

BIOCONVERSION OF CELLULOSIC WASTE INTO BIOETHANOL AS BIOFUEL

Thesis

by

NISHA SHARMA

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This is to certify that the thesis entitled, “**Bioconversion of cellulosic waste into bioethanol as biofuel**” 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 **Ms Nisha Sharma (F-2008-07-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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CERTIFICATE-II

This is to certify that the thesis entitled, “**Bioconversion of cellulosic waste into bioethanol as biofuel**” submitted by **Ms Nisha Sharma (F-2008-07-D)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfillment 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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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **“Bioconversion of cellulosic waste into bioethanol as biofuel”** submitted to Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP) by **Ms Nisha Sharma (F-2008-07-D)** in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY**.

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Needless to say, errors and omissions are mine.

Place: Nauni, Solan
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Nisha Sharma

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8.	Extracellular cellulase and xylanase production using untreated and pretreated <i>C. deodara</i> forest waste by <i>M. thermophila</i> SH1 (a) Untreated (b) steam (c) dil. H ₂ SO ₄ (d) NaOH + H ₂ O ₂ (e) dil. HCl (f) NH ₃	211-212
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ABBREVIATIONS

%	: Percent
β	: Beta
&	: 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
CFU	: Colony forming units
cm	: Centimeter
CMCase	: Carboxymethylcellulose
DEAE	: Diethylaminoethyl
DNSA	: Dinitrosalicylic acid
DNA	: Deoxyribonucleic acid
dNTPs	: deoxyribonucleotide triphosphates
E.C.	: Enzyme Commission
EG	: Endoglucanase
EtBr	: Ethidium bromide
Fig.	: Figure
FPase	: Filterpaperase
g	: Gram
g/L	: Gram per litre
h	: hour
i.e.	: That is
HMF	: Hydroxymethyl fufurals
HPLC	: High performance liquid chromatography
IU	: International units

kDa	: Kilodalton
L	: Litre
mg	: Miligram
mJ	: Mili joules
mg/ml	: Miligram per Millilitre
min	: Minute
ml	: Millilitre
mM	: Milimolar
MW	: Molecular weight
N	: Normal
nm	: Nano meter
O.D.	: Optical density
PCR	: Polymerase Chain Reaction
ppm	: Parts per million
PYC	: Peptone yeast extract
psi	: Per square inch
RNase	: Ribonuclease
rpm	: Revolution per minute
rRNA	: Ribosomal ribonucleic acid
SmF	: Submerged Fermentation
sp.	: Species
SSF	: Solid State Fermentation and Simultaneous saccharification and Fermentation
SHF	: Separate hydrolysis and fermentation
SSCF	: Simultaneous saccharification and co- fermentation
TGY	: Tryptone glucose yeast extract
U/g	: Unit per gram of substrate
UV	: Ultra violet
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

Chapter-1

INTRODUCTION

The world is facing a reduction of global fossil fuels resources, like petroleum, natural gas, or charcoal, while energy requirements are progressively growing up. Fossil fuels should be replaced, at least partially by biofuels once the current fuel supply is suspected to be unsustainable in the foreseen future. In fact, the search for sustainable alternatives to produce fuel and chemicals from non-fossil feedstocks has attracted considerable interest around the world, to face the needs of energy supply and to response to climate change issues. Alternative resources of energy are being explored in order to reduce oil dependence and increase energy production by exploring of solar, wind, hydraulic and other natural phenomena. Besides these sources of energy, also biomass possesses a potential target for fuel and power production. Thus biomass can efficiently replace petroleum-based fuels for a long term (Mussatto *et al.* 2010). Bioethanol is one of the products that can be obtained via biorefinery using bio-based resources. It is one of the most attractive biofuels, since it can be easily produced in large amounts and used as a “green” fuel (Xavier *et al.*, 2010).

Since bioethanol is an oxygenated fuel, it can be easily blended with gasoline and can be widely used for transportation purpose across the globe. The use of biofuels can significantly lower the emission of exhaust gases thereby resulting in a clean and eco-friendly environment (Idi and Mohamad, 2011). Biofuels produced from biomass have the ability to reduce CO₂ emission as the plants use CO₂ to grow. The organic/biological materials obtained from forest, agricultural residues and other wastes from food and agro industries are usually referred as biomass. Bioethanol is produced by the action of microorganisms on the fermentable sugars present in the biomass. The first generation bioethanol is produced mainly from different food crops such as corn, wheat, sugarcane, rice and barley. The usage of these crops for ethanol production led to shortage for food and currently renewable biomass such as lignocellulosic material has

emerged as an attractive low cost material for ethanol production, so called second generation bioethanol (Tiang *et al.*, 2012).

Lignocellulose consists of three main components: cellulose, hemicellulose (both referred as carbohydrate polymers) and lignin (aromatic polymer). Cellulose is the most abundant polymer in the world. Lignocellulosic biomass makes about 50% of the total biomass in the world with an estimated annual production of 10-50 billion tons. Cellulose ($C_6H_{12}O_6$) is a polysaccharide consisting of a linear chain of several hundred to over ten thousand β (1-4) linked D-glucose units. It is the most common form of carbon in biomass, accounting for 40% - 60% by weight depending on the type of biomass. Hemicellulose is a complex polysaccharide made from a variety of five and six carbon sugars. It has a random, amorphous structure with little strength; as such it is easily hydrolyzed by chemicals or enzymes. It consists of several matrix polysaccharides such as arabinoxylans. Lignin is a highly complex, three dimensional polymer of different phenyl propane units which are bound together by ether and carbon-carbon bonds. It is the most abundant and important polymeric organic substance in plants (Sanchez and Cardona 2008).

Pre-treatment of lignocellulose is a prerequisite step carried out to increase the surface area of the feed stock, remove barriers made by lignin, to increase porosity of cellulose and to release the sugars from cellulose and hemicellulose by enzymatic hydrolysis (Han *et al.*, 2012). The pretreatment can be classified into physiochemical, chemical and biological processes. This is a main processing challenge in the ethanol production from lignocellulosic biomass. Inefficient pre-treatment processes might result in non-hydrolysable residue formation and incomplete hydrolysis that affect the fermentation process adversely (Kodali *et al.*, 2006).

The pre-treatment process also generates primary inhibitors such as hydroxymethyl furfural (5-HMF), furfural and vanillins etc. which may further be degraded to produce secondary inhibitors. The inhibitors greatly affect the fermentation process by microbial inhibition (Ask *et al.*, 2013). Therefore

detoxification is employed for specific removal of inhibitors from lignocellulosic hydrolysates prior to fermentation by employing different physical, chemical and biological methods and making the ethanol fermentation an economical process (Canilha *et al.*, 2012).

Enzymatic hydrolysis of lignocellulosic biomass is considered as the most efficient and least polluting methods for generating fermentable hexose and pentose sugars from lignocellulosics. Hydrolytic enzymes comprised of cellulase and xylanase are involved in the natural degradation of cellulose and hemicellulose, the major polysaccharides of plant cells. The enzymatic complex can convert the cellulose to oligosaccharides and glucose. While xylanase in conjunction with other accessory enzymes, act synergistically to degrade xylan part of biomass to component sugars. Microorganisms such as fungi and bacteria are important producers of cellulases and xylanases, but the production economics of bioethanol is largely dependent on cost of hydrolytic enzymes. Since substrate cost accounts for a major fraction of the cost of cellulase and xylanase production, therefore the use of cheap biomass resources as cultivation media can help to reduce enzyme prices making it a cost effective process (Ali *et al.*, 2013).

Microorganisms, termed ethanologens convert the sugars saccharified from biomass to bioethanol. Enzymatic hydrolysis and fermentation process after pretreatment can either be performed separately or simultaneously, which are referred as Separate Hydrolysis and Fermentation (SHF) or Simultaneous Saccharification and Fermentation (SSF) and simultaneous saccharification and Co-fermentation (SSCF) processes.

In recent years, growing attention has been devoted to the conversion of lignocellulosic biomass into fuel ethanol, considering the liquid fuel as an alternative to fossil fuel. Therefore, keeping in view the ever increasing importance of biofuels, the present study had been undertaken with the following objectives:

- i) To isolate, screen and identify the cellulolytic and hemicellulolytic microorganisms.
- ii) To optimize cellulase and xylanase production.
- iii) To bioconvert cellulosic waste into bioethanol.

Chapter-2

REVIEW OF LITERATURE

Bioethanol, the liquid combustible fuel has become the most promising alternative substitute for gasoline. Lignocelluloses have a great potential as a biomass source for bioethanol production. Lignocellulosic materials such as agricultural residues, forestry residues, woody biomass and herbaceous energy crops are regarded as potential long- term alternative feedstocks for bioethanol production, since it is the most abundant reproducible resource on the earth (Keshwani, 2010). The basic structure of all lignocellulosic biomass consists of three basic polymers: cellulose, hemicellulose and lignin (Demirbas, 2005). Cellulose provide wood's strength and comprise ~ 40-50% of dry wood. A second major wood chemical constituent is hemicellulose which is also known as polyose and it usually account for 25-35% of the mass of dry wood (Mohan *et al.*, 2006). The lignins are highly branched, substituted, mono-nuclear aromatic polymers in the cell wall of certain biomass, especially woody species and are often bound to adjacent cellulose fibres to form a lignocellulosic complex. This complex and the lignins alone are often quite resistant to conversion by microbial systems and many chemical agents. The lignin contents on a dry basis in both softwoods and hardwoods generally range from 20% to 40% by weight (Yaman, 2004). The celluloses and hemicelluloses can be hydrolysed into monomer sugars and then used as feedstock for microbial fermentation for the accompanying production of value added biofuels and other chemicals. These complex polymers i.e., cellulose and hemicellulose can be hydrolysed chemically or enzymatically. In chemical hydrolysis predominant applied chemicals are H_2SO_4 and $NaOH$ while enzymatic hydrolysis is carried out by enzymes (Khan, 2010). Pretreatment is an important tool for practical cellulose conversion processes. The goal of any pretreatment technology is to alter or remove structural and compositional impediments to hydrolysis in order to improve the rate of enzyme hydrolysis and increase yields of fermentable sugars from cellulose or hemicellulose (Mosier *et al.*, 2005). Enzymatic hydrolysis of native lignocellulose usually results in

solubilization of 20% of originally present glucan. Some form of pretreatment to increase amenability to enzymatic hydrolysis is included in most process concepts for biological conversion of lignocellulose (Zang and Lynd, 2004). Utility cost of enzymatic hydrolysis is low as compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions and does not have corrosion problem (Sun and Cheng, 2002). Enzymatic hydrolysis is attractive because it produces better yields than acid catalyzed hydrolysis and enzyme manufacturers have recently reduced costs substantially using modern biotechnology (Pan *et al.*, 2010).

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 fibrous, insoluble, crystalline polysaccharide. It is a major polysaccharide constituent of plant cell walls, composed of repeating D-glucose units linked by β -1,4-glucosidic bonds (Jagtap and Rao, 2005) and being the most abundant carbohydrate polymer on earth (Guo *et al.*, 2008). The repeating units are cellobiose, since every other unit is rotated 180° around the main axis of cellulose chain. The parallel chains are held together by hydrogen bonds to form fibrils. Despite of cellulose is a hydrophilic molecule, it is insoluble in water. Cellulose has crystalline and less-ordered i.e. amorphous parts, which exhibit less resistance.

2.1.1.2 Biochemistry of cellulose degradation

Cellulase is an extracellular enzyme that degrades the cellulose to soluble form which can be assimilated and metabolized by the microorganism. The cellulase is distinguished from other glucoside hydrolases by its ability to hydrolyze β -1, 4-glucosidic bonds between glucosyl residues. The cellulase production is induced by cellulose or its derivative and is inhibited by hydrolyzing product which is glucose. Cellulase enzymes, which can hydrolyze cellulose forming glucose and other commodity chemicals, can be divided into three types:

- i. Endoglucanase (endo-1, 4- β -D-glucanase, EG, EC 3.2.1.4) ;
 - ii. Cellobiohydrolase or Exoglucanase (exo-1, 4- β -D-glucanase, CBH, EC 3.2.1.91) ; and
 - iii. β -glucosidase or cellobiase (1, 4- β -D-glucosidase, BG, EC 3.2.1.21)
- (Li *et al.*, 2006 and Gao *et al.*, 2008).

Cellulose degradation is a complex process and requires the synergistic action of the three enzymatic components consisting of endoglucanases, exoglucanases and β -glucosidases (Kumar *et al.*, 2008).

1. Endo-1, 4- β -D-glucanase (EC 3.2.1.4.)

These catalyse random cleavage of internal bonds of the cellulose chain to shorter chains within the macromolecules (Kumar *et al.*, 2008).

2. Exo-1, 4- β -D-glucanase (EC 3.2.1.91.)

These are the cellulases that attack the non-reducing ends, releasing cellobiose (Kumar *et al.*, 2008).

3. 1, 4- β -D-glucosidase

β -glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose (Kumar *et al.*, 2008).

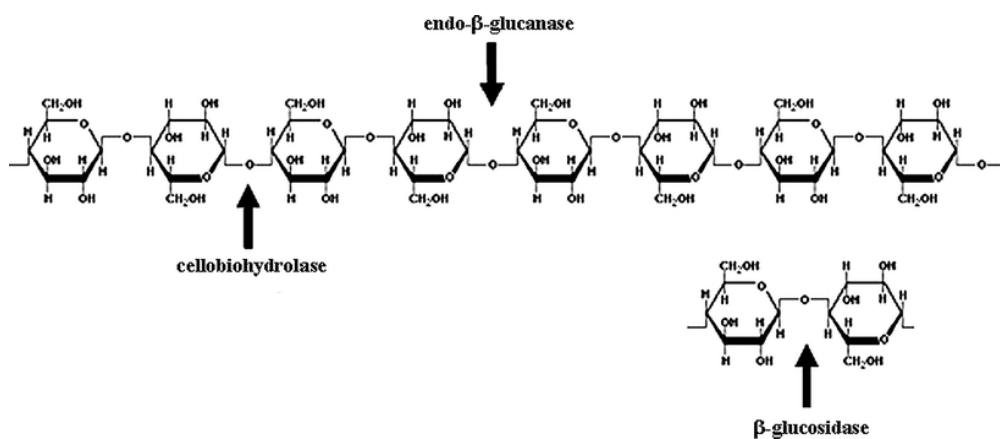
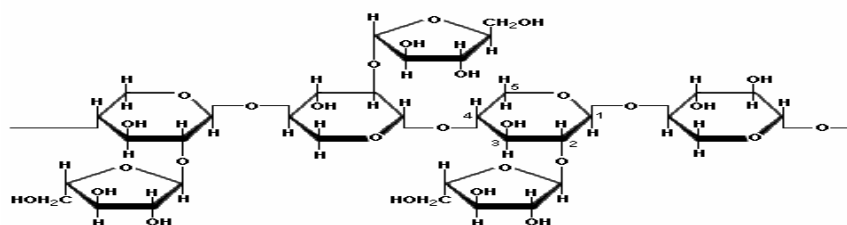


Fig. 1. Polymeric chemical structure of cellulose and site of action of endoglucanase, cellobiohydrolase and β -glucosidase (Kumar *et al.*, 2008)

2.1.1 Structure and chemistry of hemicelluloses

Hemicelluloses are a group of polysaccharides. Their amount is usually between 11-37%. Due to their branched structure they are easily hydrolysed to their monomer components consisting of xylose, mannose, glucose, galactose, arabinose and small amount of glucuronic acid and methyl glucuronic acid (Taherzadeh and Karimi, 2007).



Hemicellulose is a complex carbohydrate structure that consists of different polymers like pentoses (like xylose and arabinose), hexoses (like mannose, glucose and galactose) and sugar acids (Hendriks and Zeeman, 2009). Hemicellulose makes hydrogen bonds to cellulose micro-fibrils, forming a network that provides the structural backbone to the material (Mosier *et al.*, 2005).

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2.1.1.2 Biochemistry of hemicelluloses

Xylans are the major hemicelluloses in wood from angiosperms, where they account for 15-30% of the total dry weight. In the gymnosperms however, xylans contribute only 7-12% of the total dry weight. The structure of xylans is complex and their complete biodegradation requires the concerted action of xylanases enzymes (Gupta *et al.*, 2009).

Xylan is the most abundant non-cellulosic polysaccharide present in both hardwoods and annuals plants, and accounts for 20-35% of the total dry weight in tropical plant biomass. In temperate softwoods, xylans are less abundant and comprise about 8% of the total dry weight. Xylan is found mainly in the secondary cell wall and is considered to be forming an interphase between lignin and other polysaccharides. It is likely that xylan molecules covalently link with lignin phenolic residues and also interact with polysaccharides such as pectin and glucan. In simple forms xylans are linear homopolymers that contain d-xylose monomers linked through β -1-4-glycosyl bonds (Garg *et al.*, 2009).

In order to efficiently depolymerise xylan to the components monosaccharides, a mixture of different enzymatic functionalities are required including.

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) (Dodd and Cann, 2010).

One of the critically important enzymatic activities required for the depolymerisation of xylans is endo-1,4- β -xylanase activity. These enzymes cleave the β -1, 4 glycoside linkage between xylose residues in the backbone of xylans. Xylanases have been classified into GH families 5,7,8,10,11 and 43 on the basis of their amino acids sequences, structural fold and mechanisms for catalysis (Cantarel *et al.*, 2009).

2.2.1 ISOLATION AND SCREENING OF CELLULOLYTIC AND XYLANOLYTIC MICROORGANISMS

Efficiency of cellulases enzyme is one of determining factors in bioethanol from biomass production. However, it could be hampered by the presence of xylan. Employing microbes that can produce both cellulase and xylanase may help to overcome the current challenges in bioethanol production. Biodiversity of microorganisms in tropical forests offers more possibility for obtaining the potential microbes for this purpose.

Agustini *et al.* (2012) collect, screen, characterize and identify 553 isolates from forest ecosystems. This qualitative screening showed that out of which 304 isolates were positive producing cellulase and 323 isolates were positive producing xylanase. Eight potential isolates which showing greater cellulose- and xylanolytic indexes were subjected to enzyme activity tests and DNA analyses for species identification.

In a study conducted by Huang *et al.* (2012), 207 strains of aerobic and facultatively anaerobic cellulolytic bacteria were isolated from the gut of *Holotrichia parallela* larvae. These bacterial isolates were assigned to 21 genotypes by amplified ribosomal DNA restriction analysis (ARDRA). A partial 16S rDNA sequence analysis and standard biochemical and physiological tests were used for the assignment of the 21 representative isolates. Our results show that the cellulolytic bacterial community is dominated by the *Proteobacteria* (70.05%), followed by the *Actinobacteria* (24.15%), the *Firmicutes* (4.35%), and the *Bacteroidetes* (1.45%). At the genus level, Gram-negative bacteria including *Pseudomonas*, *Ochrobactrum*, *Rhizobium*, *Cellulosimicrobium*, and *Microbacterium* were the predominant groups, but members of *Bacillus*, *Dyadobacter*, *Siphonobacter*, *Paracoccus*, *Kaistia*, *Devosia*, *Labrys*, *Ensifer*, *Variovorax*, *Shinella*, *Citrobacter*, and *Stenotrophomonas* were also found.

Three cellulase producing isolates termed EHP1, EHP2 and EHP3 were isolated by Ibrahim and Ahmed, (2007) from Egyptian hot springs and soil samples. 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 avicel, CMC, cellobiose, and xylan, but there was no detectable activity on *p*-nitrophenyl- α -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.

Cellulase and xylanase producing bacteria from buffalo rumen were grown under anaerobic conditions at room temperature for 48 hours, using rumen fluid medium. Thirty two bacterial isolates were obtained, and screened for cellulase and xylanase production through incubation at room temperature for 72 hours on selective agar media containing respectively CMC and xylan. 19 and 4 colony isolates were positive for cellulase and xylanase production, respectively. The ratio of hydrolysis zone to the colony diameter was calculated. 6 isolates showed high hydrolysis ratio. Bacterial species were identified using 16S rDNA amplification. Comparison of the 1500-bp PCR products of the 6 isolates with the GenBank database using BLAST program showed that these isolates were matched to *Corynebacterium glutamicum* (90%), *Bacillus pumilus* (98%), *Bacillus altitudinis* (99%), *Staphylococcus* sp. (99%) *Corynebacterium casei* (99%) and *uncultured bacteria* (98%) respectively (Booppata *et al.*, 2011).

Waeonukul *et al.* (2007) isolated *Bacillus circulans* B6 which was cultivated in mineral salt medium containing avicel as a sole source of carbon under on aerobic condition. The bacterium produced extracellular cellulolytic and xylanolytic enzymes. The bacterium produced multiple cellulases and xylanases, which showed at least 12 types of CMCase and 11 types of xylanases on zymogram analysis. A study was conducted to find out the effective cellulytic bacteria for biodegradation of solid kitchen and agricultural wastes as organic manure or compost. Bacterial strains of *Moraxella* sp., *Cellulomonas* sp. and

Planococcus sp. were isolated from soil and cultured on nutrient agar media. Changes of temperature and pH, CO₂ release, crude fiber loss, protein, sugar and fat content as well as the activity of endoglucanase and cellobiase of waste were noted for selecting the most effective strain. In comparison to *Cellulomonas* sp. and *Planococcus* sp., inoculation of *Moraxella* sp. enhanced the degradation of kitchen and agricultural waste, shown by the increased CO₂ release (54.3 and 37.62 mg), crude fiber loss (46.86% and 45.11%), total sugar reduction (72.52% and 74.27%), fat reduction (65.20% and 61.22%), endoglucanase (0.097 mg/h/ml) and cellobiase (0.82 mg/hr/ml) activities. Inoculation of *Cellulomonas* sp. strain (53.89% and 77.96%) showed high protein reduction in comparison with inoculation of *Moraxella* sp. strain (20.04% and 63.42%) for kitchen and agricultural wastes. The overall findings of this investigation demonstrate the effectiveness of *Moraxella* sp. as a useful strain for bioconversion of solid organic waste (Barman *et al.*, 2011)

The cellulase producing bacteria was isolated from cow dung and characterized by morphological and biochemical analysis. Further partial purification of cellulase was carried out by dialysis and ammonium sulphate precipitation and also to determine molecular weight by SDS-PAGE and was found to be 32.5 kilodaltons. On performing zymogram staining, the cellulolytic property was confirmed (Shanmugapriya *et al.*, 2012) Forty nine out of the 250 different microbes (165 belong to fungi and 85 to bacteria) were tested for the production of cellulase enzyme and among these *Trichoderma viride* was found to be a potential strain in the secondary screening (Gautam *et al.*, 2012). Seventeen fungal species belonging to three genera i.e. *Trichoderma*, *Aspergillus* and *Penicillium* were isolated from different sources, screened and compared for their ability to degrade cellulose. Cellulolytic fungi were evaluated after 7 days for the production of cellulolytic enzymes by staining with 1% Congo red. The diameter of clear zone on fungal plates, gave an indication of cellulase activities. Fungal species were grouped as high and low cellulolytic isolates on the basis of cellulase activity using Index of Relative Enzyme Activity (ICMC). Fungal species i.e. *T. harzianum*, *T. viride*, *T. koningii*, *A. japonicus*, *A. nidulans* ver. *dentatus* *P. lanosum*, *P. expansum* and *P. oxalicum* gave the highest cellulase

activity. Whereas, *A. flavus*, *A. raperi*, *A. acculeatus*, *A. tamarri*, *A. niger*, *A. terreus*, *A. nidulans*, *P. citrinum* and *P. simplicissimum* showed least or no enzyme activity (Khokhar *et al.*, 2012).

Eight isolates of cellulose-degrading bacteria (CDB) were isolated from four different invertebrates (termite, snail, caterpillar, and bookworm) by enriching the basal culture medium with filter paper as substrate for cellulose degradation. To indicate the cellulase activity of the organisms, diameter of clear zone around the colony and hydrolytic value on cellulose Congo red agar media were measured. CDB 8 and CDB 10 exhibited the maximum zone of clearance around the colony with diameter of 45 and 50mm and with the hydrolytic value of 9 and 9.8, respectively. The enzyme assays for two enzymes, filter paper cellulase (FPC), and cellulase (endoglucanase), were examined. 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 (Gupta *et al.*, 2012). It has been established that cellulolytic enzymes play an important role in natural degradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa. In industry, these enzymes have found novel approach in the production of fermentable sugars, organic acids, detergents and ethanol production (Levy *et al.*, 2002 & Van Wyk and Mohulatsi, 2003). Isolation of mesophilic filamentous fungi from soil at incubation temperature of 25°C was done 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.

Julio *et al.* (2002) isolated 87 bacterial strains among which five strains were selected for their ability to produce either cellulases or xylanases and were

cultivated on soyabean (*Glycine max*) residue. *Bacillus subtilis* BL62 showed the highest specific cellulase activity, 1.08 UI/mg proteins within 24 hours of growth. Whereas *Bacillus subtilis* BL53 showed the highest specific activity for xylanase, 5.19 UI/mg protein within 72 hours of cultivation. Four bacterial colonies (*Bacillus* sp., two *Pseudomonas* sp. and *Proteus* sp.) and two fungal species (*Aspergillus niger* and *Aspergillus fumigatus*) were isolated and checked for cellulase production (zone formation) with the help of 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 and among fungi *A. niger* showed higher cellulase activity (0.1 IU/ml and 0.1 IU/ml) for coir waste and saw dust as substrate (Sajani *et al.*, 2010).

Yin *et al.* (2010) isolated a cellulase-producing bacterium from soil and identified as *Cellulomonas* sp. YJ5. Maximal cellulase activity was obtained after 48 h of incubation at 30°C in a medium containing 1.0% carboxymethyl cellulose (CMC), 1.0% algae powder, 1.0% peptone, 0.24% (NH₄)₂SO₄, 0.20% K₂HPO₄, and 0.03% MgSO₄·7H₂O. A cellulolytic and thermophilic anaerobe was isolated by Shuichi *et al.* (2003) from soil. Analysis of the PCR-based 16S rRNA gene sequence showed that the bacterium was closely related to *Moorella thermoacetica*. Carboxymethyl cellulase and xylanase activities were detected in the culture broth. This strain has a potential to convert cellulosic biomass to acetate directly.

Emtiazi *et al.* (2007) isolated *Paenibacillus* strains from different soils on N-free media. The cellulase positive *Paenibacillus* were selected by reduction of congored color on CMC medium. Three of these isolates with CMCase activities were identified as *Paenibacillus* strain E, H and SH. This nitrogen fixing strains with cellulase activities *Paenibacillus*, grow well on N-free media with sucrose or mannitol as the only sources of carbon and produced 3.16 and 1.3 mM ammonium, respectively. *Paenibacillus* strain E produced 4 u ml G1 CMCase when it was grown on CMC as the only source of carbon.

Myat *et al.* (2008) isolated wild type *Aspergillus niger* strains from different vegetable sources. All strains were studied by cultural, morphological

characteristics and microscopic examinations. The cellulase production (reducing sugar released) was measured by UV spectrophotometer using with 3,5-dinitrosalicylic acid (DNS) method. Among two different carbon sources, *Aspergillus niger* from sugar cane bagasse on Czapeck-Dox medium at 1% saw dust was found to be optimal for maximum cellulase producing activity. The cellulolytic ability of fungi from native environmental source was screened by Jahangeer *et al.* (2005).

Out of 115 fungal cultures isolated from environmental sources including soil, air and infected plant, 78 (67.83%) were found to possess cellulose degrading ability. Cellulolytic 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. Alkaline cellulases from 54 fungal isolates obtained from mangrove leaves and mangrove wood litters were screened as endophytes and wood litter fungi respectively. 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 (Ravindran *et al.*, 2010).

The indigenous fungi viz., *Trichoderma viride* Pers. ex Gray, *Trichoderma reesei* Rifai and *Trichoderma harzianum* Rifai were selected and analyzed on the basis of extent of hydrolyzing zones for the evaluation of their enzymatic activity in solid state fermentation on PDA and plate screening medium (PSM) at pH 4, 5 and 6. PSM at pH 4 depicted best results for all the strains tested. Strains FCBP-142 and FCBP-232 of *Trichoderma viride*, strains FCBP-271 and FCBP-364 of *Trichoderma reesei* and strains FCBP-210 and FCBP-325 of *Trichoderma harzianum* were considered best in their extent of hydrolyzing ability and were selected for evaluation of their cellulolytic activity

through submerged fermentation using 3, 5-dinitrosalicylic acid (DNS) method. The maximum enzymatic activity was achieved after 72 hours of incubation at $30 \pm 2^\circ\text{C}$ at initial pH 4 (Shafique *et al.*, 2009).

Cordeiro *et al.* (2002) isolated *Bacillus caldoxylolyticus* from soil. Studies on the enzyme characterization from liquid cultures grown on beechwood xylan revealed that the enzyme retained 100% of activity for 2 h 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 the crude enzyme solution for 24 hours at 25°C and at pH 5.5 to 8.0, a decrease of about 12% of its original activity was observed.

Roy and Rowshanul (2009) isolated twelve isolates from soil samples of different areas and studied for detection of xylanase activity. One of the strains was identified as *Bacillus cereus* on the basis of the nucleotide sequence of the 16S rRNA gene which produces xylanase extracellularly. Xylanase-producing strains were also isolated from drain water on xylan-agar media and were screened and 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 Abedin, 2002).

Nair *et al.* (2008) isolated 70 fungal strains were collected from soils. 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. All strains produced cellulase along with xylanase in solid state fermentation, while 70% of the strains produced cellulase-free xylanase during submerged fermentation. About 104 different strains of *Aspergillus niger* were isolated from soil by Khan *et al.* (2003) on xylene agar malt extract medium. The *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. Maximal xylanase productivity was obtained by GCBT-35 (225 IU/ ml). The

mycelia dry weight was 9.54 g/l while mycelia were small in size and rounded in shape.

88 actinomycetes were isolated from 20 samples of soils. Among these, 69 isolates were found positive for xylanase production. Of 69 isolates, 3 (SN32, SN77, and SN83) produced more than 125 IU/ml xylanase. Modern genetic tools were used for revealing the identities of these potent xylanase producers. The selected isolates were categorized under the genus *Streptomyces* based on their cultural and morphologic characteristics. Genetic diversity among these species of *Streptomyces* was established based on restriction length fragment polymorphism and random amplified polymorphic DNA analysis. The closest phylogenetic neighbours according to the 16S rRNA gene-sequence data for the three isolates SN32, SN77, and SN83 were *Streptomyces cyaneus*, *S. tendae*, and *S. caelestis*, respectively (Ninawe *et al.*, 2006).

Fifty-three litter degrading fungal species were isolated by nylon litterbag technique. The production of endo-b-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 (Palaniswamy *et al.*, 2008).

Xylanolytic thermophilic microorganisms were isolated from hot springs. 9 of these xylanolytic isolates belonged to the genus *Anoxybacillus*, and grew optimally at about 50-60 °C on nutrient agar. Based on the DNA-DNA hybridization study, SDS-PAGE profile, and biochemical and physiological features, I3, CT1Sari, and BT2.1 isolates are new strains of *Anoxybacillus gonensis*; I4.2 and B9.3 isolates are new strains of *A. voinovskiensis*; and I4.1, AC26, ACT14, and ACT2Sari isolates are new strains of *A. kestanbolensis*. The presence of xylanase activities, their optimum temperature, pH stability, and optimum pH were also investigated. The isolates ACT2Sari and ACT14 had the highest temperature optima (75 °C), and I3, CT1Sari, BT2.1, ACT2Sari, and

ACT14 had the highest pH optima (pH 9.0) of xylanase. The xylanases of I3, CT1Sari, BT2.1, I4.1, ACT2Sari, AC26, and ACT14 were optimally active both at alkaline pH and elevated temperature, and xylanases of I4.1, ACT2Sari, AC26, and ACT14 also were stable at alkaline pH (Inan *et al.*, 2011).

2.2.2 Mutation of potential cellulolytic and xylanolytic microorganisms to enhance their enzyme production

Mutations are changes to the nucleotide sequence of the genetic material of organisms. Mutations can be caused by copying errors in the genetic material during cell division, induced by exposure to ultraviolet or ionizing radiation, chemical mutagens such as ethyl methanesulfonate, ethidium bromide and N-methyl-N'-nitro-N-nitrosoguanidine.

The wild strain of *Rhizopus oryzae* PR7 MTCC 9642 was subjected to classical mutagenesis by suspending 5 hyphal discs (0.5 cm) in 10ml of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) solutions of various concentrations (125-1000µg). The *in situ* cellulolytic activity of the colonies of the mutant strains on the plates were measured by using alcoholic iodine solution and the highest enzyme producing mutant was selected. Out of 50 mutants, strain A7 was selected that showed about 33% increase in endoglucanase synthesis utilizing orange bagasse as sole carbon source in a shake flask screen (Bandyopadhyay *et al.*, 2012).

The fungal strain, *Aspergillus niger* was subjected to mutations involving treatment of Ethidium bromide (EtBr) for the production of cellulases. After mutagenesis, ten mutant strains (GNEB1 to GNEB10) were selected and their cellulase activities were assayed. Five of them (GNEB1, GNEB4, GNEB5, GNEB8, GNEB9) had significantly stronger ability to produce enzymes than that of normal wild type, and they were also very stable for a long period up to 8 generations to produce cellulase. Mutant Strain GNEB1 exhibited maximum Activities of cellulases, Filterpaperase (FPase-3.91 IU/ml), Carboxymethyl cellulase (CMCase-3.15 IU/ml) and β - glucosidase (0.87 IU/ml). These levels were, respectively, 2.3, 2.1 and 4.5 fold higher than those in parent strain (FPase-

1.68 IU/ml, CMCase-1.48 IU/ml and β -glucosidase-0.87 IU/ml) (Narasimha *et al.*, 2012).

Indigenous strain of *Trichoderma viride* FCBP-142 was selected to develop over-producer of cellulases and subjected to mutagenesis with ultra violet (UV) and chemical ethyl methane sulfonate (EMS). Among 178 survivals after UV irradiation, bigger zones of clearing on agar plates appeared around 81 colonies of putative mutant strains of native fungus with maximum of 87 IU/ml by Tv-UV-5.6 strain in comparison to parental strain (53 IU/ml). For EMS treatment, the enzyme production by the most active mutant Tv-Ch-4.3 showed even higher-level of cellulase activity (122.66 IU/ml) in contrast to the UV and parental strains (Shafique *et al.*, 2011). The mutant strain *Trichoderma reesei* WX-112 with high cellulase activity was isolated. The mutant's ability to produce cellulase increased 1.95 times after the treatment with UV and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Hao *et al.*, 2006)

Fawzi *et al.* (2011) subjected the wild cellulase producing fungal strain *Chaetomium cellulolyticum* NRRL 18756 to various doses of gamma irradiation (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 KGy). Among all the mutants tested, M-7 obtained at 0.5 KGy of *C. cellulolyticum* strain showed highest extracellular CMCase production in a yield 1.6-fold exceeding that of the wild type. The improvement of a thermophilic fungal strain *Humicola insolens* for cellulase production was done by Javed *et al.* (2011). *H. insolens* TAS-13 has the potential to produce 1.00 U/ml/min CMCase, 0.43 U/ml/min FPase and 0.30 U/ml/min β -glucosidase, which was improved through UV and chemical mutagenesis like *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), nitrous acid (HNO₂), ethyl methyl sulphonate (EMS) or ethidium bromide (EtBr). A mutant strain TAS-13UV-4 NG-5 proved to be the best mutant, which produced 43.19% CMCase, 60.15% FPase and 59.78% β -glucosidase more than the first generation mutant TAS-13UV-4. Furthermore, this mutant was highly stable upto many generations possessing cellulolytic ability.

Among 328 mutant strains of *Cellulomonas* sp. TSU-03, the mutant M23, NTG mutant, gave the highest value of cellulase activity 2008 U/mg protein)

followed by mutant M17 (1884 U/mg protein) in CMC medium. *Cellulomonas* sp. strain M23 produced the highest cellular growth (28.09 ± 2.28 g/L) and FPase, CMCase as well as, β -glucosidase activities at 325, 2420 and 152 U/mg protein, respectively. Under optimal condition, the cellulase activity achieved from strain M23 is 1.28 and 1.30 fold higher than cellulase from mutant M17 and wild type, respectively. After being subcultured 12 times, the cellulase production of the mutant M23 was stable (Sangkharak *et al.*, 2012).

Spores of *Aspergillus* sp. SU14 were treated repeatedly and sequentially with Co60 γ -rays, ultraviolet irradiation, and N methyl-N'-nitro-N-nitrosoguanidine. One selected mutant strain, *Aspergillus* sp. SU14-M15, produced cellulase in a yield 2.2- fold exceeding that of the wild type. Optimal conditions for the production of cellulase by the mutant fungal strain using solid-state fermentation were examined. The medium consisted of wheat-bran supplemented with 1% (w/w) urea or NH_4Cl , 1% (w/w) rice starch, 2.5mM MgCl_2 , and 0.05% (v/w) Tween 80. Optimal moisture content and initial pH was 50% (v/w) and 3.5, respectively, and optimal aeration area was 3/100 (inoculated wheat bran/container). The medium was inoculated with 25% 48 hr seeding culture and fermented at 35°C for 3 days. The resulting cellulase yield was 8.5-fold more than that of the wild type strain grown on the basal wheat bran medium (Kim *et al.*, 2011).

The strain improvement of *Bacillus coagulans* and *Geobacillus stearothermophilus* for thermostable cellulase production with higher enzyme activity was done. For strain improvement UV radiations, NTG and Sodium azide were used as mutagenic agents. NTG was found to be best mutagenic agent among all in term of producing the mutants with highest cellulase activity. Mutant strain C11 exhibited the highest cellulase specific activity at 45 U/mg followed by C15 (39 U/mg) in case of *B. coagulans* while Mutant strain S18 exhibited the highest cellulase specific activity at 69 U/mg followed by S12 (62 U/mg) in case of *G. stearothermophilus* (Sharma *et al.*, 2012).

A wild strain of *Bacillus pumilus* was investigated for cellulase production and putative mutants of this strain were screened for catabolite

repression insensitivity after chemical mutagenesis using ethyl methanesulphonate (EMS) as a mutagenic agent. Out of four classes of mutants studied, III and IV classes exhibited cellulase production upto 6.2 mg/g and 11.4 mg/g of dry cell mass respectively. These mutants were referred to as catabolite repression-insensitive when compared to the wild strain which exhibited a total repression of cellulase synthesis under the same conditions (Kotchoni and Shonukan, 2002). A mutant of *Bacillus pumilus* was obtained after chemical mutagenesis and screened for cellulase production. This mutant named Bp CRI6 was selected for its ability to produce cellulase under catabolite repression. Cellulase yield by Bp CRI6 was four times higher than that of the wild type under optimum growth conditions (25°C, pH 6.5 and Ca^{2+} 1mM). The mutant strain was stable and grew rapidly in liquid and solid media (Kotchoni *et al.*, 2003).

Singh *et al.* (1995) observed the enhancement of more than three fold in the activity of xylanase and xylosidase enzymes by mutagenesis of *Fusarium oxysporum* DSM 841 spores with UV or N-methyl- N'- nitro- N-nitrosoguanidine treatments. The hyperxylanolytic mutant (NTG-19) was found to secrete high levels of xylanolytic enzymes on commercial xylan as well as on several agricultural residues, of which wheat bran supported maximum enzyme yields. Further increase in enzyme secretion (25%) was observed with addition of 0.2% olive oil, an inexpensive source of fatty acids.

Chadha *et al.* (1999) observed that UV/NTG mutagenesis of *T. lanuginosus* BS1 aleurospores/ protoplasts resulted in hyper xylanase producing mutants. A morphological colour mutant RB 524 produced approximately 2.5 fold higher xylanase (2506.0 U/ml) as compared to the parent strain (1018.1 U/ml). The regulation of xylanase synthesis by mutant strain *T. lanuginosus* MC134 was studied by Kuttanpillai *et al.* (2009) that showed on oat spelts xylan a 1.5 fold higher xylanase production than the parent strain SK, on various carbohydrates and induction in glucose grown cells. In the growth experiments the highest production of xylanase was observed in the presence of xylan, however an appreciable amount of the enzyme, about 10% was also produced during growth on xylose. Xylobiose was found to be the most efficient xylanase

inducer in the glucose grown cells. Its induction efficiency was followed by xylose, beechwood and birchwood xylan.

Haq *et al.* (2004) studied the effects of time course, incubation temperature, medium pH and volume of fermentation medium on the production of xylanase for enhanced biosynthesis of xylanase by a chemically mutated strain of *Aspergillus niger* GCBCX-20. 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. Irfan *et al.* (2011) conducted a study to improve the enzyme production from *Aspergillus niger* using UV mutation. Submerged fermentation was carried out in 250ml Erlenmeyer flask using Vogel's media at 30°C for six days. Results of this study revealed that UV-mutation enhanced CMCase activity up to two times while FPase activity up to three times as compared to the parental strain.

Nicolas *et al.* (2006) observed 1.17 times increase in hemicellulolytic and cellulolytic activity by mutant strains from *Aspergillus niger* UAM- GS1 which was produced by UV radiation. Mutant strains GS1-S059 and GS1-S067 significantly increased their level of mannanase, xylanase and cellulase production, compared to parental strain, improving their potential industrial applications.

2.2.3 Production of cellulase and xylanase

2.2.3.1 Production of cellulase and xylanase under submerged fermentation (SmF)

The conversion of lignocellulosic biomass to fermentable sugars through biocatalyst cellulase and xylanase derived from cellulolytic and hemicellulolytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution.

