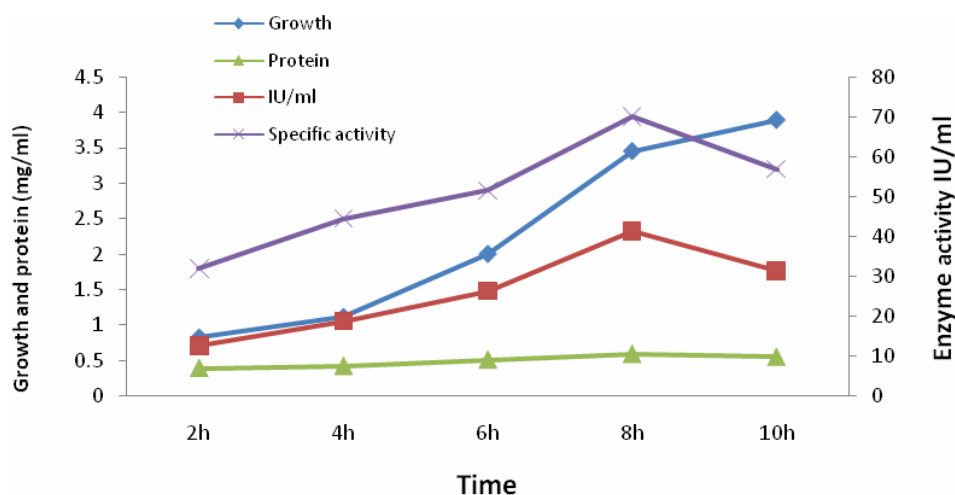


Fig. 108. Xylanase production in bioreactor at agitation rate of 100 rpm

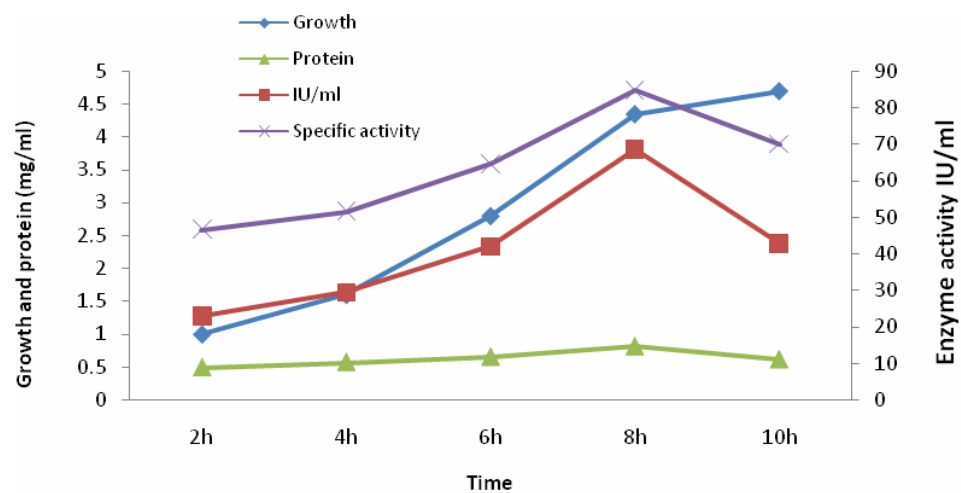


Fig. 109. Xylanase production in bioreactor at agitation rate of 200 rpm

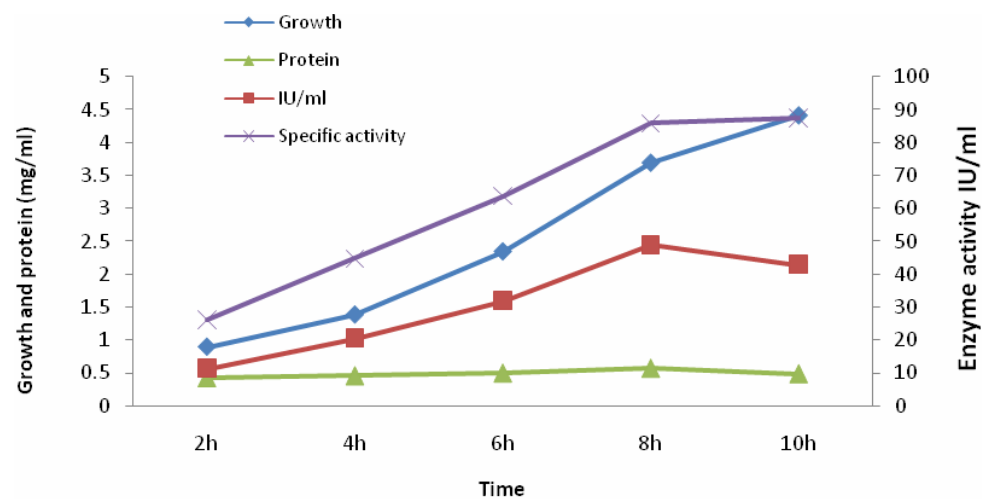


Fig. 110. Xylanase production in bioreactor at agitation rate of 300 rpm

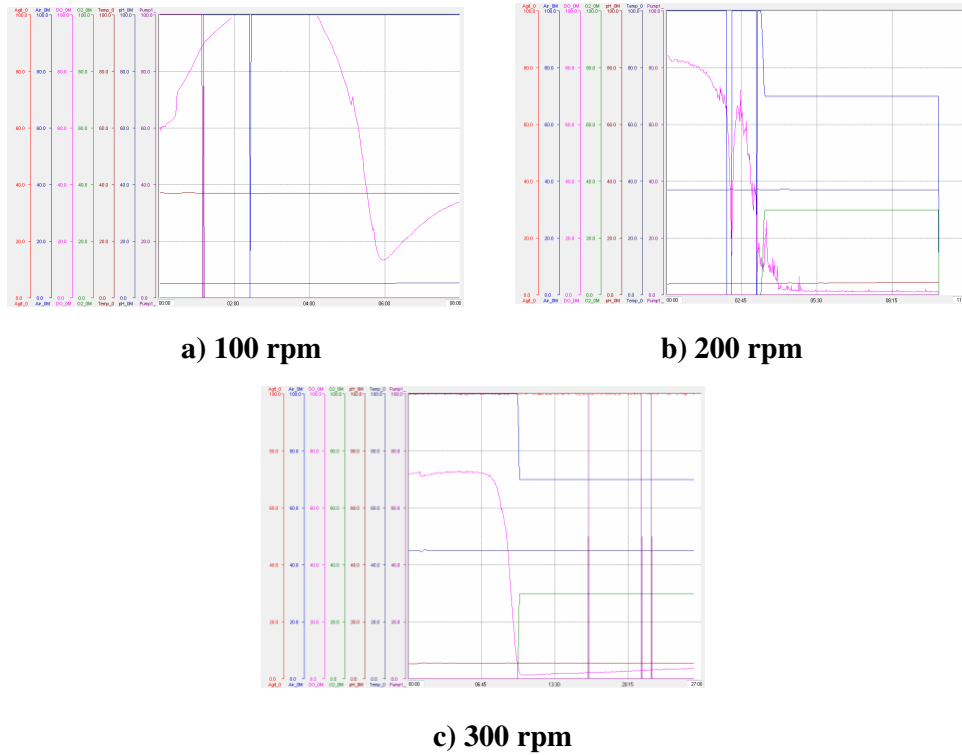


Fig. 111. Physiological behaviour of *Bacillus amyloliquefaciens* SH8 in a stirred tank bioreactor