Shabeb *et al.* (2010) tested different factors for cellulase productivity by *Bacillus subtilis* KO strain using carboxymethyl cellulose clear zone (CMCZ) and dinitrosalicylic acid (DNSA) techniques. Cellulase enzyme revealed its best production; 32 IU/ml by CMCZ technique and 228 µg/ml broth medium by DNSA technique as well as 525 µg/ml broth medium of protein content after 24 h incubation period. At 45°C, cellulase productivity was 361 IU/ml by CMCZ and 344 µg/ml broth medium by DNSA with 301 µg/ml broth medium of protein content. Moreover, maximum cellulase productivity was 351 IU/ml by CMCZ and 420 µg/ml broth medium by DNSA with 601 µg/ml broth medium protein content when molasses broth medium was supplemented with cellulose and tryptone.

Marinobacter sp. (MSI032) isolated from the marine sponge *Dendrilla nigra* was optimized by Shanmughapriya *et al.* (2009) for the production of extracellular cellulolytic enzyme (CMCase) by submerged fermentation. Initial experiments showed that the culture medium containing 1% maltose as carbon source and 1% peptone and casein as nitrogen source supported maximal enzyme production at 27°C and at a pH of 9.0. Further optimization carried out showed the maximal enzyme production was supported by the presence of 2% NaCl and 10 mM Zn²⁺ ions in the production media. The production of enzyme cellulase occurred at 48 h of incubation which proved the importance of this strain for cellulase production in large scale. Further, the enzyme was purified to 12.5-fold with a 37% yield and a specific activity of 2,548.75 U/mg. The purified enzyme displayed maximum activity at mesophilic temperature (27–35°C) and at a broad pH range with optimal activity at pH 9.0. The purified enzyme was stable even at a higher alkaline pH of 12.0 which is greater than the pH stability that has not been reported in any of the cellulolytic isolates studied so far.

Jaradat *et al.* (2008) observed the highest crude cellulase enzyme activity (432 U/l) after 3 days of incubation at pH 7 and 60 °C in a medium that was supplemented with 0.5% glucose, 0.2% starch, and 0.2% NH₄Cl by *Streptomyces* strain J2. However, enzyme production and activity were strongly decreased at 45°C and acidic pH. Six marine strains of actinomycetes were screened for their

carboxymethyl cellulase (CMCase) productivity. *Streptomyces ruber* was chosen to be the best producing strain. The highest enzyme production (25.6 IU/ml) was detected at pH 6 and 40°C after 7 days of incubation. Results showed that a high concentration of KH_2PO_4 and a low concentration of MgSO_4 had a significant effect on enzyme production. Rice straw was used as a low cost source of cellulose. It was found that 30 g/l rice straw was the suitable concentration for maximum enzyme production (Nermeen *et al.*, 2010).

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). A filamentous fungus *Trichoderma harzianum* IOC-3844 was used for the production of cellulases from a pretreated sugarcane bagasse (namely, cellulignin), by submerged fermentation and revealed that the fungal strain produced high contents of endoglucanase activity (6,358 U/l) after 72 hours of process, and further relevant β -glucosidase and FPase activities (742 and 445 U/l, respectively.). The crude enzyme extract demonstrated appropriate characteristics for its application in cellulose hydrolysis, such as high thermal stability at up to 50°C, accessory xylanase activity, and absence of proteolytic activity towards azocasein (De Castro *et al.*, 2010).

Milala *et al.* (2005) studied the production of cellulolytic enzymes by *Aspergillus niger* in submerged culture with millet, guinea corn straw, rice husks and maize straw as substrates. Optimal cellulase secretion by *A. niger* was achieved at a time of 72 h in maize straw and rice husk media. 96 hours and 120 h were the growth period in millet and guinea corn straws respectively. Substrate concentration of five percent (5%) w/v and pH 3 resulted in optimal enzyme secretion. Gautam *et al.* (2010) compared the production of cellulase by *A. niger* on three different carbon sources. Maximum cellulase enzyme activity (Filter paper activity, endoglucanase and β -glucanase) were obtained from the culture containing cellulose. The production of cellulases from *Aspergillus heteromorphus*, by submerged fermentation using wheat straw as substrate was

investigated. 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 *Aspergillus 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).

Aspergillus humicola, one of the major cellulase-producing fungi was used by Nipa *et al.* (2006), in their study for carboxymethylcellulase (CMCase) production using Winstead's basal broth supplanted with cellobiose octaacetate (OCA), a synthetic carbon source. Under all conditions, the enzyme biosynthesis was remarkably increased when the inducer OCA was added to the production medium containing carboxymethylcellulose (CMC). Maximum enzyme production (1.62 U/ml) was achieved in OCA-containing at 37°C. The enzyme production was highest at initial pH 5.5 and after 7 days incubation. The enzyme exhibited maximum activity at 40°C with a reaction pH 5.5. CMCase activity was inhibited by its own substrate CMC at concentration higher than 1.0%. The study clearly demonstrated that COA is a good inducer for extracellular CMCase production by the fungus.

The cellulolytic enzyme-endoglucanase activity against coir fibre, a major biowaste by bacteria such as *Cellulomonas*, *Bacillus* and *Micrococcus* spp. isolated from coir retting effluents of estuarine 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* spp 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. From the overall result, it was observed that, among the three bacterial strains, the enzyme activity was more in *Cellulomonas* sp, followed by *Bacillus* and *Micrococcus* spp. in varying pH and temperature (Immanuel *et al.*, 2006).

Narasimha *et al.* (2006) compared the production of cellulase (filter paper activity, endoglucanase and β -glucosidase) by *Aspergillus niger* on three media in liquid shake culture. The culture filtrate of this organism exhibited relatively highest activity of all three enzymes and extracellular protein content at 7-day interval during the course of its growth on Czapek-Dox medium supplemented with 1.0% (w/v) cellulose. Urea as a nitrogen source and pH 5.0 were found to be optimal for growth and cellulase production by *Aspergillus niger*. Among various soluble organic carbon sources and lignocelluloses tested in this study, carboxymethylcellulose and sawdust at 1% supported maximum production of all three enzymes by *Aspergillus niger*. Teoh and Mashitah (2010) addressed the cellulase production by *Pycnoporus sanguineus* grown in shake flask culture using a statistical approach. Optimum condition was obtained in 70% (v/v) palm oil mill effluent supplemented with 6 g/l sterilized palm pressed fibers at pH 6.77 and 350 rpm with CMCase, FPase and β -glucosidase activities and net changes of biomass and suspended solid at 50.11, 29.01, 5.58 IU/ml and 2.49 g/l, respectively.

Ahmed *et al.* (2009) revealed that *Trichoderma harzianum* produces cellulase enzyme. The optimal pH, temperature and incubation time for cellulases production was found to be 5.5, 28°C and 120 h respectively. Cellulase production was increased by the effects of glucose, carboxymethylcellulose (CMC), corn cobs, birchwood xylan and wheat bran as carbon sources for cellulase production. Sadia *et al.* (2005) reported that endoglucanase was produced by the fungus *Trichoderma harzianum*, E-58 strain in Vogel's medium with different carbon sources like xylan, CMC, corncobs and glucose for 5- days at 180 rpm at 28°C in orbital shaker. Optimum pH and temperature of incubation for enzyme activity were 5.5 and 60°C respectively. Maximum enzyme activity

of endoglucanase was found to be 0.798 IU /ml by using xylan as a carbon source. The cellulase production was carried out by using corn cob residue from xylose manufacture as substrate on *Trichoderma reesei* ZU-02, it was found that on the same cellulose basis, the cellulase activity and yield produced on corn cob residue were comparable with that on purified cellulose. Under batch process, the optimum concentration of substrate was 40 g/l and the optimum C/N ratio was 8.0. Cellulase and β -glucosidase activity were 5.48 IU/ml (222.8 IU/g cellulase) and 0.25 IU/ml (10.2 IU/g cellulose), respectively, after four days in submerged fermentation. (Liming and Xueliang., 2003).

Haq *et al.* (2006) described the production of cellulolytic enzymes and hemicellulolytic enzyme along with total extracellular protein by *A. niger* and *T. viride* using submerged fermentation. Enzymes were investigated by mono-and co-cultures of *A.niger* and *T. viride* using wheat bran as substrate with supply of Eggins and Pugh salt medium. Co-culture gave 30-50% higher production than mono-cultures of *A. niger* and *T. viride*.

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 (R²) of 0.995 (Rashid *et al.*, 2009).

Subramaniyam and Prema (2000) used cellulose fibres as substrate and found that alkalophilic *Bacillus* SSP-34 produced more than 100 IU/ml of xylanase activity with only small amount of cellulolytic activity i. e. CMCase 0.4 IU/ml, FPase 0.2 IU/ml at 50⁰C and at neutral pH. Xylanase excreted by *Bacillus* sp. CCMI-966 exhibited activity from pH 5.0-6.5 with the optimum at pH 6.0. The specific activity of purified xylanase was 137 U/mg (Pereira *et al.*, 2000).

The production of cellulase free and alkali stable xylanase in high titre from newly isolated *Bacillus pumilus* SV-85S using cheap and easily available agro-residue wheat bran was studied by Nagar *et al.* (2010). Optimization of fermentation conditions enhanced the enzyme production to 2995.20 ± 200.00 IU/ml which was 9.91 fold higher than the activity under optimized basal medium (302.2 IU/ml).

The effect of medium composition and environmental condition on the production of cellulase by *Aspergillus terreus* was investigated using shake flask culture with oil palm empty fruit bunch (OPEFB) as substrate. The highest activity of FPase (0.76 U ml⁻¹), CMCase (8.64 U ml⁻¹) and β -glucosidase (6.81 U ml⁻¹) was obtained in medium containing 6 g L⁻¹ yeast extract and 10 g L⁻¹ delignified OPEFB fiber. In fermentation with the addition of Tween 80 (2 ml L⁻¹) as surfactant, the production of cellulase was increased by two-fold as compared to fermentation without surfactant. Cellulase production by *A. terreus* was also enhanced with the addition of calcium chloride (3 mM) and magnesium sulfate (5 mM) in the medium. Optimum pH and temperature for cellulase production by *A. terreus* was 5.5 and 28°C, respectively. Cellulase production in agitated shake flask fermentation at 200 rpm was four times higher as compared to fermentation in static flask (Shahriarinoor *et al.*, 2011).

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 120h of incubation. NaNO₃ increased xylanase production 5.4 fold as compared to the control. Optimum initial pH was found to be 4.5-5.0 (Bakri *et al.*, 2008). Maximum xylanases activity (35U/ml) from *A. terreus* after 96 hr at 35°C and pH 6 in 1% oat spelt xylan was reported by Silas *et al.*, (2008). The xylanase was purified to homogeneity by gel filtration on sephacryl S-200. Shah and Madamwar (2005) studied the production of xylanase by *A. foetidus* MTCC 4898 using submerged fermentation under shaking condition. Maximum xylanase yield (210U/ml) with

negligible cellulase activity (≤ 0.1 FPU/ml) at 30°C using 1% birchwood xylan as substrate in 3 days of cultivation was obtained. The enzyme was stable at 40°C but retained only 36% of its activity at 50°C after 3 h. They concluded that presence of KCl, CaCl₂, glycerol, xylan and trihalose significantly improved thermostability at 50 and 60°C. The growth conditions including incubation time, temperature, agitation rate and initial pH of the medium that affected xylanase production by *A. carneus* M34 was studied by Fang *et al.*, 2007. They observed that xylanase activity (22.2 U/ml) was verified using the predicted optimal conditions. The optimal temperature and pH of the crude xylanase activity was observed at 60°C and acidic pH, respectively. Sustained xylanase activity in the crude extract was also detected over a broad range of pH from 3-10.

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 (4.75U/mL) was recorded at 72 h.

Knob and Carmona (2008) investigated the production of xylanase by the fungus *Penicillium sclerotiorum* under submerged cultivation. Oat spelt 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 minutes and when it was incubated at 45 and 50°C, the half-life was 8 minutes and shorter than 4 min, respectively. High pH stability was verified from pH 3.5 to 7.5. Nadia *et al.* (2010) studied the ability of different *Streptomyces* strains to produce many useful products such as xylanase, reducing sugar and microbial protein. Wheat straw and corn cobs were used as substrates in liquid medium for enzymes and protein production. *Sterptomyces lividans* was the most active and producer strains on the static cultured using xylan or wheat straw as the best substrate for enzyme production were studied. Maximum level of xylanase activity was observed at 20g/l wheat straw with *S. Lividans*, while the optimum pH of xylanase formation was observed at pH 7.0

Khuchareonphaisan *et al.* (2008) reported a thermophilic fungus *Thermomyces lanuginosus* to produce a high level xylanase when cultivated in the medium containing xylan and corn-cob as a carbon source. *T. lanuginosus* produced a single xylanase with molecular masses in range of 22.0 to 29.0 kDa. The cultural conditions for the production of thermostable xylanase by the thermophilic fungus *T. lanuginosus* in both submerged and solidstate fermentation. They observed that carbon and nitrogen sources mainly influenced the enzyme yield under submerged conditions in shake flasks. The xylanase of *T. lanuginosus* was found to be inducible by xylan and xylose. The medium used for the optimal production (31.2 g/l corncobs, 30.2 g/l yeast extract and 5.0 g/l KH_2PO_4) yielded 36,200 nkat/ml within 7 days of cultivation.

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. NaNO_3 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 (Bakri *et al.*, 2008).

2.2.3.2 Production of cellulase and xylanase under solidstate fermentation

Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented products may be used directly as the enzyme source (Tengerdy, 1996). A large number of microorganisms including bacteria, yeast and fungi produce different groups of enzymes in SSF. Agro-industrial residues are generally considered the best substrate for the SSF processes. The major factors that affect microbial synthesis of enzymes in a SSF system include: selection of a suitable substrate and microorganism, pretreatment of the substrate, particle size of the substrate, water content and a_w of the substrate, relative humidity, type and size and inoculum, control of temperature of fermenting matter/ removal of

metabolic heat, period of cultivation, maintenance of uniformity in the environment of SSF system and the gaseous atmosphere i.e., oxygen consumption rate and carbon dioxide evolution rate (Pandey *et al.*, 1998). SSF offers distinct advantages over submerged fermentation including economy of space, simplicity of media, no complex machinery, greater product yields and reduced energy demand (Sanghi *et al.*, 2008).

Odeniyi *et al.* (2009) isolated a *Bacillus coagulans* strain from palm fruit husk and was tested for abilities to hydrolyse plant structural polysaccharides through the depolymerising activities of carboxymethylcellulase and polygalacturonase. The two enzymes were produced using solid substrate fermentation. Both had a working pH range of 4 - 9 with an optimum pH of 6.0 for the carboxymethylcellulase and 7.0 for the polygalacturonase. The respective enzymes remained active when allowed to stand at 27°C for 1 hour over a wide pH and temperature range maintaining maximum activity at an optimum temperature of between 50°C and 60°C respectively. Ray *et al.* (2007) optimized the environmental and nutritional parameters for fermentation to enhance cellulase production by two bacterial strains isolated from fish gastrointestinal tracts. And they found that cellulase yield was highest (26 IU in *B. subtilis* and 20.2 IU in *B. circulans*) in solid-state fermentation (SSF). Enzyme production in both the isolates increased in an optimum pH range of 7.0 to 7.5. Maximum production of cellulase was obtained at 40°C. To standardize the fermentation period for cellulase production, production rate was measured at 12-hours intervals up to 120 hours. Enzyme production increased for 96 h of fermentation in both strains, and decreased thereafter. The enzyme production increased with increased inoculum size up to 3.0 percentage points. Asparagine as the nitrogen source was most effective in *B. subtilis* CY5, while beef extract proved useful in optimizing enzyme production by *B. circulans* TP3.

A novel cellulase producing actinomycetes, *Streptomyces* sp was isolated from decayed fruit waste and optimized the physicochemical parameters for cellulase production. Production of cellulase enzyme by a *Streptomyces* sp S7 was detected on cellulose agar medium after 4 days of incubation at 28 °C that

exhibited a clear zone of 25mm around the colony. The highest crude enzyme production was observed at pH 5 and temperature of 40°C in a medium that was supplemented with fruit waste as carbon source. It could be concluded that *Streptomyces sp S7* is a powerful cellulase producer strain (Rahna and Ambili, 2011).

Kapoor *et al.* (2010) aimed at the production of cellulase enzyme using wheat straw as substrate from *Chaetomium sp.* under Solid State Fermentation (SSF) condition. The culture parameters, such as, duration of incubation, incubation temperature, pH, carbon and nitrogen sources and additives, were optimized for enhancing cellulase yield. The optimal level of each parameter for maximum cellulase production by the fungus was determined. Results showed that cellulose production was highest with wheat straw containing production medium supplemented with sucrose, malt extract and CaCl₂ and the incubation temperature, time and pH were 35°C, 3rd day and 6.5, respectively

Cellulase production in solid-state fermentation was carried out by Liu and Yang (2007) using the waste from the vinegar industry as the substrate for *Trichoderma koningii* AS3.4262. 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. 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. Iqbal *et al.* (2010) investigated the potential of a filamentous fungus, *Trichoderma harzianum* for hyper-production of third most demanded industrial enzyme carboxymethyl cellulase using cheap and easily available agro-industrial residue wheat straw as growth supporting substrate under still culture solid state fermentation technique. Production of carboxymethyl cellulase was substantially enhanced through media optimization process. To promote carboxymethyl cellulase production, they evaluated the effect of several kinetic parameters like pretreatment, substrate concentration, initial moisture content,

pH, incubation temperature and inoculum size on carboxymethyl cellulose production. Samples were harvested after every 24 h to study the profile of cellulase enzyme produced by the fungus on proximally analyzed wheat straw. By optimizing the SSF medium containing 2 % HCl pretreated wheat straw; maximum carboxymethyl cellulase activity ($480 \pm 4.22 \mu\text{M} / \text{mL}/\text{min}$) was recorded after 7th day of incubation at pH 5.5; temperature, 35°C; moisture, 40 % and inoculum size, 10 %, using optimum substrate concentration (3%).

An experiment was conducted by Alam *et al.* (2005) on solid state bioconversion of lignocellulosic material oil palm biomass (OPB) generated from palm oil industries as waste for evaluating enzyme production through a filamentous fungus *Trichoderma harzianum* and the results revealed that the higher cellulase activity of 0.0413 units was achieved at the 3rd day of fermentation. The production and optimization of process parameters for the synthesis of cellulase from *Trichoderma viride* in solid state fermentation (SSF) was studied by Ahmed *et al.* (2010) using an agricultural wheat straw as substrates. The maximum cellulase enzyme activity $398.10 \pm 2.43 \mu\text{M}/\text{mL}/\text{min}$ was achieved when proximally analyzed lignocellulosic substrate wheat straw inocubated 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.

Sun *et al.* (2010) evaluated the feasibility of using apple pomace for cellulase production by *Trichoderma* sp. under solid state fermentation. The 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.

Kang *et al.* (2004) investigated the production of cellulase and hemicellulases from *Aspergillus niger* KK2 in solid state fermentation (SSF) by using different ratios of rice straw and wheat bran. When *Aspergillus niger* KK2 was grown on rice straw alone as a solid support in SSF, the maximum FPase

activity was 19.5 IU/g in 4 days. Also, CMCase (129 IU/g), β -glucosidase (100 IU/g), xylanase (5070 IU/g) and β -xylosidase (193 IU/g) activities were concurrently obtained after 5-6 days of fermentation. The production of cellulolytic enzymes by *Aspergillus niger* on lignocellulosic substrates groundnut fodder, wheat bran, rice bran and sawdust in solid state fermentation in a laboratory scale was compared by Chandra *et al.* (2007). Czapek Dox liquid broth amended with cellulose (0.5%) was used to moisten lignocellulosic solid supports for cultivation of *Aspergillus niger*. The production of filter paperase, carboxymethyl cellulase and β -glucosidase were monitored at daily intervals for 5 days. The peak production of the enzymes occurred within 3 days of incubation. Among solid supports used in the study, wheat bran was the best solid matrix followed by groundnut fodder in production of cellulolytic enzymes in solid state fermentation. Groundnut fodder supported significant production of FPase (2.09 FPU/g), CMCase (1.36 U/g) and β -glucosidase activity (0.0117 U/g) in solid state fermentation. Five *Aspergillus* sp. and standard strain *Trichoderma viride* were grown on the agriculture wastes and CMCase, FPase, Avicelase and soluble protein were determined. *T. viride* produces the highest CMCase on wheat straw (555U/ml), while the highest FPase (141U/ml) and Avicelase (46U/ml) were produced on wheat bran. The isolated strain *Aspergillus* MAM-F35 gave the highest CMCase (487U/ml), FPase (79U/ml) and Avicelase (35U/ml) on WS. However, the isolated strain *Aspergillus* MAM-F23 gave the highest CMCase (309U/ml) on rice straw, while the highest Avicelase (45U/ml) on wheat straw. So, the highest cellulases were produced on the agriculture wastes in the order wheat straw > wheat bran > rice straw > corn cobs (Abo-state *et al.*, 2010)

Archana and Satyanarayana (1997) studied the production of extracellular thermostable cellulase-free xylanase (EC 3.2.1.8) by *Bacillus licheniformis* A99 in solid-state fermentation (SSF). Wheat bran was found to be the best substrate [16.3U g⁻¹ dry bacterial bran (DBB)] among a few easily available lignocellulosics tested. The production of xylanase reached a peak in 72 h (16.8 U g⁻¹ DBB). A high level of enzyme (17.1 U g⁻¹ DBB) was produced in wheat bran moistened with tap water at a substrate-to-moistening agent ratio of 1:2.5

(w/v). The optimum temperature for xylanase production was 50°C. An elevated water activity suited the enzyme production most. Maximum enzyme production was recorded in an aerated glass column reactor (19.13 U g⁻¹ DBB). Repression by glucose was not evident in SSF even at 6% while it was seen in submerged fermentation (SmF) at or beyond 2%. Cultivation in large enamel trays yielded a xylanase titer (15–17 U g⁻¹ DBB in 72 h) that was comparable to that in flasks. The enzyme production was 22-fold higher in SSF than that in SmF. This is the first report on xylanase production by a thermophilic bacterial strain in SSF.

Sanghi *et al.* (2007) revealed that 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 72 h 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. Under optimized conditions, xylanase production in SSF (8,964 U of xylanase/g dry wheat bran) was about two fold greater than in submerged fermentation. Xylanase production from *Bacillus megaterium* was enhanced using solid state fermentation by Sindhu *et al.* (2006) with respect to the use of solid substrate, moistening solution, moisture content, inoculum, sugars, soyabean meal, amino acids, and extraction with surfactant. An increase of $\approx$ 423-fold in xylanase production and complete suppression of CMCase production was achieved over submerged liquid fermentation. Biobleaching using this cellulase-free xylanase, 8 U/g of oven dried pulp of 10% consistency, showed 8.12% and 1.16% increase in brightness and viscosity, 13.67% decrease in kappa number, and 31% decrease in chlorine consumption at the CD stage. Cellulase and xylanase production by co-culture *Bacillus coagulans* and *Bacillus licheniformis* by changing the moisture level of substrate in solid state fermentation (SSF) was studied by Sharma *et al.* (2006). At optimum moisture level, there was considerable increase in extracellular enzyme production, thus, consequently increasing the degradation of forest waste. An increase upto 1024% in biodegradation index of dilute sulphuric

acid pretreated *Cedrus deodara* wood was noticed at substrate: moisture ratio of 1:2.

Maciel *et al.* (2008) produced xylanase by *Aspergillus niger* LPB 326 cultivated on lignocellulosic substrate composed by sugarcane bagasse and soyabean meal in solid state fermentation. The best xylanase activity was obtained in a medium containing 10g of sugarcane bagasse and soyabean meal in the ratio 65 and 35% respectively, moistened to 85% of initial water content with a nutrient salt solution composed of (g/l): CuSO_4 -0.4, KH_2PO_4 -1.5 and CuSO_4 -.0012 and incubated for four days at 30°C. The effects of solid substrates initial moisture content, moistening medium, temperature and incubation time on xylanase production by *Aspergillus niger* DFR-5 and the highest activity (2596 IU/g) dry substrate was achieved in medium that contained wheat bran (WB) and soyabean cake (SBC) at ratio of 70:30, was moistened to 70% with MSS-2 mineral salt solution and incubated for efficient recovery of enzyme from moldy WB-SBC medium was reported by Pal and Khanum (2010). Xylanase production was optimized by Lakshami *et al.* (2009) by a newly isolated *Aspergillus terreus* MTCC 8661 using palm fibre in solid state fermentation (SSF). Different fermentation parameters such as incubation, temperature, moisture content, medium, pH, particle size, incubation time, inoculums, xylose and sodium nitrate concentration were investigated at the individual and interactive level by the Taguchi methodology. All selected fermentation parameters influenced xylanase production. Moisture content, incubation time and inoculums concentration were the major (8.5%) influential parameters on xylanase production at the individual level.

The lignocellulosic coffee by-products such as coffee pulp, coffee cherry husk, silver skin and spent coffee for their efficacy as a sole carbon sources for the production of xylanase in solid state fermentation using *Penicillium* sp. (FR 303) was reported by Murthy and Naidu (2010). Among the residues, coffee cherry husk was observed to produce maximum xylanase activity of 9,475U/g.

The production of xylanase from thermophilic fungus *Scytalidium thermophilum* by solid-state fermentation using *Jatropha curcas* was assessed by

Joshi and Khare, (2010). The seed cake was efficiently utilized by *S. thermophilum* for its growth during which it produced good amount of heat stable extracellular xylanase. The solid state fermentation conditions were optimized for maximum xylanase production. Under the optimized conditions viz. deoiled seed cake supplemented with 1% oat-spelt xylan, adjusted to 9.0, moisture content 1:3w/v inoculated with 1×10^6 spores per 5g cake and incubated at 45°C, 1455U xylanase/g deoiled seed cake was obtained. The xylanase was useful in biobleaching of paper pulp. Solid state fermentation of deoiled cake appears a potentially viable approach for its effective utilization.

2.3 ESTIMATION OF HOLOCELLULOSE, CELLULOSE AND LIGNIN CONTENT OF LIGNOCELLULOSIC BIOMASS

The chemical composition of lignocellulosic compound differs considerably and is influenced by genetic and environmental factors. However, cellulose, hemicelluloses and lignin are comprised primarily of lignocellulosic materials. As cellulose account for roughly 50 – 70% of the dry mass of most biomass species, with hemicelluloses and lignin accounting for approximately 30 - 50 and 10 - 30%, respectively as well as ash and minor components (e.g. waxes and protein) make up balance. Cellulose is a linear polymer of glucose unit which condensed through α -1,4-linkages and is usually arranged in microcrystalline structures, which is very difficult to dissolve or hydrolyze under natural conditions. The degree of polymerization (DP) of cellulose chains range from 500 to 25,000. Unlike, hemicellulose is a heteropolysaccharide composed of different sugar in addition to glucose especially xylose but also including mannose, galactose, rhamose and arabinose. Hemicellulose is more soluble than cellulose and frequently branched with DP of 100 to 200 (Leschine, 1995). Xylan is the most common hemicelluloses component of grass and wood. Lignin is a highly irregular and insoluble polymer consisting of phenylpropanoid subunits, namely r-hydroxyphenyl (H-type), guaiacyl (G-type) and syringyl (S-type) units. Lignin has no repeating subunits, thereby making the enzymatic hydrolysis of this polymer extremely difficult (Kuhad, 1997).

Supantamart *et al.* (2009) pretreated *Eucalyptus* and *Acacia* chip by steam explosion under different conditions of temperature between 200 and 210°C and operating time from 2-6 min. The explosion chips were then washed with hot water. The effects of steam explosion conditions have been assessed by measuring chemical compositions (cellulose, hemicelluloses and lignin) in solid. Steam explosion pretreatment removes the major part of the hemicelluloses, and makes the high ratio cellulose in the solid material for further enzyme hydrolysis. At log (Ro) 3.84 (210°C, 4 min), the Eucalyptus fibers contained the significant lowest hemicelluloses and the highest of celluloses at 3.64 %DW and 53.94 %DW, respectively. Cellulose and hemicellulose contents of algal biomass were assessed by Ververis *et al.* (2007) and was found 7.1% and 16.3%, respectively, whereas for citrus peels cellulose content ranged from 12.7% to 13.6% and hemicellulose from 5.3% to 6.1%. For all materials, lignin and ash content was 2% or lower, rendering them suitable for use as paper pulp supplements.

The chemical constituents of oil heat treated *Acacia hybrid* was studied at temperatures of 180, 200 and 220°C for the time 30, 60 and 90 min. The variation occurred in the chemical contents for both the sapwood and heartwood. The holocellulose contents decrease from 71.5 to 63.1% and 73.4 to 64.0% for sapwood and heartwood respectively. The cellulose contents decreased from 47.1 to 37.7% for the sapwood and 48.9 to 38.1% for heartwood. The hemicellulose contents increases from 24.4 to 25.4% in the sapwood and 24.5 to 25.9% for the heartwood. Lignin contents increased 20.8 to 24.0% for the sapwood and 22.4 to 24.9% in the heartwood for treatment temperature from 180 to 220°C (Khalid *et al.*, 2010).

Longui *et al.* (2012) investigated the influence of extractives, lignin and holocellulose contents by TAPPI standards T204 (2004) and TAPPI T222 (2004), respectively. The hemp fibers contained 73-77% w/w cellulose, 7-9% w/w hemicellulose and 4-6% w/w lignin, while the hemp shives contained 48% w/w cellulose, 21-25% w/w hemicellulose and 17-19% w/w lignin. Among the four investigated cultivars, Felina contained least lignin, while Futura and Fasamo contained least cellulose (Thomsen *et al.*, 2005).

2.4 EFFECT OF PRETREATMENT ON LIGNOCELLULOSIC BIOMASS FOR ENZYME PRODUCTION

The goal of biomass pretreatment is to improve the enzymatic digestibility of pretreated lignocellulosic biomass. Many substrate factors, such as substrate accessibility, lignin content, particle size and so on, contribute to its recalcitrance. Cellulose accessibility to hydrolytic enzymes is believed to be the most important substrate characteristic limiting enzymatic hydrolysis. Cellulose solvents effectively break linkages among cellulose, hemicellulose and lignin, and also dissolve highly-ordered hydrogen bonds in cellulose fibers accompanied with great increases in substrate accessibility (Sathitsuksanoh *et al.*, 2012). The inherent properties of native lignocellulosic materials make them resistant to enzymatic attack. To achieve enzymatic degradation in production of ethanol by enzymatic hydrolysis, a pretreatment process is necessary (Taherzadeh and Karimi, 2009).

Pretreatment is required to alter macroscopic and microscopic size of biomass and structure as well as its submicroscopic structural and chemical composition and to facilitate rapid and efficient hydrolysis of carbohydrate of fermentable sugars. The pretreatment aims to increase pore size and reduce cellular crystallinity. In acid-catalysed pretreatment, the mainly a part of the lignin is removed and hemicellulose has to hydrolysed using hemicelluloses. Hence, pretreatment is necessary to expose the cellular fibres to the enzymes or to at least make the cellulose more accessible to the enzymes (Petrova and Ivanova, 2010). Pretreatment can be carried out in different ways such as mechanical pretreatment, steam explosion, ammonia fibre explosion, alkali or acid pretreatment and biological pretreatment. Cost effective pretreatment of cellulosic biomass is a major challenge of cellulose-bioethanol technology research and development.

2.4.1 Physical Pretreatment

Physical pretreatment of lignocelluloses typically involves size comminution by grinding, milling or chipping. The goal is to reduce the crystallinity of the cellulose fibers in the biomass. Size reduction of lignocelluloses is also necessary to eliminate mass and heat transfer limitations

during the hydrolysis reactions (Schell and Harwood, 1994). The size of the resulting materials is typically 10–30 mm after chipping and 0.2–2 mm after milling or grinding. The first step for ethanol production from agricultural solid wastes is comminution through milling, grinding or chipping. This reduces cellulose crystallinity and improves the efficiency of downstream processing. Wet milling, dry milling, and compression milling are usually done (Sun and Cheng, 2002).

Milling (cutting the lignocellulosic biomass into smaller pieces) is a mechanical pretreatment of the lignocellulosic biomass. The objective of a mechanical pretreatment is a reduction of particle size and crystallinity. The reduction in particle size leads to an increase of available specific surface and a reduction of the degree of polymerization (DP). The milling causes also shearing of the biomass. The increase in specific surface area, reduction of DP, and the shearing, are all factors that increase the total hydrolysis yield of the lignocellulose in most cases by 5–25% (depends on kind of biomass, kind of milling, and duration of the milling), but also reduces the technical digestion time by 23–59% (thus an increase in hydrolysis rate). A particle size reduction below 40 mesh however has little effect on the hydrolysis yield as well as hydrolysis rate of the biomass (Chang and Holtzaple, 2000) Milling causes both an increased methane (5–25%) and ethanol yield and also increases the hydrolysis rate. As no production of inhibitors (like furfural and HMF (hydroxymethylfurfural)) are produced, milling is suited for both methane and ethanol production. It has however a high energy requirement (Ramos, 1993) and was found therefore not economically feasible as pretreatment in 1987 (Fan *et al.*, 1987).

Bridgeman *et al.* (2007) studied the effects of size reduction of switchgrass achieved via ball milling. The study reported that for particle sizes smaller than 90 μm , cellulose content was 13.4% lower than for larger sized particles. The losses in lignin and hemicellulose were appreciably less (3.43% and 4.74%, respectively). These results indicate that extensive size reduction is undesirable as it causes significant carbohydrate losses which ultimately results

in less reducing sugars and reduced ethanol yield. Mani *et al.* (2004) examined the energy requirements for size reduction of switchgrass using a hammer mill. At 8% moisture content, energy requirements increased linearly as particle size reduced and at 12% moisture content, the energy requirements tended to level off for particle sizes less than 2 mm. The study noted that energy requirements for size reduction of switchgrass were higher than that of corn stover, barley straw and wheat straw at the same moisture content. Additionally, it was reported that higher moisture content biomass required greater energy inputs for size reduction. Igathinathane *et al.* (2008) reported a significant increase in cutting energy requirements for switchgrass with high moisture (51%) in comparison to low moisture (9%) using a shear based knife grid for size reduction using a linear grid of knives.

Digestibility of cellulosic biomass has been enhanced by the use of high energy radiation methods, including γ -ray, ultrasound, electron beam, pulsed electrical field, UV, and microwave heating. The action mode behind the high energy radiation could be one or more changes of features of cellulosic biomass, including increase of specific surface area, decrease of the degrees of polymerization and crystallinity of cellulose, hydrolysis of hemicellulose, and partial depolymerization of lignin. However, these high energy radiation methods are usually slow, energy-intensive, and prohibitively expensive. They also appear to be strongly substrate-specific. As a result, high energy radiation techniques lack commercial appeal based on current estimation of overall cost (Zheng *et al.*, 2009). Irradiation of cellulose by α -rays, which leads to cleavage of β -1, 4-glycosidic bonds and gives a larger surface area and a lower crystallinity, has also been tested. This method is far too expensive, however, to be used in a full-scale process. 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 Cellulosin TP 25 from *Trichoderma viride*, which possesses endo- β -D-xylanase and β -D-xylosidase activities as well as cellulase activities.

The effect of gamma-ray irradiation on enzymatic hydrolysis of spent enokitake mushroom substrate containing corncob meal and rice bran as major components was studied by Tomoko *et al.* (2007). Almost all the lignin component remained in the bottle though 34% of the original substrate was consumed. 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 Cellulosin TP 25 from *Trichoderma viride*, which possesses endo- β -D-xylanase and β -D-xylosidase activities as well as cellulase activities.

Steam explosion is a promising method of pretreatment which makes biomass more accessible to cellulase attack (Neves *et al.*, 2007). This method of pretreatment without the use of any catalyst is promising and the biomass fractionates to yield levulinic acid, xylitol and alcohols (Balat *et al.*, 2008). In this method the biomass is heated using high pressure steam (20-50 bar, 160-290°C) for a few minutes, the reaction is then stopped by sudden decompression to atmospheric pressure (Sanchez and Cardona, 2008). When steam is allowed to expand within the lignocellulosic matrix it separates the individual fibres. The recovery of xylose (45-65%) makes steam-explosion pretreatment economically attractive (Hamelinck *et al.*, 2005). The advantages of steam explosion are low energy requirement compared to mechanical comminution (70% more energy required) and involves no recycling or environmental cost. It is considered the

most cost effective option for hardwood and agriculture residues, but is less effective for softwood (Vessia, 2010).

Viola *et al.* (2008) showed the ethanol production using Eel grass (*Zostera marina*), a submerge sea grass. Eel grass was optimally treated with steam explosion at 180°C for 15 min, impregnation with oxalic acid (2% by wt), which could activate a synergic mechanism where it bind to metal ions and help in structure destabilisation oxalate anions capture metal ion from eel grass fibres, making carbohydrate structure more available for enzyme attack. Steam explosion for pretreatment of sunflower stalks before enzymatic hydrolysis at a temperature in the range of 180-230°C was reported by Ruiz *et al.* (2008). The highest glucose yield was obtained in steam pretreated sunflower stalks at 220°C, while the highest hemicellulose recovery was obtained at 210°C pretreated temperature. Steam explosion for production of ethanol from several lignocellulosic materials with *Kluyveromyces marxinus* was reported by Ballesteros *et al.* (2004). The poplar and eucalyptus chips were treated at 210°C for 4 min, wheat straw at 190°C for 8 min. *Brassica carinata* residue at 210°C for 8 min and sweet sorghum bagasse at 210°C for 2 min.

Laser *et al.* (2002) compared the performance of LHW and steam pretreatment for sugarcane bagasse, which was subsequently used for ethanol production by SSF. They performed the treatment in a 25-liter reactor at 170-230°C for 104 min with 1% to 8% solid concentrations. It has been found that poplars (*Populus* sp.) representing an important source of short rotation energy crops steam-explosion was found to be effective method for improving enzymatic saccharification of cellulose content of poplar wood (Excoffer *et al.*, 1991).

The effect of microwave irradiation to change ultra structure of cellulose, degrades lignin and hemicelluloses in rice straw and increase enzymatic susceptibility was studied by Xiang *et al.* (2003). Compared with conduction/convection heating, microwave uses ability of direct interaction between a heated object and an applied electromagnetic field yield to create heat. Therefore, heating is volumetric and rapid when microwave is used to treat lignocellulose. *Geotrichum* sp. F₃ revealed maximum xylanase titers in Nakamura

medium, pH 4.0, temperature 55°C, inoculum size 10%, substrate concentration 2.25% after seven days of incubation. Optimization of fermentation conditions enhanced the enzyme production to 77.54 IU/ml as compared to initial production i.e. 10.12 IU/ml. When enzyme production mode was switched over to SSF by using microwave irradiated sawdust of *C. deodara* by *Geotrichum sp.* F3, xylanase production was enhanced dramatically to 112.890 U/g (Sharma *et al.*, 2012).

Rose and Inglett (2010) treated maize bran with microwave irradiation (160-200 for 2-20 min) to release feruloyated arabino-xylo-oligosaccharides (AXOS). Lower temperatures and shorter treatment times were consisted with low AXOS yields, while high temperatures and longer reaction times also resulted in low yield which were contaminated with increasing levels of monosaccharides, free ferulic acid and furfural, occurred with treatment at 18°C for 10 min or 200°C for 2 min.

2.4.2 Chemical Pretreatment

Chemical pretreatment of lignocelluloses includes the use of ozone, acids, alkali, organic solvents and peroxides. Ozonolysis is carried out at room temperature and is effective at lignin removal without the formation of toxic by-products (Vidal and Molinier, 1988). However, the large ozone requirement makes the process expensive (Sun and Cheng, 2002). Dilute acid pretreatment with sulfuric acid has been studied extensively and is efficient at removal of hemicelluloses but fails to effectively remove lignin. While dilute acid pretreatments are known to improve enzymatic hydrolysis, their cost is relatively high compared to physico-chemical pretreatments.

Dilute alkali pretreatments using sodium hydroxide targets intermolecular bonds between lignin and hemicelluloses and improves the porosity of the biomass (Tarkow and Feist, 1969). Other studies on dilute alkali pretreatments have examined the use of ammonia water and hydrated lime. Ladisch *et al.* (1983) showed that pretreatment of lignocelluloses with cadoxen, a cellulose solvent resulted in 90% conversion of cellulose into glucose. The use of organic

solvents such as methanol, ethanol, acetone and ethylene glycol along with inorganic and organic acids as catalysts has also been studied (Wood and Saddler, 1988; Chum *et al.*, 1988; Thring *et al.*, 1990). Dilute acid pretreatment of switchgrass for bioethanol production was first examined by Wyman *et al.* (1992). The pretreatment was conducted at 140°C for 1 h using sulfuric acid at low concentrations (up to 0.5% v/v). Enzymatic hydrolysis of the resulting biomass yielded up to 70% conversion of cellulose into glucose over a five-day period. Since the removal of hemicellulose during dilute acid pretreatment correlates with enhanced reactivity of cellulose to enzymes (Torget *et al.*, 1990), several studies have used percentage of xylose recovery as a means of optimizing dilute acid pretreatments. Wu and Lee (1997) used a two-stage dilute sulfuric acid pretreatment with an acid concentration of 0.0785% (w/w) to successfully remove 100% of hemicellulose from switchgrass. Esteghlalian *et al.* (1997) determined that up to 90% of xylose was recovered as a result of pretreatment at 180°C with an acid concentration of 1.2% (w/w). It was also noted that temperature was a more significant parameter than acid concentration. Chung *et al.* (2005) reported optimal conditions of 1.2% (w/w) at 180°C for sulfuric acid pretreatment of switchgrass and determined that 90% of cellulose in the pretreated biomass was converted into reducing sugars during a 72 h enzymatic hydrolysis. Dien *et al.* (2006) reported yields greater than 80% for both glucan and non-glucan sugars for dilute sulfuric pretreatment of switchgrass with an optimal acid concentration of 1.2% (w/v). Although pretreatment temperature was not optimized, the study indicates higher conversion rates at 150°C in comparison to 120°C.

Dilute acid pretreatment of switchgrass does not significantly impact lignin removal. Wu and Lee (1997) noted that high lignin content could lead to increased enzyme consumption due to irreversible adsorption of cellulase enzymes to lignin. Hence, they proposed combining dilute acid pretreatment with an ammonia percolation step to remove lignin. Their results indicate that with this additional step, 20% more lignin can be removed to significantly increase enzymatic digestibility of the pretreated biomass. Kong *et al.* (1992) noted that alkali remove acetate groups from hemicellulose which result in reduced steric

hindrance to enzyme molecules in addition to saponification of ester groups that crosslink lignin and hemicelluloses. Kim and Lee (1996) reported up to 99% delignification using a combined ammonia–hydrogen peroxide percolation pretreatment at 170°C. They reported reagent loadings of 0.28 g loading of hydrogen peroxide g/1 biomass, and 10% (w/w) of ammonia. Their results indicated that the enzymatic digestibility of the resulting pretreated biomass was higher than that of pure alpha cellulose. This could be a sign of significant reduction in crystallinity and increase in porosity of cellulose in the pretreated biomass. Kurakake *et al.* (2001) used ammonia water (25–28%) for the pretreatment of switchgrass (2 ml/g biomass) for 20 min at 120°C and noted a 3–5- fold improvement in reducing sugar production over untreated switchgrass after a 24-h enzymatic hydrolysis. Isci *et al.* (2008) pretreated switchgrass in 30% aqueous ammonium hydroxide with liquid–solid ratios of 5 and 10 ml/g and residence times of 5 and 10 days. The study reported a 40–50% reduction in lignin content and 50% reduction in hemicelluloses content as a result of pretreatment. Lime (calcium hydroxide) pretreatment of switchgrass was investigated by Chang *et al.* (1997). With a lime loading of 0.1 g/1 of dry switchgrass, a pretreatment time of 2 h at 100°C, the study showed that a 72-h enzymatic hydrolysis of pretreated biomass yielded five times higher reducing sugars than untreated.

Acid pretreatment is considered as one of the most important techniques and aims for high yields of sugars from lignocellulosics. It is usually carried out by concentrated or diluted acids (usually between 0.2% and 2.5%w/w) at temperature between 130°C and 210°C. Sulphuric acid is widely used for acid pretreatment among various types of acid such as hydrochloric acid, nitric acid and phosphoric acid (Cardona *et al.*, 2009). Acid pretreatment can utilize either dilute or concentrated acids to improve cellulose hydrolysis (Balat *et al.*, 2008). The acid medium attacks the polysaccharides, especially hemicelluloses which are easier to hydrolyze than cellulose. However acid pretreatment results in the production of various inhibitors like acetic acid, furfural and 5-hydroxymethylfurfural. These products are growth inhibitors of microorganisms. Hydrolysates to be used for fermentation therefore need to be detoxified. Mosier

et al. (2005) reported higher hydrolysis yield from lignocellulose pretreated with diluted H₂SO₄ compared to other acids. A saccharification yield of 74% was obtained from wheat straw when subjected to 0.75%v/v of H₂SO₄ at 121°C for 1 h (Saha *et al.*, 2005).

Dilute sulfuric acid pretreatment of the *Jatropha curcas* fruit hull at high temperatures (140°C to 180°C) performed in a 110-mL stainless steel reactor was investigated to enhance the enzymatic digestibility of its lignocellulosic components. Carbohydrates accounted for 43% of the dry matter of the *J. curcas* fruit hull biomass. The goal of the study was to optimize the pretreatment conditions (acid concentration, time, and temperature) in order to obtain the highest sugar yield after subsequent enzymatic hydrolysis. The optimal pretreatment conditions are 30-min incubations at a temperature of 178°C with a sulfuric acid concentration of 0.9% (w/v). Using these pretreatment conditions for a fruit solid loading of 9.52% followed by a 24-h enzymatic hydrolysis resulted in a liberation of 100% of all pentoses present (71% yield and 29% degradation to furfural) and 83% of the hexoses (78% yield and 5% degradation to 5-hydroxymethylfurfural). Acid + steam pretreated *P. roxburghii* needles has emerged as a suitable substrate for cellulase production by *A. niger* F7 reaching upto 146.14 U/g. (Sharma *et al.*, 2012).

Sun and Cheng (2005) reported the pretreatment of rye straw and Bermuda grass for ethanol production by enzymatic hydrolysis at 121°C with different sulphuric acid concentrations (0.6, 0.9, 1.2, and 1.5% w/w) and residue times (30, 60 and 90 min). It has been reported that *Eucalyptus grandis* impregnated with 0.087 and 0.175% (w/w) H₂SO₄ at 200-210°C for 2-5min by Emmel *et al.*, (2003). The best conditions for hemicellulose – recovery was obtained at 210°C for 2 min, while a lower pretreatment temperature of 200°C was enough to obtain the highest yield of cellulose conversion (90%) by enzymatic hydrolysis. Eklund *et al.* (1995) studied steam pretreatment of willow with the addition of SO₂ or H₂SO₄ in order to recover both cellulose and hemicellulose. The maximum glucose yield, 95% was obtained when willow was treated with 1% SO₄ at 200°C. However, the yield of xylose recovery by SO₂ was

not as high as pretreatment with dilute sulphuric acid. Guo *et al.* (2008) evaluated potential of silver grass (xylan and glucan rich) for ethanol production; pretreatment with dilute H₂SO₄ gave highest yield of xylose (70-75%) which was similar to bagasse. Also, silver grass gave higher level of fermentability than bagasse using hydrolysate because less acetic acid is formed. Depending upon the substrate and the conditions used, between 80-95% of the hemicellulosic sugars can be recovered by dilute acid pretreatment from the lignocellulosic feedstock (Jeffries and Jin, 2000).

Alkaline pretreatment processes utilize lower temperatures and pressures compared to other pretreatment technologies. Alkali pretreatment may be carried out at ambient conditions. Alkaline pretreatment of lignocellulosic digests the lignin matrix and makes cellulose and hemicellulose available for enzymatic degradation (Pandey *et al.*, 2000). Alkali treatment of lignocellulose disrupts the cell wall by dissolving hemicelluloses, lignin and silica by hydrolyzing uronic and acetic esters, and by swelling cellulose. Crystallinity of cellulose is decreased due to swelling. By this process, the substrate can be fractionated into alkali-soluble lignin, hemicelluloses and residue, which makes it easy to utilize them for more valuable products. The end residue (mainly cellulose) can be used to produce either paper or cellulose derivatives (Cardona *et al.*, 2009). Hydroxides of sodium, potassium, calcium and ammonium are used in this process.

Kaushal *et al.* (2012) observed the maximum enzyme titers i.e 787.89 U/g of cellulase and 669.08 U/g of xylanase in alkali (NaOH) pretreated *Celtris australis* as compared to untreated biomass which was 450.02 U/g and 398.03 U/g of cellulase and xylanase respectively when tap water was used as moistening agent. The efficiency of enzymatic saccharification of the wheat straw was increased by the combination of grinding and sodium hydroxide (NaOH) treatment. The optimal pretreatment condition was to grind the wheat straws into the sizes of 120 meshes followed by treatment with 1.0% NaOH for 1.5 h (121°C/15psi). Under this condition, the cellulose content of wheat straw was increased by 44.52%, while the content of hemicellulose and lignin was decreased by 44.15% and 42.52%, respectively. Cellulase produced by

Penicillium waksmanii F10-2 was used to hydrolyze the pretreated wheat straw and the optimal condition was determined to be 30 h of enzymatic reaction under the temperature of 55°C, pH 5.5 and substrate concentration of 3% (Han *et al.*, 2012).

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. Significant factors for efficient sodium hydroxide pretreatment on canola residues were optimized by using the response surface method (RSM). Based on the fundamental experiments, the real values of factors at the center (0) were determined as follows; 70 of temperature, 10% of sodium hydroxide concentration and 18 h of reaction time in the experiment design using central composition rotatable design (CCRD). A statistical model predicted that enzyme digestibility (theoretical maximum yields of sugar) and the removal yield of lignin were 59.34% and 36.38%, respectively at the following optimized reaction conditions: 73.39 of temperature, 8.95% of ammonia concentration and 21.85 h of reaction time. Saccharification was performed at 50°C with Celluclast (cellulase) and Novozyme 188 (β -glucosidase) as biocatalysts in an enzyme loading test. Finally, conversion of glucose was 90.17% at the optimized conditions (Yoo *et al.*, 2012).

The microwave assisted alkali pretreatment and enzymatic hydrolysis of banana pseudostem (BPS) for the production of bioethanol was optimized by Chittibabu *et al.* (2011). when BPS was pretreated by 10 % NaOH with 4:1 liquid to solid ratio at 90°C for 8 min, the yield of reducing sugars reached 84% by enzymatic hydrolysis of 110 h with cellulase enzyme loading of 30 FPU/g of solid.

Among different pretreatments, a modified NaOH + H₂O₂ treatment was found best for the biodegradation of pine needles. During biodegradation, highest biodegradation index (BI) and percent hydrolysis were 54.56 and 12.18 while cellulase and xylanase synthesis ranged upto 200.7U/g in NaOH + H₂O₂ pretreated needles using modified BSM under SSF as compared to tap water (Tandon *et al.*,2012). Pine needles, which are highly resistant to biodegradation, can be degraded successfully with *A. niger* after suitable pretreatment. Modified alkali pretreatment [NaOH +H₂O₂ ; ratio 9:1] in 2 h followed by steam explosion for 15 min has been found the best pretreatment for pine needles hydrolysis by *A. niger* (Sharma *et al.*,2011).

Significant factors for efficient sodium hydroxide pretreatment on canola residues were optimized by using the response surface method (RSM). Based on the fundamental experiments, the real values of factors at the centre (0) were determined as follows; 70°C of temperature, 10% of sodium hydroxide concentration and 18 h of reaction time in the experiment design using central composition rotatable design (CCRD). A statistical model predicted that enzyme digestibility (theoretical maximum yields of sugar) and the removal yield of lignin were 59.34% and 36.38%, respectively at the following optimized reaction conditions: 73.39 of temperature, 8.95% of ammonia concentration and 21.85 h of reaction time. Saccharification was performed at 50 with Celluclast (cellulase) and Novozyme 188 (β -glucosidase) as biocatalysts in an enzyme loading test. Finally, conversion of glucose was 90.17% at the optimized conditions (Yoo *et al.*, 2012).The effectiveness of sulphuric acid, sodium hydroxide, hydrogen peroxide, and ozone pretreatments for enzymatic conversions of cotton stalks by was studied by Silverstein *et al*, (2007). They found that sodium hydroxide pretreatment resulted in the highest level of delignification (65% with 2% NaOH in 90 min. at 121°C) and cellulose conversion (60-80%). Zhao *et al.* (2007) reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose compared with H₂SO₄ pretreatment. Compared with acid or oxidative reagents alkali treatment appears to be the most effective

method in breaking the ester binds between lignin, hemicellulose and cellulose and avoiding fragmentation of hemicelluloses polymers.

Acharya *et al.* (2008) focussed on 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 (2 N 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.

Qi *et al.* (2009) optimized the enzymatic conversion of widely available alkaline peroxide pretreated lignocellulosic biomass-wheat straw (WS), 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. Different substrates *viz.* rice husk, millet straw, guinea corn stalk and sawdust were used in fermentation for the evaluation of cellulase activity secreted by *Aspergillus candidus* by Milala *et al.* (2009). The substrates were pretreated with 5% NaOH (alkaline treatment) and autoclaved. From the fermentation studies, rice husk, millet straw and guinea corn stalk feed substrates showed the highest cellulase activity of 7.50, 6.88 and 5.84 IU, respectively. The effect of pH showed that optimal pH for maximum cellulase activity varied in each of the substrates used. Rice husk and millet straw had maximum enzyme activity at pH 5, while guinea corn stalk and sawdust had maximum activity at pH 3 and 4, respectively.

Ammonia fibre/ freeze explosion (AFEX) pretreatment involves liquid ammonia and steam explosion. The AFEX is a process in which ground, pre-

wetted lignocellulosic material at a moisture content of 15-30% is placed in a pressure vessel with liquid ammonia (NH_3) at a loading of about 1-2 kg NH_3 /kg dry biomass. Pressures exceeding 12 atm are required for operation at ambient temperature. It is simple and has short process time. This system does not directly liberate any sugars, but allows the polymers (hemicellulose and cellulose) to be attacked enzymatically and it reduces the sugars. Ammonia fibre explosion (AFEX) is an alkali process where ligno-cellulosic material is subjected to anhydrous ammonia under elevated temperature and pressure with quenching through decompression. Common with all alkali treatments, the use of ammonia as a swelling agent for water ingress and disruption therefore does not cause depolymerisation of the cellulose/hemi-cellulose fraction, reducing the efficiency of conversion in the subsequent enzymatic hydrolysis. Increased efficiency is seen in low lignin content materials, therefore AFEX is advantageous for herbaceous and agricultural residues, with little produced in terms of inhibitory elements. AFEX also has the benefit of reduced downstream processing prior to fermentation. The drawback of the process is the loss of efficiency in higher lignin content materials such as softwood chips and paper residues, reducing flexibility in the biorefinery feedstocks. In addition, AFEX has difficulty in solubilising smaller fractions such as hemi-cellulose (Hayes 2009). In the lower lignin content materials (~5 - 15% as seen in Bermuda grass and bagasse (Sun and Cheng 2002)) following AFEX treatment, greater the 90% hydrolysis was obtained. As the lignin fraction increased, such as in newspaper (18- 30%) and aspen chips (25%) the total reducing sugar yield decrease to between 40 and 50% (Balat *et al.*, 2008; Hayes, 2009). As AFEX does not directly cause hydrolysis, but allows the easing of enzymatic hydrolysis. Through the use of the AFEX system, the reduction of enzyme requirement is achieved. AFEX is currently in development and undergoing optimisation with the ammonia recycling to make the process viable. Aqueous ammonia is applied to biomass at temperatures between 160 and 180°C. In studies with corn stover, approximately 50% of the xylan and >75% of the lignin was removed, which enhanced enzymatic digestibility of the remaining solids. A modification of this process is to soak biomass in ammonia at mild reaction conditions (ambient-80 °C for several hours to several days), a process known as ammonia soaking or soaking in aqueous

ammonia (SAA). Ammonia soaking of corn stover resulted in 85% of xylan and all of the glucan remaining in the solid fraction after treatment, but over half of the lignin was removed (Kim and Lee, 2005). Over 86% of the remaining solids were hydrolyzed by commercial cellulase at a loading of 15 FPU/g glucan. In addition, ARP is able to remove lignin from hardwoods (Yoon *et al.* 1995). However, ARP resulted in minimal improvement (> 10%) in enzymatic hydrolysis of cellulose in softwood pulp sludge (Kim *et al.* 2000).

2.4.3 Hydrolysis

To make the sugar monomers available for fermentation the cellulose and hemicellulose chains have to be further hydrolyzed after the pretreatment. There are a lot of microorganisms that produce a variety of enzymes that are able to do that, known as the cellulase systems. There exist both anaerobic and aerobic bacteria and fungi that produce cellulase systems.

The aerobic fungus *Trichoderma reesei* produced the first high-efficiency cellulase used in lab-scale and commercial applications in 1950 (Reese *et al.* 1950). Today, *Trichoderma reesei* is still the most commonly used fungus since it is well understood and very efficient at hydrolyzing cellulose. A cellulase system, which is a mixture of hydrolytic enzymes, is required to saccharify cellulose into glucose monomers. Cellulose is hydrolyzed by this system of enzymes that acts synergistically and hydrolyses the β - (1, 4) glycosidic linkages. An endoglucanase, endo-1,4- β -D-glucanase; randomly cleaves the cellulose to produce free chain ends. An exoglucanase, also known as a cellobiohydrolase, exo- 1, 4- β -D-glucanase, cleaves cellobiose units from the non-reducing ends. The soluble disaccharide is then hydrolysed by β -D-glucosidase to glucose monomers (Tolan and Foody 1999; Ting *et al.* 2009).

Cellulases usually have two domains: a catalytic domain (CD) and a cellulose binding module (CBM), which are connected by a linker region. In *Trichoderma reesei*, the CBM is made up of 35 amino acids and the linker region contains many serine and threonine 16 residues. CBMs increase cellulase activity by increasing the amount of CDs on the biomass. The role of the linker region

however, is unknown. At the start of enzymatic hydrolysis, the amorphous non-crystalline regions of the biomass are attacked by cellulase due to their ease of accessibility. As the total amount of non-crystalline regions begins to decrease, the rate of enzymatic hydrolysis begins to drop; this is the rate-limiting factor for enzymatic hydrolysis. This occurs because the decrease in non-crystalline regions means an increase in crystalline regions, which reduces accessibility to glycosidic bonds. Apart from biomass recalcitrance, cellulase efficiency is limited by: end-product inhibition, thermal deactivation, non-specific binding to lignin and irreversible adsorption of the enzymes to the substrate (Ting et al. 2009). Anaerobic bacteria occur for example in the soil, in decaying plant material, in sewage plants and in ruminant animals and in the guts of termites, where they live in symbiosis with their hosts and process plant material for them. Common anaerobic cellulolytic bacteria are various *Clostridium* species. Aerobic bacteria are found in various habitats e.g. in water, in the soil, on decaying plant material or in animal feces. *Cellulomonas* and *Streptomyces* are two frequent genera of cellulolytic aerobic bacteria. Aerobic fungi play an important role in the degradation of plant material and are found nearly throughout the nature. A well known aerobic cellulolytic fungus is *Aspergillus niger*. Anaerobic cellulolytic fungi are less common and there exist only six recognized genera of them: *Anaeromyces*, *Caecomyces*, *Cyllamcyces*, *Neocallimastix*, *Orpinomyces* and *Piromyces*. They live in the intestinal tract of large herbivorous animals, like elephants, horses, cows or sheep (Doi, 2008).

Enzymes are added to the biomass, acting as catalysts to break down the glycosidic bonds of the polysaccharides. The mechanism involved in the enzymatic degradation of lignocellulose depends on the chemical nature and physical structure of the substrate. Structural features of the substrate that affect enzymatic hydrolysis are crystallinity, specific surface area, accessibility to enzyme adsorption, degree of polymerization and the unit cell dimensions of the lignocellulose (Taherzdeh and Karimi 2008). Several enzymes are needed to work together synergistically to break down the various polysaccharides. Enzymes are estimated to contribute to 10-20% of the total cost of bioethanol production. On a commercial scale, the cost of enzymes needs to be lowered in

order for ethanol production to be cost effective. This would require enzymes with the best activity in order to minimize the amount needed and keep the cost at a minimum.

2.5 BIOETHANOL PRODUCTION

The saccharified biomass is used for fermentation by several microorganisms. After the hydrolysis the sugars have to be fermented to ethanol. The hydrolysate now contains various hexoses, mainly glucose, and pentoses, mainly xylose, and depending on the substrate and the pretreatment method various by-products. Currently the fermentation is mostly done with *S. cerevisiae* (and to some extent with *Zymomonas mobilis*), because of its well known characteristics, robustness and high ethanol yield. However, *S. cerevisiae* can only ferment hexoses and not the pentoses. The pentoses can be fermented in an additional step by another organism, or *S. cerevisiae* can be genetically engineered so that it is able to ferment pentoses as well (Lynd *et al.* 2002). Besides a high yield, an important issue with fermentation is the tolerance of the fermenting organisms to the product itself. A strategy to overcome this is to have a system where the ethanol is removed continuously to keep the concentrations low. Other problems are inhibitory compounds that are produced during the thermochemical pretreatment. As mentioned above they can be reduced by an additional detoxification step, but this is expensive. Another way would again be to improve the tolerance of *S. cerevisiae* towards inhibitors. There are several technologies applied to obtain ethanol from lignocellulose. In this study we try to compare the productivity and efficiency of three technologies: Separate hydrolysis and fermentation, Simultaneous saccharification and fermentation and Simultaneous saccharification and Co-fermentation.

2.5.1 SHF (Separate Hydrolysis and Fermentation)

The terminology —separate hydrolysis and fermentation (SHF) refers to a process in which enzymatic hydrolysis (saccharification) of polysaccharides and the microbial fermentation are performed sequentially in separate units. The major advantage of this method is that the enzymatic hydrolysis and fermentation can be carried out at their own optimum conditions: 45-50°C for enzymatic

hydrolysis with cellulase and β -glucosidase, and 30-37°C for ethanol fermentation with fermentative microorganisms (Olsson *et al.*, 2006; Saha *et al.*, 2005). However, the drawbacks of SHF process include: (1) the inhibition of the released sugars, mainly cellobiose and glucose, on cellulase activity. At a cellobiose concentration as low as 6 g/l, the activity of cellulase is reduced by 60%. Although glucose decreases the cellulase activity as well, the inhibitory effect of glucose is lower than that of cellobiose. On the other hand, glucose is a strong inhibitor for β -glucosidase. At a level of 3 g/l of glucose, the activity of β -glucosidase is reduced by 75% (Philippidis *et al.*, 1994). (2) high probability of contamination since separate vessels are used for hydrolysis and fermentation. The hydrolysis process is long and the released sugars as carbon source provide the opportunity of contamination with naturally-occurring microbes. A possible source of contamination could be the enzyme preparation. In practice, it is difficult to sterilize enzymes in large scale, since it must be filter-sterilized owing to the denaturation of enzymes in an autoclave (Taherzadeh and Karimi, 2007). In addition, it is not feasible to add antibiotics in hydrolysis reactor because antibiotics may affect the growth and fermentation of microorganisms in the subsequent fermentation step. Iogen Corporation, a major manufacturer of enzymes in Canada, developed a SHF process comprising a dilute acid catalyzed steam explosion and the removal of the major part of acetic acid released during the pretreatment, the use of *Saccharomyces cerevisiae* as a fermenting organism, distillation of broth, bioethanol dehydration and disposal of stillage in landfill.

Kathiresan *et al.* (2011) analyzed ethanol production by SHF using 10 marine strains of yeasts. Of the species tested, *Pichia salicaria* exhibited the maximum ethanol production of 12.3 $\pm$ 3.1 g/l. This was further studied in the culture filtrate supplemented with different concentrations (0.01, 0.1, 1, 2 and 4% w/v) of sawdust as substrate that was pre-processed with phosphoric acid, and then incubated for different durations (24, 48, 72, 96 and 120 h). The ethanol production by *P. salicaria* was found maximum (26.2 $\pm$ 8.9 g/l) in 72 h of fermentation in the culture broth supplemented with 2% sawdust.

Ethanol production was evaluated under SHF from wheat straw (WS) hemicellulose acid hydrolysate using an adapted and parent strain of *Pichia stipitis*, NRRL Y-7124. The treatment by boiling and overliming with $\text{Ca}(\text{OH})_2$ significantly improved the fermentability of the hydrolysate. Ethanol yield and productivity were increased 2.490.10 and 5.790.24 folds, respectively, compared to neutralized hydrolysate. Adaptation of the yeast to the hydrolysate resulted further improvement in yield and productivity. The maximum yield was 0.4190.01 gp gs, equivalent to 80.490.55% theoretical conversion efficiency (Nigam, 2001). Wheat straw consists of 48.57% cellulose and 27.70% hemicellulose on dry solid (DS) basis and has the potential to serve as a low cost feedstock for production of ethanol. Dilute acid pretreatment at varied temperature and enzymatic saccharification were evaluated for conversion of wheat straw cellulose and hemicellulose to monomeric sugars. The maximum yield of monomeric sugars from wheat straw (7.83%, w/v, DS) by dilute H_2SO_4 (0.75%, v/v) pretreatment and enzymatic saccharification (45⁰C, pH 5.0, 72 h) using cellulase, β -glucosidase, xylanase and esterase was 565 mg/g. The yield of ethanol (per litre) from acid pretreated enzyme saccharified wheat straw (78.3 g) hydrolyzate by recombinant *Escherichia coli* strain FBR5 was 19 g with a yield of 0.24 g/g DS. Detoxification of the acid and enzyme treated wheat straw hydrolyzate by overliming reduced the fermentation time from 118 to 39 h in the case of separate hydrolysis and fermentation (35⁰C, pH 6.5) and increased the ethanol yield from 13 to 17 g/l (Saha *et al.*, 2005). Acid-hydrolysis of cellulosic pyrolysate to glucose and its fermentation to ethanol were investigated by Zhang *et al.* (2006). The maximum glucose yield (17.4%) was obtained by the hydrolysis with 0.2 mol/l sulfuric acid using autoclaving at 121⁰C for 20 min. The fermentation by *Saccharomyces cerevisiae* of a hydrolysate medium containing 31.6 g/l glucose gave 14.2 g/l ethanol after 24 h, whereas the fermentation of the medium containing 31.6 g/l pure glucose gave 13.7 g/l ethanol after 18 h.

Marques *et al.*, 2008 evaluated the possibility of converting recycled paper sludge (RPS) into valuable products. The approach used was based on the enzymatic conversion of major sludge components (cellulose and xylan) and

sequential (separate hydrolysis and fermentation— SHF) fermentation of the resulting sugars to ethanol. In the enzymatic hydrolysis step using Celluclast 1.5 L supplemented with Novozyme 188, a degree of saccharification of 100% was achieved. In relation to ethanol production using the yeast *Pichia stipitis* CBS 5773, a slightly higher conversion yield was attained on SHF, corresponding to an ethanol concentration of 19.6 g /L, but 179h were needed. Using two-sequential steps of acid hydrolysis (10% sulfuric acid) and yeast (*Candida shehatae*; xylose-fermenting yeast) fermentation, bioconversion of water hyacinth (*Eichhornia crassipes* to liquid ethanol has been performed. The maximum ethanol yield coefficient of 0.19 g /g WH with the productivity of 0.008 g /l /h was achieved (Isarankura, 2007).

Acid pretreatment and enzymatic hydrolysis was used to evaluate to produce more sugar, to be fermented to ethanol. Separated hydrolysis and fermentation (SHF) studies were carried out to produce ethanol from water hyacinth leaves. The abilities of three different yeast cells, *Saccharomyces cerevisiae* TISTR 5048, *Saccharomyces cerevisiae* KM 1195 and *Saccharomyces cerevisiae* KM 7253, on ethanol production were compared. Dilute sulfuric acid pretreatment and enzymatic hydrolysis were conducted to select the optimum pretreatment conditions for further enzymatic hydrolysis research. The optimum dilute acid pretreatment conditions included T = 135°C, t = 30 min, and sulfuric acid concentration = 0.1 M. The cellulosic residue was enzymatically hydrolyzed in 0.05 M citrate phosphate buffer (pH 5.0) using the mixture of enzymes cellulase, xylanase and pectinase. The maximum enzymatic saccharification of cellulosic material (76.8%) was achieved after 24 h incubation at 50°C. SHF of water hyacinth leaves by *Saccharomyces cerevisiae* KM 7253 resulted lowest yield of ethanol. SHF by monoculture of *Saccharomyces cerevisiae* KM 1195 achieved the highest yields of ethanol. Further improvement in ethanol production was accomplished with the co-culture of *S. cerevisiae* TISTR 5048 and *Candida tropicalis* TISTR 5045 in the ratio of 1:1 which contributed to the highest increase in ethanol production. In this case, the ethanol concentration of 3.39 (g/l), percentage of the theoretical ethanol yield of 96.07%, the ethanol yield

of 0.25 g/g and the volumetric productivity of 0.221 g/l/h were obtained (Sornvoraweat and Kongkiattikajorn, 2010).

2.5.2 SSF (Simultaneous Saccharification and Fermentation)

Simultaneous saccharification and fermentation (SSF) integrates enzymatic hydrolysis and microbial fermentation into one step: the sugars released from enzymatic hydrolysis are immediately consumed by the fermentative microorganism for ethanol production. By SSF, enzymatic hydrolysis rates can be maximized by reducing the product (sugar) inhibition. SSF gives higher reported ethanol yields from cellulose than SHF and requires lower amounts of enzyme (Eklund and Zacchi, 1995; Sun and Cheng, 2002). Another advantage of SSF is that the risk of contamination is reduced due to the low sugar concentration and ethanol accumulation in the fermentation system. Furthermore, the number of vessels required for SSF is reduced in comparison to SHF, resulting in lower capital cost of the process (Taherzadeh and Karimi, 2007). SSF has the drawback that the enzymatic hydrolysis and fermentation have to be performed under the comprised conditions, particularly with respect to pH and temperature. The optimum temperature and pH of enzyme activity and fermentation are always different. Hydrolysis is usually the rate-limiting step in SSF, and the optimal temperature of enzyme reaction is typically higher than that of fermentation. Therefore, several thermotolerant bacteria and yeasts, e.g., *Candida acidothermophilum* and *Kluyveromyces marxianus* have been proposed for SSF in order to raise the temperature close to the optimum temperature of enzyme reactions (Ballesteros *et al.*, 2004; Golias *et al.*, 2002). Inhibition of ethanol accumulated during the SSF process on enzymes and microorganisms may also be a disadvantage of SSF. It was reported that the enzyme activities are reduced by 25% when ethanol concentration is 30 g/l (Wyman and Abelson, 1996).

Vintila *et al.* (2011) compare the productivity and efficiency of two technologies: simultaneous hydrolysis (saccharification) and fermentation (SSF) and separated hydrolysis and fermentation (SHF) applied to hydrolyze and ferment different types of cellulose from agriculture. The biomass was pretreated

using alkaline and steam combined method. The cellulases used for hydrolysis are *Trichoderma* cellulases Onozuka from Merk, *Aspergillus* cellulases from Fluka, and cellobiase from Novo. The results indicate that SSF is more efficient than SHF in terms of total production time, energy consumption and total production costs. Significant progress has been made with respect to increasing the substrate loading, decreasing the yeast concentration and co-fermentation of both hexoses and pentoses during SSF. Presently, SSF process for *e.g.* wheat straw hydrolyzate can be expected to give final ethanol concentrations close to 40 g/L with a yield based on total hexoses and pentoses higher than 70% (Olofsson *et al.*, 2008).

Wyman *et al.* (1992) reported significantly higher ethanol yields from dilute acid pretreated switchgrass using SSF over SHF. The study also noted ethanol yields from switchgrass were less than those from corn cobs and corn stover with SSF. Using a co-culture of *Brettanomyces clausenii* and *S. cerevisiae*, 87% (of theoretical maximum) conversion into ethanol using SSF was reported. Chung *et al.* (2005) reported comparable ethanol yields for SSF and SHF of dilute acid pretreated switchgrass (90.3% and 91.4%, respectively). Other studies have reported lower ethanol yields: 40% for SHF of AFEX pretreated switchgrass (Alizadeh *et al.*, 2005) and 72% for SSF of lime-pretreated switchgrass (Chang *et al.*, 2001).

Reshamwala *et al.* (1995) used recombinant *Klebsiella oxytoca* to ferment both glucose and xylose in the enzymatic hydrolysate of AFEX pretreated switchgrass. Their results indicate that xylose fermentation was slower and less complete than glucose fermentation and greatly reduced at higher sugar concentrations.

Ethanol production from alfalfa fiber using separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) with and without liquid hot water (LHW) pretreatment was described by Sreenath *et al.* (2001).. *Candida shehatae* FPL-702 produced 5 and 6.4 g/l ethanol with a yield of 0.25 and 0.16 g ethanol/g sugar respectively by SHF and SSF from alfalfa fiber without pretreatment. With LHW pretreatment using SSF, *C.*

shehatae FPL-702 produced 18.0 g/l ethanol, a yield of 0.45 g ethanol/g sugar from cellulosic solids or 'raffinate'. Using SHF, it produced 9.6 g/l ethanol, a yield of 0.47 g ethanol/g sugar from raffinate.

Simultaneous saccharification and fermentation (SSF) of alkaline hydrogen peroxide pretreated *Antigonum leptopus* (Linn) leaves to ethanol was optimized using cellulase from *Trichoderma reesei* QM-9414 (Celluclast from Novo) and *Saccharomyces cerevisiae* NRRL-Y-132 cells. Response surface methodology (RSM) and a three-level four-variable design were employed to evaluate the effects of SSF process variables such as cellulase concentration (20-100 FPU/g of substrate), substrate concentration (5-15% w/v), incubation time (24-72 h), and temperature (35-45°C) on ethanol production efficiency. At these conditions, the predicted ethanol yield was 3.02% (w/v) and the actual experimental value was 3.0% (w/v) (Krishna and Chowdary, 2000).

Saccharomyces cerevisiae and *Candida tropicalis* were used separately and as co-culture for simultaneous saccharification and fermentation (SSF) of 5±20% (w/v) dry corn cobs. A maximal ethanol concentration of 27, 23, 21 g/l (w/v) from 200 g/l (w/v) dry corn cobs was obtained by *S. cerevisiae*, *C. tropicalis* and the co-culture, respectively, after 96 h of fermentation. However, theoretical yields of 82%, 71% and 63% were observed from 50 g/l dry corn cobs for the above cultures, respectively. Maximal xylitol concentration of 21, 20 and 15 g/l from 200 g/l (w/v) dry corn cobs was obtained by *C. tropicalis*, co-culture, and *S. cerevisiae*, respectively. Maximum theoretical yields of 79.0%, 77.0% and 58% were observed from 50 g/l of corn cobs, respectively. The volumetric productivities for ethanol and xylitol increased with the increase in substrate concentration, whereas, yield decreased. Glycerol and acetic acid were formed as minor by-products. *S. cerevisiae* and *C. tropicalis* resulted in better product yields (0.42 and 0.36 g/g) for ethanol and (0.52 and 0.71 g/g) for xylitol, respectively, whereas, the co-culture showed moderate level of ethanol (0.32 g/g) and almost maximal levels of xylitol (0.69 g/g) (Latif and Rajoka, 2001).

2.5.3 SSCF (Simultaneous saccharification and Co Fermentation)

Basically, simultaneous saccharification and cofermentation (SSCF) has the same mechanism with SSF. Cofermentation refers to the fermentation of both hexoses (C6) and pentoses (C5) to ethanol. The hydrolyzed hemicellulose during pretreatment and the solid cellulose are not separated after pretreatment, allowing the conversion of cellulose and hemicellulose to be carried out in a single reactor via a single step (Teixeira *et al.*, 2000). Some conventional wild type fermentative microorganisms, e.g., *Saccharomyces cerevisiae*, are only capable of utilizing glucose as the carbon source for ethanol production, while some can consume both hexoses and pentoses, e.g., *Pichia stipitis*. However, the growth and fermentation of *Pichia stipitis* require expensive media, making the fermentation with *Pichia stipitis* less cost-effective. Therefore, one important requirement is an efficient microorganism capable of fermenting a wide range of substrates (pentoses and hexoses) as well as to tolerate stress conditions. There have been multiple efforts to develop these recombinant microorganisms to allow co-fermentation of both pentose and hexose sugars (Deanda *et al.*, 1996; Dien *et al.*, 1997; Eliasson *et al.*, 2000; Moniruzzaman *et al.*, 1997; Zhang *et al.*, 1995). Particularly, three main microbial platforms, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli*, have emerged and their performance has been demonstrated in pilot studies (Zaldivar *et al.*, 2001). Co-utilization of several sugars, especially xylose and glucose, is essential for economically feasible processes with high ethanol productivity. However, the major problem during xylose/glucose co-fermentation is that xylose is used very slowly until after glucose is completely consumed.

Nakamura *et al.* (2008) demonstrated an effective co-fermentation process using xylose- and cellobiose-assimilating recombinant *Saccharomyces cerevisiae*. The recombinant yeast is able to consume xylose during xylose/cellobiose co-fermentation as rapidly as during glucose fermentation. After 72 h, 95.6% of xylose was consumed, despite the cofermentation conditions, and the ethanol yield was 0.358 g-ethanol/g total sugar. Genetically engineered *Z. mobilis* strains were also investigated for their co-fermentation potential. Krishnan *et al.* (2000) employed an immobilized recombinant *Z. Mobilis* strain to co-ferment xylose and

glucose. In batch experiments it was shown that fermentation results were comparable for concentrated acid rice straw hydrolyzate and a synthetic control medium. Continuous experiments in a fluidized bed reactor were performed with the control medium at dilution rates between 0.24 and 0.5/h and excellent volumetric ethanol productivities of 6.5– 15.3 g/l/h were achieved. However, xylose conversion varied from 10% to 90%. The two main sugars in the agricultural by-product corn stover are glucose and xylose. Co fermentation of glucose and xylose at high content of water-insoluble solids (WIS) without detoxification is a prerequisite to obtain high ethanol concentration and to reduce production costs. A recombinant strain of *Saccharomyces cerevisiae*, TMB3400, was used. TMB3400 co-fermented glucose and xylose with relatively high ethanol yields giving high final ethanol concentration. The ethanol productivity increased with increasing concentration of pretreatment hydrolysate in the yeast production medium and when SSF was performed in a fed-batch mode (Ohgren *et al.*, 2006).

Zymomonas mobilis was genetically improved for pentose utilization to increase the final ethanol concentration. It showed good fermentation ability on both soluble sugar mixture and lignocellulose. Nearly all the glucose and xylose in sugar mixture can be consumed, corresponding to 86% of theoretic ethanol yield. Simultaneous saccharification and co-fermentation (SSCF) of NaOH pretreated corncob was then carried out in a high dry matter (DM) loading of 15– 25w/v%. At the DM loading of 15%, the suitable operating conditions were determined, i.e., *Z. mobilis* loading of 0.30 g dry weight/L at 30 °C (pH5.5), under which the ethanol concentration reached 49.2 g/L. Higher final ethanol concentrations were obtained when SSCF was operated at the fed-batch mode. Several amounts of substrate (1% to 10%) were added, and the highest final ethanol concentration (60.5 g/L) was obtained at 10% DM addition (Rongxin *et al.*, 2012).

Seven different recombinant xylose utilizing *Saccharomyces cerevisiae* strains were evaluated for their fermentation performance in hydrolysates of steam pretreated corncobs. Two strains, RHD-15 and KE6-12 with highest

ethanol yield and lowest xylitol yield, respectively. With a fed-batch SSCF in combination with prefermentation and a feed of substrate and enzymes 47 and 40 g/l of ethanol corresponding to 68% and 58% of the theoretical ethanol yield. The strain KE6-12 was able to completely consume xylose within 76 h during the fermentation of hydrolysate in a 10 m³ demo scale bioreactor. The potential of SSCF is improved in combination with prefermentation and a feed of substrate and enzymes. It was possible to successfully reproduce the fed-batch SSCF at demo scale producing 4% (w/v) ethanol which is the minimum economical requirement for efficient lignocellulosic bioethanol production process (Koppram *et al.*, 2013).

Tang *et al.* (2011) reported the use of simultaneous saccharification and cofermentation (SSCF) of lignocellulosic residues from commercial furfural production (furfural residue, FR) and corn kernels to compare different nutritional media. An ethanol concentration of 73.1 g/L at 120 h, which corresponded to a 101.1% ethanol yield based on FR cellulose and corn starch, was obtained in SSCF of 7.5% FR and 14.5% corn kernels with mineral-salt medium. SSCF could simultaneously convert cellulose into ethanol from both corn kernels and FR.

11 types of grasses were selected as feedstocks for the ethanol production by simultaneous saccharification and cofermentation (SSCF). The enzymes, cellulase and xylanase, were utilized for hydrolysis and the yeasts, *Saccharomyces cerevisiae* and *Pichia stipitis*, were applied for cofermentation at 35°C for 7 days. From the results, the highest yield of ethanol, 1.14 g/L or 0.14 g/g substrate equivalent to 32.72% of the theoretical values was obtained from Sri Lanka ecotype vetiver grass and it gave the yield of ethanol at 1,091.84 L/ha/year, whereas the leaves of dwarf napier grass showed the maximum yield of 2,720.55 L/ha/year (0.98 g/L or 0.12 g/g substrate equivalent to 30.60% of the theoretical values) (Wongwatanapaiboon *et al.*, 2012).

Poplar wood pretreated by steam explosion was used as the feedstock of simultaneous saccharification and cofermentation (SSCF) process for ethanol production. For cofermentation, the mixed culture of recombinant *Escherichia*

coli (KO11) and *Saccharomyces cerevisiae* resulted in superior ethanol production than its single culture counterparts. The ethanol yield increased with decreasing concentration of reducing sugars. The maximum ethanol production was 8.97 g/l and 13.98 g/l in 5% and 10% substrate, respectively, and the corresponding yield was 53.8% and 41.9%, respectively (Chen *et al.*, 2011).

Use of Recombinants for improved ethanol yield

Xylose is the second most abundant carbohydrate in the lignocellulosic biomass hydrolysate. The fermentation of xylose is essential for the bioconversion of lignocelluloses to fuels and chemicals. However the wild-type strains of *Saccharomyces cerevisiae* are unable to utilize xylose. Many efforts have been made to construct recombinant yeast strains to enhance xylose fermentation over the past few decades. Xylose fermentation remains challenging due to the complexity of lignocellulosic biomass hydrolysate. A modified genome shuffling method was developed to improve xylose fermentation by *S. cerevisiae*. Recombinant yeast strains were constructed by recursive DNA shuffling with the recombination of entire genome of *P. stipitis* with that of *S. Cerevisiae* (Zhang and Geng, 2012).

The robustness under heat, acetic acid, and furfural stresses was improved for ethanologenic *S. cerevisiae* using genome shuffling. Recombinant yeast strain R32 could grow at 45°C, and resist 0.55% (v/v) acetic acid and 0.3% (v/v) furfural at 40°C. When ethanol fermentation was conducted at temperatures ranging from 30 to 42°C, recombinant strain R32 always gave high ethanol production. After 42 h of fermentation at 42°C, 187.6 ± 1.4 g/l glucose was utilized by recombinant strain R32 to produce 81.4 ± 2.7 g/l ethanol, which were respectively 3.4 and 4.1 times those of CE25. After 36 h of fermentation at 40°C with 0.5% (v/v) acetic acid, 194.4 ± 1.2 g/l glucose in the medium was utilized by recombinant strain R32 to produce 84.2 ± 4.6 g/l of ethanol. The extent of glucose utilization and ethanol concentration of recombinant strain R32 were 6.3 and 7.9 times those of strain CE25. The ethanol concentration produced by recombinant strain R32 was 8.9 times that of strain CE25 after fermentation for 48 h under 0.2% (v/v) furfural stress at 40°C (Lu *et al.*, 2011).