4.6.2 Mathematical Modeling of *B. amyloliquefaciens* SH8 for Cell Growth

Most of the growth processes are explained in terms of Monod or logistic equations (unstructured models). Logistic equation is simple for calculation of fermentation parameters related to biological and geometrical significance using sigmoid profiles independent of substrate concentration (Venkatesh *et al.* 1993, Garcia-Ochoa *et al.* 1995). The sigmoidal growth pattern of *Bacillus amyloliquefaciens* was analyzed using logistic equation for its variation against time during log phase. The idea of microbial growth kinetics has been dominated by an empirical model originally proposed by Monod (1942). The Monod model introduced the concept of a growth limiting substrate.

$$\mu = \mu_{\max} S / (K_s + S) \text{ (Batch growth)}$$

$$1/\mu = K_s / \mu_{\max} \cdot 1/s + 1/\mu_{\max}$$

Where μ = specific growth rate, $\mu_{\max}$ = maximum specific growth rate, S = substrate concentration, K_s = substrate saturation constant (i.e. substrate concentration at half $\mu_{\max}$).

Bacillus amyloliquefaciens SH8 showed a conventional growth pattern as shown in figure during batch fermentation in which exponential phase lasted for 8h and was followed by stationary phase upto 10h. The production of xylanase was started in 2h and obtained maximum 8h. Xylanase production increased rapidly during late exponential phase and early stationary phase, while it was slowed down during late stationary phase as shown in Fig. 112.

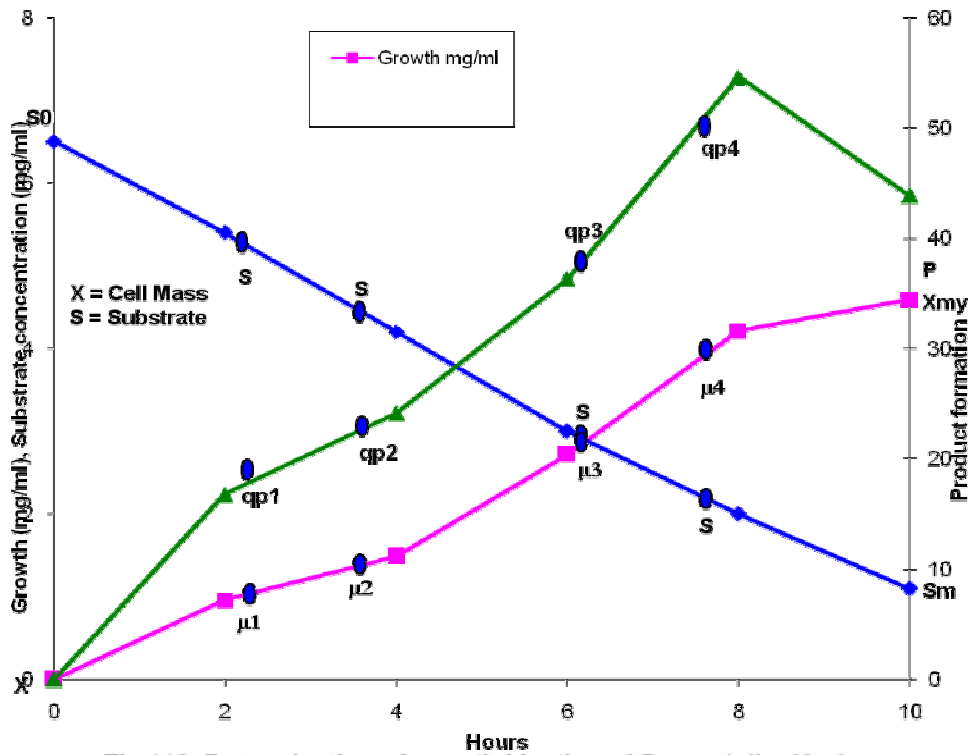


Fig. 112. Determination of growth kinetics of *B. amyloliquefaciens* SH8 in stirred tank bioreactor

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters $\mu_{\max}$ and K_s . In addition to this, Monod also related the yield coefficient ($Y_{x/s}$) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (q)

$$Y_{x/s} = \frac{dx}{ds}$$

$$Y_{x/s} = \frac{X_m - X_0}{S_0 - S_m}$$

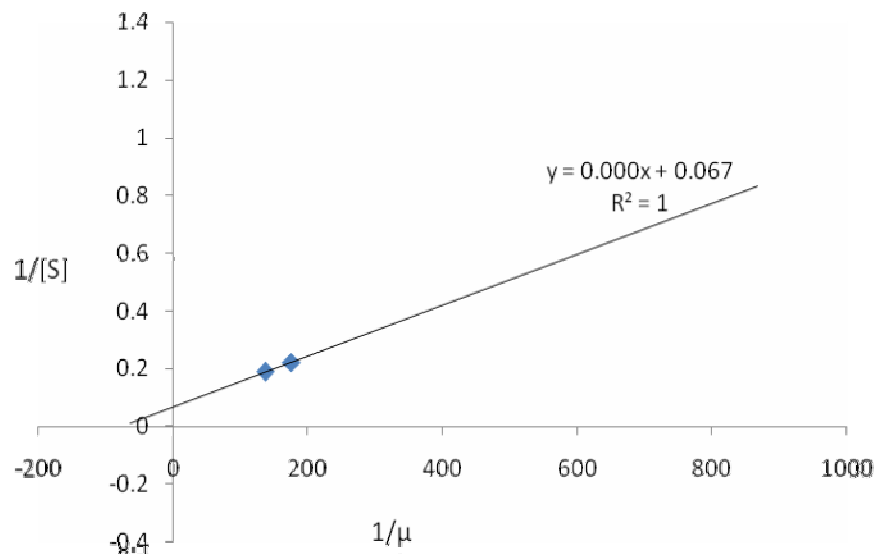


Fig. 113. Comparison between experimental results of specific growth rate (μ) and Substrate utilization [S] and model prediction for biomass

Model prediction for cell growth was followed with the experimental results with coefficient of determination, $R^2 = 1.0$ (Fig. 113), which revealed that this kinetic model is appropriate for predicting the experimental cell growth.

$$Y_{x/s} = dx/ds$$

$$Y_{x/s} = 0.96$$

$$1/\mu_{\max} = 0.06$$

$$\mu_{\max} = 14.92 \text{ min}^{-1}$$

$$K_S/\mu_{\max} = 0.0006 \text{ from slope}$$

$$K_S = 0.00892 \text{ mg/ml}$$

Table 68. Growth and production kinetic parameters for xylanase production by *B. amyloliquefaciens* SH8 in Bioreactor

	μ	$1/\mu$	[S]	$1/[S]$	q_p
μ_1	0.0073	136.90	5.3	0.188	0.086
μ_2	0.0057	175.40	4.5	0.222	0.077
μ_3	0.0040	250.00	2.8	0.357	0.044
μ_4	0.0015	666.66	2.0	0.500	0

4.6.2.1 Kinetic Analysis for Xylanase Production

The Luedeking-Piret equation (Luedeking and Piret 1959) was applied for the kinetic modeling of xylanase production. The Luedeking-Piret model indicates that the rate of xylanase production (q_p) is linearly proportional to the *B. amyloliquefaciens* SH8 cell growth rate and cell biomass as shown in Fig. 123.

$$q_p = \alpha\mu + \beta$$

where, α = growth associated constant and β non growth associated constant, both may vary under different fermentation conditions.