Saccharomyces strain 1400 (pLNH32) was genetically engineered to ferment xylose by expressing genes encoding a xylose reductase, a xylitol dehydrogenase and a xylulose kinase. The recombinant efficiently fermented xylose alone or in the presence of glucose. Xylose-grown cultures had very little difference in xylitol accumulation, with only 4 to 5 g/l accumulating, in aerobic, micro-aerated and anaerobic conditions. Highest production of ethanol with all sugars was achieved under anaerobic conditions. From a mixture of glucose (80 g/l) and xylose (40 g/l), this strain produced 52 g/l ethanol, equivalent to 85% of theoretical yield, in less than 24 h. Using a mixture of glucose (31 g/l), xylose (15.2 g/l), arabinose (10.5 g/l) and galactose (2 g/l), all of the sugars except arabinose were consumed in 24 h with an accumulation of 22 g ethanol/l, a 90% yield (excluding the arabinose in the calculation since it is not fermented). Approximately 98% theoretical yield, or 21 g ethanol/l, was achieved using an enzymatic hydrolysate of ammonia fibre exploded corn fibre containing an estimated 47.0 g mixed sugars/l. In all mixed sugar fermentations, less than 25% arabinose was consumed and converted into arabitol (Moniruzzaman *et al.*, 1997).

Saccharomyces cerevisiae 424A (LNH-ST) strain was used for fermentation of glucose and xylose. Growth kinetics and ethanol productivity were calculated for batch fermentation on media containing different combinations of glucose and xylose to give a final sugar concentration of 20 ± 0.8 g/L. Growth rates obtained in pure xylose-based medium were less than those for media containing pure glucose and glucose-xylose mixtures. A maximum specific growth rate μ_{max} of 0.291 h⁻¹ was obtained in YPD medium containing 20 g/L glucose as compared to 0.206 h⁻¹ in YPX medium containing 20 g/L xylose. In media containing combinations of glucose and xylose, glucose was exhausted first followed by xylose. Ethanol production on pure xylose entered log phase during the 12–24 h period as compared to the 4–10 h for pure glucose based medium using 2% inoculum. When glucose was added to fermentation flasks which had been initiated on a pure xylose-based medium, the rate of xylose usage was reduced indicating co substrate inhibition of xylose consumption by glucose (Govindaswamy and Vane, 2007).

Zymomonas mobilis genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) were integrated into the *Escherichia coli* chromosome within or near the pyruvate formate-lyase gene (*pif*). Integration improved the stability of the *Z. mobilis* genes in *E. coli*, but further selection was required to increase expression. Spontaneous mutants were selected for resistance to high levels of chloramphenicol that also expressed high levels of the *Z. mobilis* genes. Analogous mutants were selected for increased expression of alcohol dehydrogenase on aldehyde indicator plates. These mutants were functionally equivalent to the previous plasmid-based strains for the fermentation of xylose and glucose to ethanol. Ethanol concentrations of 54.4 and 41.6 g/liter were obtained from 10% glucose and 8% xylose, respectively. The efficiency of conversion exceeded theoretical limits (0.51 g of ethanol/g of sugar) on the basis of added sugars because of the additional production of ethanol from the catabolism of complex nutrients. Further mutations were introduced to inactivate succinate production (*frd*) and to block homologous recombination (*recA*) (Ohta *et al.*, 1991).

The ethanol fermenting genes such as pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh* II) were cloned from *Zymomonas mobilis* and transformed into three different cellulolytic bacteria, namely *Enterobacter cloacae* JV, *Proteus mirabilis* JV and *Erwinia chrysanthemi* and their cellulosic ethanol production capability was studied. Recombinant *E. cloacae* JV was found to produce 4.5% and 3.5% (v/v) ethanol, respectively, when CMC and 4% NaOH pretreated bagasse were used as substrates, whereas recombinant *P. mirabilis* and *E. chrysanthemi* with the same substrates could only produce 4%, 3.5%, 1%, and 1.5 % of ethanol, respectively. The recombinant *E. cloacae* strain produced twofold higher percentage of ethanol than the wild type. The recombinant *E. cloacae* strain could be improved further by increasing its ethanol tolerance capability through media optimization and also by combining multigene cellulase expression for enhancing ethanol production from various types of lignocellulosic biomass so that it can be used for industrial level ethanol production(Piriya *et al.*, 2012). The T7-expression system has been very useful for protein expression in *Escherichia coli*. A T7- expression transposon was constructed, which allowed

simple construction of T7-expression *Zymomonas mobilis*. This transposon contained the T7 RNA polymerase being driven by the *gap* promoter from *Z. mobilis*. The T7-expression *fadK* genomes were introduced into *Z. mobilis* ATCC 31821 in order to increase ethanol production. The recombinant bacteria were named as *Z.M.F-1*, *Z.M.F-2*, *Z.M.F-3*, and *Z.M.F-4*. However, *Z.M.F-4* had the highest ability of producing ethanol by selection. Compared with *Z. mobilis* ATCC 3182, there was 7% increase in ethanol production for *Z.M.F-4* with corn hydrolysates as fermentation medium. The 16 mmolL⁻¹ supplement of calcium chloride could significantly improve the ethanol production. This was also clearly demonstrated by a variety of kinetic parameter values over time in *Z.M.F-4* under high sugar osmotic stress. Calcium chloride not only increased the fermentation ability but also improved the stability of cell membrane (Zeng *et al.*, 2010).

A study conducted by Gold *et al.* (1996) describes the expression of the *Zymomonas mobilis* genes coding for pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase (*adh*) in *Lactobacillus casei* 686. To promote transcription, the promoter and ribosome binding site (RBS) from the *Lactococcus lactis* subsp. *lactis*-derived vector, pMGE36e, were inserted upstream of the *pdh* gene. The former sequences were positioned such that translation of *pdh* was coupled to translation of an 81-base pair open reading frame terminating within the *pdh* initiation site. The recombinant plasmid (pRSG02) was electroporated into *L. casei*, and transformants were obtained. Northern analysis confirmed the production of a 3.1-kb transcript corresponding to the predicted size of the PET operon. Western blot analyses revealed that the recombinant strain expressed both enzymes. The recombinant produced more than twice the ethanol produced by the parental *L. casei* strain.

Ethanol Technologies and Trade

The future of ethanol fuels appears to be bright, as countries around the world have begun to pursue aggressive ethanol strategies in recent years. One of the issues surrounding the future of ethanol comes from the prospect of international trade in ethanol. While one of the major justifications for ethanol fuels seems to be a desire to maintain a secure domestic supply, importation of

ethanol could provide the fuel to many countries at a greatly reduced cost and on the other hand it ensure poor food crops yields as farmers turned to produce fuel crops . However, most countries have high importation tariffs on ethanol to make imported fuel uncompetitive with the domestic supply.

Biofuels represent an alternative source of energy that should gradually decrease our dependence on crude oil. A rapid development of their use in combustion engines is above all the consequence of their very positive balance of emissions. The possibility of use of biofuels in conventional combustion engines is given by their physico-chemical properties. Bioethanol is one of biofuels that can be used in spark-ignition engines. However, because of its lower heating value, it is necessary to change the mixing ratio fuel/air. Changes in chemical composition of biofuels or, better to say, in ratios between carbon, hydrogen, and other elements (e.g. oxygen), can significantly reduce not only the volume of noxious substances but also of carbon dioxide (CO₂) that is one of final products of the process of complete combustion (Polacar, 2012). To enable the use of these biofuels in conventional engines without any extensive modifications of their construction, it is necessary that their physicochemical properties would be as much as possible similar to those of petroleum-derived fuels and it can be said that just the bioethanol is one of those products that are the most prospective and suitable for combustion in both spark and compression ignition engines. Bioethanol (ethyl alcohol) is the product of fermentation that belongs to the group of light alcohols. It is the final product of those fermentation processes, which transform raw materials containing biodegradable wastes and residues, e.g. simple sugars (sugar beet), starch (maize, cereals, and potatoes), or ligno-cellulose biomass (waste materials from wood-processing industry) into utility products (Demirbas, 2005).

In 2003, Volkswagen released its first flex-fuel vehicle, capable of running on fuel with any blend of ethanol and gasoline. Research into production of ethanol from cellulose-based feedstocks could allow producers to extract ethanol from corn stalks, sugarcane leaves, and other forms of organic waste. These technologies are likely to reduce feedstock costs, which could make

ethanol fuels considerably more affordable, especially in developed countries. Dedini SA, a Brazilian ethanol-engineering firm, announced in June 2003 that it had developed such technologies to extract ethanol from sugarcane leaves in addition to cane juice. It estimates that this technology could double the amount of ethanol produced per hectare of sugarcane (Knights, 2003).

Benefits to be Gained

Whilst the recent controversy on the use of high ethanol content fuel dominated the daily press, the main arguments that have long been advanced in favour of fuel ethanol include environmental benefits, the development of regional industries, and supplementation of the national fuel supply. Ethanol production from agricultural crops including wheat and sugarcane is also regarded as 'renewable' and sustainable. Another reason why ethanol fuels are so attractive is that they represent an environment-friendly technology that is available today. While ethanol fuelled automobiles are probably not likely to be the permanent solution to environmental concerns in automotive fuels, in the very least, they provide a temporary solution while research into fuel cells and other advanced technologies are under development. By investing in such practical technologies today, governments around the world enjoy significant environmental progress as well as domestic economic development, and realize two goals that often appear at odds with one another. As countries around the world begin to take note of the benefits provided by ethanol fuels and develop their own programmes, it is important to consider what the future might hold for ethanol fuels and how emerging technologies and policies can help guide this development in a desirable direction. There are still many issues to be addressed in considering the future of ethanol fuels. Many critics have charged that the energy balance of ethanol fuels is flawed, arguing that the energy inputs exceed the energy content of the final product. However, a study released by the United States Department of Agriculture in July of 2002 refutes these claims and finds that ethanol has an output: input energy ratio of 1.34:1 (Shapouri *et al.*, 2002). Furthermore, another study found that the energy balance of gasoline is actually negative, giving ethanol a 1.42:1 output energy ratio compared to gasoline. Perhaps even more important than the energy balance from an

environmentalist perspective is the amount of carbon released in the production of the fuel as compared to its final energy content. If agricultural processes and transportation of ethanol release large enough quantities of carbon dioxide, running a vehicle on ethanol could potentially result in higher carbon emissions than a clean diesel or gasoline engine. Thus, some have argued that ethanol fuels should be evaluated based on their carbon dioxide balance, indicative of the production process. For example, due to the photosynthetic efficiency advantage of sugarcane over corn and a large ethanol transportation infrastructure already established, ethanol in Brazil has a much lower carbon dioxide emissions: final energy ratio, than ethanol produced in the United States. At present, there is no distinction made between the two types of ethanol fuels, despite the fact that the Brazilian ethanol reduces considerably more carbon dioxide emissions. Recognition of this dissimilarity is essential to making ethanol fuels sustainable and maximizing the environmental benefits of its use (Lindqvist, 2003).

Conflicting Needs

The biofuels produced from the renewable resources could help to minimize the fossil fuel burning and CO₂ production. Biofuels produced from biomass such as plants or organic waste could help to reduce both the world's dependence on oil and CO₂ production. These biofuels have the potential to cut CO₂ emission because the plants they are made from use CO₂ as they grow (Osamu and Carl, 1989). Biofuels and bioproducts produced from plant biomass would mitigate global warming. This may due to the CO₂ released in burning equals the CO₂ tied up by the plant during photosynthesis and thus does not increase the net CO₂ in the atmosphere. Additionally, biofuel production along with bioproducts can provide new income and employment opportunities in rural areas. 21st Century is looking for a shift to alternate industrial feedstock and green processes to produce these chemicals from renewable biomass resources. 'First generation' biofuels can offer some CO₂ benefits and can help to improve domestic energy security. But concerns exist about the sourcing of feedstocks, including the impact it may have on biodiversity and land use and competition with food crops. A 'first generation' biofuel (i.e. biodiesel , bio-ethanol, and biogas) is characterized either by its ability to be blended with petroleum-based

fuels, combusted in existing internal combustion engines, and distributed through existing infrastructure, or by the use in existing alternative vehicle technology like FFVs (“Flexible Fuel Vehicle”) or natural gas vehicles. The production of 1st generation biofuels is commercial today, with almost 50 billion liters produced annually. There are also other niche biofuels, such as biogas which have been derived by anaerobic treatment of manure and other biomass materials. However, the volumes of biogas used for transportation are relatively small today. However, the first generation biofuels seems to create some skepticism to scientists. There are concerns about environmental impacts and carbon balances, which sets limits in the increasing production of biofuels of first generation. The main disadvantage of first generation biofuels is the food-versus-fuel debate, one of the reasons for rising food prices is due to the increase in the production of these fuel. Additionally it is claimed that biodiesel is not a cost efficient emission abatement technology. Therefore, for the abatement of GHG, it is recommended to have more efficient alternatives based on both renewable and conventional technologies. The lignocellulosic feedstock can offer the potential to provide novel biofuels, the biofuels of the ‘second generation’ (Simpson *et al.*, 2007). The examples of 2nd generation biofuels are cellulosic ethanol and Fischer–Tropsch fuels. The production of 2nd generation biofuels is non-commercial at this time, although pilot and demonstration facilities are being developed. Therefore it is anticipated that, these 2nd generation biofuels could significantly reduce CO₂ production, do not compete with food crops and some types can offer better engine performance. When commercialized, the cost of second generation biofuels has the potential to be more comparable with standard petrol, diesel, and would be most cost effective route to renewable, low carbon energy for road transport. Therefore due to many advantages and disadvantages of the 1st generation biofuels and obvious advantages of 2nd generation biofuels, the approaches to integral utilization of biomass for sustainable development are more reasonable, where all parts of the plant such as leaves, bark, fruits, and seeds can be utilized to useful products.

Third generation biofuels derived from microalgae are considered to be a viable alternative energy resource that is devoid of the major drawbacks

associated with first and second generation biofuels (Nigam and Singh, 2010). Microalgae are able to produce 15–300 times more oil for biodiesel production than traditional crops on an area basis. Furthermore compared with conventional crop plants which are usually harvested once or twice a year, microalgae have a very short harvesting cycle (≈ 1 – 10 days depending on the process), allowing multiple or continuous harvests with significantly increased yields (Schenk *et al.*, 2008). The idea of using microalgae as a source of biofuel is not new, but it is now being taken seriously because of the rising price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning of fossil fuels (Chisti, 2007). The utilization of microalgae for biofuels production offers the following advantages over higher plants: (1) microalgae synthesize and accumulate large quantities of neutral lipids (20–50 % dry weight of biomass) and grow at high rates; (2) microalgae are capable of all year round production, therefore, oil yield per area of microalgae cultures could greatly exceed the yield of best oilseed crops; (3) microalgae need less water than terrestrial crops therefore reducing the load on freshwater sources; (4) microalgae cultivation does not require herbicides or pesticides application; (5) microalgae sequester CO₂ from flue gases emitted from fossil fuel-fired power plants and other sources, thereby reducing emissions of a major greenhouse gas (1 kg of dry algal biomass utilise about 1.83 kg of CO₂); (6) wastewater bioremediation by removal of NH₄⁺, NO₃⁻, PO₄⁻³ from a variety of wastewater sources (e.g. agricultural run-off, concentrated animal feed operations, and industrial and municipal wastewaters); (7) combined with their ability to grow under harsher conditions and their reduced needs for nutrients, microalgae can be cultivated in saline/brackish water/coastal seawater on non-arable land, and do not compete for resources with conventional agriculture; (8) depending on the microalgae species other compounds may also be extracted, with valuable applications in different industrial sectors, including a large range of fine chemicals and bulk products, such as polyunsaturated fatty acids, natural dyes, polysaccharides, pigments, antioxidants, high-value bioactive compounds, and proteins (Mata *et al.*, 2010; Um and Kim, 2009).

It is important to consider the carbon dioxide released during ethanol production, and the effects of drastically increasing agricultural production in

order to meet an increased demand for the fuel (increased agriculture necessitates more land use change, more pesticides, and more fertilizer) all of which bring a host of different environmental concerns. Scientists debate the extent to which agricultural capacity can be expanded to meet a global demand for ethanol, especially as the rapidly growing population in the developing world struggles to feed itself. While all of these externalities can be addressed to yield an ethanol fuel that is better for the environment than our conventional fossil fuel-based consumption, it is important to consider them and not blindly accept the environmental benefits of ethanol fuels just because they are cleaner at the tailpipe. Most mainstream environmental groups support fuels ethanol as a significant step toward slowing or stopping global climate change. However, fuel ethanol production can threaten the environment if it is not done sustainably.

Fuel ethanol produces greenhouse gas emissions during their manufacture. The sources of these emissions are: fertilizers and agricultural processing, transportation of the biomass, processing of the fuels and transport and delivery of biofuels to the consumer. Some fuel ethanol production processes produce far fewer emissions than others; for example sugar cane cultivation requires fewer fertilizer inputs than corn cultivation, therefore sugar cane bioethanol reduces greenhouse gas emissions more effectively than corn derived bioethanol.

In addition to converting these feedstock waste products into electricity, research into production of ethanol from cellulose-based feedstocks (woody materials, stalks, and leaves) could allow these agricultural residues to be converted into more ethanol. Additional research into ethanol extraction from cellulose feedstocks promises to reduce ethanol costs by running production on the relatively cheap waste products of the agriculture and timber industries. Additionally, the use of cellulose-based feedstocks appeases concerns that a reliance on ethanol fuels could divert food from the rapidly growing world population by providing farmers with a secondary flow of revenue; the technology could instead make food more affordable to the world's poor since crop displacement has been minimal and has little effect on food prices, ethanol advocates says.

RECOMMENDATIONS

- 1) The governments should develop ethanol trading programmes that will enhance the affordability of ethanol fuels in comparison to gasoline and diesel.
- 2) The governments should use tradable permits as a means of encouraging car manufacturers to sell alternative fuelled vehicles.
- 3) The governments should introduce a well reasoned policies and technological advancement in ethanol fuels that could guide a smooth transition away from fossil fuels in the transportation sector.
- 4) The use of bio-ethanol as alternative to conventional petroleum products require a substantial shift in public attitudes probably towards an acceptance of paying a premium for motor biofuels, and any new engine technology. Such changes would require change of the fuel market; governments should provide subsidies and tax incentives and possibly a pollution or carbon tax.
- 5) Governments should encourage and support research into ethanol extraction from cellulose feed stocks as it promises to reduce ethanol costs by running production on a relatively cheap waste products of the agriculture and timber industries.
- 6) The use of cellulose- based feed stocks appeases concerns that a reliance on ethanol fuels could divert food from the rapidly growing world population. The governments should provide farmers with a secondary flow of revenue; the technology could instead make food more affordable to the world's poor.
- 7) A combination of well-reasoned government policies and technological advancements in ethanol fuels could guide a smooth transition away from fossil fuels to ethanol fuel use in the transportation sector. An environmental externalities should continue be incorporated into policy consideration and the fledgling industry emerges, ethanol fuels are likely to become and increasing attractive fuel alternative in the foreseeable future.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled “**Bioconversion of cellulosic waste into bioethanol as biofuel**” was conducted in laboratory of Microbiology section, Department of Basic Sciences, Nauni, Solan (H.P.) – India, during the year 2009-2013. 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 cellulolytic and xylanolytic microorganisms from soil
- 3.2 Secondary screening of hypercellulase and hyperxylanase producers to enhance their enzyme production by mutation
- 3.3 Identification of hypercellulolytic and hyperxylanolytic enzyme producing isolates
- 3.4 Optimization and production of cellulase and xylanase under submerged fermentation
- 3.5 Cost effective production of cellulase and xylanase by using lignocellulosic biomass under submerged (SmF) and solidstate fermentation (SSF)
- 3.6 Partial Purification of cellulase and xylanase enzymes
- 3.7 Scale up of enzymes in a bioreactor
- 3.8 Bioethanol production under SHF (Separate hydrolysis and fermentation), SSF (Simultaneous saccharification and fermentation) and SSCF (Simultaneous saccharification and co fermentation) using different microorganisms and recombinants.
- 3.9 Estimation of inhibitors (5-HMF) using high performance liquid chromatography (HPLC)
- 3.10 Scale up of bioethanol production in a stirred tank bioreactor

3.1 ISOLATION AND SCREENING OF CELLULOLYTIC AND XYLANOLYTIC MICROORGANISMS FROM SOIL

3.1.1 Collection of soil samples

Soil samples were collected and pooled together in clean polythene bags from different sites of Himachal Pradesh viz. Solan, Shimla and Kangra district and brought to the laboratory.

3.1.2 Statistical analysis

Three stage random sampling technique was used for collection of samples to isolate microorganisms from soil. Three districts viz. Solan, Shimla and Kangra were randomly selected from Himachal Pradesh. In the second stage, three places were selected randomly from Distt. Solan and three places were selected randomly from Distt. Shimla and three places from Distt. Kangra. In the third stage, from each place 3-6 sites were selected randomly as given in the following table:

Districts	Name of places	Number of sites
Solan	Nauni	3
	Kumarhatti	5
	Dharampur	6
	Total no. of sites	14
Shimla	Sanjoli	4
	Panthghatti	5
	Kusumpati	4
	Total no. of sites	13
Kangra	Palampur	6
	Dharamshala	5
	Dehra	6
	Total no. of sites	17

3.1.3 Enrichment of soil samples

To 100g of soil sample, 2 % of cellulose powder was added and water was sprinkled in the petriplate and mixed thoroughly. The petriplates containing the above mixture were incubated at 35 ± 2 °C for one week.

3.1.4 Isolation of cellulolytic and xylanolytic microorganisms

One gram of enriched soil sample was serially diluted from 10^{-2} to 10^{-8} times using sterilized 9 ml dilution blanks. Each of diluent (0.1 ml) was placed on

the surface of Riviere's and PYC (Peptone Yeast Extract) medium in a petriplate and evenly spread with the help of a spreader and the plates were incubated at $35\pm 2^{\circ}\text{C}$ for 5 days. The pure line cultures were obtained by streak plate method and bit method for bacteria and fungus respectively. These pure line cultures of all isolates were maintained on nutrient agar slants and preserved in refrigerator at 4°C for further use. The periodic subculturing was done once in a month.

- ❖ Chemical composition of Riviere's medium (Riviere's, 1961) NaNO_3 - 1.0 g ; $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ - 1.18 g ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.5 g ; KH_2PO_4 - 0.9 g ; KCl- 0.5 g ; Yeast extract powder - 0.5 g ; Cellulose powder - 10.0 g , Distilled water - 1000 ml, pH - 6.8 (For fungus pH – 5.5).
- ❖ Chemical composition of PYC medium , CMC - 10.0 g ; peptone - 5.0 g ; Yeast extract - 5.0 g ; K_2HPO_4 - 1.0 g ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.2 g ; NaCl - 5.0 g ; Cellulose - 10.0 g , Distilled water -1000 ml, pH – 7.0.

These media were used for the isolation of cellulolytic and xylanolytic microorganisms.

3.1.5 Screening of cellulolytic and xylanolytic microorganisms

The microbial isolates i.e. isolated from soil of section 3.1.4 were screened for the production of extracellular enzymes i.e. cellulase - CMCase, FPase and β -glucosidase and xylanase.

3.1.5.1 Production of extracellular enzymes - cellulase and xylanase by microbial isolates

Each bacterial isolate was grown in 100 ml of nutrient broth at $35\pm 2^{\circ}\text{C}$ for 24 h. As soon as the substantial growth was observed in the broth, the optical density was set to 1.0 using autoclaved distilled water. Whereas for fungus, different fungal cultures were grown in malt extract agar media petriplates and were kept for 5-7 days in incubator for full plate growth. As soon as full plate of fungus was observed, 10 ml of autoclaved distilled water was poured and scratched. The spore suspension thus formed contained 1×10^7 spores /ml. 5 ml of inoculum was added to each 45 ml of Riviere's broth containing 1% cellulose for cellulase and 1% xylan for xylanase in 250 ml of Erlenmeyer flasks and the

flasks were incubated for 5 days at $35\pm 2^{\circ}\text{C}$ for bacteria and at $28\pm 2^{\circ}\text{C}$ for fungus at 120 rpm.

After incubation, the culture contents were centrifuged at 10,000 rpm for 15 min (4°C). The supernatant was collected. The following quantitative tests were performed with the supernatant to screen out the hypercellulolytic and hyperxylanolytic producers among different isolates.

3.1.5.1.1 Cellulase Assays

3.1.4.1.1.1 CMCase assay (Reese & Mandel, 1963)

Reagents

- i) 1 % CMC in citrate buffer (0.055M, pH 5.0)
- ii) Dinitrosalicylic acid (DNSA) Reagent: NaOH : 1.0 g, Phenol : 0.2 g, Sodium potassium tartarate : 20.0 g, Sodium sulphate : 0.05g, DNSA reagent : 1.0g, Distilled water : 100ml
- iii) Standard solution of glucose (0.4mg/ml)

Procedure

The reaction mixture contained 0.5ml of 1% of CMC in citrate buffer (0.055M, pH-5) and 0.5ml of diluted enzyme (supernatant). Reaction mixture was incubated at 50°C for 30 min. After incubation 3ml of DNSA reagent was added. Tubes were immersed in boiling water bath and removed after 15 min when colour development was complete. Control was run with all the components except the enzyme. Tubes were cooled at room temperature and O.D was read at 540nm in spectrophotometer against the reagent blank i.e. 1ml of distilled water and 3ml of DNSA reagent. The standard curve was made from the stock solution of glucose (0.4 mg/ml). The enzyme activity was expressed in terms of International Unit (IU) and Specific Activity (SA).

- I. One International Unit (IU) of enzyme activity represents μ moles of glucose released/min/ml of enzyme.
- II. Specific Activity (SA) represents μ moles of glucose released/min/mg of protein.

3.1.5.1.1.2 Filter Paperase (FPase) assay (Reese & Mandel, 1963)

Reagents

- i) Strips of filter paper (Whatmann no.1)
- ii) 0.055M citrate buffer (pH 5.0)
- iii) Dinitrosalicylic acid (DNSA) Reagent
- iv) Standard solution of glucose (0.4 mg/ml)

Procedure

To 50 mg of filter paper strips (Whatman no. 1), 0.5 ml of citrate buffer (0.055M, pH-5) and 0.5ml of culture supernatant was added. Reaction mixture was incubated at 50°C for 30 min. After incubation 3ml of DNSA reagent was added. Tubes were immersed in boiling water bath and removed after 15 min when colour development was complete. Control was run with all the components except the enzyme. Tubes were cooled at room temperature and O.D was read at 540nm in spectrophotometer against a reagent blank i.e. 1ml of distilled water and 3ml of DNSA reagent. The standard curve was prepared from the stock solution of glucose (0.4mg/ml). The enzyme activity was expressed in terms of International Unit (IU) and Specific Activity (SA).

- I. One International Unit (IU) of enzyme activity represents μ moles of glucose released/min/ml of enzyme.
- II. Specific Activity (SA) represents μ moles of glucose released/min/mg of protein.

3.1.5.1.1.3 β -Glucosidase assay (Berghem and Petterson, 1973)

Reagents

- i) 1mM p -nitrophenyl β -D-glucopyranoside in 0.055M sodium acetate buffer (pH 5.0)
- ii) 1M sodium carbonate solution
- iii) Standard solution of p -nitrophenol (80 μ g/ml)

Procedure

Reaction mixture contained 1ml of 1mM of p -nitrophenyl β -D-glucopyranoside in 0.055M sodium acetate buffer and 100 μ l of culture

supernatant. After incubation at 40°C for 10 min, 2ml of 1M of Na₂CO₃ was added to the reaction mixture to stop the reaction. Reaction mixture was heated in boiling water bath for 15 min and after heating it was diluted with 10ml of distilled water. p-nitrophenol liberated was determined from the absorbance at 400nm against reagent blank i.e. 1ml of distilled water and 2ml of 1M of sodium carbonate. The standard curve was prepared from the stock solution of p-nitrophenol (80 µg/ml). The enzyme activity was expressed in terms of International Unit (IU) and Specific Activity (SA).

- I. One International Unit (IU) of enzyme activity represents µmoles of p-nitrophenyl β-D-glucopyranoside released/min/ml of enzyme.
- II. Specific Activity (SA) represents µmoles of p-nitrophenyl β-D-glucopyranoside released/min/mg of protein.

3.1.5.1.2 Xylanase Assay (Miller, 1959)

Reagents

- i) 1.0 g Oatspelt xylan in 100 ml of 0.055 M citrate buffer at pH 4.0
- ii) DNSA reagent
- iii) Standard solution of xylose (0.4 mg/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 the enzyme. The reaction mixture was incubated at 45°C for 10 min. After the incubation, 3 ml of DNSA reagent was added and the mixture was then heated on boiling water bath for 30 min. After cooling down at room temperature, absorbance of reaction mixture was read at 540 nm.

- 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.

3.1.5.1.3 Protein assay (Lowry *et al.*, 1951)

Reagents

- i) 1N Folin Ciocalteu's Phenol reagent
- ii) 2% sodium carbonate in 0.1M sodium hydroxide solution
- iii) 1% sodium potassium tartarate solution
- iv) 1% copper sulphate solution
- v) 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.
- vi) Bovin Serum Albumin (BSA) as standard (10-100 µg/ml)

Procedure

To 0.1 ml of culture supernatant, 2.5 ml of Lowry's alkaline reagent was added, mixed and allowed to stand for 10 min. Diluted (1N) Folin Ciocalteu's reagent (0.25 ml) was added. The contents were shaken quickly and allowed to stand for 30 min for maximum colour development. Absorbance of reaction mixture was read at 670nm against a reagent blank. The contents of protein in culture supernatant were estimated from standard curve which was prepared by using Bovin Serum Albumin (BSA) in concentration of 10-100 µg/ml.

Best enzyme producer bacterial isolates were selected on the basis of highest cellulase and xylanase production. After the screening of cellulolytic and xylanolytic microorganisms, out of 84 bacterial isolates 7 hypercellulolytic i.e. N₁₃, S₁, N₅, N₁₂, S₁₂, NS₁ and S₇ and 3 hyperxylanolytic i.e. SL₈, Kd₁ and N₁₁ were selected for further study.

3.2 SECONDARY SCREENING OF HYPERCELLULASE AND HYPERXYLANASE PRODUCERS TO ENHANCE THE ENZYME PRODUCTION BY INDUCING MUTATION

The selected bacterial isolates were subjected to mutation by the physical mutagen i.e., ultraviolet irradiation (UV) and chemical mutagen i.e., ethidium bromide (EtBr) with an apparent aim to get mutants secreting higher enzyme production.

3.2.1 Mutation with Ultraviolet irradiation (UV)

For the present study, UV light of laminar air flow [Phillips TUV 30W/G30.T8 Ultra volt] was used for the mutation. 24 h old culture of bacterial isolate (10 ml) was transferred to a sterilized petriplate and each of sample was exposed to UV light. Before exposing sample to the UV irradiation, 1 ml of sample was withdrawn for determining the initial population in terms of colony forming units on BSYEM medium containing 0.5% of xylan (for xylanase producer) and cellulose (for cellulase producer). The sample was withdrawn after 15, 30, 45, 60 and 75 min of UV exposure and subsequently plated on to the BSYEM medium in order to get the survival count. The UV dose given to the samples was calculated as given below:

$$\begin{aligned}\text{Intensity} &= \text{Volt (mW)/ cm}^2 \\ \text{Voltage used} &= 30 \text{ W} \\ \text{Distance} &= 15 \text{ cm} \\ \text{UV dose} &= \text{Intensity} \times \text{time (seconds)}\end{aligned}$$

UV exposure time (min)	Calculated dose (mJoules/cm ²)
15	0.00012
30	0.00024
45	0.00036
60	0.00048
75	0.00064

3.2.2 Mutation with Ethidium Bromide (EtBr)

One ml of culture was withdrawn from a 24 h old culture of bacteria grown in 10 ml of basal salt yeast extract in order to determine the initial population in terms of colony forming units. EtBr was mixed well with culture and incubated at room temperature. One ml sample was removed for plating after 60 min of exposure. Different concentrations of EtBr (0.1-2.0 mg/ml) was evaluated for selecting a concentration that would result in 1 percent survival. This concentration was used for carrying out mutation at 30, 60, 90 and 120 min interval in order to optimize the time period that would results in 3log kill of cells (0.1-1.0% survival).

3.2.3 Screening of mutants

Survivors were screened on the basis of their capacity of enzyme production with respect to their parent strain. Some isolates showed decrease while others showed an increase in enzyme production after mutation. Among them N₁₂ (M) and Kd₁ (M) showed hypercellulase and xylanase production respectively and thus were selected finally for further enzyme production studies.

3.2.4 Hereditary stability studies of mutants

The selected mutants i.e. N₁₂ (M) and Kd₁ (M) were studied for their stability for cellulase and xylanase production consecutively for 8 generations. The mutants after every fermentation were inoculated on PYC and TGY media and used for inoculating next fermentation for respective enzyme assays.

3.3 MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF CELLULASE AND XYLANASE PRODUCING ISOLATES

3.3.1 Phenotypic Characterization

The Conventional morphological and biochemical tests as described by Aneja (2003) were performed with isolates as given below:

3.3.1.1 Morphological Test

Cell morphology, Gram reaction, shape and culture conditions of different isolates were studied.

3.3.1.2 Physiological and Biochemical test

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

3.3.2 Genotypic Identification

Screened bacterial isolates parent and mutant were identified at genomic level by using 16S r RNA gene technique as given below:

3.3.2.1 Isolation of Genomic DNA

Genomic DNA of N₁₂ (W), N₁₂ (M), Kd₁ (W) and Kd₁ (M) were isolated by using standard protocol of DNA prep kit (Bangalore Genei, India Pvt. Ltd. make). The bacterial strains N₁₂ (W), N₁₂ (M), Kd₁ (W) and Kd₁ (M) 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 3 h. 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 RNAase 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.

3.3.2.2 Agarose gel Electrophoresis

3.3.2.2.1 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

3.3.2.2.2 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	

3.3.2.2.3 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)

3.3.2.2.3 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

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

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

3.3.2.2.4 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)	:	5.0µl
dNTP 2mM	:	2.5 µl
Primer (F)	:	1.0 µl

Primer (R)	:	1.0 μ l
Taq Polymerase	:	0.2 μ l
Glycerol	:	0.5 μ l
Water	:	12.8 μ l
DNA	:	1 μ l
MgCl ₂	:	1 μ l

The universal primers used for amplification were 8F: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'

3.3.2.2.5 Agarose gel Electrophoresis

Same as section 3.3.2.2

3.3.2.2.6 PCR Clean up

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

1. The PCR product (100 μ l) was transferred 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 through 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 25 μ l 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 N₁₂ (W), N₁₂ (M), Kd₁ (W) and Kd₁ (M) were sequenced by commercially available services of (Xcelris, Ahmdabad, India). The sequences so obtained after sequencing were used to identify N₁₂ (W), N₁₂ (M), Kd₁ (W) and Kd₁ (M) by BLASTN bioinformatics tool.

3.3.2.2.7 Identification

On the basis of 16S r RNA gene technique, bacteria Kd₁ (W) and Kd₁ (M) and N₁₂ (W) and N₁₂ (M) were identified as *B. altitudinis* (W) and *B. altitudinis* (M), and *B. stratosphericus* (W) and *B. stratosphericus* (M) respectively. The sequences so obtained of all the four strains (W and M) were submitted in National Centre for Biotechnology Information (NCBI) and assigned accession number for parent and wild strains were *B. altitudinis* Kd₁ (W) - |KC995115|, *B. altitudinis* Kd₁ (M) - |KC995117|, *B. stratosphericus* N₁₂ (W) - |KC995116| and *B. stratosphericus* N₁₂ (M) - |KC995118| respectively.

3.3.2.2.8 Clustal W2 analysis

Translated nucleotide sequences were then analyzed for the confirmation of mutation by using tool (www.ebi.ac.uk/services).

3.4 OPTIMIZATION OF PROCESS PARAMETERS FOR CELLULASE AND XYLANASE ENZYMES

Cellulase and xylanase from selected mutants were optimized using conventional approach by varying different fermentation variables i.e. medium, incubation time, pH, temperature, inoculum size, different carbon source and concentration of carbon source were evaluated using classical approach of one factor at a time as given below:

3.4.1 For bacterial isolates under SmF

3.4.1.1 Extracellular cellulase production by *B. stratosphericus* N₁₂ (M)

Different growth parameters were optimized for extracellular cellulase production for *B. stratosphericus* N₁₂ (M)

3.4.1.1. A Effect of Media

3.4.1.1.A.1a Inoculum preparation

B. stratosphericus N₁₂ (M) was grown in 100 ml of nutrient broth and was incubated at 35±2°C for 24 h. As soon as the substantial growth was observed in the broth, the optical density was set to 1.0 using nutrient broth as blank.

3.4.1.1. A.1b Production of Cellulase

5 ml of 1.0 O.D inoculum was added to each 45 ml of each different medium (given below) in 250 ml of Erlenmeyer flasks and the flasks were incubated at $35\pm 2^{\circ}\text{C}$ under shaking conditions for 5 days. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C . The supernatant was collected and stored at 4°C in refrigerator for further use. The quantitative tests were performed with the supernatant.

3.4.1.1.A.1c Composition of Media Used

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

CMC - 10.0 g ; peptone - 5.0 g ; Yeast extract - 5.0 g ; K_2HPO_4 - 1.0 g ; $\text{Mg SO}_4\cdot 7\text{H}_2\text{O}$ - 0.2 g ; NaCl - 5.0 g ; Cellulose - 10.0 g , Distilled water -1000 ml, pH – 7.0.

2. **Basal Salt Medium** (Maniatis *et al.*, 1982)

$(\text{NH}_4)_2\text{SO}_4$ - 6.0 g ; KH_2PO_4 -3.0 g ; NaCl -0.5 g ; NH_4Cl -1.0 g ; Cellulose -10.0 g ; 1 M CaCl_2 - 2.0 ml ; and 1 M MgSO_4 - 0.01 ml added separately after autoclaving , Distilled water -1000 ml, pH-5.5

3. **Modified Basal Salt Medium** (Sharma *et al.*, 2005)

Na_2HPO_4 - 0.6 g ; KH_2PO_4 - 0.3 g ; NaCl - 0.05 g ; NH_4Cl - 0.1 g ; $\text{CO}(\text{NH}_2)_2$ - 10.0 g ; Yeast extract - 10.0 g ; Peptone - 0.1 g ; Cellulose -10.0 g ; 1 M CoCl_2 -0.02 ml ; 1M NaNO_3 - 0.02 ml ; 1 M CaCl_2 - 2.0 ml ; and 1 M MgSO_4 - 0.01 ml added separately after autoclaving , Distilled water -1000 ml pH-5.5

4. **Okoshi *et al.* Medium** (Okoshi *et al.*, 1990)

Peptone - 3.0 g ; KH_2PO_4 - 1.0 g ; $\text{Na}_2\text{HPO}_4\cdot 12\text{H}_2\text{O}$ -2.5 g ; $\text{Mg SO}_4\cdot 7\text{H}_2\text{O}$ - 0.2 g ; Yeast extract - 1.0 g ; Cellulose -10.0 g, Distilled water -1000 ml, pH - 6.8.

Li & Gao Medium (Li & Gao, 1997)

Mg SO₄.7H₂O - 0.02 g ; KNO₃ - 0.075 g ; K₂HPO₄ - 0.05 g ; FeSO₄.7H₂O - 0.002 g ; CaCl₂.2H₂O - 0.004 g ; Peptone - 0.2 g ; Cellulose - 10.0 g , Distilled water -1000 ml, pH-7.0

5. Mandel and Reese Media (Vyas & Vyas, 2005)

NaNO₃: 0.18g, KH₂PO₄: 0.2g, Urea: 0.03g, MgSO₄. 7H₂O: 0.03g, CaCl₂: 0.03g, Yeast Extract: 0.01g, FeSO₄. 7H₂O: 0.5mg, MnSO₄. H₂O: 0.2mg, CoCl₂: 0.2mg, ZnCl₂: 0.18mg, Cellulose: 1g, in 100 ml of distilled water, pH-6.8

3.4.1.1A.1d Enzyme Assays

3.4.1.1A.1d (i) CMCase Assay (Grajek, 1987) as given in section 3.1.5.1.1.1

3.4.1.1A.1d(ii) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.5.1.1.2

3.4.1.1A.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.5.1.1.3

3.4.1.1A.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.5.1.3

3.4.1.1A.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. PYC (Peptone Yeast Extract) medium was selected for further studies.

3.4.1.1.B Effect of Incubation Time

3.4.1.1. B.1a Inoculum Preparation

Same as given in 3.4.1.1A.1a

3.4.1.1. B.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of PYC in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different incubation time ranging from 24h, 48h.....144h 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.

3.4.1.1 B.1c Enzyme Assays

3.4.1.1B.1d (i) CMCase Assay (Grajek, 1987) as given in section 3.1.5.1.1.1

3.4.1.1B.1d (ii) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.5.1.1.2

3.4.1.1B.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.5.1.1.3

3.4.1.1B.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.5.1.3

3.4.1.1 B.1f Statistical Analysis

Same as given in 3.4.1.1A.1f

Best incubation time showing optimum cellulase production i.e. 48 h for *B. stratosphericus* N12 (M) was selected for further studies.