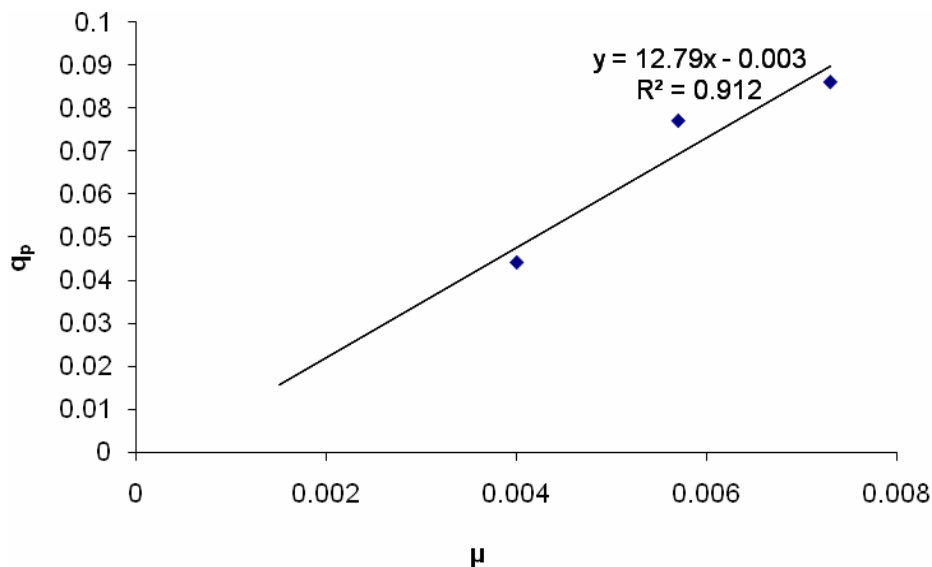


Fig. 114. Comparison between experimental results and model prediction for product formation

The comparison between model predictions and the experimental data of the product formation during batch fermentation is shown in Fig 114. Results indicate that this model is evidently very appropriate for describing the product formation rate (coefficient of determination, R^2 was 0.912). The growth-associated constant (α) was 0.003 IU/mg cells, which illustrates that the rate of xylanase production is during the exponential growth phase of *B. amyloliquefaciens* SH8. The non-growth associated constant (β) was 0.0046 IU/mg/h which indicates that xylanase production was during the stationary growth phase of *B. amyloliquefaciens*. The biological meaning of the parameters

K_S and $\mu_{\max}$, which are used to characterize microbial growth for, given growth conditions, is necessary. The maximum specific growth rate $\mu_{\max}$ is a characteristic of all organisms and it is related to their ability to reproduce themselves in nutritional conditions of plenty. It is simply defined as the increase of biomass per unit time under optimal feeding conditions, in which there are not limiting nutrients. While the interpretation of $\mu_{\max}$ as the maximum specific growth rate is straight forward, the biological meaning of K_S is less obvious. The empirical constant K_S is the substrate concentration at which organisms are substrate limited to a growth rate of half the prevailing maximum value. This constant is closely related to the mechanism of transport of the substrate over the cell membrane, so it depends on cell membrane properties and intracellular conditions, on the type of transporter proteins in the cell and on the substrate properties. For this reason, the value of K_S can be interpreted as a reflection of the affinity of the cell towards the substrate S.

The values of these model parameters depend primarily on properties of the organism itself, which are determined by its genome. Moreover, because of the adaptive ability of organisms, both $\mu_{\max}$ and K_S can vary with the environmental conditions, for example with the nature of the growth medium and the duration of exposure to it, with temperature and pressure. For these reasons the values of $\mu_{\max}$ and K_S have to be estimated for each specific couple cell-substrate and under constant conditions of temperature, pressure and medium composition.

In the present study rarely reported hypercellulolytic and xylanolytic bacteria and fungi isolated from compost are unique in a sense as these have been found thermophilic as well as acidophilic in nature and capable of producing high titers of cellulase and xylanase under optimized conditions using classical (OFAT) approach as well as advanced statistical tools (RSM) utilizing inexpensive agricultural residue i.e. wheat bran/wheat straw inturn rendering it a cost effective process. Cellulase and xylanase encoding genes of potential isolates have been isolated and sequenced with the high possibility of designing clones for further relevant industrial applications. Enzyme scale up studies in a

bioreactor (7.5 L capacity) has been done with an aim of establishing the commercial production of these potential isolates. Purification of hydrolytic enzymes as well as their enzyme kinetics have been explored to strengthen the use of these important enzymes at commercial scale, thus fulfilling the main objectives of our study i.e. industrial application of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost.

Chapter-5

SUMMARY AND CONCLUSION

In the present investigation entitled **“Purification of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost for industrial application”**, an attempt has been made for the isolation of most efficient cellulolytic and xylanolytic bacteria and fungi from compost from mid hills of Himachal Pradesh located in sub temperate zone of northern Himalayas, their screening to select hyperenzyme producing strains, optimization of various growth and nutritional parameters, optimization using response surface methodology under SmF, purification and characterization of cellulase and xylanase, sequencing of cellulase and xylanase genes encoding cellulolytic and xylanolytic bacteria and fungi and scale up of xylanase enzyme in a bioreactor.

In total, 102 isolates including 93 bacteria (30 from Solan, 24 from Shimla, 22 from Mandi and 17 from Kangra district) and 9 fungi (2 isolates each from Solan, Shimla, Mandi and Kangra district) had been isolated from the compost from four districts of Himachal Pradesh viz. Solan, Shimla, Mandi and Kangra. Different physiochemical and biochemical tests were performed with these bacterial and fungal isolates for their tentative identification, eventually confirming the identification of potential enzyme producers at molecular level using 16S rRNA for bacterial isolates and ITS 5.8 S rRNA technique for the best selected fungal isolates depending upon their highest enzyme synthesizing capacity. A hyper extracellular cellulase producing bacterial strain BM-1 was identified as *B. subtilis*, while hyper extracellular xylanase producing bacterial strains SH8 and SH0 were identified as *B. amyloliquifaciens* and *B. tequilensis*, respectively. The hypercellulolytic and xylanolytic fungi T2 and W2 were identified as *T. longibrachiatum* and *R. oryzae*. These isolates were selected for further enzyme optimization study.

After quantitative analysis of enzyme produced by different bacterial strains, under SmF, *B. subtilis* BM-1 showed the maximum extracellular cellulase

production on Reverie media i.e. 1.33 IU/ml and minimum production of extracellular cellulase was ascertained in SH8 i.e. 0.03 IU/ml. While *B. amyloliquifaciens* SH8 and *B. tequilensis* SH0 showed the maximum extracellular xylanase production on Reverie media i.e. 14.40 and 13.30 IU/ml, respectively and minimum production of extracellular xylanase was observed in M6 i.e. 0.40 IU/ml. The isolates *B. subtilis* BM-1, *B. amyloliquifaciens* SH8 and *B. tequilensis* SH0 on the basis of their highest cellulase and xylanase activity were selected for the further optimization under SmF. Optimization of different factors i.e. media, temperature, pH, inoculum size, carbon sources, different concentration of best selected carbon source and incubation time for the production of extracellular cellulase and xylanase from hypercellulolytic and xylanolytic bacterial strains *B. subtilis* BM-1, *B. amyloliquifaciens* SH8 and *B. tequilensis* SH0 were carried out under submerged fermentation.