3.4.1.1. C Effect of Temperature

3.4.1.1. C. 1a Inoculum Preparation

Same as given in 3.4.1.1A.1a

3.4.1.1. C.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of PYC medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different temperatures ranging from 25, 30,...., 50°C at 120 rpm. After 48 h, 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.

3.4.1.1 C.1c Enzyme Assays

3.4.1.1C.1d (i) CMCase Assay (Grajek, 1987) as given in section 3.1.5.1.1.1

3.4.1.1C.1d (ii) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.5.1.1.2

3.4.1.1C.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.5.1.1.3

3.4.1.1C.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.5.1.3

3.4.1.1 C.1f Statistical Analysis

Same as given in 3.4.1.1A.1f

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

3.4.1.1 D Effect of pH

3.4.1.1.D.1a Inoculum Preparation

Same as given in 3.4.1.1 A.1a

3.4.1.1.D.1b Production of cellulase

5 ml of inoculum was added to 45 ml of PYC medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at 30°C with different pH ranging from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 at 120 rpm. After 48 h, 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.

3.4.1.1.D. 1c Enzyme assays

3.4.1.1D.1d (i) CMCase Assay (Grajek, 1987) as given in section 3.1.5.1.1.1

3.4.1.1D.1d (ii) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.5.1.1.2

3.4.1.1D.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.5.1.1.3

3.4.1.1D.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.5.1.3

3.4.1.1D.1.f Statistical Analysis

Same as given in 3.4.1.1A.1f

pH showing optimum cellulase production i.e.8.0 was selected for further studies.

3.4.1.1. E Effect of Different Inoculum Size

3.4.1.1. E.1a Inoculum Preparation

Same as given in 3.4.1.1A.1a

3.4.1.1. E.1b Production of Cellulase

PYC medium (pH-8.0) was taken in each of 250ml of Erlenmeyer flasks. The flasks were incubated at 30⁰C with different inoculum size ranging from 1.0%, 2.5%, 5.0 %....17.5 % at 120 rpm. After 48 h, 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.

3.4.1.1 E.1c Enzyme Assays

3.4.1.1E.1d (i) CMCase Assay (Grajek, 1987) as given in section 3.1.5.1.1.1

3.4.1.1A.1d (ii) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.5.1.1.2

3.4.1.1E.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.5.1.1.3

3.4.1.1E.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.5.1.3

3.4.1.1 E.1f Statistical Analysis

Same as given in 3.4.1.1A.1f

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

3.4.1.1. F Effect of Different Carbon Sources

3.4.1.1. F.1a Inoculum Preparation

Same as given in 3.4.1.1A.1a

3.4.1.1. F.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of PYC medium (pH-8.0) in each of 250ml of Erlenmeyer flasks. The flasks were incubated at 30⁰C with different substrates (hexoses) ranging from Cellulose, Mannose, Dextrose, Lactose, Maltose and Starch at 120 rpm. After 48 h, 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.

3.4.1.1. F.1c Enzyme Assays

3.4.1.1F.1d (i) CMCase Assay (Grajek, 1987) as given in section 3.1.5.1.1.1

3.4.1.1F.1d (ii) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.5.1.1.2

3.4.1.1F.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.5.1.1.3

3.4.1.1F.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.5.1.3

3.4.1.1. F.1f Statistical Analysis

Same as given in 3.4.1.1A.1f

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

3.4.1.1. G Effect of Different Concentrations of Carbon Source

3.4.1.1. G.1a Inoculum Preparation

Same as given in 3.4.1.1A.1a

3.4.1.1. G.1b Production of Cellulase

5 ml of inoculum was added to 45 ml of PYC medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different concentrations of

lactose ranging from 0.50%, 1.0%....3.0 % at 120 rpm. After 48 h, 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.

3.4.1.1. G.1c Enzyme Assays

3.4.1.1G.1d (i) CMCase Assay (Grajek, 1987) as given in section 3.1.5.1.1.1

3.4.1.1G.1d (ii) Filter Paperase (FPase) Activity (Grajek, 1987) as given in section 3.1.5.1.1.2

3.4.1.1G.1d (iii) β -Glucosidase Activity (Grajek, 1987) as given in section 3.1.5.1.1.3

3.4.1.1G.1e Protein Estimation (Lowry *et al.*, 1951) as mentioned in section 3.1.5.1.3

3.4.1.1. G.1f Statistical Analysis

Same as given in 3.4.1.1A.1f

Different concentrations of lactose for optimum cellulase production were used and optimum i.e. 2.0% was selected for further studies.

3.4.2.1 Extracellular xylanase production by *Bacillus altitudinis* Kd₁ (M)

Different growth parameters were optimized for extracellular xylanase production for *B. altitudinis* Kd₁ (M)

3.4.2.1.A Effect of Media

3.4.2.1.A.1a Inoculum Preparation

B. altitudinis Kd₁ (M) 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.

3.4.2.1.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.

3.4.2.1.A.1c Composition of Media

1. Bacillus Xylose Salt Medium

Yeast extract - 5.0 g ; NaCl - 1.0 g ; NH₄Cl - 1.0 g ; KH₂PO₄ - 0.5 g ; Mg SO₄.7H₂O - 0.5 g ; CaCl₂.2H₂O - 0.05 g ; Xylose - 10.0 g , Distilled water-1000 ml, pH-7.0

2. Xylan Medium (Annamalai *et al.*, 2009)

Yeast extract - 5.0 g ; Peptone - 10.0 g ; Mg SO₄.7H₂O - 0.2 g ; NaCl - 3.0 g ; K₂HPO₄ - 1.0 g ; NaCO₃ - 10.0 g ; Xylose - 10 g, Distilled water-1000 ml, pH-7.1

3. Basal Salt Medium (Maniatis *et al.*, 1982)

(NH₄)SO₄ - 6.0 g ; KH₂PO₄ - 3.0 g ; NaCl - 0.5 g ; NH₄Cl - 1.0 g ; Xylose - 10.0 g ; 1 M CaCl₂ - 2.0 ml ; and 1 M MgSO₄ - 0.01 ml added separately after autoclaving, Distilled water-1000 ml, pH-6.8

4. TGY Medium (Garg *et al.*, 2009)

Yeast extract - 5.0 g ; Tryptone - 5.0 g ; K₂HPO₄ - 1.0 g ; Xylose - 10.0 g , Distilled water-1000 ml, pH-7.0

5. Emmerson Medium (Garg *et al.*, 2009)

Yeast extract - 5.5 g ; Peptone - 5.0 g ; Mg SO₄.7H₂O - 0.02 g ; K₂HPO₄ - 0.1 g ; Xylose - 10 g, Distilled water-1000 ml

6. X. Heck media (Heck and Hertz, 2002)

MgSO₄-0.2 g, KH₂PO₄- 1.0g, K₂HPO₄-1.0g, NH₄NO₃-1.0g, CaCl₂-0.02 g, FeCl₂-0.05g, Distilled water-1000 ml.

3.4.2.1.A.1d Enzyme assay

3.4.2.1.A.1d (i) Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.4.2.1.A.1e Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.4.2.1.A.1f Statistical analysis

Same as given in 3.4.1.1A.1f

Best medium showing optimum xylanase production i.e. TGY medium was selected for further studies.

3.4.2.1.B.1 Effect of Incubation Time

3.4.2.1.B. 1a Inoculum Preparation

Same as given in section 3.4.2.1.A.1a

3.4.2.1.B. 1b Production of Xylanase

5 ml of inoculum was added to 45 ml of TGY in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different incubation time ranging from 24h, 48h.....144 h 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.

3.4.2.1.B.1c Enzyme assay

3.4.2.1.B.1c (i) Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.4.2.1.B.1d Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.4.2.1.B.1e Statistical analysis

Same as given in 3.4.1.1A.1f

Best incubation time showing optimum xylanase production i.e. 48h for *B. altitudinis* Kd1 (M) was selected for further studies.

3.4.2.1.C Effect of Temperature

3.4.2.1.C.1a Inoculum Preparation

Same as given in section 3.4.2.1.A.1a

3.4.2.1.C.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of TGY medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different temperatures ranging from 25, 30, ..., 50°C at 120 rpm. After 48 h, 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.

3.4.2.1.C.1c Enzyme assay

3.4.2.1.C.1c (i) Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.4.2.1.C.1d Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.4.2.1.C.1e Statistical analysis

Same as given in 3.4.1.1A.1f

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

3.4.2.1.D Effect of pH

3.4.2.1.D.1a Inoculum Preparation

Same as given in section 3.4.2.1.A.1a

3.4.2.1.D.1b Production of Xylanase

5 ml of inoculum was added to 45 ml of TGY medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at 30°C with different pH ranging from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 at 120 rpm. After 48h, 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.

3.4.2.1.D.1c Enzyme assay

3.4.2.1.D.1c (i) Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.4.2.1.D.1d Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.4.2.1.D.1e Statistical analysis

Same as given in 3.4.1.1A.1f

pH showing optimum xylanase production i.e. 5.5 was selected for further studies.

3.4.2.1.E Effect of Different Inoculum Size

3.4.2.1.E.1a Inoculum Preparation

Same as given in section 3.4.2.1.A.1a

3.4.2.1.E.1b Production of Xylanase

TGY medium (pH 5.5) was taken in each of 250ml of Erlenmeyer flasks and inoculated with different inoculum size ranging from 2.5%, 5.0 %....17.5 %. Flasks were incubated at 30⁰C at 120 rpm. After 48h, 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.

3.4.2.1.E.1c Enzyme assay

3.4.2.1.E.1c (i) Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.4.2.1.E.1d Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.4.2.1.E.1e Statistical analysis

Same as given in 3.4.1.1A.1f

Inoculum size showing optimum xylanase production i.e. 12.5 % was selected for further studies

3.4.2.1.F Effect of Different Carbon Sources

3.4.2.1. F.1a Inoculum Preparation

Same as given in section 3.4.2.1.A.1a

3.4.2.1.F.1b Production of Xylanase

6.25 ml of inoculum was added to 43.75 ml of TGY of pH 5.5 in each of 250ml of Erlenmeyer flasks. The flasks were incubated at 30⁰C with different substrates that are xylan, xylose, arabinose, maltose, dextrose and D- ribose at 120 rpm. After 48 h, 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.

3.4.2.1.F.1c Enzyme assay

3.4.2.1.F.1c (i) Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.4.2.1.F.1d Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.4.2.1.F.1e Statistical analysis

Same as given in 3.4.1.1A.1f

The best carbon source i.e. xylose showing optimum xylanase production was selected for further studies.

3.4.2.1. G Effect of Different Carbon Concentrations

3.4.2.1. G.1a Inoculum Preparation

Same as given in section 3.4.2.1.A.1a

3.4.2.1.G.1b Production of Xylanase

6.25 ml of inoculum was added to 43.75 ml of TGY (pH-5.5) in each of 250ml of Erlenmeyer flasks. The flasks were incubated at 30⁰C with different concentrations of xylose ranging from 0.5%, 1.0%, 1.5%....3.0 % at 120 rpm. After 48 h, 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.

3.4.2.1.G.1c Enzyme assay

3.4.2.1.G.1c (i) Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.4.2.1.G.1d Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.4.2.1.G.1e Statistical analysis

Same as given in 3.4.1.1A.1f

2.5% xylose concentration showing optimum xylanase production was selected for further studies.

3.4.2.2 Percent (%) increase in enzyme activity after optimization of different parameters

$$\% \text{ increase in enzyme activity} = \frac{\text{Final enzyme activity} - \text{Initial enzyme activity}}{\text{Initial enzyme activity}} \times 100$$

3.5 COST EFFECTIVE PRODUCTION OF CELLULASE AND XYLANASE BY USING INEXPENSIVE LIGNOCELLULOSIC WASTE AS SUBSTRATE

Keeping in view the highly expensive market cost of xylan and cellulose, an attempt had been made to replace these conventional carbon sources with cheap lignocellulosic substrates as given below:

3.5.1 Selection of lignocellulosic forest waste as substrate

Lignocellulosic biomass holds remarkable potential for conversion into commodity products presenting dual advantage of sustainable resource supply and environmental quality. Lignocellulosic materials are renewable, low cost and are abundantly available. The following lignocellulosic forest wastes were selected for enzyme production studies depending upon their wide abundance and easy availability in local forests/adjoining areas.

Sr. No.	Botanical name	Vernacular name
1.	<i>Eucalyptus sp.</i>	Eucalyptus
2.	<i>Populus deltoides</i>	Poplar
3.	<i>Dandracalamus strictus</i>	Bamboo
4.	<i>Cedrus deodara</i>	Devdar
5.	<i>Pinus roxburghii</i>	Chir
6.	<i>Pinus wallichiana</i>	Kail

3.5.2 Chipping of biomass

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. The completely dried biomass was stored in air tight containers for further use.

3.5.3. Pretreatments of Lignocellulosic Biomass

3.5.3.1 Acid (2.5 %HCl) pretreatment

200 g of each selected biomass was dipped in 2.5% HCl. After 4 h, it was thoroughly washed with tap water until it becomes neutral. After washing it was dried at 60°C overnight and stored in air tight containers.

3.5.3.2 Alkali (2.0 % NaOH +H₂O₂) pretreatment

200 g of each selected biomass was dipped in 2.0% NaOH + H₂O₂ in 9:1. After 4 h, it was thoroughly washed with tap water until it becomes neutral. After washing it was dried at 60°C overnight and kept in air tight containers.

3.5.3.3 Acid (2.5% H₂SO₄) pretreatment

200 g of each selected biomass was dipped 2.5% H₂SO₄. After 4 h it was thoroughly washed with tap water until it becomes neutral. After washing it was dried at 60°C overnight and kept in air tight containers.

3.5.3.4 5.0% Ammonia (NH₃)

200 g of each selected biomass was dipped 5% NH₃. After 4 h it was thoroughly washed with tap water until it becomes neutral. After washing it was dried at 60°C overnight and kept in air tight containers.

3.5.3.5 Steam

200 g of each selected biomass was moistened with distilled water and followed by 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.

3.5.4 Analytical studies of different components of lignocellulosic biomass by standard methods of technical association of pulp and paper industry (TAPPI)

3.5.4.1 Alcohol-Benzene Extractives (T 6m-59- Anonymous, 1974)

2 g of oven dried wood meal placed in a porous thimble. The thimble was then placed in soxhlets apparatus and extracted with alcohol: benzene mixture in ratio of 1:2 for 6 h. The porous thimble was then taken out and allowed to dry in open air and finally in oven at 50⁰C till constant weight. The solubility was then determined by calculating the loss in weight of the sample and expressed in percentage.

3.5.4.2 Lignin Extraction (T12 M-59-Anonymous, 1988)

2 g of oven dried wood meal preextracted with alcohol benzene was treated with 15 ml of 72% sulphuric acid for 2 h at 18-20⁰C with constant stirring. The material was then transferred to 1 litre flask and the acid concentration was brought down to 3% by adding 545 ml of distilled water. The solution was refluxed for 4 h and then allowed to settle. The contents were then filtered, washed with hot water and dried in an oven at 50⁰C till constant weight. The lignin content was expressed in percentage.

3.5.4.3 Holocellulose extraction (T 9m-54-Anonymous, 1993)

4g of oven dried wood meal pre-extracted with alcohol-benzene (explained in section 3.7.1) was taken in a conical flask of 250 ml and 160 ml of distilled water was added. The contents were then treated with 15 g of sodium chlorite (NaClO₂) and 10 drops of acetic acid at 70-80⁰C on a water bath for 1 h. The process was repeated 4 times till wood sample becomes white. The contents were then filtered, washed with water and finally with acetone. The sample was then dried at 50⁰C till constant weight. The holocellulose content was expressed in percentage.

3.5.5 Production of cellulase and xylanase using lignocellulosic biomass as carbon source

3.5.5.1 Production of cellulase and xylanase by bacteria under SmF

3.5.5.1.1 Cellulase production by *B. stratosphericus* N₁₂ (M)

3.5.5.1.1a Inoculum preparation

100 ml nutrient broth was seeded with 10% *B. stratosphericus* N₁₂ (M) (O.D. 1.0) culture in 250 ml Erlenmeyer flasks and was kept at 30 ±2°C at 120 rpm for 24 h.

3.5.5.1.1b Production of cellulase using lignocellulosic forest wastes

To 5 g of each untreated and pretreated biomass, 100 ml of PYC medium was added in 250 ml Erlenmeyer flask and was autoclaved. After autoclaving, the flasks were inoculated with 10% of inoculum and incubated at kept 30±2°C for 3 days.

3.5.5.1.1c Enzyme assays

3.5.5.1.1c(i) CMCase assay (Reese & Mandel, 1963) as given in section 3.1.5.1.1.1

3.5.5.1.1c (ii) Filter Paperase (FPase) Activity (Reese & Mandel, 1963) as mentioned in section 3.1.5.1.1.2

3.5.5.1.1c (iii) β-Glucosidase Activity (Berghem and Petterson, 1973) as given in section 3.1.5.1.1.3

3.5.5.1.1d Protein estimation (Lowry *et al.*, 1951) as given in section 3.1.5.1.3

3.5.5.1.1e Statistical Analysis

Same as given in 3.4.1.1A.1f

3.5.5.1.2 Xylanase production by *B. altitudinis* Kd₁ (M)

3.5.5.1.2.1a Inoculum preparation

100 ml nutrient broth was seeded with 12.5% *B. altitudinis* Kd₁ (M) (O.D. 1.0) culture in 250 ml Erlenmeyer flasks and was kept at 30±2°C at 120 rpm for 24 h.

3.5.5.1.2.1b Production of xylanase using lignocellulosic forest wastes

To 5 g of each untreated and pretreated biomass, 100 ml of TGY was added in 250 ml Erlenmeyer flask and was autoclaved. After autoclaving, the flasks were inoculated with 12.5% of inoculum and incubated at kept 30±2°C for 3 days.

3.5.5.1.2.1c Xylanase activity (Miller, 1959) as given in section 3.1.5.1.2

3.5.5.1.2.1d Protein estimation (Lowry et al., 1951) as mentioned in section 3.1.5.1.3

3.5.5.2 Production of cellulase and xylanase by fungi under SSF

Myceliophthora thermophila SH1 [Accession no. JX124712], a thermophilic fungus used as a source of cellulase and xylanase in the present study. It had been isolated from hot springs of northern Himalayas region of Himachal Pradesh and maintained on Malt extract agar slants at 4°C in a refrigerator (Pathania, 2011) .

3.5.5.2.1 Cellulase and xylanase production by *M. thermophila* SH1

To 5 g of each untreated and pretreated biomass, 10 ml of moistening agent viz. Vogel's medium was added (in the ratio of 1:6 i.e., substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 2 ml of *Myceliophthora thermophila* SH1 having 1×10^7 spores/ml and incubated at 50°C for 8 days in static phase.

3.5.5.2.2 Extraction of cellulase and xylanase enzyme by Repeated Extraction method (Bollag & Edelstein, 1993)

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.

3.5.5.2.2.1 Enzyme assays

3.5.5.2.2.1a Cellulase Assay

3.5.5.2.2.1a (i) CMCase activity (Reese & Mandel, 1963)

As given in section 3.1.5.1.1.

3.5.5.2.2.1a (ii) Filter Paperase (FPase) Activity (Reese & Mandel, 1963)

As mentioned in section 3.1.5.1.1.2

3.5.5.2.2.1a (iii) β -Glucosidase Activity (Berghem and Petterson, 1973)

As mentioned in section 3.1.5.1.1.3

3.5.5.2.2.1b Protein estimation (Lowry *et al.*, 1951)

As mentioned in section 3.1.5.1.3

3.5.5.2.2.1c Statistical analysis

As mentioned in section 3.4.1.1A.1f

3.5.4.2.2.1d Xylanase Assay (Miller, 1959)

As mentioned in the section 3.1.5.1.2

3.6 PARTIAL PURIFICATION OF CELLULASE AND XYLANASE FROM POTENTIAL MICROORGANISMS

3.6.1 Partial Purification of Cellulase

3.6.1.1 Partial Purification of cellulase of *B. stratosphericus* N₁₂ (M)

3.6.1.1.1 Inoculum Preparation and Production of Cellulase

The culture of *B. stratosphericus* N₁₂ (M) having O.D. 1.0 was separately inoculated in PYC medium broth (pH-8.0) @ 10%. Inoculated flasks were kept at $30 \pm 2^\circ\text{C}$ at 120 rpm for 48 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.

3.6.1.1.2 Partial Purification by Ammonium sulfate precipitation

Different concentrations of ammonium sulfate i.e 0-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90% were evaluated to attain saturation point for each of cellulase subunits i.e CMCase, FPase and β -glucosidase. The preparations were kept at 4°C for overnight and then centrifuged which resulted in separation of pellets and supernatants. CMCase and FPase were precipitated at 30-60% and β -glucosidase at 0-30% level of saturation of ammonium sulfate. Precipitates of each subunit so obtained were dissolved in phosphate buffer (0.1 M, pH 6.9) separately and were refrigerated until further use.

3.6.1.1.3 Dialysis

Dissolved precipitates of CMCase, FPase and β -glucosidase were dialyzed against same buffer overnight at 4°C using dialysis membrane of cut-off 14 kDa.

3.6.1.2 Partial Purification of Cellulase of *Myceliophthora thermophila* SH1

3.6.1.2.1 Production of cellulase

5 g of NaOH+H₂O₂ pretreated, *Populus deltoides* wood moistened with 20ml of Vogel's media was taken and autoclaved. Added 10% of seven days old *M. thermophila* SH1 culture scratched with 10 ml of autoclaved distilled water and fixing their inoculum size 1×10⁷ spores/ml and kept for 8 days at 50°C. The crude enzyme (200ml) was harvested by Repeated Extraction Method, centrifuged and stored at 4°C.

3.6.1.2.2 Partial Purification by Ammonium sulfate precipitation

Same as mentioned in section 3.6.1.1.2

3.6.1.2.3 Dialysis

Same as in section 3.6.1.1.3

3.6.2 Partial purification of Xylanase

3.6.2.1 Partial Purification of Xylanase of *B. altitudinis* Kd₁ (M)

3.6.2.1.1 Inoculum Preparation and Production of Cellulase

The culture of *B. altitudinis* Kd₁ (M) having O.D. 1.0 was separately inoculated in TGY medium broth (pH-5.5) @ 12.5%. Inoculated flasks were kept at 30 ± 2°C at 120 rpm for 48 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.

3.6.2.1.2 Partial Purification by Ammonium sulfate precipitation

Different concentrations of ammonium sulfate i.e 0-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90% were evaluated for locating saturation point of enzyme xylanase with constant stirring. The

preparations were kept at 4°C for overnight and then centrifuged which resulted in separation of pellets and supernatants. Xylanase was precipitated at 0-70% level of saturation of ammonium sulfate respectively. Precipitates so obtained were dissolved separately in phosphate buffer (0.1 M, pH 6.9 and were refrigerated until further use

3.6.2.1.3 Dialysis

Same as in section 3.6.1.1.3

3.6.2.2 Partial Purification of Xylanase of *Myceliophthora thermophila* SH1

3.6.2.2.1 Production of Xylanase by *Myceliophthora thermophila* SH1

5 g of NaOH+H₂O₂ pretreated, *Populus deltoides* wood, moistened with 20ml of Vogel's media was taken and autoclaved. Added 10% of seven days old *M. thermophila* SH1 culture scratched with 10 ml of autoclaved distilled water and fixing their inoculum size 1×10^7 spores/ml and kept for 8 days at 50°C. The crude enzyme (200ml) was harvested by Repeated Extraction Method, centrifuged and stored at 4°C.

3.6.4.2 Partial Purification of Xylanase by Ammonium sulfate precipitation

Same as mentioned in section 3.6.2.1.2

3.6.4.3 Dialysis

Same as in section 3.6.1.1.3

3.7 SCALE UP OF CELLULASE AND XYLANASE PRODUCTION IN A STIRRED TANK BIOREACTOR

3.7.1 Cellulase production by *B. stratosphericus* N₁₂ (M)

3.7.1.1 Inoculum Preparation

B. stratosphericus N₁₂ (M) was grown in 300 ml of nutrient broth and incubated at 30±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.

3.7.1.2 Batch Fermentation

Batch cultivation for the production of cellulase 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 35°C and pH 8.0. Carbon and nitrogen sources used for batch fermentations were 1.0% *P. deltooides* (biomass), K₂HPO₄ – 1.0 g/L, NaCl – 5.0 g/L, peptone – 5.0 g/L, yeast extract – 5.0 g/L, MgSO₄·7H₂O – 0.2 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 bioreactor was inoculated with 10% inoculum under aseptic conditions. The agitation speed 200 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4.....10 h

3.7.1.3 Cellulase assay

3.7.1.3.a CMCase assay (Reese & Mandel, 1963) as given in section 3.1.5.1.1.1

3.7.1.3a (ii) Filter Paperase (FPase) Activity (Reese & Mandel, 1963) as mentioned in section 3.1.5.1.1.2

3.7.1.3a (iii) β -Glucosidase Activity (Berghem and Petterson, 1973) as given in section 3.1.5.1.1.3

3.7.1.3b Protein estimation (Lowry *et al.*, 1951) as given in section 3.1.5.1.3

3.7.2 Xylanase production by *B. altitudinis* Kd₁ (M)

3.7.2.1a Inoculum Preparation

B. altitudinis Kd₁ (M) was grown in 300 ml of nutrient broth and incubated at 30±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.

3.7.2.1b Batch Fermentation

Batch cultivation for the production of cellulase 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 30± 2°C and pH 5.5. Carbon and nitrogen sources; for batch

fermentations were 1.0% *P. deltoides* wood, tryptone - 5.0 g/L, yeast extract – 5.0g/L, K₂HPO₄ – 1.0g/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 bioreactor was inoculated with 10% inoculum under aseptic conditions. The agitation speed 200 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4.....10 h

3.7.2.1c Xylanase Assay (Miller, 1959)

As mentioned in the section 3.1.5.1.2

3.7.2.1d Protein estimation (Lowry *et al.*, 1951) as given in section 3.1.5.1.3

3.8 BIOCONVERSION OF LIGNOCELLULOSIC FOREST WASTE INTO ETHANOL

Bioethanol production from forest waste was carried out by opting for different processes of fermentation i.e. separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF) as given below:

3.8.1 Substrate: *Populus deltoides* wood, chipped, grinded and alkali pretreated as mentioned in section 3.5.3.2

3.8.2 Source of hydrolytic enzymes

Partially purified cellulase and xylanase of potential microorganisms as prepared under section 3.6.1 and 3.6.2 were used for hydrolysis by mixing them as given below:

3.8.2.1 Inhouse enzyme cocktail

The inhouse enzymes which were prepared in section 3.6.1 and 3.6.2 had been mixed in the ratio of (2:2:1) i.e 2 ml of cellulase from *M. thermophila* SH1 (CMCase: 1.752 IU, FPase: 1.193 IU and β -glucosidase: 2.571 IU) + 2 ml of cellulase from *B. stratosphericus* N₁₂(M) (CMCase: 2.384 IU, FPase: 2.249 IU and β -glucosidase: 2.451 IU) and 0.5 ml of xylanase from *B. altitudinis* Kd₁ (M)

(71.49 IU) + 0.5 ml of xylanase from *M. thermophila* SH1 (12.59 IU) and enzymatic dose was adjusted @ 1ml/g of biomass for hydrolysis.

3.8.2.2 Commercial enzymes as control

Commercial enzymes i.e. celluclast 1.5 L (Cellulase from *Trichoderma reesei*, C2730, Sigma Aldrich), Novozyme 188 (Cellobiase from *Aspergillus niger*, C6105, Sigma Aldrich) and Pantopan Mono BG (Xylanase from *Thermomyces lanuginosus*, X2753, Sigma Aldrich) were used as control to compare the results of hydrolysis of biomass with inhouse enzyme preparation. Commercial enzymes were mixed in the ratio of 1:1:1 i.e. 1ml celluclast 1.5L (CMCase: 29.75 IU, FPase: 29.75 IU), 1ml Novozyme 188 (β -glucosidase: 20.51 IU) + 1ml Pantopan Mono BG (xylanase: 22.64 IU) and used @ 1 ml/g of biomass.

3.8.3.1 Separate hydrolysis and fermentation (SHF)

SHF was carried out under four different strategies in order to ascertain the effect of pretreatment and inhibitors on final ethanol concentration.

3.8.3.1.1 Hydrolysis

5 g of untreated *P. deltoides* wood biomass was taken in 250 ml of flask. To this 50 ml of 2% NaOH +H₂O₂ (9:1) was added and kept at 65⁰C in water bath for 3 h. After 3 h supernatant was separated out from solid biomass by centrifugation at 10,000 rpm for 20 min and reducing sugars were estimated in the supernatant (Miller, 1959). Enzymatic saccharification of the alkaline peroxide pretreated left over solid biomass was done by adding hydrolytic enzymes at 1ml/g of residue. After 72 h saccharified biomass was centrifuged at 10,000 rpm for 20 min and clear hydrolysate was collected. The wood hydrolysate so prepared was used for fermentation in four different ways as given below.

Method 1: The saccharified syrup was pooled together with supernatant obtained after alkaline hydrogen peroxide pretreatment making the total volume to 100 ml. Reducing sugars so obtained were analysed (Miller, 1959).

Method 2: The saccharified syrup was pooled together with supernatant of alkali pretreated wood, followed by detoxification by overliming with Ca(OH)_2 . Reducing sugars so obtained were estimated (Miller, 1959).

Method 3: The supernatant from alkaline hydrogen peroxide pretreatment was decanted off, only enzymatic wood hydrolysate was used for fermentation without detoxification. Reducing sugars were assayed. (Miller, 1959).

Method 4: The supernatant from alkaline hydrogen peroxide pretreatment was decanted off and detoxified enzymatic wood hydrolysate was used for fermentation.

3.8.4.1.2 Detoxification by overliming with Ca (OH)_2

Calcium hydroxide was added to the alkaline peroxide pretreated and enzyme saccharified hydrolyzate (50°C, pH-5.0, 72 h) to increase the pH to 10.5. At high pH inhibitors were precipitated out with calcium hydroxide. The whole mixture was stirred for 30 min at 90°C, allowed to cool slowly to room temperature and then adjusted back to pH 6.0 with HCl. It was then centrifuged (15,000g ×, 30 min) to remove precipitate formed before using as substrate for fermentation. After removing the precipitates sugar estimation was done (Miller, 1959).

3.8.4.1.3 Fermentation

3.8.4.1.3.1 Ethanologens used:

Fermenting microbes i.e. *Saccharomyces cerevisiae* I (MTCC-3089) procured from MTCC Chandigarh -India, *Saccharomyces cerevisiae* II (DSM-1334) procured from DSM- Germany, *Pichia stipitis* (NCIM 3498), *Candida shehatae* (NCIM 3500) and *Zymomonas mobilis* (NCIM 5134) were procured from NCIM- Pune, India were used for fermentation in the experiments.

Growth: The inoculum of above mentioned microorganisms was prepared by growing cells aerobically in 250 ml flask containing 100 ml of the growth medium as mentioned below in a rotary shaker incubator for 24 h at 25±2°C to make 1.O.D. culture.

3.8.4.1.3.2 Growth Media:

3.8.4.1.3.2.1 For *Saccharomyces cerevisiae* (MTCC 3089 and DSM 1334)

Yeast extract	- 3.0g,
Peptone	- 10.0g,
Dextrose	- 20.0g,
Distilled water	- 1 liter.

3.8.4.1.3.2.2 For *Pichia stipitis* (NCIM 3498) and *Candida shehatae* (NCIM 3500)

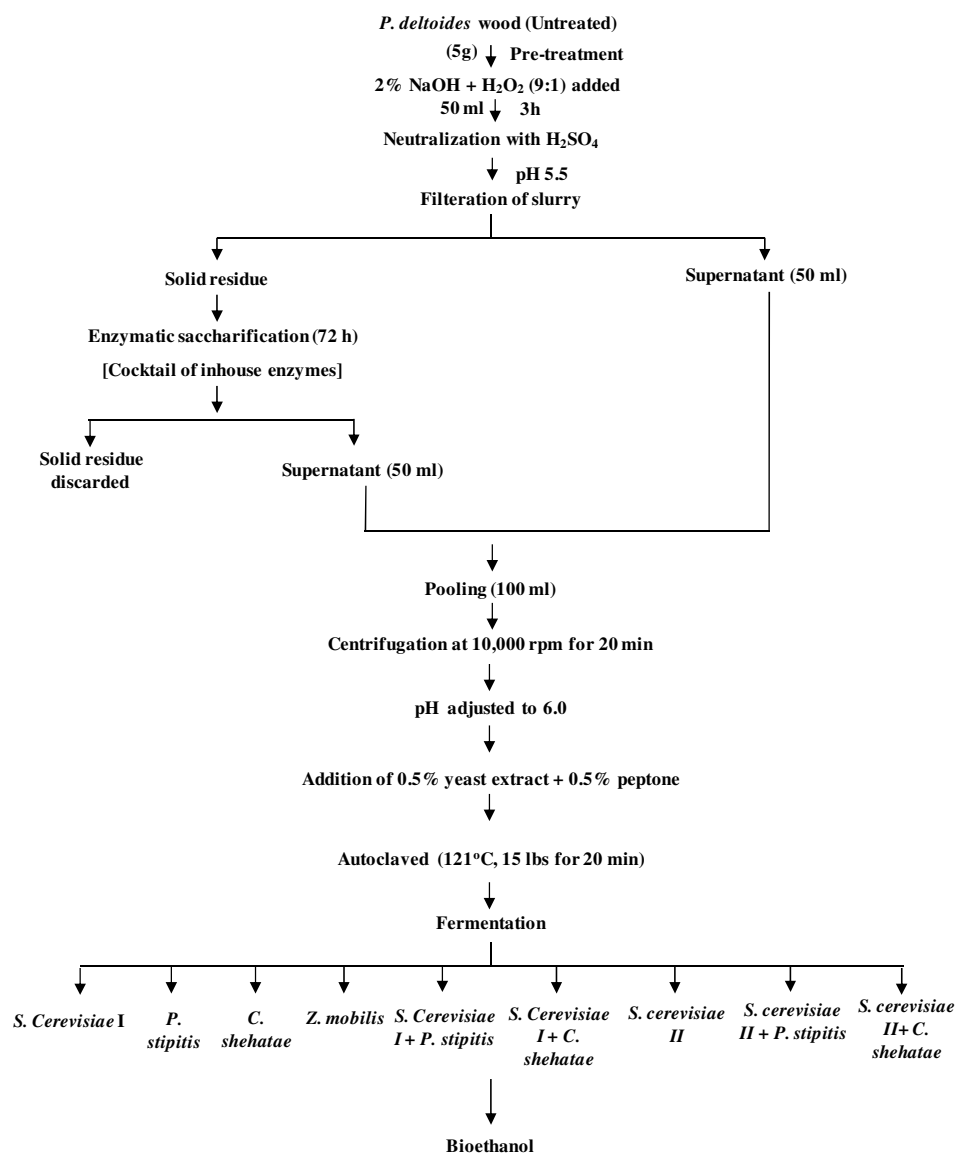
Malt extract	- 0.3g
Glucose	- 1.0g
Yeast extract	- 0.3g
Peptone	- 0.5g
Distilled water	- 1 liter

3.8.4.1.3.2.3 For *Zymomonas mobilis* (NCIM 5134)

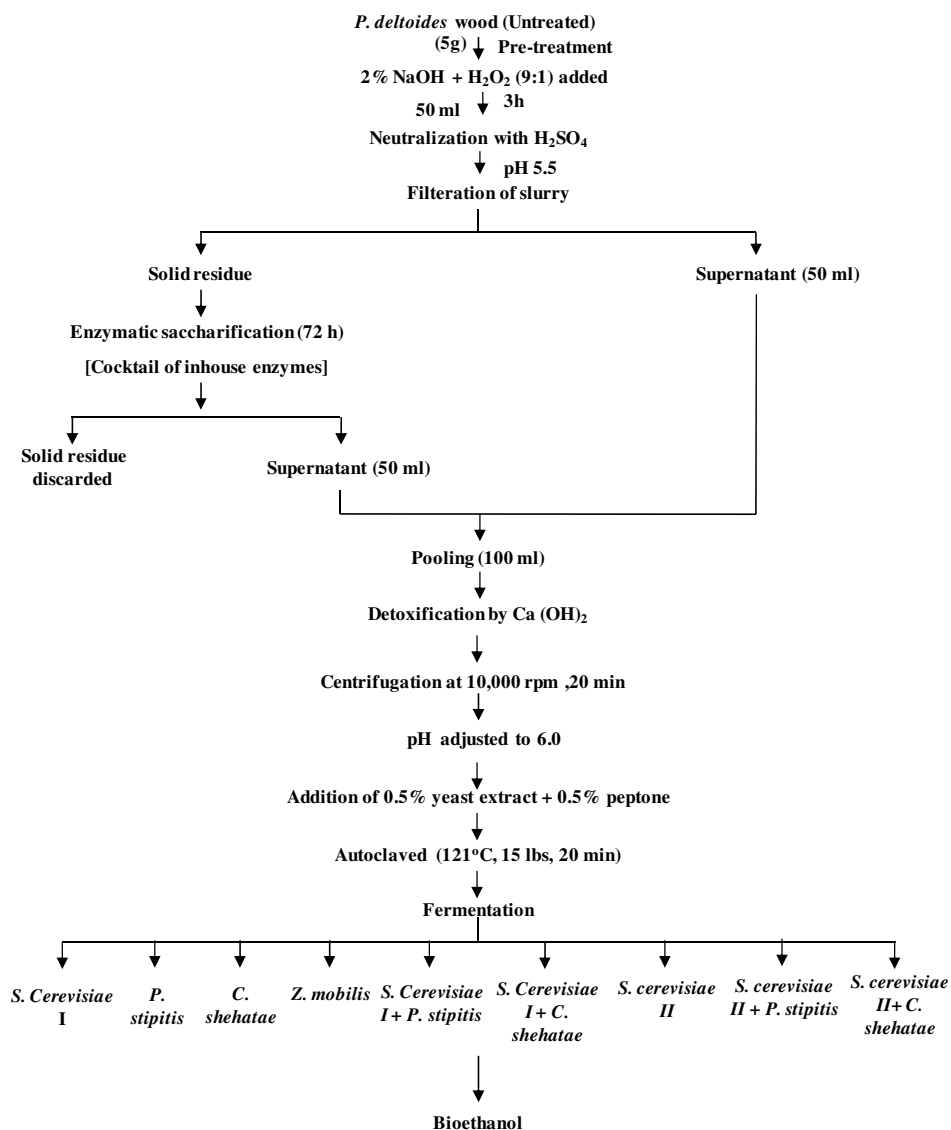
Beef extract	- 10.0g
NaCl	- 5.0g
Peptone	- 10g
Glucose	- 2%
Distilled water	- 1 liter

3.8.4.1.3.3 Ethanol Fermentation

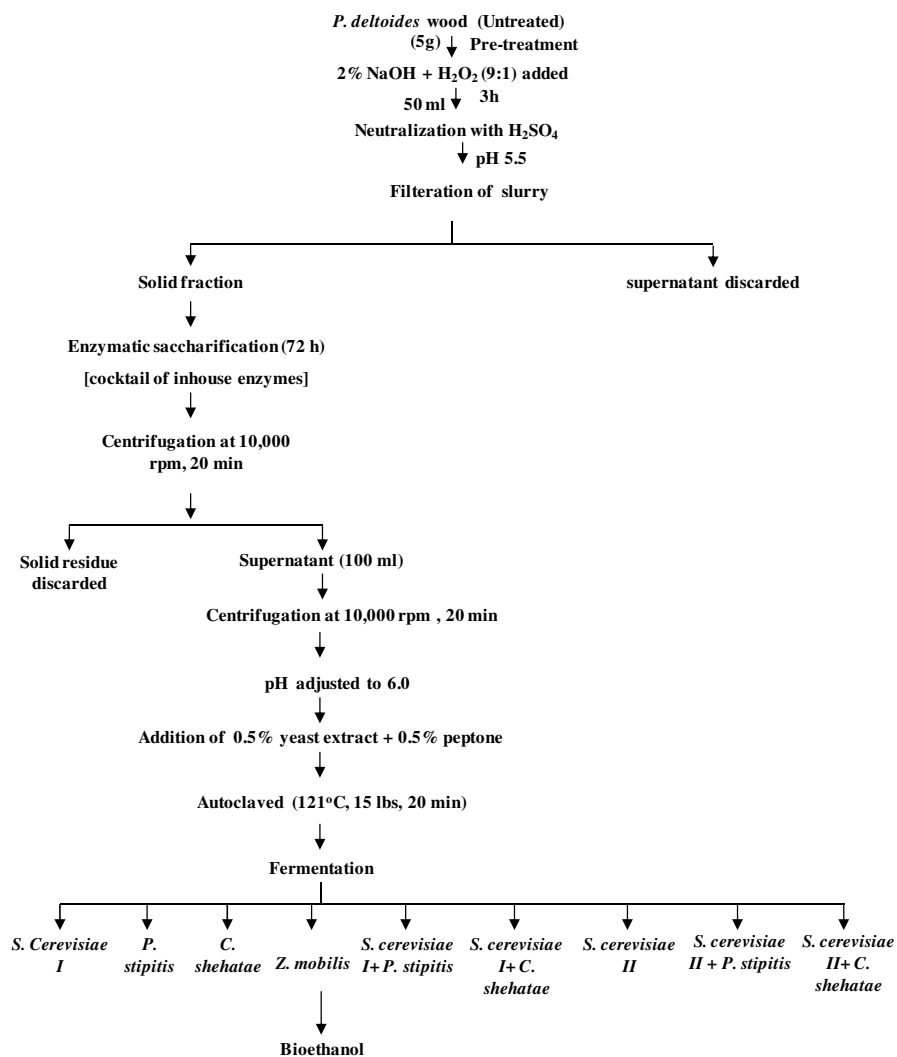
To the calcium hydroxide treated (detoxified) and non-detoxified (in second case) supernatant 0.5 % yeast extract and 0.5% peptone was added and autoclaved at 121⁰C, 15 lbs for 20 min. To the fermentation media 10 % (10.D) inoculum *Saccharomyces cerevisiae*-I, *Saccharomyces cerevisiae*-II , *Pichia stipitis* , *Candida shehatae*, *Zymomonas mobilis* , *S. cerevisiae*-I + *P. stipitis* , *S.*



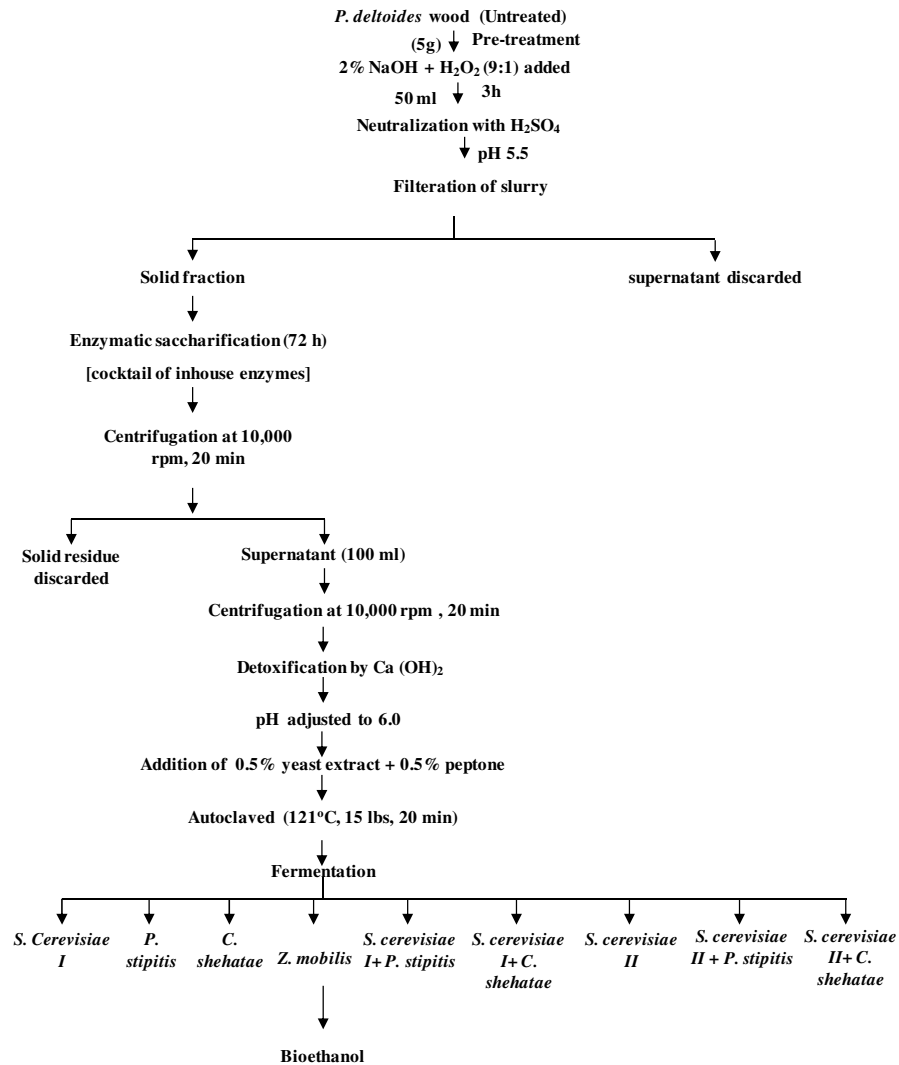
Scheme of separate hydrolysis and fermentation for bioethanol production from pretreated *P. deltooides* wood: Method-I



Scheme of separate hydrolysis and fermentation for bioethanol production from pretreated *P. deltooides* wood: Method-II



Scheme of separate hydrolysis and fermentation for bioethanol production from pretreated *P. deltooides* wood: Method -III



Scheme of separate hydrolysis and fermentation for bioethanol production from pretreated *P. deltooides* wood: Method-IV

cerevisiae-I + C. shehatae, *S. cerevisiae-II + P. stipitis* and *S. cerevisiae-II + C. shehatae* was added and kept for 72 h (3 days) at 25°C.

3.8.4.1.3.4 Ethanol estimation

34.0 g of potassium dichromate was dissolved in 500ml of distilled water. To this 375 ml of concentrated sulphuric acid was added, mixed thoroughly and allowed to cool. Final volume was made 1000ml by adding distilled water. To the distillation flask 29 ml of distilled water and 1 ml of sample was added. On the other side to the 50 ml volumetric flask 25 ml of potassium dichromate was added. Distillation was set at 60°C and tap water was turned ON. To the 25 ml of potassium dichromate, 20 ml of distilled sample was collected and it became total 45 ml. To this 45 ml solution 5 ml of distilled water was added and total volume became 50 ml and was incubated at 60°C for 20 min. After that O.D. was measure at 600 nm against blank (Caputi *et al.*, 1969).

Bioethanol was estimated in terms of g/l of fermented liquor and g/g of biomass on dry weight basis. Fermentation efficiency was calculated using the following formula:

$$\text{Fermentation efficiency} = \frac{\text{ethanol produced (g/g)}}{\text{theoretical yield of ethanol}} \times 100$$

Theoretical yield was referred as standard value of 0.511 g/g of sugars.

3.8.5 Simultaneous Saccharification and Fermentation (SSF)

3.8.5.1 Substrate: *P. deltooides* wood chipped, grinded and alkaline hydrogen peroxide pretreated as mentioned in section 3.5.3.2

3.8.5.2 Source of hydrolytic enzymes:

Inhouse enzyme cocktail and commercial enzymes as control used for hydrolysis were the same as mentioned in section 3.3.2

3.8.5.2.3 Process:

5 g of untreated *P. deltooides* wood was taken in each of 9 sets of 250 ml flask. To these flasks 100 ml of 2% NaOH + H₂O₂ (9:1) was added separately and

kept at 65°C in water bath for 3 h. The pH was maintained 6.0, to this 0.5% yeast extract and 0.5% peptone was added, autoclaved at 121°C, 15 lbs or 20 min. To the cooled autoclaved slurry in each of flask, hydrolytic enzymes @ 5mg/5mg were added, simultaneously with fermenting microorganisms (24 h old, 1.0 O.D.) i.e. *Saccharomyces cerevisiae-I*, *Saccharomyces cerevisiae-II*, *Pichia stipitis*, *Candida shehatae*, *Zymomonas mobilis*, *S. cerevisiae-I* + *P. stipitis* , *S. cerevisiae-I* + *C. shehatae* , *S. cerevisiae-II* + *P. stipitis* and *S. cerevisiae-II* + *C. shehatae* and these flasks were kept for fermentation at 32°C for 72 h.

3.8.5.2.4 Ethanol Estimation

Same as section 3.8.4.1.3.4

3.8.6 Simultaneous Saccharification and Co-Fermentation (SSCF)

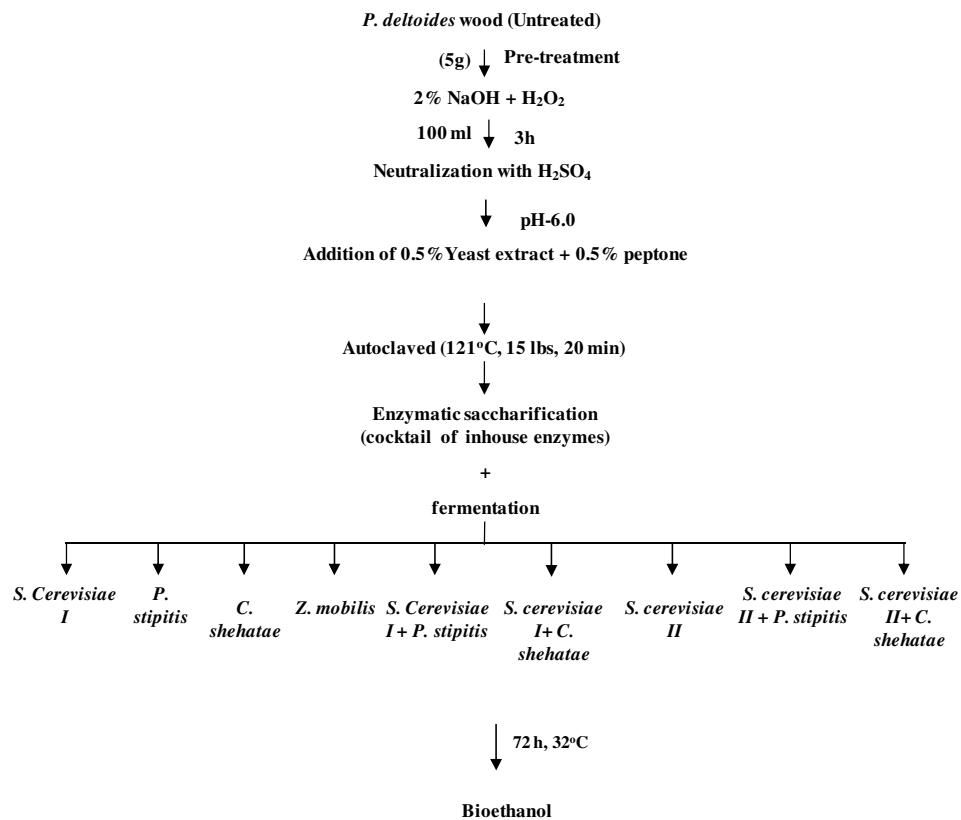
3.8.6.1 Substrate: *P. deltoides* wood chipped, grinded and alkaline hydrogen peroxide pretreated as mentioned in section 3.5.3.2

3.8.6.2 Microorganism used:

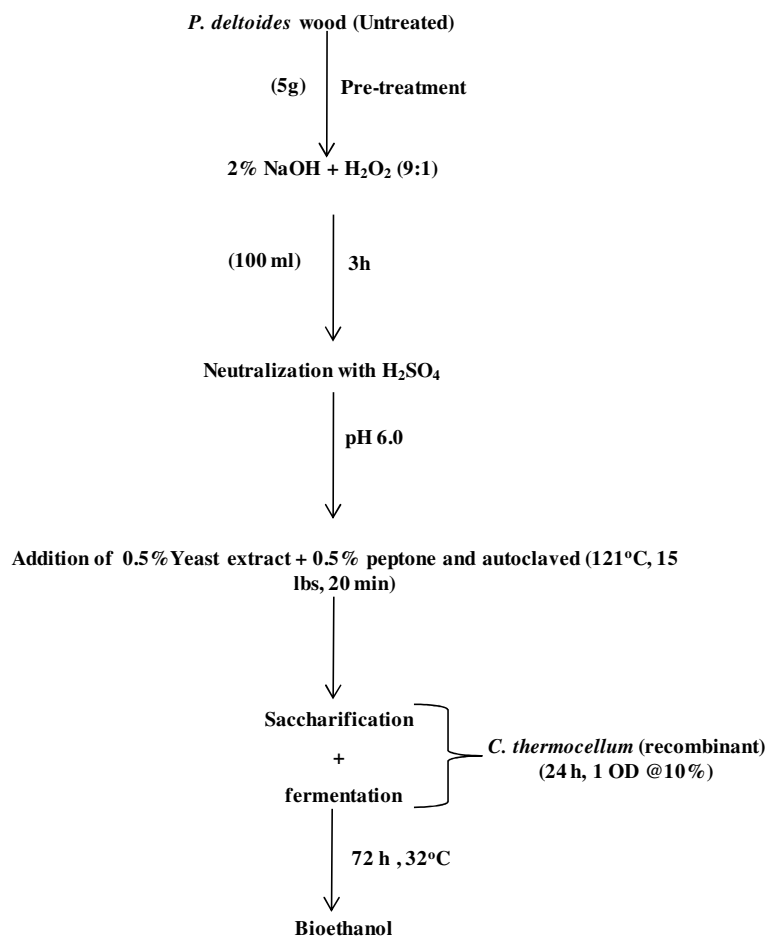
A recombinant strain of *Clostridium thermocellum* strain DSM 1313 [Accession No. ICP002416] procured from DSM- Germany was used.

3.8.6.2 Process

5 g of untreated *P. deltoides* wood was taken in each of 9 sets of 250 ml flask. To these flasks 100 ml of 2% NaOH + H₂O₂ (9:1) was added and kept at 65°C in water bath for 3 h. The pH was maintained 6.0, to this 0.5% yeast extract and 0.5% peptone was added, autoclaved at 121°C, 15 lbs or 20 min. To the cooled autoclaved slurry a recombinant capable of saccharification as well as fermentation i.e. *Clostridium thermocellum* strain DSM 1313 [Accession No. ICP002416] was added (24 h old, 1.0 O.D.) @ 10% and kept for fermentation at 32°C for 72 h.



Scheme of simultaneous saccharification and fermentation process for bioethanol production from pretreated *P. deltoides* wood



Scheme of simultaneous saccharification and Co-fermentation for bioethanol production from pretreated *P. deltoides* wood

3.8.6.3 Ethanol Estimation

Same as section 3.8.4.1.3.4

3.9 Estimation of inhibitors (5-HMF) at different bioethanol production steps

3.9.1 Qualitative estimation of inhibitors

Presence of furfurals and 5- HMF was checked by performing Molish's Test.

Molish's Test: Molisch's test was used to detect the presence of inhibitors in the samples. A sample solution (2 ml) was taken in a test tube, to this 1 drop of Molisch's reagent (10% α -naphthol in ethanol) was added. 2 ml of conc. H_2SO_4 poured down the side of the test tube, so that it forms a layer at the bottom of the tube. The color at the interface between two layers was observed and compared with a control test.

3.9.2 Quantitative estimation of 5-HMF using High performance Liquid Chromatography (HPLC)

Inhibitors generated during hydrolysis of biomass usually slow down the fermentation by ethanogens. So in the present study, one of important inhibitor i.e. 5-HMF has been tested as given below:

3.9.2.1 Extraction of samples for analysis of 5- HMF

5g of untreated *P. deltoides* wood biomass was taken in 250 ml Erlenmeyer flask. To this biomass pretreatment with 2% NaOH + H_2O_2 (9:1) was given for 3h. After 3h pH was maintained 5.5 with H_2SO_4 and sample (supernatant) was taken for HPLC analysis to detect the inhibitors i.e 5-hydroxy methyl furfurals. Supernatant and solid biomass were separate out by centrifugation at 10,000rpm for 20 min. Solid biomass was allowed to enzymatic saccharification at 50⁰C temperature for 72 h and then sample was withdrawn for HPLC analysis. Enzymatic hydrolysate was pooled with the supernatant separated after pretreatment. Sample was taken with and without pooling for HPLC study. Detoxification was done with Ca (OH) ₂ to neutralize the inhibitors. Sample with

and without detoxification was withdrawn for HPLC analysis. Fermentations were performed with detoxified and non-detoxified supernatant. HPLC analysis was also performed after fermentation. HPLC study of samples collected at each step of bioethanol production was done.

3.9.2.2 HPLC Conditions

Column:	Ultra C18 (Restek Corp.), 250mm × 4.6 mm, 5µm
Mobile Phase A:	90: 10 water: methanol, 10mM ammonium formate
Mobile phase B:	10: 90 water: methanol, 10mM ammonium formate
Gradient:	0-5 min at 100% A, to 100% B at 10 min, 10 min hold
Flow:	0.5 mL /min
Temperature:	ambient
Detector:	UV@ 280 nm
Injection volume:	10µL
Standard dilution:	500ppm

3.9.2.3 Formula to calculate the concentration of 5-HMF

$$\text{Concentration of 5-HMF} = \frac{\text{Area of Sample}}{\text{Area of Standard}} \times \text{Standard dilution (500 ppm)}$$

3.10 Scale up of bioethanol production in a stirred tank bioreactor under Simultaneous Saccharification and Fermentation mode

Fermenting microorganisms used:

The co-culture of *S. cerevisiae* II+ *P. stipitis* was selected for ethanol production based upon its best performance in shake flask culture.

3.10.1.1 Inoculum Preparation

The strains i.e *S. cerevisiae* II and *P. stipitis* were grown in 150 ml of YEPD (Yeast extract, peptone and dextrose) and MGYD (Malt extract, glucose, yeast extract and peptone) broth respectively and incubated at 25±2 °C for 24 h.

As soon as the substantial growth of the strain was observed in the broth and optical density was set to 1.0 using broth as blank.

3.10.1.2 Enzyme preparation

Cocktail of inhouse enzymes was prepared in large volume by mixing as given below:

20 ml cellulase from *M. thermophila* SH1

20 ml cellulase from *B. stratosphericus* N₁₂ (M)

5 ml of xylanase from *B. altitudinis* Kd₁ (M)

5 ml of xylanase from *M. thermophila* SH1 used for the saccharification @ 1 ml/g of biomass i.e. 50 ml by mixing.

3.10.1.3 Batch Fermentation

Batch cultivation for the production of ethanol 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 32± 2°C and pH 5.5. Carbon and nitrogen sources; for batch fermentations were 50g *P. deltooides* (biomass), yeast extract – 0.5% and 0.5% peptone. 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 bioreactor was inoculated with 10% inoculum of *S. cerevisiae* II + *P. stipitis* and 50 ml of prepared enzyme solution under aseptic conditions. The fermentation was carried out under anaerobic conditions. The agitation speed 100 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4.....12 h and ethanol estimation was done.

3.10.2 Ethanol Estimation

Same as section 3.8.6.1.3.4

Chapter-4

RESULTS AND DISCUSSION

The results emanating from the present investigation entitled “**Bioconversion of cellulosic waste into bioethanol as biofuel**”, which was 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 2009-13. The facts and statistics used for analysis pertaining to different parameters and characters were statistically analyzed and significance of results had been verified. This chapter deals with the experimental results and discussion and most likely causes and effects, underlying patterns, resulting in the predictions, evidence or even a line of reasoning, which support 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 primary screening of cellulolytic and xylanolytic microorganisms from soil
- 4.2 Secondary screening of hypercellulase and hyperxylanase producing microorganisms by inducing mutation to enhance their enzyme production
- 4.3 Identification of screened hypercellulolytic and hyperxylanolytic isolates
- 4.4 Isolation and screening of cellulolytic and xylanolytic fungi from soil
- 4.5 Optimization and production of cellulase and xylanase under submerged fermentation
- 4.6 Cost effective production of cellulase and xylanase by replacing conventional carbon source with lignocellulosic biomass under submerged (SmF) and solidstate fermentation (SSF)
- 4.7 Partial purification of cellulase and xylanase enzymes
- 4.8 Scale up of enzymes in a bioreactor

- 4.9 Bioethanol production under SHF (Separate hydrolysis and fermentation), SSF (Simultaneous saccharification and fermentation) and SSCF (Simultaneous saccharification and co fermentation) using different microorganisms and recombinants.
- 4.10 Quantitative analysis of inhibitors formed during bioethanol production by High Performance Liquid Chromatography (HPLC)
- 4.11 Scale up of bioethanol production in a stirred tank bioreactor

4.1 ISOLATION AND SCREENING OF HYPER CELLULOLYTIC AND XYLANOLYTIC BACTERIA

4.1.1 Isolation of Cellulolytic and Xylanolytic Bacteria from Soil

The soil samples for isolation of cellulolytic and xylanolytic microorganisms was collected from different sites of Solan, Shimla and Kangra districts of Himachal Pradesh (Fig 1)

In total, 84 bacteria isolated from the pooled soil samples in PYC medium having pH 6.8 have been enlisted in Fig 2a, b, c, d, e, f, and g with their morphological characteristics. Out of 84 bacteria, 36% were isolated from the soil samples collected from 4-6 sites of Solan district, 25% from Shimla district and 39% from Kangra district of Himachal Pradesh. Bacteria isolated from forest soil showed a great variation in their morphological characteristics i.e. colour, texture and shape. 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. Among these 84 isolates, 61% were found to be gram +ve and the rest 39% were found to be gram -ve. Among all isolates, 59% were rod shaped while 41% were spherical in shape.

Fig. 3a, b, c, d, e, f, g, h, i and j represents the biochemical characteristics of 84 bacterial isolates. Different biochemical tests were performed such as 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 84 bacterial isolates. Among 84 bacterial isolates, 73 %



Fig. 1. Isolation of cellulolytic and hemicellulolytic microorganisms from selected sites of mid hills of Himachal Pradesh - Sub Temperate Zone of Northern Himalayas

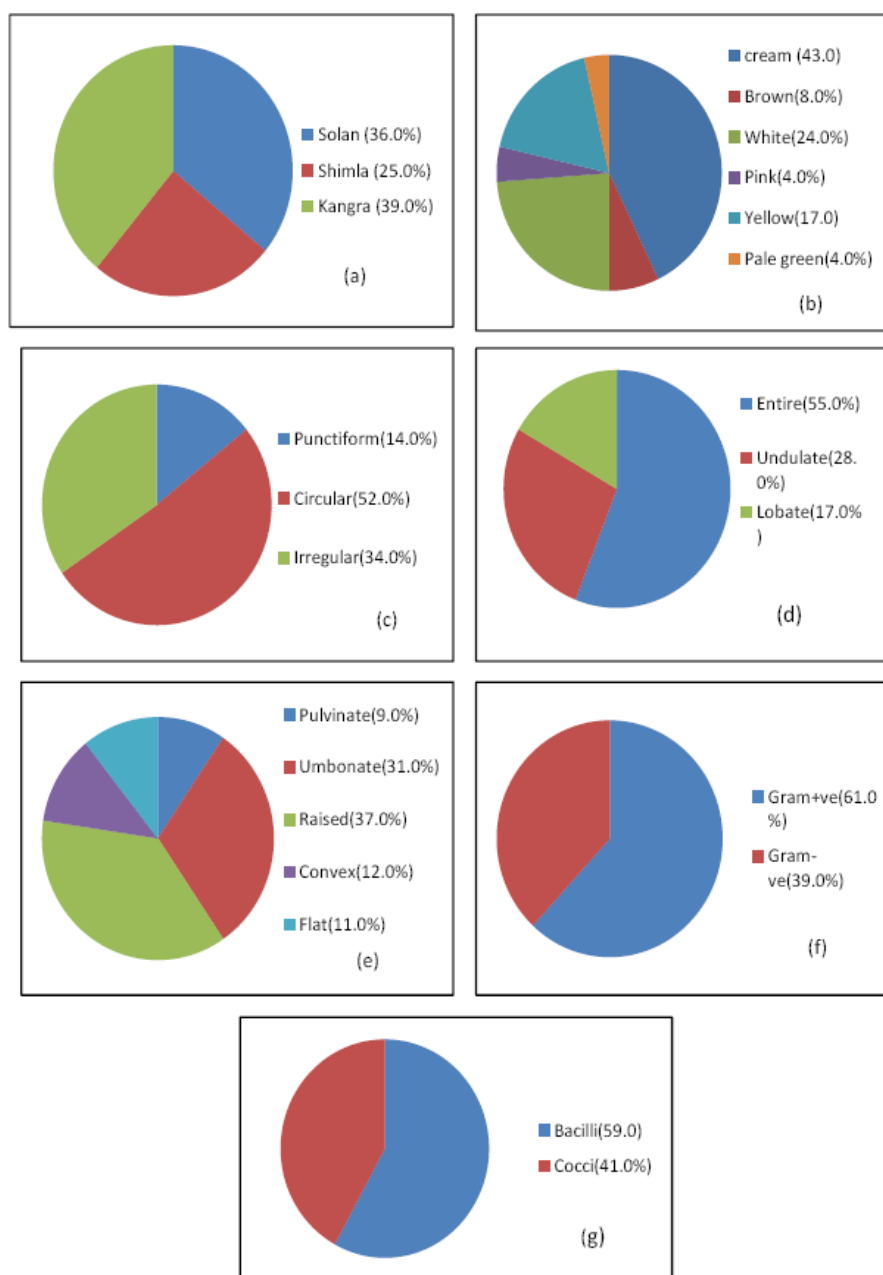


Fig 2. Morphological description of cellulolytic and xylanolytic bacterial isolates on the basis of (a) Site (b) colour (c) Elevation (d) Form (e) Margin (f) Gram's staining (g) Shape

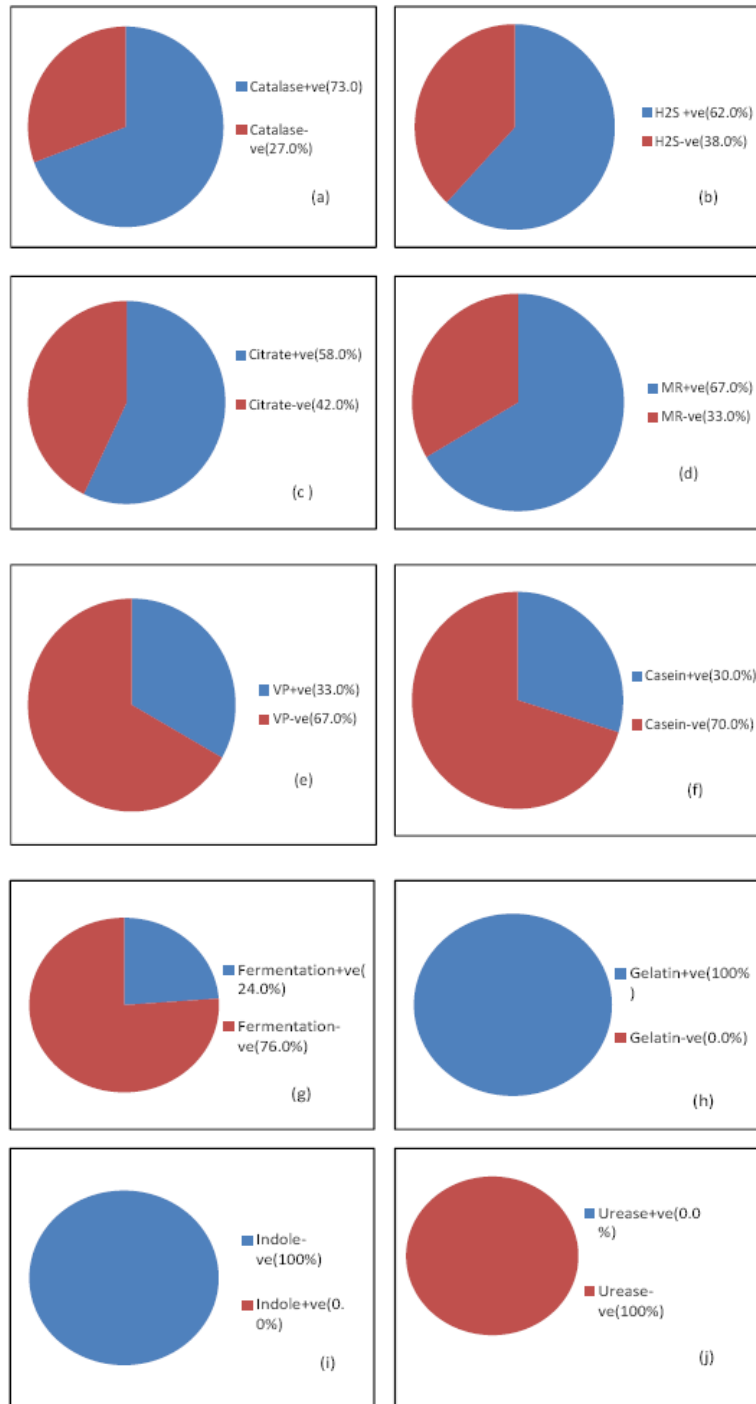


Fig 3. Biochemical characteristics of cellulolytic and xylanolytic bacterial isolates (a) Catalase test (b) H₂S production (c) Citrate utilization (d) MR- test (e) VP-test (f) Casein test (g) Fermentation of glucose (h) Gelatin Hydrolysis (i) Indole test (j) Urease test

isolates were catalase positive and 27% were negative. While 62% were found +ve for H₂S production, 58% for citrate utilization, 67% for MR, 33% for VP test, 30% for casein and 24% for fermentation of carbohydrates. For gelatin hydrolysis, all strains showed positive test, while for indole test and urease test, all exhibited negative test. Depending upon their morphological, physiological and biochemical characteristics, these bacteria were tentatively identified as *Bacillus* spp. and *Coccus* spp.

Soil is a rich habitat of potential biodegrading microorganisms. Thus, isolation of hypercellulolytic and hyperxylanolytic microorganisms has been done in the present study from the soil considering it a valuable niche of microorganisms. Cellulose is the main building block of plants and have major fraction of organic carbon in soil. Microorganisms which live in soil are accountable for recycling of this organic carbon to the environment (Wang *et al.*, 2009). Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. The habitats where cellulosic substrates are present are the best sources for isolation of cellulolytic microorganisms (Das *et al.*, 2010).

In the present study cellulolytic and xyranolytic bacterial strains were isolated from forest soil enriched with decomposing litter from three different districts of Himachal Pradesh. These areas were selected because they represent pristine dense forest and have not been influenced by agricultural practices. There was a predominance of Gram +ve isolates among bacteria with the marked prevalence from the order *Bacilliales*.

The isolation of *Bacillus* strains with cellulolytic and hemicellulolytic activity has also been reported in prospection of *Bacillus coagulans* and *Bacillus licheniformis* isolated from soil and degrading wood logs by Sharma *et al.* (2005). Researchers studying on cellulolytic activity have isolated various bacteria from different environmental sources. Hatami *et al.* (2008) isolated aerobic cellulolytic bacteria from forest and farming soils and determined their ability to decompose cellulose. Yin *et al.* (2010) isolated *Cellulomonas* sp. YJ5

from soil samples and cellulase activity was determined after 48 h of incubation. Das *et al.* (2010) isolated eight bacterial strains from cow dung samples and they found that maximum amount of cellulase producing bacterial isolate was *Bacillus* sp. Otajevwo and Auyi (2010) isolated *Pseudomonas* sp. and *Serratia* sp. from soil samples, which have greater ability to produce cellulase enzyme.

Seven primary cellulolytic isolates were isolated from forest soil. Among them, VG-4 consortium had a complete cellulose degrading potential since it showed high endocellulase, xylanase and filter paper degrading capacities (Ghio *et al.*, 2012). Several cellulolytic strains were isolated from the gut of termite in three different media. Each strain showed significant cellulolytic activity in congo red assay. They were identified like *Citrobacter*, *Enterobacter* and *Cellulomonas* on the basis of biochemical tests (Upadhyaya *et al.*, 2012). The cellulase producing bacteria was also isolated from cow dung and characterized by morphological and biochemical analysis and identified primarily as genus, *Bacillus* species (Shanmugapriya *et al.*, 2012). *Bacillus*, *Flouribacter*, *Acidobacterium* were isolated from the rice field soil (Weber, 2010).

4.1.2 Preliminary screening of Cellulolytic and Xylanolytic Bacteria from Soil

The production of extracellular enzymes i.e. cellulase and xylanase from different bacterial isolates had been depicted in Table 1. Among all, N₁₃ showed the highest total cellulase activity of 2.138 IU (CMCase 1.577 IU, FPase 0.088 IU and 0.473 IU β -glucosidase) followed by NS₁ showing enzyme activity of 1.689 IU (CMCase 1.200 IU, FPase 0.068 IU and β -glucosidase 0.421 IU) whereas N₉ showed the lowest cellulase activity i.e. 0.018 IU. Among 84 bacteria, seven isolates were primarily selected for the final screening on the basis of their higher cellulase activity i.e. N₅ (1.503 IU), N₁₂ (1.476 IU), N₁₃ (2.138 IU), NS₁ (1.689 IU), S₁ (1.639 IU), S₇ (1.135 IU) and S₁₂ (1.220 IU).

Maximum xylanase activity was exhibited by SL₈ (61.54 IU) followed by Kd₁ (31.72 IU) and the least xylanase activity was noted in SL₇ (2.930 IU).

Table1. Preliminary screening of bacterial isolates for hypercellulase and hyperxylanase production

S. No.	Isolate name	Protein (mg/ml)	CMCase activity		FPase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)		Protein (mg/ml)	Xylanase		Total enzyme (cellulase + xylanase)	Total protein (mg/ml)
			Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity		Enzyme activity	Specific activity		
1	N ₁	1.50	0.388	0.258	0.122	0.081	0.026	0.017	0.536	0.357	1.09	19.98	18.33	20.51	2.59
2	N ₂	1.83	0.014	0.007	0.111	0.060	0.526	0.287	0.651	0.355	1.23	17.04	13.85	17.69	3.06
3	N ₃	1.52	0.021	0.013	0.044	0.028	0.368	0.242	0.433	0.284	1.08	22.38	20.72	22.81	2.60
4	N ₄	1.89	0.148	0.078	0.066	0.034	0.052	0.027	0.266	0.140	1.09	22.30	20.45	22.56	2.98
5	N ₅ *	1.83	0.740	0.404	0.126	0.068	0.637	0.348	1.503	0.821	1.00	15.45	15.45	16.95	2.83
6	N ₆	1.24	0.444	0.358	0.124	0.100	0.000	0.079	0.568	0.537	1.34	19.71	14.70	20.37	2.58
7	N ₇	1.62	0.555	0.342	0.111	0.068	0.210	0.129	0.876	0.540	1.46	17.31	11.85	18.18	3.08
8	N ₈	1.76	0.004	0.002	0.028	0.015	0.131	0.074	0.163	0.092	1.80	11.45	6.36	11.61	3.56
9	N ₉	1.40	0.002	0.001	0.016	0.011	0.000	0.053	0.018	0.066	1.67	16.51	9.88	15.60	3.07
10	N ₁₀	1.26	0.003	0.002	0.055	0.043	0.052	0.041	0.110	0.087	1.54	17.84	11.58	17.95	2.80
11	N ₁₁ *	2.56	0.005	0.001	0.002	0.0007	0.657	0.256	0.664	0.259	1.98	29.64	14.96	30.30	4.54
12	N ₁₂ *	2.72	1.274	0.468	0.093	0.034	0.109	0.040	1.476	0.542	1.09	17.31	15.88	18.78	3.81
13	N ₁₃ *	3.16	1.577	0.499	0.088	0.027	0.473	0.149	2.138	0.676	1.45	14.12	9.73	16.25	4.61
14	NS ₁ *	2.92	1.200	0.410	0.068	0.023	0.421	0.144	1.689	0.578	1.57	11.72	7.46	13.40	4.49
15	NS ₂	3.40	0.600	0.176	0.104	0.030	0.236	0.069	0.940	0.276	1.32	15.78	11.95	16.72	4.72
16	NS ₃	2.48	0.177	0.071	0.078	0.031	0.105	0.042	0.360	0.145	1.89	12.52	6.62	14.02	4.45
17	NS ₄	2.40	0.696	0.290	0.088	0.036	0.004	0.001	0.788	0.328	1.23	13.32	10.82	14.10	3.63
18	NS ₅	3.28	0.962	0.293	0.120	0.036	0.000	0.029	1.082	0.329	1.09	16.78	15.39	17.86	4.37
19	NS ₆	1.00	0.010	0.010	0.032	0.032	0.007	0.007	0.049	0.049	1.04	16.85	16.20	18.04	2.64
20	Kd ₁ *	3.36	0.148	0.044	0.066	0.019	0.104	0.030	0.318	0.094	2.34	31.72	13.55	32.03	5.70
21	Kd ₂	3.12	0.377	0.120	0.120	0.038	0.131	0.041	0.628	0.201	1.89	15.71	8.31	16.33	5.01
22	Kd ₃	3.52	0.229	0.065	0.097	0.027	0.001	0.0002	0.327	0.092	1.90	22.11	11.63	22.43	5.42
23	Kd ₄	3.24	0.012	0.003	0.055	0.016	0.210	0.064	0.277	0.085	1.98	14.98	7.56	15.25	5.22
24	Kd ₅	4.32	0.348	0.080	0.068	0.015	0.215	0.049	0.631	0.146	1.53	12.12	7.92	12.75	5.85
25	Kd ₆	2.90	0.296	0.102	0.111	0.038	0.184	0.063	0.591	0.203	0.67	5.461	8.14	4.05	3.57
26	Kd ₇	1.85	0.237	0.128	0.115	0.062	0.078	0.042	0.430	0.232	0.98	7.593	7.74	8.02	2.83
27	Kd ₈	2.56	0.170	0.066	0.053	0.020	0.010	0.003	0.233	0.091	1.04	14.65	14.08	14.88	3.60
28	Kd ₉	2.48	0.177	0.071	0.078	0.031	0.105	0.042	0.360	0.145	1.07	5.328	4.97	5.68	3.55
29	Kd ₁₀	2.96	0.181	0.061	0.095	0.032	0.021	0.007	0.297	0.100	0.89	6.194	6.95	6.48	3.85
30	Kd ₁₁	1.22	0.006	0.004	0.004	0.003	0.012	0.009	0.022	0.018	0.56	3.263	5.82	3.28	1.78

S. No.	Isolate name	Protein (mg/ml)	CMCase activity		FPase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)		Protein (mg/ml)	Xylanase		Total enzyme (cellulase + xylanase)	Total protein (mg/ml)
			Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity		Enzyme activity	Specific activity		
31	Kd ₁₂	1.24	0.007	0.003	0.014	0.011	0.010	0.008	0.031	0.025	2.10	25.57	12.17	25.60	3.34
32	Kd ₁₃	1.26	0.004	0.003	0.036	0.028	0.008	0.006	0.048	0.038	2.14	23.97	11.20	24.01	3.40
33	Kd ₁₄	1.00	0.008	0.008	0.043	0.043	0.010	0.010	0.061	0.061	0.67	5.72	8.53	5.78	1.67
34	Kd ₁₅	1.71	0.036	0.021	0.067	0.039	0.009	0.005	0.112	0.065	1.07	16.51	15.42	16.62	2.78
35	Kd ₁₆	1.62	0.048	0.029	0.059	0.036	0.008	0.004	0.115	0.070	1.15	6.128	5.32	6.23	2.77
36	KD1	1.52	0.040	0.026	0.031	0.020	0.026	0.017	0.097	0.063	1.04	12.78	12.28	12.87	2.56
37	KD2	0.70	0.012	0.017	0.008	0.011	0.151	0.215	0.171	0.244	1.04	12.38	11.90	12.55	1.74
38	KD3	1.14	0.044	0.038	0.066	0.057	0.006	0.005	0.116	0.101	1.23	17.78	14.45	17.89	2.37
39	KD4	1.24	0.003	0.002	0.087	0.070	0.009	0.007	0.099	0.079	1.11	16.58	14.93	16.67	2.35
40	KD5	1.52	0.004	0.002	0.012	0.007	0.010	0.006	0.026	0.017	0.97	7.19	7.41	7.21	2.49
41	KD6	1.00	0.010	0.010	0.023	0.023	0.008	0.008	0.041	0.041	2.24	35.96	15.84	36.00	1.24
42	KD7	1.83	0.033	0.018	0.090	0.049	0.009	0.004	0.132	0.072	0.99	8.92	9.01	9.05	2.82
43	KD8	1.36	0.023	0.016	0.015	0.011	0.010	0.007	0.048	0.035	1.30	11.85	9.11	11.89	2.66
44	Kp ₁	1.00	0.010	0.010	0.032	0.032	0.007	0.007	0.049	0.049	1.20	12.89	10.74	12.93	2.20
45	Kp ₂	1.00	0.004	0.004	0.027	0.027	0.006	0.006	0.037	0.037	0.69	9.87	14.30	9.90	1.69
46	Kp ₃	1.26	0.036	0.028	0.042	0.033	0.010	0.007	0.088	0.069	1.06	12.90	12.16	12.98	2.32
47	Kp ₄	1.56	0.009	0.005	0.011	0.007	0.012	0.007	0.023	0.014	2.89	23.00	7.95	23.02	4.45
48	KG1	1.40	0.008	0.005	0.042	0.030	0.014	0.010	0.056	0.040	1.45	12.00	8.27	12.05	2.80
49	KG2	1.22	0.003	0.002	0.028	0.022	0.009	0.007	0.040	0.032	1.87	9.080	4.85	9.12	3.09
50	KG3	1.72	0.001	0.005	0.028	0.016	0.010	0.005	0.038	0.022	1.23	13.89	11.29	13.92	2.95
51	KG4	0.82	0.005	0.006	0.064	0.078	0.289	0.352	0.358	0.436	1.67	11.98	7.17	12.33	2.49
52	KG5	0.90	0.007	0.007	0.042	0.046	0.009	0.010	0.051	0.056	1.45	23.78	16.40	23.83	2.35
53	S ₁ *	1.80	1.096	0.608	0.148	0.082	0.395	0.219	1.639	0.910	1.45	7.650	5.27	9.28	3.25
54	S ₂	0.94	0.466	0.495	0.082	0.087	0.078	0.082	0.626	0.665	2.00	4.789	2.39	5.40	2.94
55	S ₃	1.60	0.004	0.002	0.046	0.028	0.158	0.098	0.208	0.130	2.34	15.87	6.78	16.07	3.94
56	S ₄	1.50	0.037	0.024	0.053	0.035	0.009	0.006	0.099	0.066	1.00	16.79	16.79	16.85	2.50
57	S ₅	1.34	0.016	0.011	0.084	0.062	0.012	0.008	0.112	0.083	1.90	10.98	5.77	11.09	3.24
58	S ₆	2.07	0.037	0.017	0.066	0.031	0.010	0.004	0.113	0.054	0.967	23.09	24.05	23.20	3.03

S. No.	Isolate name	Protein (mg/ml)	CMCase activity		FPase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)		Protein (mg/ml)	Xylanase		Total enzyme (cellulase + xylanase)	Total protein (mg/ml)
			Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity		Enzyme activity	Specific activity		
59	S ₇ *	1.76	0.002	0.001	0.053	0.030	1.080	0.613	1.135	0.644	0.876	21.08	24.22	22.21	2.63
60	S ₈	1.72	0.045	0.026	0.057	0.033	0.710	0.412	0.812	0.472	1.02	12.08	11.84	12.89	2.74
61	S ₉	1.83	0.017	0.009	0.060	0.032	0.026	0.014	0.103	0.056	1.11	10.09	9.09	10.19	2.94
62	S ₁₀	1.66	0.013	0.007	0.053	0.031	0.922	0.555	0.988	0.595	1.34	23.09	17.23	24.07	3.00
63	S ₁₁	1.50	0.037	0.024	0.053	0.035	0.009	0.006	0.099	0.066	1.45	22.75	15.68	24.04	3.11
64	S ₁₂ *	1.34	0.012	0.008	0.086	0.064	1.122	0.837	1.220	0.910	1.78	9.76	5.48	10.98	3.12
65	S ₁₃	1.86	0.015	0.008	0.053	0.028	0.057	0.030	0.125	0.067	1.23	13.98	11.36	14.10	3.09
66	S ₁₄	1.80	0.259	0.143	0.054	0.030	0.009	0.005	0.322	0.178	1.22	14.09	11.54	14.41	3.02
67	S ₁₅	2.19	0.027	0.012	0.060	0.027	0.004	0.001	0.091	0.041	1.90	11.54	6.07	11.63	4.09
68	S ₁₆	2.19	0.162	0.073	0.057	0.026	0.006	0.002	0.225	0.102	0.256	9.87	38.55	10.09	2.44
69	S ₁₇	3.00	0.016	0.005	0.005	0.001	0.009	0.003	0.030	0.010	0.987	7.732	7.88	7.76	3.98
70	S ₁₈	1.68	0.036	0.021	0.008	0.004	0.010	0.005	0.054	0.032	0.543	8.759	16.22	8.80	2.22
71	S ₁₉	1.74	0.340	0.195	0.113	0.064	0.014	0.008	0.467	0.268	0.654	6.879	10.56	7.33	2.39
72	S ₂₀	1.92	0.170	0.088	0.055	0.028	0.210	0.109	0.435	0.226	0.678	8.437	12.58	8.86	2.59
73	S ₂₁	2.04	0.011	0.005	0.008	0.003	0.315	0.154	0.334	0.163	0.123	4.422	35.95	4.75	2.16
74	SL ₁	2.52	0.281	0.111	0.071	0.028	0.184	0.073	0.536	0.212	0.234	2.959	12.64	3.48	2.75
75	SL ₂	1.68	0.150	0.089	0.165	0.098	0.095	0.056	0.410	0.244	0.987	15.65	15.96	16.06	2.66
76	SL ₃	1.89	0.222	0.117	0.231	0.122	0.012	0.006	0.465	0.246	1.00	18.98	18.98	19.44	2.89
77	SL ₄	1.46	0.136	0.093	0.157	0.107	0.034	0.023	0.327	0.223	0.78	4.195	5.37	4.51	2.24
78	SL ₅	2.19	0.254	0.115	0.240	0.109	0.023	0.010	0.517	0.236	0.67	4.395	6.55	4.90	2.86
79	SL ₆	2.34	0.354	0.151	0.328	0.140	0.023	0.009	0.705	0.301	0.45	6.059	13.46	6.75	2.79
80	SL ₇	2.13	0.360	0.169	0.216	0.101	0.047	0.022	0.623	0.292	0.56	2.930	5.23	3.55	2.69
81	SL ₈ *	1.86	0.234	0.125	0.186	0.101	0.035	0.018	0.455	0.244	2.97	61.54	20.72	61.99	4.83
82	SL ₉	2.10	0.136	0.064	0.145	0.069	0.095	0.045	0.376	0.179	1.98	22.64	11.43	23.01	4.08
83	SL ₁₀	1.05	0.096	0.091	0.121	0.115	0.191	0.181	0.408	0.388	0.56	3.795	6.76	4.19	1.61
84	SL ₁₁	1.92	0.384	0.200	0.213	0.110	0.022	0.011	0.619	0.322	0.57	5.694	9.98	6.30	2.49

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

Specific activity : enzyme activity/mg of protein.