The best yield of extracellular cellulase production by *B. subtilis* BM-1 i.e. 4.05 IU/ml was obtained in the Basal salt medium after 72h at pH 6, temperature 45°C, inoculum size 10.0%, carbon source-wheat bran and 0.75% wheat bran. After optimization of different process parameters, cellulase activity from the hypercellulolytic bacteria was enhanced from 1.33 to 4.05 IU/ml and percent increase in the enzyme activity had gone upto 204.51% which was significantly higher. Similarly, Strain *B. amyloliquifaciens* SH8 exhibited maximum xylanase activity i.e. 46.50 IU/ml in Basal salt medium after 72h, pH 5.5, temperature 45°C, inoculum size 10%, carbon source-wheat bran and 1.25% wheat bran, while strain *B. tequilensis* SH0 showed highest xylanase activity i.e. 41.30 IU/ml in Basal salt medium at 96h, pH 5.5, temperature 45°C, inoculum size 10%, carbon source-wheat bran and 1.25% wheat bran concentration. After optimization of different process parameters, xylanase activity from the hyperxylanolytic *B. amyloliquifaciens* SH8 and *B. tequilensis* SH0 was enhanced from 14.40 to 46.50 IU/ml and 13.30 to 41.30 IU/ml, respectively and percent increase in the enzyme activity had gone upto 222.91% and 210.52 %, respectively. Therefore production of cellulase and xylanase from potential bacteria utilizing wheat bran is the main highlight of this study.

Response surface methodology (RSM) was employed to optimize medium components for the production of thermostable xylanase of *B. amyloliquifaciens* SH8 using inexpensive agricultural residue wheat bran as a carbon source. Plackett-Burman design was applied to evaluate the effect of 19 physicochemical variables (different nitrogen sources, salt additives, pH, inoculum size, temperature, incubation time). Tryptone, NH_4Cl , ZnSO_4 and CaCl_2 were screened as the significant factor positively affecting the xylanase production to PBD. A central composite design (CCD) quadratic response surface was applied to explicate the concentrations of these four factors that significantly affected the xylanase production in submerged fermentation. Subsequently, a second-order polynomial was determined by multiple regression analysis. The high xylanase activity and low production cost may further broaden this application to industrial scale. In addition, applicability of the statistical approach has proved useful and powerful tool for the development of optimal cultural conditions and to gather sufficient information with a minimum number of trials.

As far as fungi are concerned *T. longibrachetum* T2 and *R. oryzae* W2 evinced maximum extracellular cellulase (3.91 and 4.20 IU/ml) and xylanase yield in Vogel medium (14.87 and 15.56), respectively and minimum extracellular cellulase and xylanase yield was observed in S2 i.e. 1.69 and 6.23 IU/ml. The different parameters for hypercellulolytic and xylanolytic fungal isolates viz. alkali pretreatment, acid pretreatment and steam pretreatment with selected agricultural and forest residues i.e. wheat straw, rice straw, pine wood, pine needles and bamboo wood were optimized under SSF. The best screened wheat straw was habituated for further optimization of hyper cellulase and xylanase production viz. pH, temperature; moisture level and incubation time for fungal strains *T. longibrachetum* T2 and *R. oryzae* W2 under solid state fermentation.

Lignocellulosic substrate i.e. wheat straw (*Triticum aestivum* straw) was screened as best substrate among fungal isolates *T. longibrachetum* T2 and *R. oryzae* W2 for the production of cellulase and xylanase. Optimization of different environmental factors for *T. longibrachetum* T2 i.e. alkali pretreated wheat

straw, pH 5.0, temperature 30°C, moisture ratio of 1:4.5 and incubation time of 7 days for the cellulase and xylanase production under SSF had yielded cellulase titers of 173.20 U/gds from 93.20 U/gds resulting in an impressive increase of 63.65% in cellulase activity, while xylanase had yielded 669.0 U/gds from 356.60 U/gds resulting in significant increase of 95.96% xylanase activity. Similarly, optimization of bioprocess parameters of *R. oryzae* W2 in untreated biomass at pH 5.0, and temperature at 30°C, moisture ratio of 1:4.5 and incubation time of 5 days for the cellulase and xylanase production under SSF had yielded cellulase titers of 179.40 U/gds from 106.40 U/gds resulting in an impressive increase 107.38% in cellulase activity, while xylanase had yielded 709.0 U/gds from 362.50 U/gds resulting in significant increase of 125.64% in xylanase activity. Inexpensive agricultural residue of wheat straw has emerged as a suitable substrate for high titers of cellulase and xylanase turning it as a cost effective process.

For purification of enzymes from potential bacteria and fungi the culture filterates of *B. amyloliquifaciens* SH8, *B. tequilensis* SH0 and *T. longibracheatum* T2 were subjected to two step purification strategies. The xylanase enzyme was initially concentrated by lyophilization and then applied to ion exchange chromatography and the pooled fractions showing highest enzyme activity were subjected to gel exclusion column chromatography, an effective purification steps for xylanase enzyme. Multiple bands ranging from 14.3 kDa to 97.4 kDa were obtained in both the bacterial and fungal isolates in ion exchange and gel exclusion chromatography after SDS gel electrophoresis.

In *B. amyloliquifaciens* SH8, xylanase was purified 1.65 fold with 63.35% yield and final specific activity of 90.12 IU/mg of protein in ion exchange chromatography. The enzyme activity of partially purified xylanase was 4.88 fold with 22.90% yield and final specific activity of 265.30 IU/mg of protein in gel exclusion chromatography. Partially purified xylanase of *B. tequilensis* SH0 was 1.82 fold with 57.69% yield and final specific activity of 72.95 IU/mg of protein in ion exchange chromatography. The enzyme activity of partially purified xylanase was 5.30 fold with 21.53% yield and final specific activity of 211.53

IU/mg of protein in gel exclusion chromatography. Similarly, in case of fungal isolate *T. longibracheatum* T2, final recovery in ion exchange chromatography of partially purified xylanase was 1.82 fold with 61.93% yield and final specific activity of 46.58 IU/mg of protein. The enzyme activity of partially purified xylanase was 5.23 fold with 23.87% yield and final specific activity of 133.89 IU/mg in gel exclusion chromatography.

The partially purified xylanase of *B. amyloliquifaciens* SH8, *B. tequilensis* SH0 and *T. longibracheatum* T2 were characterized by studying the effect of pH, temperature, metal ions, substrate concentration, K_m and V_{max} . The partially purified xylanase was tested for the optimum pH for the enzyme activity. pH optima for both the bacterial isolates was found 6.0. Partially purified enzymes of both the bacterial isolates had broad range activity between pH 4.0-10.0 with optimum peak at 6.0. Similarly, the optimum enzyme activity of *T. longibracheatum* T2 was at pH 5.0.