* } Hypercellulase and hyperxylanase producer

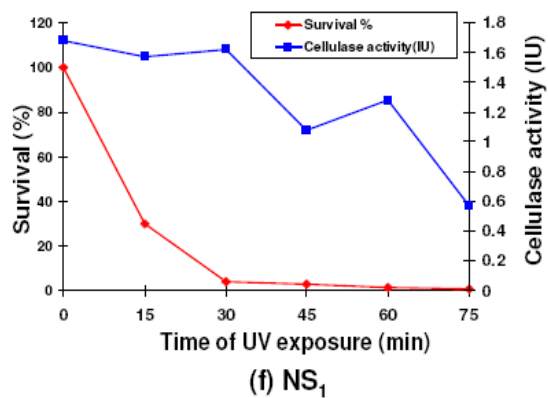
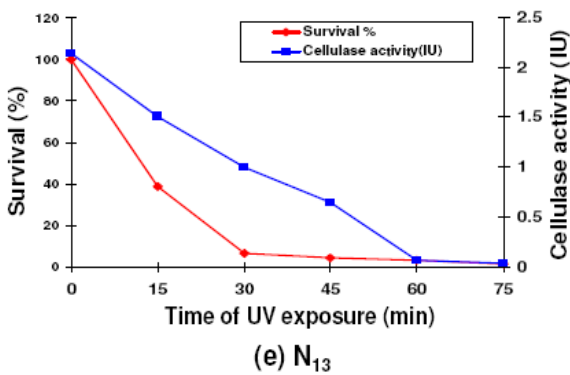
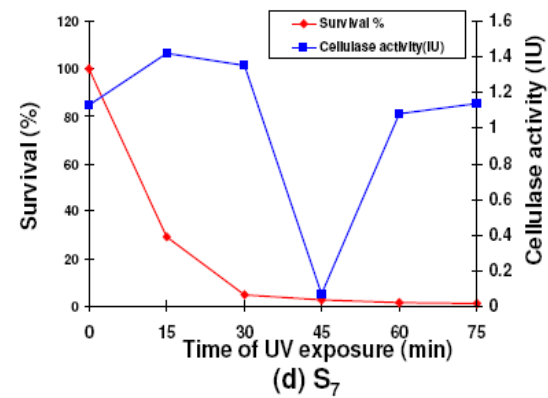
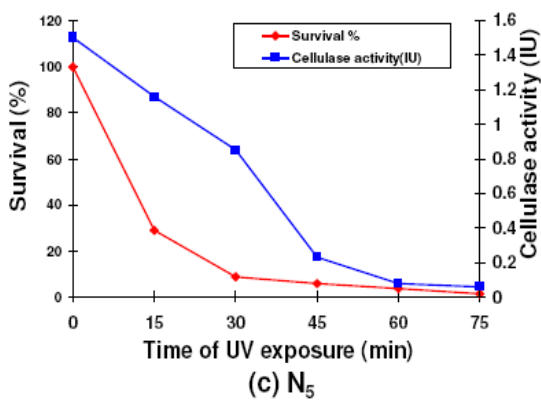
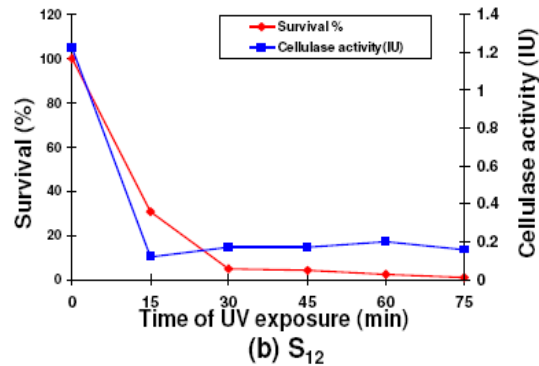
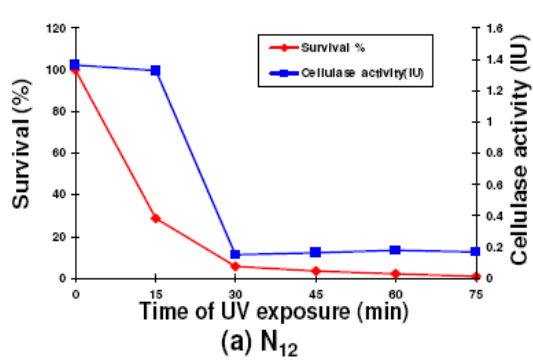
In case of xylanase production, three isolates with higher activity were selected for further screening i.e. N₁₁ (29.64 IU), Kd₁ (31.72 IU) and SL₈ (61.54 IU).

Seven different bacterial strains were isolated and screened for cellulase production in submerged fermentation process by Irfan *et al.* (2012). Among them, ASN2 showed maximum yield for cellulase production. This strain was further characterized by biochemical and morphological tests and identified as *Cellulomonas* sp. ASN2 Twenty eight cellulolytic bacteria isolated from peat soils were tested for their cellulase activities. Four bacterial isolates (S3B40, S3B32, S3B37, and AB16) that have high cellulase activities were selected for degrading cellulose based on CMC congo red test. These showed cellulase activity of 0.193, 0.014, 3.082 and 0.021 (U/ml) respectively (Lisdiyanti *et al.*, 2012). Jaradat *et al.* (2008) observed the highest crude cellulase enzyme activity (432 U/l) after 3 days of incubation by *Streptomyces* strain J2. Maximum xylanase production level of 188.0 ± 20.0 and 5.6 ± 1.1 U/ml were achieved by *Bacillus pumilus* M1 and *Bacillus pumilus* M2 in a liquid medium containing 3% corn cobs as a sole carbon source (Yasinok *et al.*, 2008). The activities of the nine strains screened as cellulase producers in the preliminary screening. At 72 h of incubation, EWBC-M exhibited the highest cellulase activity (0.1271 ± 0.002 IU), which is significantly different from the other. The minimum cellulase activity was exhibited by EWBC-I with 0.0345 ± 0.002 IU (Shankar *et al.*, 2011).

4.2. SECONDARY SCREENING OF HYPERCELLULASE AND HYPERXYLANASE PRODUCING ISOLATES SELECTED IN PRELIMINARY SCREENING BY INDUCING MUTATION

4.2.1 Mutation of bacterial isolates using UV irradiation

Mutagenesis is a well recognized approach to enhance the expression of enzymes. Since mutagen leads to mutation and alters many of basic characters of a strain by inducing lesions or modification in base sequences of DNA that remains unrepaired (Devehand and Gwynne, 1991). Thus an attempt was made in the present study to enhance cellulase as well as xylanase production in wild strains by treating them with physical and chemical mutagen.



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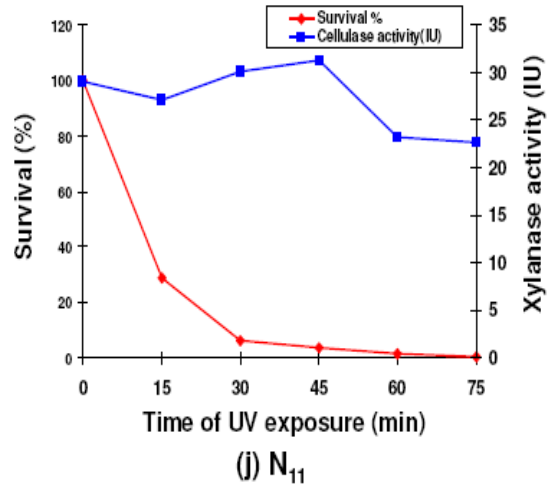
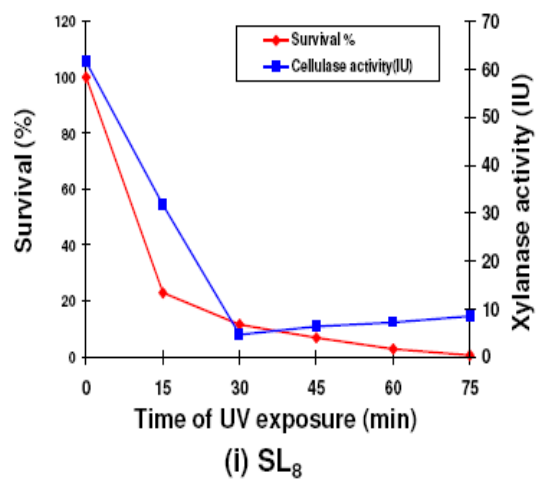
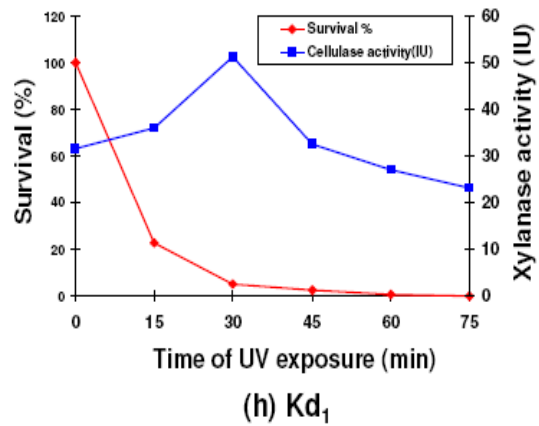
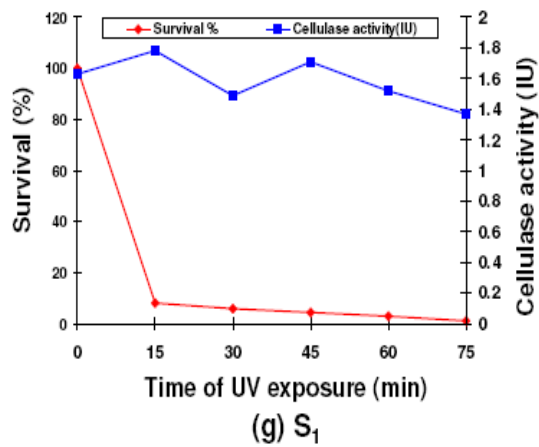


Fig 4. Induction of mutation in potential cellulase and xylanase producing bacterial isolates by using UV radiations

In the present study, 7 bacterial isolates i.e. N₁₂, S₁₂, N₅, S₇, N₁₃, NS₁ and S₁ were selected for hypercellulase production on the basis of primary screening where as 3 bacterial isolates i.e. Kd₁, SL₈ and N₁₁ were selected for hyperxylanase production. Twenty four hour old culture of bacterial isolates selected in preliminary screening in PYC medium were exposed to UV dose i.e. 0.00012 mJ/cm² for 15 min, 0.00024 mJ/cm² for 30 min, 0.00036 mJ/cm² for 45 min, 0.00048 mJ/cm² for 60 min and 0.00064 mJ/cm² for 75 min in an open petriplate. The survival count at each interval was determined on PYC medium containing 0.5% cellulose for cellulase and 0.5% xylan for xylanase producing isolates and the results have been shown in Fig 4 where UV irradiation of 75 min led to minimum survival of N₁₂ (0.81%), S₁₂ (0.92%), N₅ (1.44%), S₇ (0.93%), N₁₃ (1.33%), NS₁ (0.57%) and S₁ (1.06%), SL₈ (0.76%) and N₁₁ (0.22%) , while 60 min was found to be the best to kill maximum number of cells for Kd₁ (0.80%). Hopwood *et al.* (1985) suggested that 99.9% kill is best suited for strain improvement as the fewer survivors in the treated sample will have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture.

4.2.2 Production of cellulase and xylanase by UV mutants

Cellulase and xylanase production of mutants of different UV doses and time exposure for 120 h in PYC medium containing 0.5% cellulose in case of cellulase and 0.5% xylan in case of xylanase production. Among different isolates N₁₂ (M), S₁₂ (M), N₅ (M), N₁₃ (M) and NS₁ (M) showed decrease while isolate S₇ (M) and S₁ (M) showed increase in cellulase activity i.e. from 1.135 IU to 1.421 IU and 1.639 IU to 1.787 IU respectively.

In case of xylanase production from UV treated isolates Kd₁ (M) and N₁₁ (M) showed increase in xylanase activity ranging from 31.72 IU to 51.32 IU and 29.05 IU to 31.30 IU respectively, while isolate SL₈ (M) showed decrease in their xylanase activity (Fig 4). Based upon their results Kd₁ (M) UV irradiated was finally selected for further study.

UV rays have been considered important inducers for strain mutations. The pyrimidines are especially sensitive to modifications by UV rays absorption

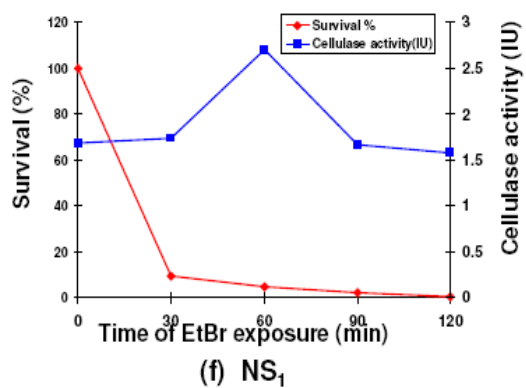
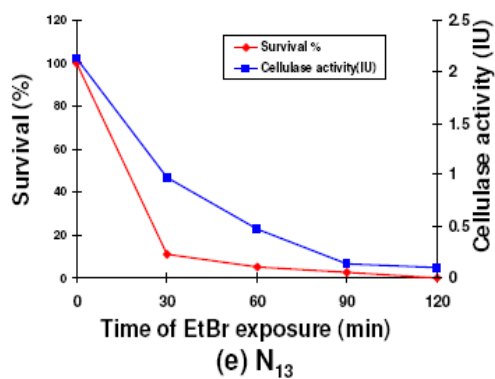
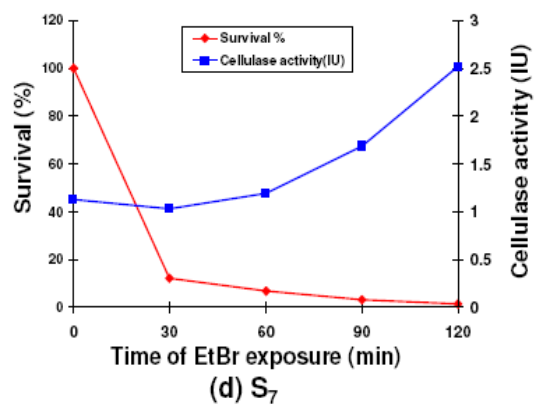
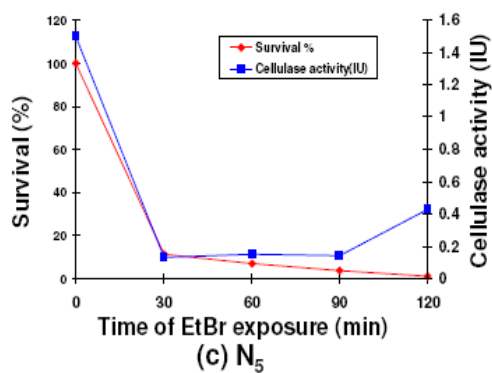
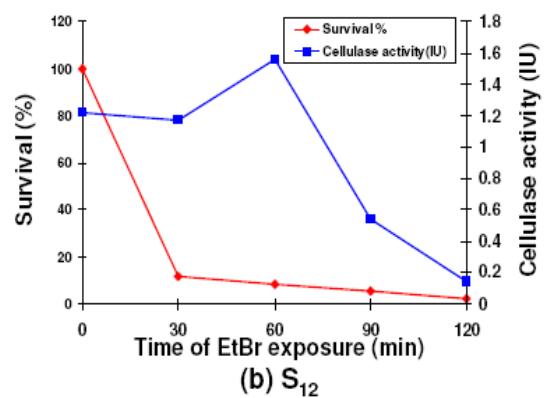
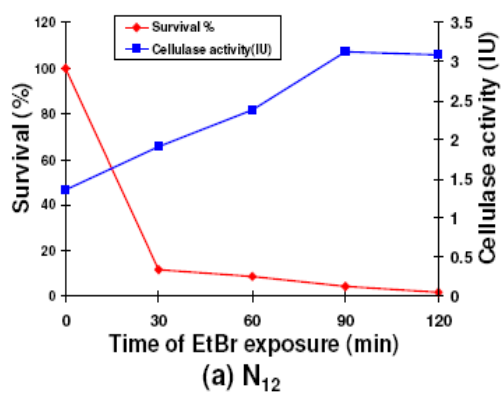
which results in the production of thymine dimers that distort the DNA helix and block future replication (Chavan *et al.*, 2012). The potential of a microorganism to mutate is an important property conferred by DNA, since it creates new variations in the gene pool (Prabakaran *et al.*, 2009). The approach has been followed for increasing enzyme yields in bacterial (Subramaniyan *et al.*, 2001; Xu *et al.*, 2005) and fungal (Singh *et al.*, 1995; Kuhad *et al.*, 1997; Bakri *et al.*, 2008) strains.

A study was conducted by Irfan *et al.* (2011) to improve the cellulase and xylanase production from *Aspergillus niger* using UV mutation. Strain showed highest CMCase (2.6 ± 0.041 IU) as well as FPase activity (2.5 ± 0.061 IU) at 45 min UV exposure time i.e. CMCase up to 2 times and FPase up to 3 times higher as compared to parent strain while xylanase activity (21.5 ± 1.1 IU) was observed maximum at 25 min of UV exposure. The strain improvement of *Bacillus coagulans* and *Geobacillus stearothermophilus* for thermostable cellulase production using UV radiations, NTG and Sodium azide were used as mutagenic agents. NTG was found to be best mutagenic agent among all in term of producing the mutants with highest cellulase activity. Mutant strain C11 exhibited the highest cellulase specific activity at 45 U/mg followed by C15 (39 U/mg) in case of *B. coagulans* while Mutant strain S18 exhibited the highest cellulase specific activity at 69 U/mg followed by S12 (62 U/mg) in case of *G. stearothermophilus* (Sharma *et al.*, 2012).

Djamel *et al.* (2009) reported hyper protease producing UV mutant (S08M4) that produced 1400 U/ml in liquid fermentation. UV mutation was also used for production of alkaline protease from *Bacillus sp.* (Solaiman *et al.*, 2005). Kadam *et al.* (2006) employed UV mutagenesis to improve strain of *Lactobacillus delburkii* for lactic acid production.

4.2.2.1 Mutation of bacterial isolates using EtBr

Twenty four hour old culture of bacterial isolates selected in primary screening in PYC medium was exposed to 2.0 mg/ml of EtBr i.e. chemical mutagen for 30, 60, 90 and 120 min in an Erlenmeyer flask. The survival count at



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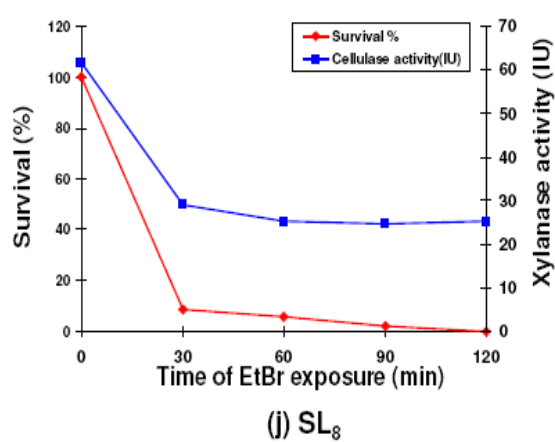
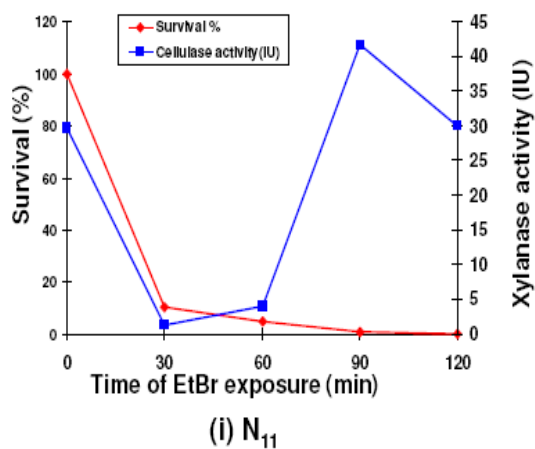
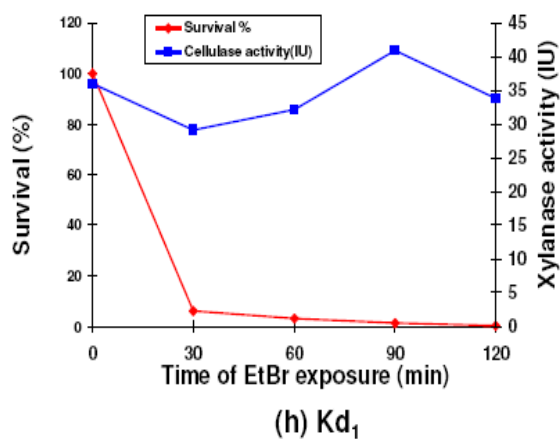
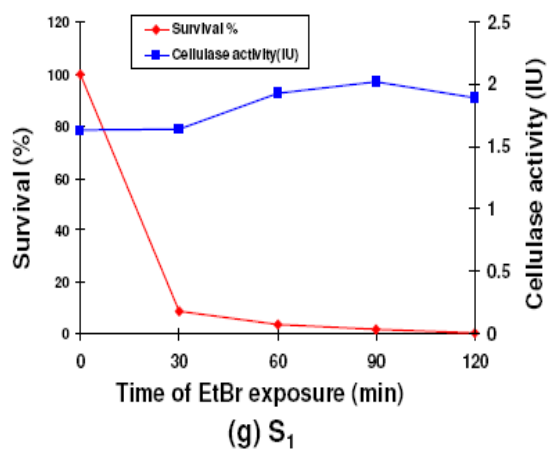


Fig 5. Induction of mutation in potential cellulase and xylanase producing isolates by using ethidium bromide (EtBr)

each interval was determined on PYC medium containing 0.5% cellulose for cellulase and 0.5% xylan for xylanase producing isolates.

Exposure of 120 min resulted in 1.84% (N₁₂), 2.26 % (S₁₂), 1.20% (N₅), 1.53% (S₇), 0.0% (N₁₃), 0.31% (NS₁) and 0.23% (S₁) percent survival of hypercellulase producers, while 0.20% (Kd₁), 0.11% (SL₈) and 0.0% (N₁₁) percent survival was noted in hyperxylanase producing isolates (Fig 5).

4.2.2.2 Production of cellulase and xylanase by EtBr mutants

Cellulase and xylanase production in case of mutants using ethidium bromide (EtBr) as mutagen have been shown in fig 5.

In case of cellulase production isolate N₁₂ (M), S₁₂ (M), S₇ (M), NS₁ (M), and S₁ (M) expressed increase in their cellulase activity while others showed a decline in cellulase activity. Isolate N₁₂ (M) showed highest increase in cellulase activity with EtBr (90 min exposure) i.e. 1.367 IU to 3.136 IU (2.29 fold increase) and was selected for further study.

In case of xylanase production mutant Kd₁ (M) produced 51.33 IU (1.61 fold increase) of xylanase and N₁₁ (M) showed 31.30 IU (1.07 fold increase), while SL₈ (M) showed a decrease in xylanase activity upon mutation.

A mutant BpCRI6 of *Bacillus pumilus* obtained after chemical mutagenesis was screened for cellulase production. Cellulase yield by BpCRI6 was four times higher than that of the wild type under optimum growth conditions (25⁰C, pH 6.5). The mutant was stable and grew rapidly in liquid and solid media (Kotchoni *et al.*, 2003). In a similar study by Sangkharak *et al.* (2012) *Cellulomonas sp.* strain TSU-03 was subjected to successive mutagenic treatment using NTG (chemical) and UV irradiation (physical). *Cellulomonas sp.* strain M23 was selected from 328 mutant strains after UV irradiation and NTG treatment and the highest value of cellulase activity 2008 U/mg was achieved.

Thermomyces lanuginosus MC 134 mutant showed a 1.5 fold increase in xylanase production on oat spelts xylan as compared to wild strain (Kuuttanpillai

et al., 2009). An alkali tolerant cellulase free xylanase producer *Pseudomonas sp.* (WLI-11) was mutated by using UV irradiation and NTG treatment and it produced xylanase (1245 U/ml) (Xu *et al.*, 2005).

After mutation with physical as well as chemical mutagens used in the present study, the overall significant percent increase or decrease in cellulase and xylanase production had been observed presented in fig 6, 7, 8 and 9. Some of the isolates after mutation showed an escalation in enzyme units, while others dipped down as compared to their respective wild strains. As far cellulase is concerned, mutants of S₇ (M) and S₁ (M) showed the 19.47 and 9.02 percent increase respectively (Fig 6).

Kd₁ (M) exhibited 61.79 and in N₁₁ (M) i.e 7.74 percent increase in xylanase production with UV irradiation (Fig 7).

Fig. 8 showed the overall percent increase / decrease in cellulase activity when bacterial isolates were subjected to chemical mutation by EtBr, N₁₂ (M) gave 129.40 percent, S₁₂ (28.03), S₇ (122.37), NS₁ (59.91) and S₁ (23.24) percent increase in cellulase activity, while others showed a decrease in it with this treatment. On the other hand in case of xylanase activity during mutation with EtBr, isolate Kd₁ (M) had an increase of 13.50 and N₁₁ for 40.89 percent, while SL₈ showed a decrease in xylanase activity as shown in fig 9.

Finally the mutants of Kd₁ (UV mutated) for xylanase production and N₁₂ (EtBr mutated) for cellulase production were selected for further study on the basis of highest enzymes secreted by them.

4.2.2.3 Hereditary stability studies of mutants

The selected mutants i.e. N₁₂ (M) for cellulase and Kd₁ (M) for xylanase production were found to stable for 8 generations. As the Table 2 showed cellulase and xylanase activities of the mutants upto 8 generations.

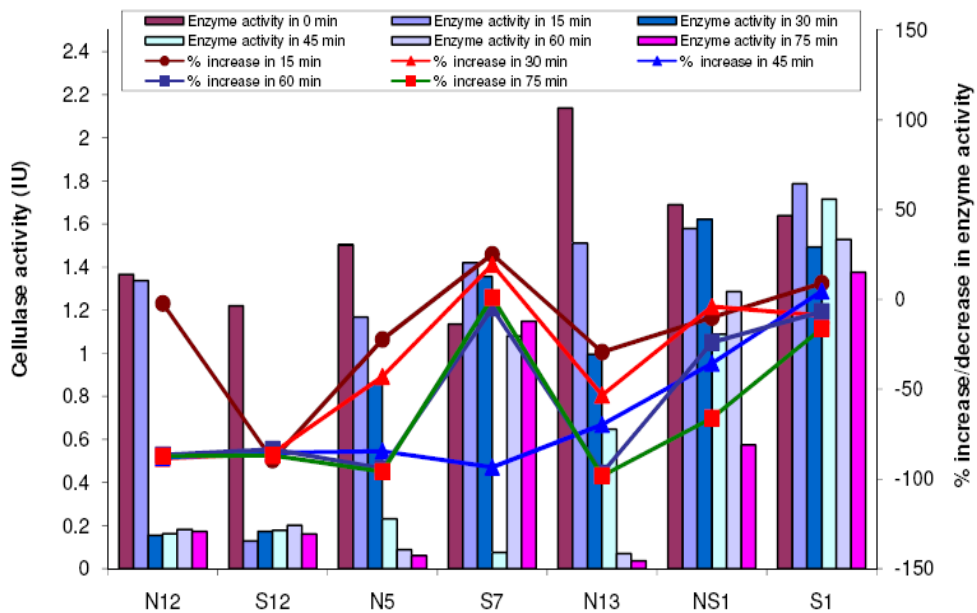


Fig 6. A comparison of cellulase activity in UV mutated potential bacterial strains in SmF

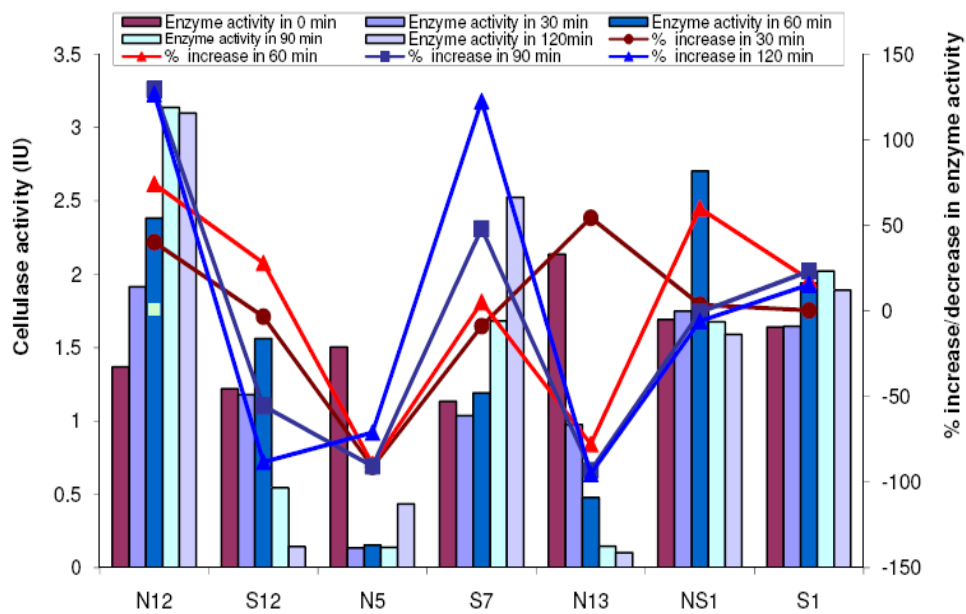


Fig 7. A comparison of cellulase activity in EtBr mutated potential bacterial strains in SmF

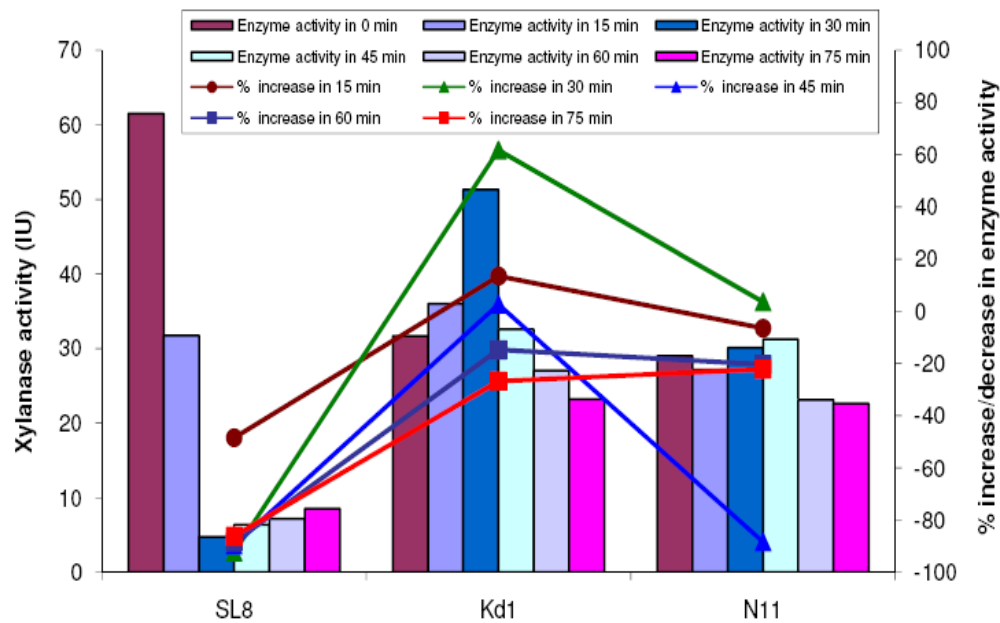


Fig 8. A comparison of xylanase activity of UV mutated potential strains in SmF

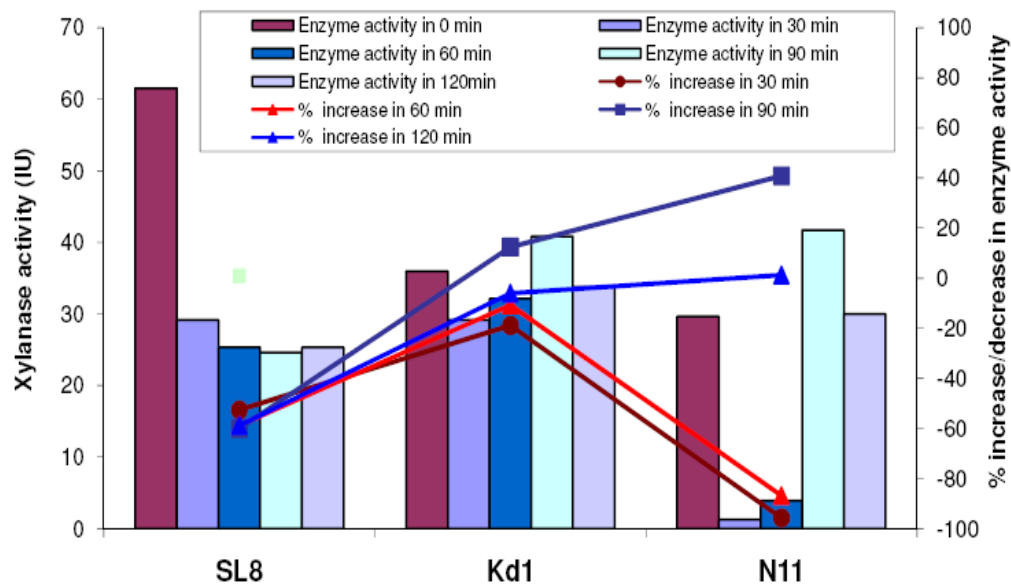


Fig 9. A comparison of xylanase activity of EtBr mutated potential bacterial strains in SmF

Table 2. Total cellulolytic and xylanolytic activities of mutants

Mutants	Total cellulase/ xylanase (IU/ml)			
	Generation-2	Generation-4	Generation-6	Generation-8
N ₁₂ (M) (cellulose activity)	5.940±0.05	5.845±0.09	5.902±0.05	5.914±0.04
Kd ₁ (M) (xylanase activity)	94.24±1.00	92.50±2.29	93.98±1.80	94.01±1.50

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

4.3.1 Phenotypic Identification of Bacterial Isolates

Initially, the bacterial isolates, N₁₂ (Wild), N₁₂ (Mutant), Kd₁ (Wild) and Kd₁ (Mutant) were tentatively identified on the basis of their phenotypic and biochemical characteristics in our research laboratory (Plate1). On the basis of phenotypic and biochemical characters N₁₂ (W), N₁₂ (M), Kd₁ (W) and Kd₁ (M) were placed under the group of Genus *Bacillus* sp. as per Bergey's Manual of Determinative Bacteriology, 7th Edn.

4.3.2 Genotypic Characterization

These four strains were identified at genomic level by using 16S rRNA gene technique. Genomic DNA of both isolates was isolated by using DNA purification kit (Banglore Genei, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). 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 2). Amplified PCR products were purified and got sequenced by the services provided by Xcelris Labs Ltd, Ahmdabad- India to confirm the results.

4.3.2.1 Nucleotide Sequencing

Following sequences of isolates N₁₂ (W), N₁₂ (M), Kd₁ (W) and Kd₁ (M) were obtained after sequence analysis.