Bacterial xylanases had revealed thermostability up to 90°C and above that activity of the enzyme was affected negatively and declined afterwards. Partially purified xylanase being thermostable till 90°C has a desirable attribute for its use in industry. Similarly, the thermal activity of partially purified xylanase of *T. longibracheatum* T2 was evaluated by incubating it under varying temperature ranging from 30°C, 40°C...80°C. The optimum thermal activity of partially purified xylanase of *T. longibracheatum* T2 was found at 60°C and which declined with further increase in temperature.

The effect of metal ions on partially purified xylanase of *B. amyloliquifaciens* SH8, *B. tequilensis* SH0 and *T. longibracheatum* T2 were evaluated. The enzyme activity of partially purified xylanase was tested by incubating it with different metal ions (5mM) and it had been determined that in both the bacterial isolates, Hg^{2+} was strong inhibitor of enzyme activity while Cu^{2+} , Na^+ and Mg^{2+} had a slight inhibition effect on enzyme activity, while stimulatory effects were obtained with Zn^{2+} , Ca^{2+} and Mn^{2+} .

The partially purified xylanase enzymes of *B. amyloliquifaciens* SH8, *B. tequilensis* SH0 and *T. longibracheatum* T2 were evaluated towards various polysaccharides as substrates for hydrolysis. The enzyme activity of partially purified enzyme was tested by incubating 1% birch wood xylan, oat spelt xylan, cellulose, avicel and carboxymethylcellulose showing its true nature and conferring it cellulase free status. The partially purified xylanase enzyme showed highest substrate specificity towards birch wood xylan followed by oat spelt xylan whereas enzyme evinced no activity towards avicel, cellulose and carboxymethylcellulose. Whereas, in *T. longibracheatum* T2, the enzyme activity of partially purified xylanase testified highest towards birch wood xylan, followed by oat spelt xylan and exhibited trivial enzyme activity towards cellulose, avicel and carboxymethylcellulose.

The effect of partially purified xylanase on the hydrolysis of birchwood xylan concentration of *B. amyloliquifaciens* SH8, *B. tequilensis* SH0 and *T. longibracheatum* T2 was appraised. The enzyme activity of partially purified xylanase was tested by incubating it with different concentration of substrate ranging from (0.2, 0.4%....2.0%) and it had been determined that in both bacterial and fungal isolates, there was a gradual increase in velocity of the enzyme reaction with increase in the substrate concentration. A rectangular hyperbola is obtained when velocity is plotted against the substrate concentration.

The Michaelis-Menten behaviour and kinetic constant for birch wood xylan substrate hydrolysis by partially purified enzyme of *B. amyloliquifaciens* SH8, *B. tequilensis* SH0 and *T. longibrachiatum* T2 were evaluated. The kinetic parameters (K_m and V_{max}) were determined using Line weaver-Burk plot at 90°C (pH 6.0) with different concentrations of birchwood xylan ranging from 2-20 mg/ml for both the bacterial isolates whereas, K_m and V_{max} of *T. longibracheatum* T2 were determined using Line weaver-Burk plot at 60°C (pH 5.0) with birch wood xylan concentration ranging from 2-20 mg/ml.

For cellulase and xylanase encoding genes the PCR results depicted that the cellulase producing bacteria *B. subtilis* BM-1 exhibited a band of 1400 bp

corresponded to a fragment of *endoglucanase* gene and showed 98 per cent homology with endoglucanase (*eglS*) gene of *B. subtilis* (Acc. no. HM470252.1). In the present investigation, when the genomic DNA of the *R. oryzae* W2 was amplified with the primers of *cel* gene, amplicon of 650 bp were observed showing the presence of *cel* gene in the selected isolate. Cellulase ability of *R. oryzae* was confirmed by amplification of *cel* gene fragments. The PCR results showed that the xylanase producing *B. amyloliquifaciens* SH8 exhibited a band of 850bp and showed 96 per cent homology with *B. amyloliquifaciens* (Acc. no. CP000560.1).

Xylanase enzyme of *B. amyloliquifaciens* SH8 was scaled up from shake flask to pilot scale in a bioreactor. The different parameters were optimized in bioreactor i.e. inoculum age, inoculum size, aeration rate and agitation rate. The maximum enzyme yield was observed at 4h inoculum age, 10% inoculum size, 1.0 vvm aeration rate and agitation of 200 rpm (66.10 IU/ml). The growth kinetics for the product formation of *B. amyloliquifaciens* SH8 was determined and it showed that the product formation is growth associated.

In the present study the isolation of hypercellulolytic and xylanolytic bacteria and fungi from compost having rare combination of desirable characters have rendered them highly suitable for economically viable enzyme production, optimization, purification, enzyme kinetics and scale up studies, thus, strongly recommending their use for relevant industrial applications.

Chapter-6

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Title of thesis : “Purification of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost for industrial application”
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Degree awarded : Ph.D.
Year of award of degree : 2012
No. of pages in thesis : 385+II
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ABSTRACT

The present investigation was carried out to isolate, screen and identify the most efficient cellulolytic and xylanolytic microorganisms from compost. Enzyme production, optimization, purification, characterization, kinetics, cellulase/xylanase gene encoding and scale up studies were performed with selected strains to recommend their use for industries. In total 102 microorganisms including 93 bacteria and 9 fungi were isolated. Among them, three bacteria SH8, SH0 and BM-1 and two fungal isolates i.e. T2 and W2 were screened for cellulase and xylanase enzyme production studies. The bacterial isolates were identified as *B. amyloliquefaciens*, *B. tequilensis* and *B. subtilis* respectively by 16S rRNA PCR technique and registered in NCBI under accession no. JX129360.1, JX129359.1 and JX129361.1 while fungi were identified as *T. longibrachiatum* and *R. oryzae* by ITS 5.8S rRNA technique and being assigned accession no. JX213811.1 and JX213812.1. in NCBI. Cellulase and xylanase enzymes were optimized through classical approach one factor at a time (OFAT) as well as response surface methodology (RSM) under submerged fermentation varying medium, pH, temperature, inoculum size, carbon source, substrate concentration and incubation time and in solid state fermentation using different pretreatments i.e. pH, temperature, moisture ratio and incubation time. The percent increase in enzyme activity obtained after optimization of different process parameters was 204.51% for cellulase of *B. subtilis* BM-1, 222.91% and 210.52% for xylanase of *B. amyloliquefaciens* SH8 and *B. tequilensis* SH0, respectively in SmF. The increase in cellulase and xylanase of *T. longibrachiatum* T2 was recorded 63.65% and 95.96% while in *R. oryzae* W2 it was 107.38% and 125.64%, respectively under SSF. The purification of hydrolytic enzymes was proceeded following multistep purification technique using ion exchange chromatography and gel exclusion chromatography and the molecular weight of partially purified xylanase enzymes of *B. amyloliquefaciens* SH8, *B. tequilensis* SH0 and *T. longibrachiatum* T2 were found in the range of 14 kDa to 93.4 kDa, respectively. The partially purified xylanase of both bacterial and fungal origin were further characterized by studying, the effect of various parameters viz. the effect of pH, temperature, metal ions, substrate specificity, substrate concentrations and their kinetic parameters were derived. The partially purified thermostable xylanase of bacterial origin was active at 90°C, pH 6.0, showed high activity on xylan containing substrates and depicted cellulase free nature. The K_m and V_{max} of *B. amyloliquefaciens* SH8 xylanase for birch wood xylan were 166.67 $\mu\text{mol/mg/min}$ and 5.83 mg/ml, while for *B. tequilensis* SH0 were 166.66 $\mu\text{mol/mg/min}$ and 10.99 mg/ml. Similarly, partially purified enzyme of *T. longibrachiatum* T2 was optimally active at 60°C, pH 5.0 and showed high activity on xylan containing substrates. The K_m and V_{max} of *T. longibrachiatum* T2 xylanase for birch wood xylan were 125.0 $\mu\text{mol/mg/min}$ and 1.55 mg/ml. Cellulase and xylanase abilities of the isolates were targeted by amplification of *eglS* and *Xyn* genes. Optimum conditions i.e. inoculum age, inoculum size, aeration rate and agitation rate were explored at pilot scale for xylanase enzyme by *B. amyloliquefaciens* SH8. The inoculum age of 4h, inoculum size @ 10% concentration, 1.0vvm and agitation rate of 200 rpm were found best for production of xylanase enzyme in 7.5 L bioreactor. Mathematical model based on experimental results for xylanase production was proposed. The production of xylanase was found growth associated. The model consists of a set of ordinary differential equations taking into account the bacterial growth, substrate utilization and xylanase production with time.

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APPENDIX - I

ANOVA for Table 11
Cellulase activity IU/ml

Source	DF	SS	MS	F
Treatment	4	0.76	0.19	187.38
Error	10	0.01	0.001	
Total	14	0.77		

ANOVA for Table 12
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	4	1010.3	252.58	374.38
Error	10	6.74	0.67	
Total	14	1017.1		

ANOVA for Table 12
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	4	864.96	216.24	486.59
Error	10	4.44	0.44	
Total	14	869.40		

ANOVA for Table 13
Cellulase activity IU/ml

Source	DF	SS	MS	F
Treatment	4	3.81	0.95	179.76
Error	10	0.05	0.005	
Total	14	3.86		

ANOVA for Table 14
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	4	818.92	204.73	7311.74
Error	10	0.28	0.02	
Total	14	819.20		

ANOVA for Table 14
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	4	812.68	203.17	5079.23
Error	10	0.40	0.04	
Total	14	813.08		

ANOVA for Table 15
Cellulase activity IU/ml

Source	DF	SS	MS	F
Treatment	7	11.53	1.64	246.87
Error	16	0.10	0.006	
Total	23	11.64		

ANOVA for Table 16
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	12	3682.20	306.87	1780.64
Error	26	4.48	0.17	
Total	38	3686.89		

ANOVA for Table 16
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	12	2771.20	230.93	7263.23
Error	26	0.82	0.03	
Total	38	2872.0		

ANOVA for Table 17
Cellulase activity IU/ml

Source	DF	SS	MS	F
Treatment	6	1.22	0.20	20.12
Error	14	0.14	0.01	
Total	20	1.36		

ANOVA for Table 18
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	6	979.79	163.30	820.39
Error	14	2.78	0.19	
Total	20	982.57		

ANOVA for Table 18
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	4	919.83	153.20	5110.14
Error	10	0.42	0.03	
Total	14	920.25		

ANOVA for Table 19
Cellulase activity IU/ml

Source	DF	SS	MS	F
Treatment	6	3.49	0.58	97.24
Error	14	0.08	0.005	
Total	20	3.57		

ANOVA for Table 20
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	8	1834.50	229.31	247.40
Error	18	16.68	0.92	
Total	26	1851.20		

ANOVA for Table 20
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	8	1602.2	200.27	89.85
Error	18	40.12	2.22	
Total	26	1642.3		

ANOVA for Table 21
Cellulase activity IU/ml

Source	DF	SS	MS	F
Treatment	7	4.50	0.64	4.82
Error	16	2.14	0.13	
Total	23	6.64		

ANOVA for Table 22
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	11	3128.80	284.44	7140.14
Error	24	0.95	0.03	
Total	35	3129.7		

ANOVA for Table 22
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	11	3483.80	316.71	10858.62
Error	24	0.70	0.02	
Total	35	3484.50		

ANOVA for Table 23
Cellulase activity IU/ml

Source	DF	SS	MS	F
Treatment	6	10.87	1.81	15.36
Error	14	1.65	0.11	
Total	20	12.52		

ANOVA for Table 24
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	6	997.35	166.22	10577.91
Error	14	0.22	0.01	
Total	20	997.57		

ANOVA for Table 24
Xylanase activity IU/ml

Source	DF	SS	MS	F
Treatment	6	1580.7	263.45	14952.81
Error	14	0.24	0.01	
Total	20	1581.0		

ANOVA for Table 31
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	3909.40	977.35	960.07
Treatment (B)	1	2323.20	2323.2	2282.12
AxB	4	156.24	39.06	38.37
Error	20	20.36	1.01	
Total	29	6409.20		

ANOVA for Table 32
Xylanase activity IU/gds

Source	DF	SS	MS	F
Treatment (A)	4	3722.30	9305.70	291.40
Treatment (B)	1	1799.30	1799.30	563.43
AxB	4	538.60	134.65	4.22
Error	20	638.69	31.93	
Total	29	5639.30		

ANOVA for Table 33
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	1013.70	2534.30	492.20
Treatment (B)	1	2392.30	2392.90	464.62
AxB	4	179.54	44.88	8.72
Error	20	102.98	5.14	
Total	29	1281.20		

ANOVA for Table 34
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	11168.0	2792.0	11122.17
Treatment (B)	1	1627.7	1627.7	6484.15
AxB	4	2415.0	603.75	240.51
Error	20	50.20	2.51	
Total	29	13042.0		

ANOVA for Table 35
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	2401.80	600.46	457.67
Treatment (B)	1	1387.20	1387.20	1057.32
AxB	4	133.08	33.27	25.36
Error	20	26.24	1.31	
Total	29	3948.40		

ANOVA for Table 36
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	3802.3	9505.90	3541.24
Treatment (B)	1	1166.9	1166.90	434.70
AxB	4	49.86	12.46	4.64
Error	20	53.68	2.68	
Total	29	3929.4		

ANOVA for Table 37
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	9988.8	2497.20	851.37
Treatment (B)	1	3709.0	3709.0	1264.50
AxB	4	203.80	50.94	17.37
Error	20	58.66	2.93	
Total	29	1396.0		

ANOVA for Table 38
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	12019.0	3004.8	10949.25
Treatment (B)	1	7866.70	7866.70	2866.53
AxB	4	1332.5	333.12	121.39
Error	20	54.88	2.74	
Total	29	12945.0		

ANOVA for Table 39
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	3133.8	783.46	270.35
Treatment (B)	1	112.91	112.91	38.96
AxB	4	3.91	0.97	0.34
Error	20	57.96	2.89	
Total	29	3308.60		

ANOVA for Table 40
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	10655.0	2663.90	9495.75
Treatment (B)	1	89.58	89.58	31.93
AxB	4	1097.8	274.45	97.83
Error	20	56.10	2.80	
Total	29	10780.0		

ANOVA for Table 41
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	1189.60	297.41	365.61
Treatment (B)	1	573.17	573.17	70.46
AxB	4	7.58	1.89	0.23
Error	20	162.69	8.1	
Total	29	1264.0		

ANOVA for Table 42
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	13369.0	3342.20	8208.45
Treatment (B)	1	896.75	896.75	220.24
AxB	4	381.18	95.29	23.40
Error	20	81.43	4.07	
Total	29	13505.0		

ANOVA for Table 43
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	6	1837.60	3062.70	1160.11
Treatment (B)	1	3369.70	3369.70	1276.39
AxB	6	1276.90	212.82	80.61
Error	28	73.92	2.64	
Total	41	2309.70		

ANOVA for Table 44
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	6	21452.0	3575.40	11657.01
Treatment (B)	1	2251.80	2251.80	7341.59
AxB	6	9312.0	1552.0	506.01
Error	28	85.88	3.06	
Total	41	24644.0		

ANOVA for Table 45
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	6	4022.30	6703.80	6463.10
Treatment (B)	1	54.510	54.51	52.56
AxB	6	142.44	23.74	22.89
Error	28	29.04	1.03	
Total	41	4044.90		

ANOVA for Table 46
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	6	44754.0	7459.10	19186.76
Treatment (B)	1	773.14	773.14	198.87
AxB	6	26.53	4.42	1.14
Error	28	108.85	3.88	
Total	41	44845.0		

ANOVA for Table 47
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	2203.30	5507.50	4035.79
Treatment (B)	1	6169.10	6169.10	4520.57
AxB	4	1570.20	392.56	287.66
Error	20	27.29	1.36	
Total	29	2979.70		

ANOVA for Table 48
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	39244.0	9811.0	19503.74
Treatment (B)	1	3787.90	3787.90	7530.02
AxB	4	1216.30	3040.80	604.49
Error	20	100.61	5.03	
Total	29	44258.0		

ANOVA for Table 49
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	6405.30	1601.30	15880.17
Treatment (B)	1	48.87	48.87	48.46
AxB	4	21.24	5.31	5.27
Error	20	20.16	1.00	
Total	29	6414.30		

ANOVA for Table 50
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	4	67701.00	16925.0	7423.08
Treatment (B)	1	2857.70	2857.70	125.33
AxB	4	398.11	99.52	4.37
Error	20	456.02	22.80	
Total	29	68073.0		

ANOVA for Table 51
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	4886.20	6980.30	7050.79
Treatment (B)	1	1121.80	1121.80	11331.28
AxB	7	3989.40	569.910	575.67
Error	32	31.68	0.99	
Total	47	6410.10		

ANOVA for Table 52
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	71295.00	10185.00	39466.97
Treatment (B)	1	12241.00	12241.00	47435.02
AxB	7	2044.10	2990.20	1131.58
Error	32	82.58	2.58	
Total	47	85588.00		

ANOVA for Table 53
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	10122.0	1446.0	5953.46
Treatment (B)	1	63.135	63.13	25.99
AxB	7	538.21	76.88	31.66
Error	32	77.72	2.42	
Total	47	10190.0		

ANOVA for Table 54
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	81959.0	11708.0	46833.60
Treatment (B)	1	9747.0	9747.0	3898.80
AxB	7	981.0	140.14	56.06
Error	32	80.0	2.50	
Total	47	83040.0		

ANOVA for Table 55
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	5851.80	8359.70	4413.37
Treatment (B)	1	1479.80	1479.80	7812.49
AxB	7	6049.70	864.24	456.27
Error	32	60.61	1.89	
Total	47	7942.60		

ANOVA for Table 56
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	102700.0	14672.0	38307.48
Treatment (B)	1	16666.0	16666.0	43515.27
AxB	7	3291.90	4702.80	1227.88
Error	32	122.56	3.83	
Total	47	122670.0		

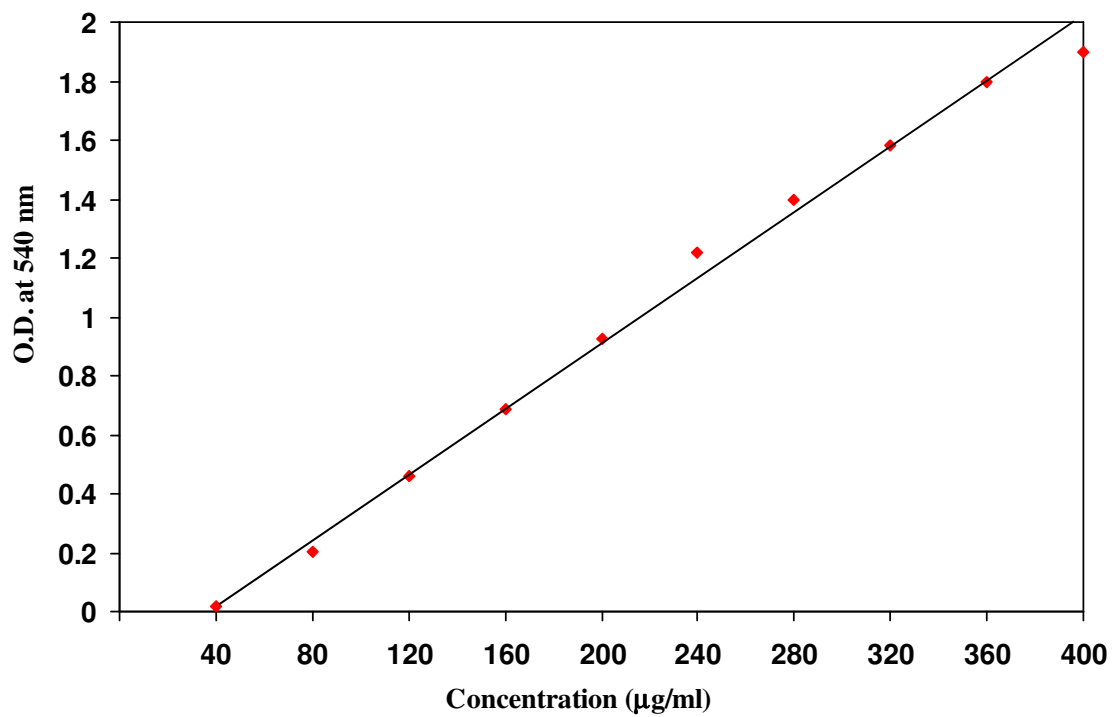
ANOVA for Table 57
Cellulase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	10213.0	1459.0	824.87
Treatment (B)	1	47.00	47.00	2.66
AxB	7	483.52	69.07	3.91
Error	32	565.99	17.68	
Total	47	10322.0		

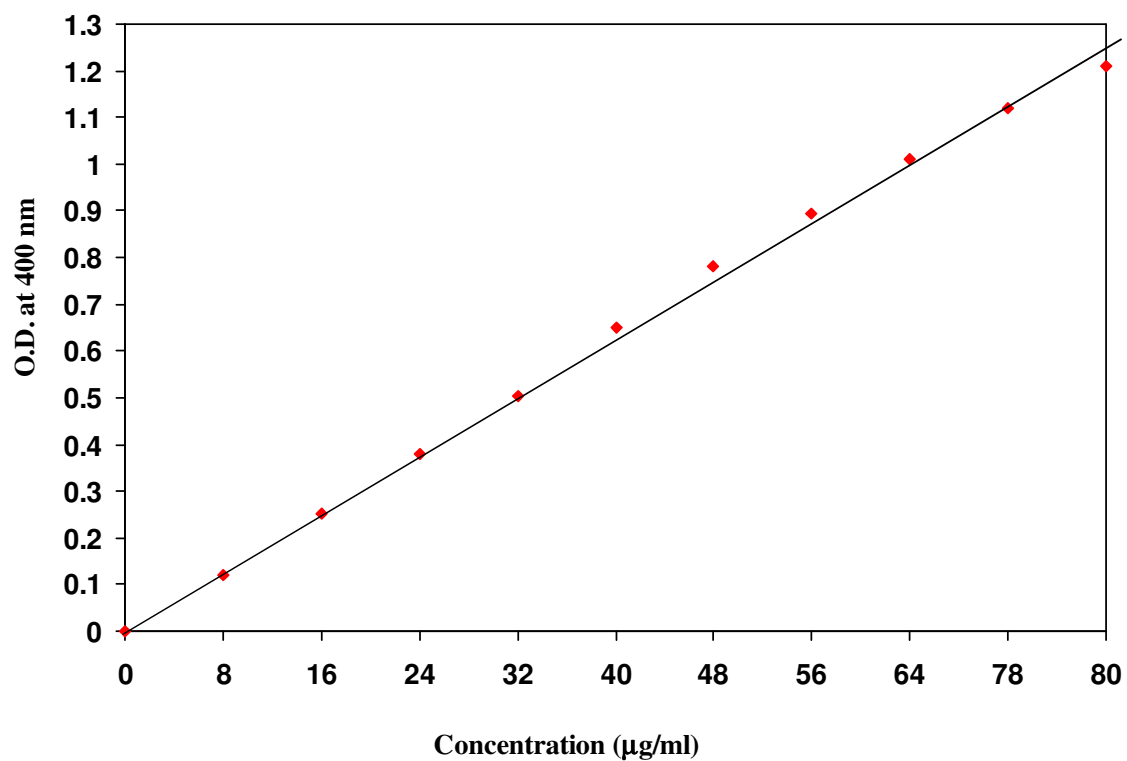
ANOVA for Table 58
Xylanase activity U/gds

Source	DF	SS	MS	F
Treatment (A)	7	177250.0	25321.0	21786.03
Treatment (B)	1	311.61	311.61	26.81
AxB	7	23.55	3.36	0.29
Error	32	371.93	11.62	
Total	47	177320.0		

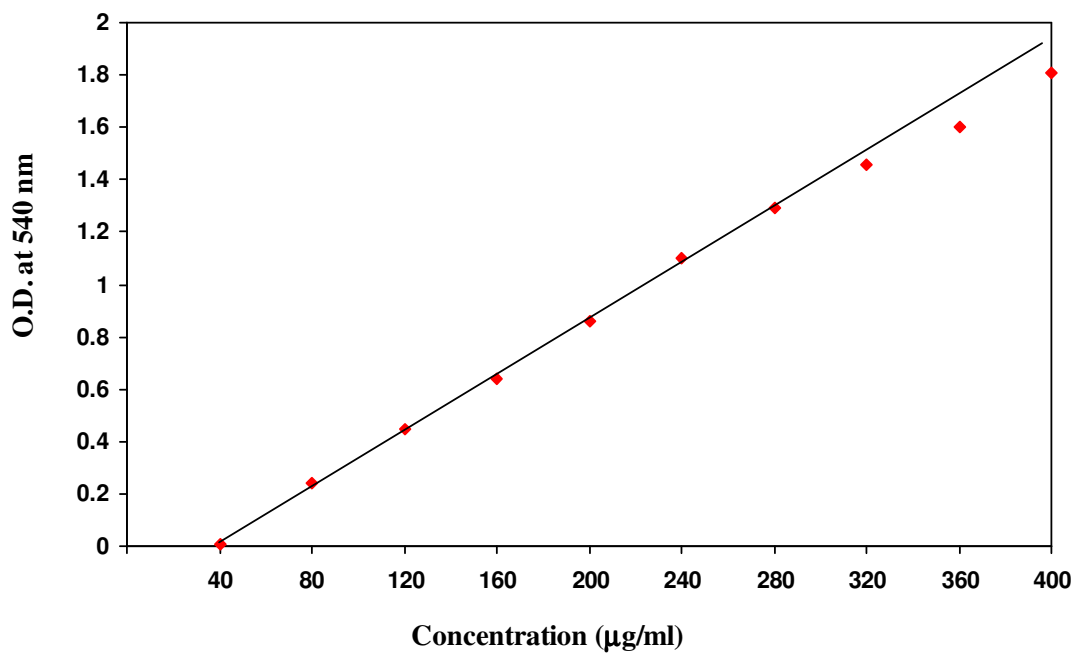
Appendix-II



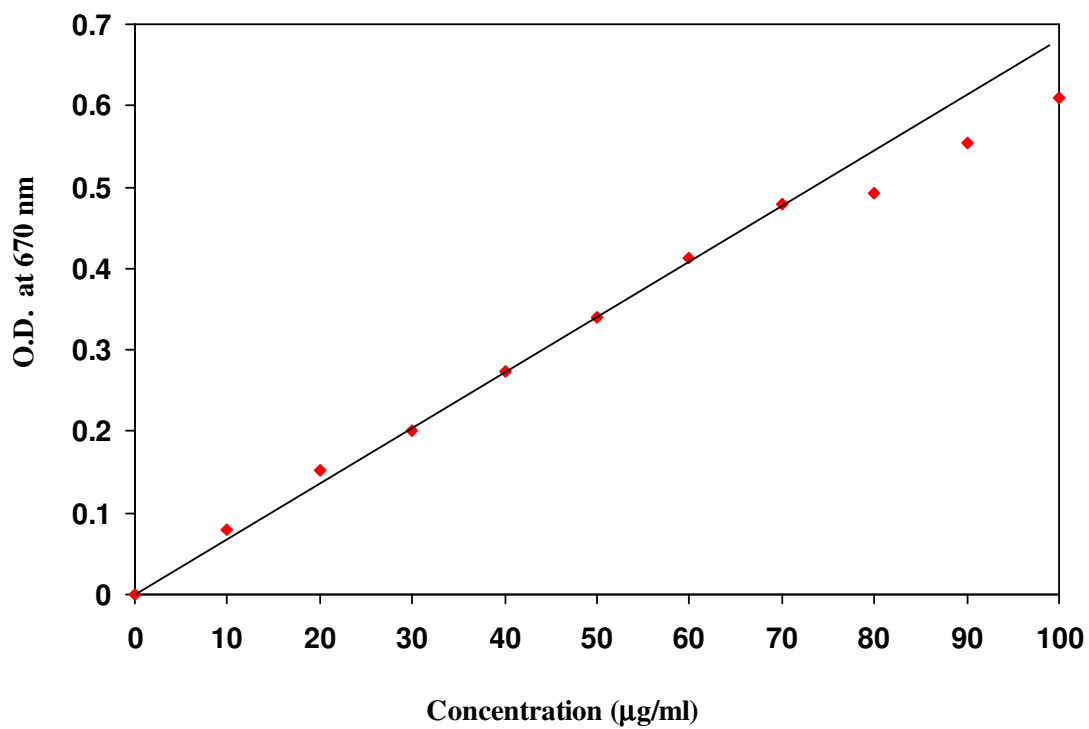
Standard curve of glucose



Standard curve of p-nitrophenol



Standard curve of xylose



Standard curve of Bovine Serum Albumin

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any other financial assistance No
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