Sequence of N₁₂ (W)

>Contig

GAAGGGAGCTTGCTCGCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACTG
CCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACC
GCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCA
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTT
TCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAAGTCTTGACCT
TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAACTTGAG
TGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGT
TAGGGGGTTTCCGCCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGT
ACGGTTCGCAAGACTGAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTGCAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACC
CTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTG
CCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAG
AACAAAGGGCTGCGAGACCGCAAGGTTTAGCCAATCCACAAATCTGTTCTCAGTTCGG
ATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCTCGGGCCTTGTACACACCGCCGTCACACCACGAGAGTTTG
CAAC

N₁₂ (W)_8F_S6980_B05_045 (Sequence with Forward primer:723 bp)

GAAGGGAGCTTGCTCGCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACTG
CCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACC
GCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCA
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTT
TCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAAGTCTTGACCT
TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAACTTGAG
TGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATT

N₁₂ (W)_1492R_S6980_C05_043 (Sequence with Reverse primer:731 bp)

GTTGCAAACCTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTACCG
CGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTG
CGATCCGAACTGAGAACAGATTTGTGGGATTGGCTAAACCTTGCGGTCTCGCAGCCCTT
TGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGTTTGTACCGGCAGTCACCTTAGAGTGCCCAACTGAATG

CTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
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CCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCC
CCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTCG
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GTGTTCTCTCCACATCTCTACGCA

Sequence of N₁₂ (M)

> Contig

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AGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGC
AAGAGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCA
GGCGGTTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG
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GTGGAGGAACACCAAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAAAG
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ATGCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACC
GCAAGGTTTAGCCAATCCACAAATCTGTTCTCAGTTCCGATCGCAGTCTGCAACTCGACTG
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N₁₂ (M) 8F_S6980_D05_041 (Sequence with Forward primer: 803 bp)

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GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAA
AGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTG
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CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
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CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTTCCGCCCTTAG
TGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGA

N₁₂ (M)_1492R_S6980_E05_039 (Sequence with Reverse primer: 686 bp)

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TCCCCACCTTCCTCCGGTTTGTACACGGCAGTCACCTTAGAGTGCCCAACTGAATGCTG
GCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA
GCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGGAAAGCCCTATCTCTA
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CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCG
TACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCT
AACACTTAGCACTCATCGTTTACGGCGTGACTACCAGGGTATCTAATCCTGTTGCTC
CCCACGCTTTTCGCTCCTCAGCGTCAGTTACAGACCAG

Sequences of Kd₁ (W)

>Contig

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CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGC
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
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AAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGT
GGCGAAGGCGACTCTCTGGTCTGTAACCTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
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CCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTTCGCAAGACTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCTTCG
GGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTAAAG
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GGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCGCAAGGTTTAGCCAAT
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TCACACCACGAGAG

Kd₁ (W)_8F_S6980_F02_006 (Sequence with Forward primer: 880 bp)

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TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGT

GCAAGAGTAACTGCTTGACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT
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 AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGT
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Kd₁ (W)_1492R_S6980_G02_004 (Sequence with Reverse primer:873 bp)

CTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGC
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 CCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACAC
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Sequences of Kd₁ (M)

>Contig
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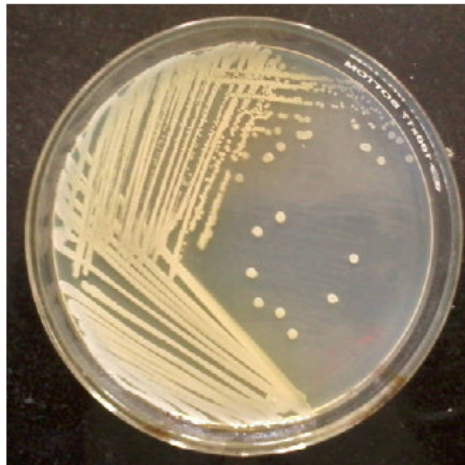
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TGACGGGGGCCCCGACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
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Kd₁ (M)_1492R_S6980_A03_031 (Sequence with Reverse primer:881 bp)

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CTCCGGTTTGTACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGAT
CAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA
CCATGCACCACCTGTCACTCTGTCCCCGAAGGGAAAGCCCTATCTCTAGGGTTGTGAGA
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CGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAG
GCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGC
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CGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCTCCACA
TCTCTACGCATTTACCGCTACACGTGGAATTCCAATCTCTCTTCTGCACTCAAGTTT
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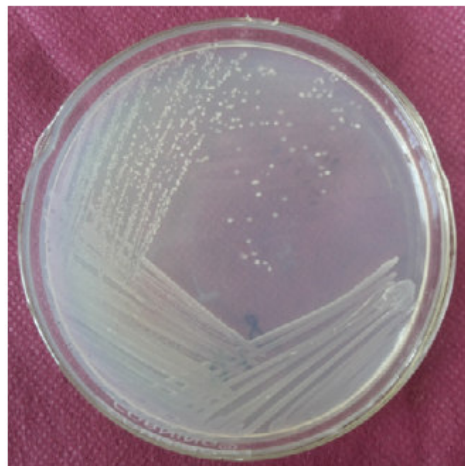
Sequences of four isolates so obtained were submitted to NCBI database and matched with already existing sequences. Sequence similarity search (mBLAST, NCBI) for the N₁₂ (Wild) and N₁₂ (Mutant) showed 99 % homology with nucleotide sequence of *Bacillus stratosphericus* strain SH150 and *Bacillus*



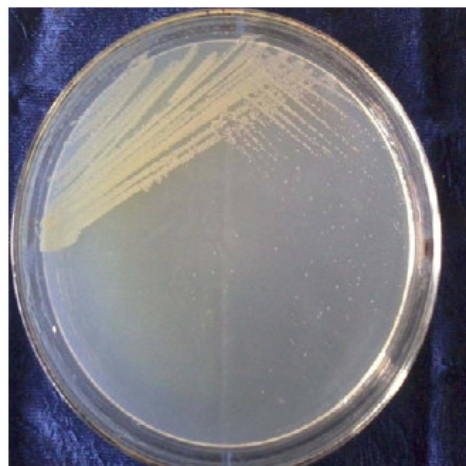
a) *B. altitudinis* Kd₁ (W)



b) *B. altitudinis* Kd₁ (M)

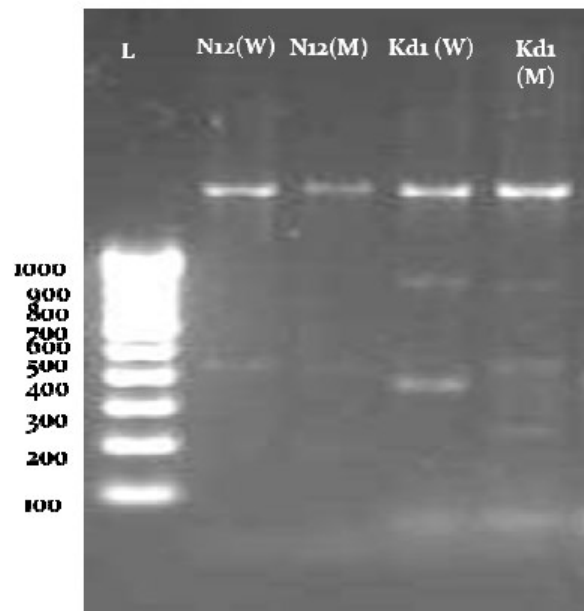


c) *B. stratosphericus* N₁₂ (W)

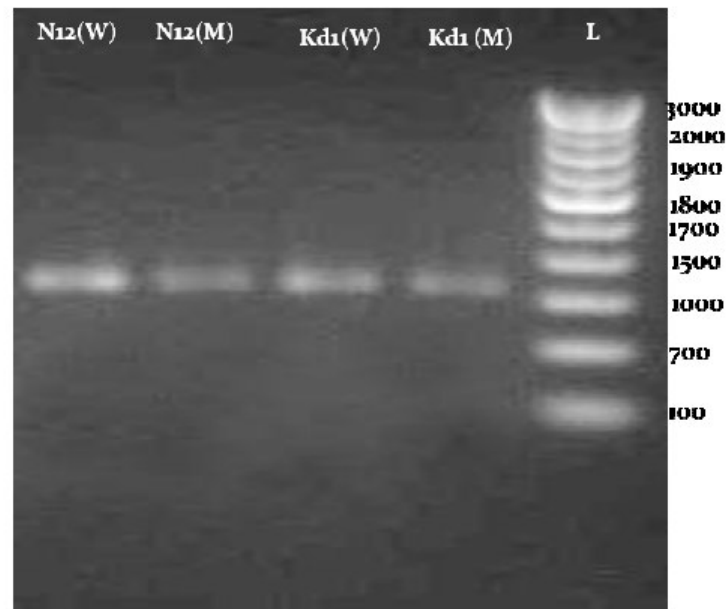


d) *B. stratosphericus* N₁₂ (M)

Plate 1. Morphology of wild (W) and mutant (M) strains of hyperenzyme producing bacterial isolates



Genomic DNA



PCR Product

Plate 2. Molecular identification of screened bacterial isolates by 16S rRNA gene technique

stratosphericus strain SH130 as revealed in Table 3 and 4. Whereas Kd₁ (Wild) showed 99% species specific alignment with *Bacillus altitudinis* strain MD02 and Kd₁ (Mutant) showed 99% species specific alignment with *Bacillus altitudinis* strain H34 (Table 5 and 6).

Table 3. Sequence alignment using BLAST and genotyping of finally screened *Bacillus stratosphericus* N₁₂ (W) by 16S rRNA gene Technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
[KC172055]	<i>Bacillus stratosphericus</i> strain SH150	2508	2508	100%	0.0	99.0%

Table 4. Sequence alignment using BLAST and genotyping of finally screened *Bacillus stratosphericus* N₁₂ (M) by 16 S rRNA gene Technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
[KC172047]	<i>Bacillus stratosphericus</i> strain SH130	2459	2459	100%	0.0	99.0%

Table 5. Sequence alignment using BLAST and genotyping of finally screened *Bacillus altitudinis* Kd₁ (W) by 16 S rRNA gene Technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
[KC247686.1]	<i>Bacillus altitudinis</i> strain MD02	2425	2425	100%	0.0	99.0%

Table 6. Sequence alignment using BLAST and genotyping of finally screened *Bacillus altitudinis* Kd₁ (M) by 16 S rRNA gene Technique

Accession No.	Sequences producing significant alignments					
	Description	Max Score	Total Score	Query coverage	E value	Max Ident
[KC441770.1]	<i>Bacillus altitudinis</i> D34	2425	2425	100%	0.0	99.0

The 16SrRNA sequences of these isolates have been registered under gene bank databases with accession numbers i.e. *Bacillus stratosphericus* N₁₂ (W) [KC995116], *Bacillus stratosphericus* N₁₂ (M) [KC995118], *Bacillus altitudinis* Kd₁ (W) [KC995115] and *Bacillus altitudinis* Kd₁ (M) [KC995117]. Phylogenetic trees of *Bacillus altitudinis* Kd₁ (W), *Bacillus altitudinis* Kd₁ (M), *Bacillus stratosphericus* N₁₂ (W) and *Bacillus stratosphericus* N₁₂ (M), with

respect to other *Bacillus sp.* bacteria as inferred by neighbor joining method have been presented in Fig 10, 11, 12 and 13.

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 by 16S rRNA technique using PCR with universal primers and species specific primer. 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).

In a similar study conducted by Yadav *et al.* (2012), culturable mesophilic (37°C) and thermophilic (60°C) cellulose degrading bacterial flora were isolated from weathered wood samples. Cellulase activities were checked on plate by Congo red staining method. Molecular characterization was determined by phylogenetic analysis of the 16S rRNA sequence by various bioinformatics tools like BLAST, ClustalW, Drawtree etc. The results showed 93% resemblance with *Enterobacter luduwigi*, *Leclercia adecorboxylata*, *Enterobacter cloacae* and Endophytic bacterium.

Two cellulolytic bacterial strains were isolated from the gut of an estuarine fish *Etroplus suratensis*. The strains were identified through basic conventional and also through 16S rRNA gene sequencing methods and they were identified as *Bacillus altitudinis* and *Bacillus licheniformis* (Sreeja *et al.*, 2013).

Sadhu *et al.* (2013) screened out cellulase producing bacteria from herbivorous animal fecal matter and the bacterium was identified as *Bacillus sp.* using 16S rDNA based molecular phylogenetic approach. A novel Gram-positive, slightly halophilic, catalase and oxidase-positive, endospore forming, motile, aerobic, rod-shaped bacterium, designated strain JSM 081003T, was isolated

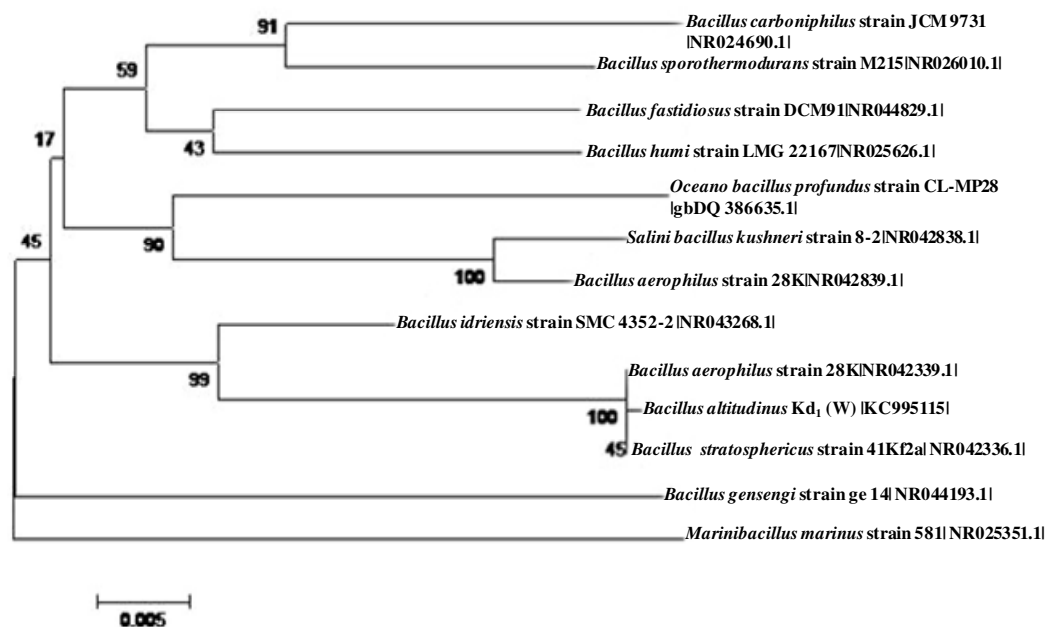


Fig10. Neighbour- joining tree constructed in Clustal X showing phylogenetic relationship of *Bacillus altitudinis* Kd₁ (W) [KC995115] among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The number at the nodes show the bootstrap values obtained with 100 replicates.

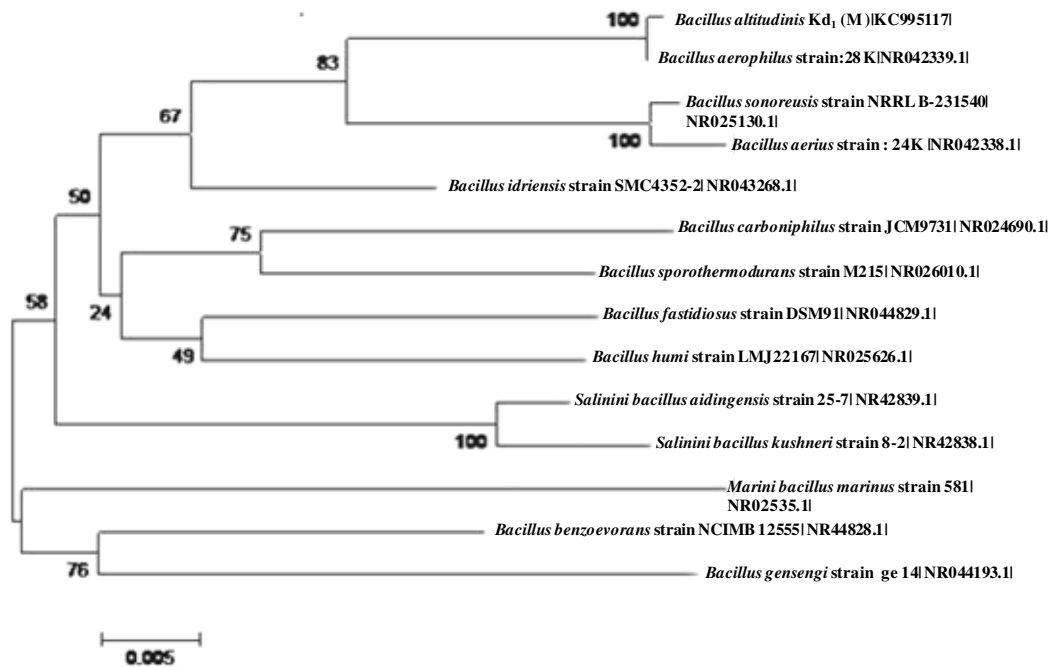


Fig 11. Neighbour- joining tree constructed in Clustal X showing phylogenetic relationship of *Bacillus altitudinis* Kd1 (M) (KC995117) among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The number at the nodes show the bootstrap values obtained with 100 replicates.

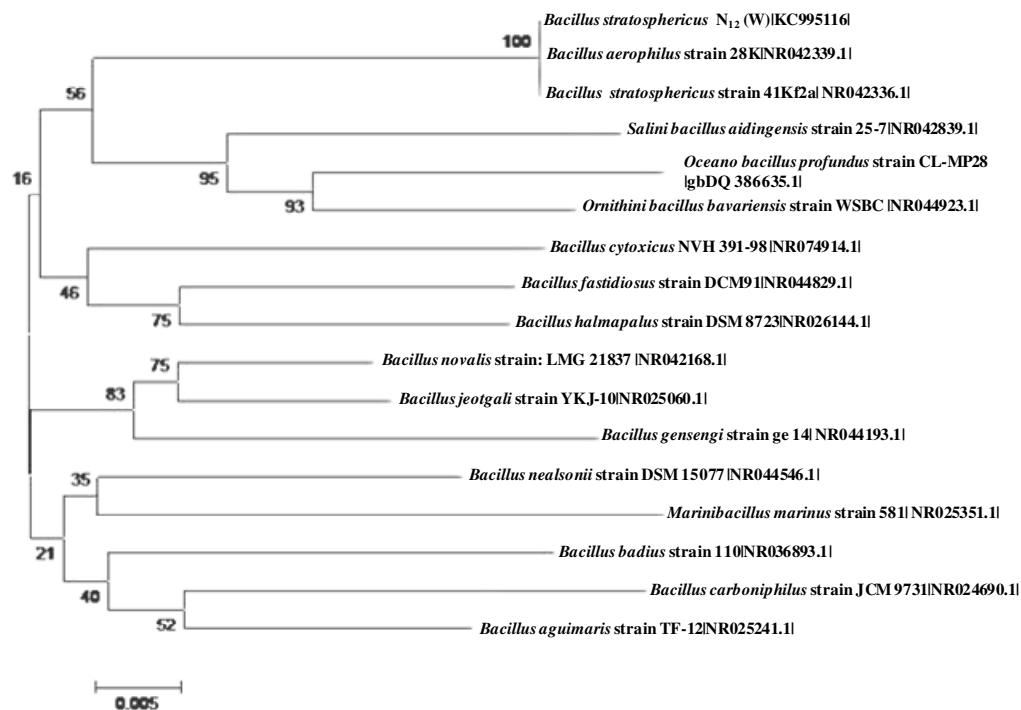


Fig 12. Neighbour- joining tree constructed in Clustal X showing phylogenetic relationship of *Bacillus stratosphericus* N₁₂ (W) |KC995116| among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The number at the nodes show the bootstrap values obtained with 100 replicates.

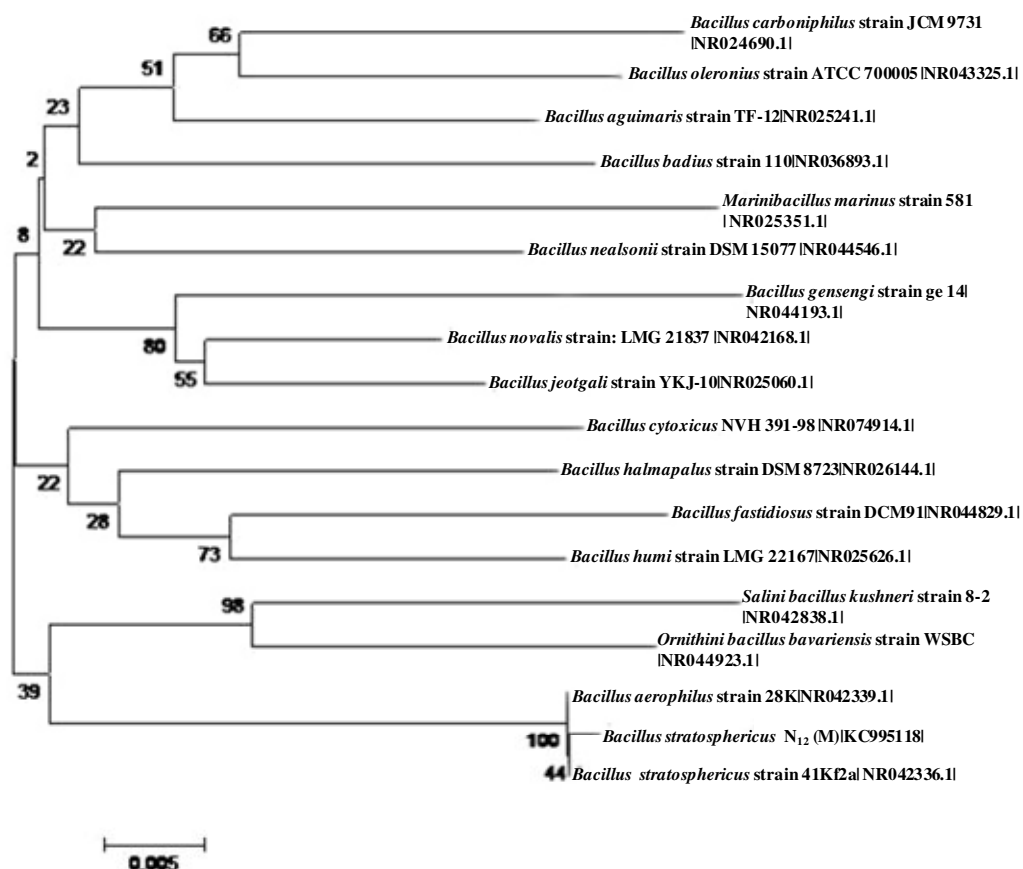


Fig 13. Neighbour- joining tree constructed in Clustal X showing phylogenetic relationship of *Bacillus stratosphericus* N₁₂ (M) [KC995118] among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The number at the nodes show the bootstrap values obtained with 100 replicates.

from non-saline forest soil. A phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain JSM 081003T should be assigned to the genus *Bacillus*, and was related most closely to the type strains of *Bacillus lehensis* (sequence similarity 99.6%), *Bacillus oshimensis* (99.4%) and *Bacillus patagoniensis* (96.6%); lower than 96.0% sequence similarity was observed with other *Bacillus* species (Chen *et al.*, 2010).

4.4 ISOLATION AND SCREENING OF CELLULOLYTIC AND XYLANOLYTIC FUNGI FROM SOIL

4.4.1 Isolation and collection of cellulolytic and xylanolytic fungi

Different types of fungi isolated from soil with their morphological and cultural characteristics have been shown in Table 7. These showed different mycelial characteristics and spore morphology which includes the spore colour and its texture. The fungal isolate NF₁ had septate mycelium, black colour and rough, globular and tightly packed conidia and was tentatively identified as *Aspergillus niger*. NF₂ due to green colour and feathery mycelium was identified as *Trichoderma harzianum*. NF₃ was having purple coloured, sickle-shaped macroconidium and had been identified as *Fusarium oxysporum*. NF₄ have non-septate, abundant cottony mycelium, blackish-brown sporangia and was identified as *Rhizopus* sp. whereas NF₅ showed green and rough mycelium and was identified as *Penicillium* sp. (Frazier, 1958).

Seventy fungal strains were isolated from dairy manure soil by Devi and Kumar (2012) and among them ten isolates showed lignocellulolytic activity. Among them *Aspergillus*, *Penicillium*, *Trichoderma*, *Alternaria* had high cellulolytic activity as compared to the others. Fifty-three litter degrading fungal species were isolated by nylon litterbag technique. The production of xylanase and protease was studied using oat spelt xylan as carbon source and all fifty-three fungal species produced fairly good xylanolytic enzyme activity (Palaniswamy *et al.*, 2008). Fungi isolated from the rumen of three different ruminants (cow, sheep, and goat) were species of *Fusarium* (21.2%), *Penicillium* (23.4%), *Aspergillus* (14.7%) and *Mucor* (40.6%). These were examined for their ability to hydrolyze cellulose (Okusanmi and Oyeleke, 2008).

Filamentous fungi isolated by Damaso *et al.*, (2012) from diverse substrates to obtain the strains with potential to produce the hydrolytic enzymes. From a total of 215 strains, seven strains from the soils, six from the plants and one from sugarcane bagasse were selected and identified as belonging to the *Trichoderma*, *Penicillium* and *Aspergillus* genera.

Narasimha *et al.* (1998) isolated *Aspergillus* and *Penicillium* spp. from soil contaminated with cotton ginning effluents. Similarly reports were made by other authors; Jahangeer *et al.* (2005) reported that majority of *Aspergillus* and *Penicillium* spp. was found to possess cellulolytic activity. The most active producer of cellulolytic enzymes was *A. terreus* compared to *P. tigrinus*, *P. ostreatus* and *F. fomentarius* (Mirzaakhmedov *et al.*, 2007).

Table 7. Isolation and collection of cellulolytic and xylanolytic fungi from soil and their morphological and cultural characteristics

Fungal Isolate	Source	Mycelium	Spore		
			Colour	Texture	Tentative identification
NF ₁	Solan	hyphae(long)	Black	Rough	<i>Aspergillus niger</i>
NF ₂	Shimla	hyphae (long)	Green	Feathery	<i>Trichoderma harzianum</i>
NF ₃	Solan	filamentous	Purple	Feathery	<i>Fusarium oxysporium</i>
NF ₄	Solan	short hyphae	Brown	Rough	<i>Rhizopus</i> spp.
NF ₅	Kangra	short hyphae	Green	Rough	<i>Penicillium</i> sp.
SH1	In house	Short hyphae	Light Brown	Rough	<i>Myceliophthora thermophila</i> SH1

4.4.2 Screening of Hypercellulolytic and Hyperxylanolytic Fungi

Table 8 depicted the screening of hypercellulolytic and hyperxylanolytic fungi isolated from soil. Among all isolates, *M. thermophila* SH1 exhibited the



Plate 3. Morphology of *M. thermophila* SH1

highest cellulase as well as xylanase production i.e. 0.692 IU (CMCase; 0.116, FPase; 0.111, β -glucosidase; 0.466) followed by *Rhizopus* sp. NF₄ i.e. 0.623 IU (CMCase; 0.304, FPase; 0.154, and β -glucosidase 0.165) and xylanase (23.00 IU) followed by *Aspergillus niger* NF₁ i.e. 18.05 IU. The lowest cellulase activity was shown by *Trichoderma harzianum* NF₂ i.e. (0.424 IU) while the least xylanase activity (5.33 IU) was shown by *Fusarium oxysporium* NF₃ and the other isolates produced cellulase and xylanase between these two extremes.

On comparison *M. thermophila* SH1 [IJX124712] a inhouse strain was selected for further study depending upon its highest cellulase as well as xylanase activity. Plate 3 represented the morphology of *M. thermophila* SH1.

Rana and Kaur, (2012) isolated three fungal strains showed good enzyme production but out of the three *Penicillium* spp. had been showed maximum activity of 2.037 U/ml/min which is followed by *Aspergillus* spp. which gave an activity of 0.057 U/ml/min and *Stachybotrys* spp. showing 0.023 U/ml/min. Bhattacharya *et al.* (2013) isolated and screened indigenous fungi for the hydrolysis of water hyacinth in the lignocellulosic conversion process. Cellulase-free xylanase producing fungi were isolated and screened from composting soil by Kamble and Jadhav (2012). In submerged conditions, 25% strains produced xylanase (45 to 205 U/ml) and negligible amount of cellulase (12 to 13 U/ml).

Palaniswamy *et al.*, (2008) reported the production of xylanase and protease using oat spelt xylan as carbon source and results showed that 53 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. Devi and Kumar (2012) reported cellulase production by *Aspergillus* sp. (3.891 IU) and *Trichoderma* sp. (3.880 IU) in the Mandal's medium supplemented with 1% cellulose.

Table 8. Screening of fungal isolates for hypercellulase and xylanase production

Sr. No.	Isolate name	Protein (mg/ml)	CMCase activity		FPase activity		β -glucosidase		Cellulase (CMCase + FPase + β -glucosidase)		Protein (mg/ml)	Xylanase		Total enzyme (cellulase + xylanase)	Total protein (mg/ml)
			Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity		Enzyme activity	Specific activity		
1.	<i>Aspergillus niger</i> NF ₁	2.40	0.333	0.138	0.072	0.030	0.169	0.070	0.574	0.239	1.24	18.05	14.55	18.62	3.64
2.	<i>Trichoderma harzianum</i> NF ₂	1.68	0.241	0.143	0.103	0.061	0.080	0.047	0.424	0.252	1.06	17.20	16.22	17.62	2.74
3.	<i>Fusarium oxysporium</i> NF ₃	1.40	0.206	0.147	0.059	0.042	0.205	0.146	0.470	0.335	1.21	5.33	4.40	5.80	2.61
4.	<i>Rhizopus sp.</i> NF ₄	1.35	0.304	0.225	0.154	0.114	0.165	0.122	0.623	0.461	1.02	9.57	9.38	10.19	2.37
5.	<i>Penicillium sp.</i> NF ₅	1.40	0.208	0.148	0.106	0.075	0.211	0.150	0.525	0.375	1.30	6.79	5.22	7.31	2.70
6.	<i>Myceliophthora thermophila</i> SH1	1.20	0.116	0.096	0.111	0.092	0.466	0.380	0.692	0.568	1.33	23.0	17.29	23.69	2.53

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

Sukumaran *et al.*, 2009 demonstrated the production of a cellulolytic “pool” (FPase, CMCase and β -glucosidase) by semi- solid fermentation using wheat bran and strains from *T. reesei* RUT30 and *Aspergillus niger* MTCC 7956. The results of the FPase, CMCase and β - glucosidase activities were 1.14 U/ml, 14.91 U/ml and 0.22 U/ml, respectively, for *T. reesei* RUT C 30 strain and for *A. niger* were 0.24, 6.77 and 2.84 U/ml, respectively. In the other study xylanase activity (35.0 IU) was observed after 96 h at 35⁰C by *Aspergillus terreus* UL 4209 strains in shake flask culture (Chidi *et al.*, 2008). Khan *et al.*, 2003 similarly screened *Aspergillus strains* i.e. isolated from soil for xylanase production under submerged fermentation and xylanase activity ranged from 30-225 IU.

4.5 OPTIMIZATION OF CELLULASE AND XYLANASE BY POTENTIAL MICRORGANISMS

4.5.1 Effect of Different Media

The data pertaining to effect of different media on cellulase and xylanase production enlisted in Table 9 and 10 and had revealed that highest cellulase activity was found in the Peptone Yeast extract Cellulose medium (PYC) i.e. 3.230 IU for *B. stratosphericus* N₁₂ (M) while least enzyme production 2.211 IU was observed in Okoshi *et al.*, medium (Fig.14). A perusal of the data revealed that maximum production in PYC was statistically significant over Modified BSM (2.580 IU) followed by Li and Gao medium (2.472 IU), Basal salt medium (2.472 IU) and Mandel and Reese medium. Similarly, the maximum xylanase activity was observed in TGY medium i.e. 56.83 IU for *B. altitudinis* Kd₁ (M) followed by Basal salt medium (51.57 IU) which was significantly higher than other media. Least xylanase production i.e. 18.80 IU was observed in *Bacillus* xylose medium (Fig. 15).

On the other hand strain *B. altitudinis* Kd₁ (M) showed maximum growth in TGY medium containing Yeast extract (0.5%), tryptone (0.5%), K₂HPO₄ (0.1%), glucose (1.0%). Highest xylanase production using defined medium may be due to the presence of nitrogen, carbohydrate and other compounds in appropriate concentration as per physiological requirement of this organism that

would have resulted in enhancing the ability of the cells to synthesize most of extracellular xylanase (Basar *et al.*, 2010).

Table 9: Effect of different media on extracellular cellulase production from *Bacillus stratosphericus* N12 (M) under SmF

Media	Cellulase(IU)								
	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
Basal salt medium	2.34	0.060	0.027	0.556	0.237	1.850	0.789	2.472	1.055
Okoshi et al., medium	1.64	0.046	0.028	0.571	0.348	1.593	0.970	2.211	1.343
Li and Gao medium	1.30	0.012	0.009	0.544	0.418	1.915	1.473	2.472	1.901
PYC medium	1.95	0.175	0.089	1.170	0.599	1.885	0.996	3.230	1.653
Mandel and Reese medium	1.70	0.010	0.005	0.561	0.322	1.873	1.076	2.445	1.403
Modified BSM	2.52	0.094	0.036	0.764	0.302	1.721	0.682	2.580	1.023
C.D. _{0.05}	0.03	0.013	0.007	0.021	0.009	0.115	0.052	0.141	0.062
S.E.(Mean)	0.01	0.006	0.003	0.010	0.004	0.053	0.024	0.064	0.028

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

Specific activity : enzyme activity/mg of protein

Table 10. Effect of different media on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

Media	Protein(mg/ml)	Xylanase activity(IU)	Specific activity
Xylan media	4.66	23.94	5.127
X.Heck media	4.05	33.86	8.350
TGY media	5.96	56.83	9.523
Emmersion media	3.39	27.62	8.140
Basal salt media	3.32	51.57	15.53
Bacillus xylose media	2.34	18.80	7.990
C.D. _{0.05}	0.138	3.728	0.899
S.E.(Mean)	0.063	1.711	0.412

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

Specific activity : enzyme activity/mg of protein

The concentration of different nutrients as well as concentration in a particular medium has influenced the production of cellulase and xylanase by

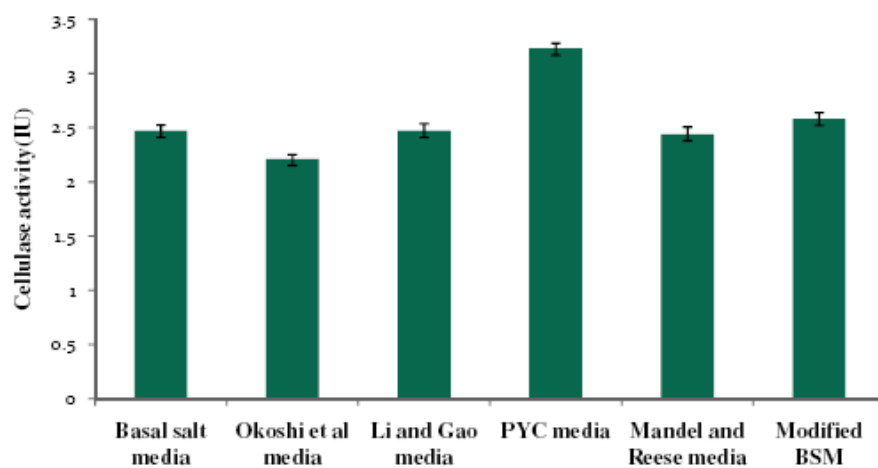


Fig 14. Effect of media on cellulase production from *B. stratosphericus* N₁₂ (M)

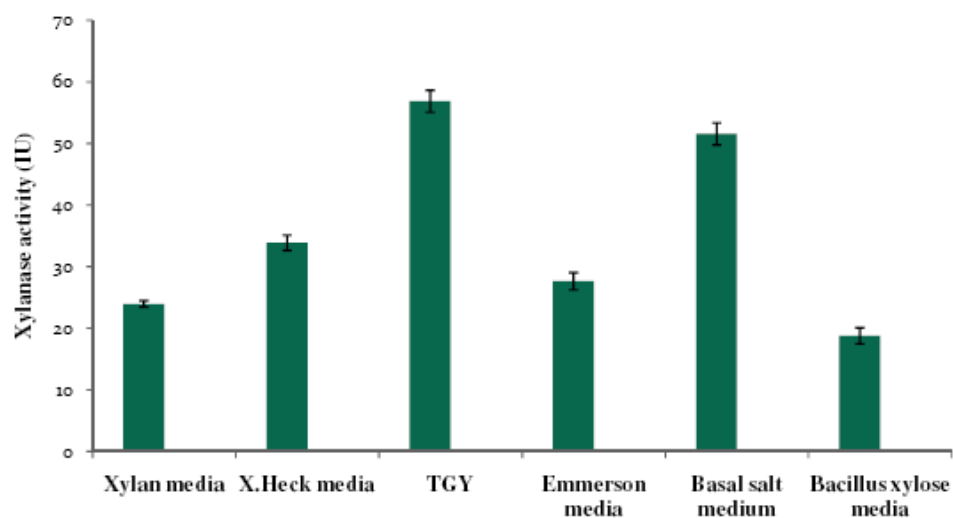


Fig 15. Effect of media on xylanase production from *B. altitudinis* Kd1 (M)

these bacterial isolates. The strain *B. stratosphericus* N₁₂ (M) showed maximum growth in the PYC medium containing carboxy-methyl cellulose (1.0%), sodium chloride (0.05%), yeast extract (0.5%), potassium hydrogen phosphate (0.3%), magnesium sulphate (0.02%) and peptone (0.5%) as growth supplements in the medium that seemed to have promoted extracellular cellulase 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.

TGY medium supported highest production of xylanase in case of *B. altitudinis* Kd₁ (M). Effect of glucose and tryptone added in TGY seemed to induce high titers of xylanase. Glucose present in medium must have served as a ready utilizable carbon source leading to higher growth of *B. altitudinis* Kd₁ (M) consequently causing more synthesis of extracellular xylanase. Beside, tryptone has stimulating effect causing highest production of xylanase as nitrogen sources play a significant role in promoting the extracellular release of enzyme.

Thus medium formulation is a crucial step for designing successful laboratory experiments to enhance the yield of enzyme. It clearly reflects that medium components must satisfy the elemental requirement for cell biomass and enzyme production.

When critically observed the respective best media for *B. stratosphericus* N₁₂ (M) and *B. altitudinis* Kd₁ (M), PYC and TGY medium had contained yeast extract and tryptone which was the most important source of nitrogen in the medium in terms of accessibility and composition to get the higher yield of cellulase and xylanase (Sharma *et al.*, 2005). Since nitrogen is a major constituent of protoplasm and building block of the enzymes. On the other hand glucose act as the best carbon source because the glucose is the good inducer of cellulase as well as xylanase production.

Paenibacillus sp. N1 showed maximum xylanase production in Basal salt medium i.e. 27.20 IU supplemented with different nutrients i.e. (NH₄)SO₄ (0.6%),

KH₂PO₄ (0.3%), NaCl (0.05 %), NH₄Cl (0.1%), Xylose (0.1%), CaCl₂ (0.02%), and 1 M MgSO₄ (0.001%) at 50⁰C in 5 days incubation (Pathania *et al.*, 2012).

Narasimha *et al.* (2006) compared the production of cellulase by *Aspergillus niger* on three media in liquid shake culture and exhibited relatively highest activity of cellulase and extracellular protein content at 7-day interval during the course of its growth on Czapek-Dox medium supplemented with 1.0% (w/v) cellulose. Maximum cellulase productivity was exhibited when molasses broth medium was supplemented with cellulose as a carbon source and (NH₄)₂SO₄ as a nitrogen source (Shabeb *et al.*, 2010). The cellulase producing bacteria were isolated from cow dung and were grown on carboxymethyl cellulose medium and three substrates i.e. coir waste, saw dust and CMC was optimized for cellulase production. Among these three substrates CMC was found to be the best substrate for cellulase production when compared with others (Shanmugapriya *et al.*, 2012).

4.5.2 Effect of pH

Table 11 and 12 limned the effect of pH of the medium for cellulase and xylanase production of *B. stratosphericus* N₁₂ (M) and *B. altitudinis* Kd₁ (M). Cellulase and xylanase production was estimated at 5.0, 5.5.....9.0. A perusal of the data revealed that maximum production of cellulase in *B. stratosphericus* N₁₂ (M) was observed at pH 8.0 i.e. 3.370 IU which was significantly higher than others statistically and minimum was observed in pH 5.0 i.e. 1.16 IU (Fig16). The highest xylanase titers were observed at pH 5.5 for *B. altitudinis* Kd₁ (M) i.e. 59.09 IU followed by 58.72 IU at pH 6.5 which were also statistically significantly higher than others and least xylanase titers were observed at pH 9.0 i.e. 25.48 IU (fig 17).

Thus *B. stratosphericus* N₁₂ (M) has emerged as an alkalophile showing highest enzyme production at pH 8.0. In contrast, *B. altitudinis* Kd₁ (M) has shown acidophilic nature by exhibiting maximum release of enzyme in acidic range i.e. 5.5.

Since pH of the medium influences the growth of microorganisms and hence the enzyme production. Each microorganism possesses a specific pH range for its growth and activity. 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).

If the cultivation of the organisms is carried out at an unfavorable pH, it may limit their growth as well as enzyme production. Enzymes have an optimum pH in which their activity is maximum and at higher and lower pH values, their activities decreases because pH of the culture medium affects the availability of certain metabolic ions and permeability of cell membranes. The preferable pH range of *B. stratosphericus* N₁₂ (M) - alkaline and *B. altitudinis* Kd₁ (M) – acidic respectively are of special interest because enzymes from microorganisms can also survive under extreme pH and that could be particularly useful for applications under highly acidic or highly alkaline reaction conditions in the industry.

Table 11: Effect of different pH on extracellular cellulase production from *Bacillus stratosphericus* N12 (M) 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
5.0	1.08	0.122	0.112	0.091	0.084	0.955	0.882	1.169	1.081
5.5	1.66	0.147	0.088	0.062	0.037	1.310	0.788	1.520	0.914
6.0	1.91	0.115	0.059	0.081	0.041	1.206	0.630	1.402	0.733
6.5	1.94	0.118	0.060	0.493	0.230	1.078	0.555	1.646	0.847
7.0	2.16	0.176	0.081	1.114	0.515	1.754	0.810	3.043	1.408
7.5	2.27	0.173	0.075	1.157	0.509	1.827	0.804	3.157	1.390
8.0	1.10	0.185	0.168	1.355	1.231	1.830	1.661	3.370	3.060
8.5	1.25	0.187	0.149	0.770	0.616	1.769	1.412	2.727	2.179
9.0	1.17	0.153	0.130	0.404	0.344	1.717	1.466	2.274	1.941
C.D. _{0.05}	0.040	0.021	0.011	0.167	0.118	0.247	0.153	0.396	0.261
S.E. (Mean)	0.019	0.010	0.005	0.079	0.056	0.117	0.072	0.188	0.124

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

Table 12. Effect of different pH on xylanase production from *B. altitudinis* Kd1 (M) under SmF

pH range	Protein(mg/ml)	Xylanase(IU)	Specific activity
5.0	6.047	56.56	9.510
5.5	6.497	59.09	9.123
6.0	6.060	58.62	9.663
6.5	4.957	58.72	11.93
7.0	6.247	58.49	9.363
7.5	6.413	49.82	7.767
8.0	6.910	43.15	6.237
8.5	3.843	28.07	7.293
9.0	3.827	25.48	6.653
CD0.05	0.7560	3.0041	1.1762
S.E.(Mean)	0.3598	1.4299	0.5598

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

Specific activity : enzyme activity/mg of protein

Song *et al.* (2001) found the optimum pH i.e. 9.0 for cellulase production by *Clostridium acetobutylicum*. Fauzi and Makky (2013) investigated the effect of pH for avicelase production from *Bacillus* sp. and the maximum enzyme activity was obtained at pH 7.0. Kumar *et al.* (2012) observed the maximum cellulase production by *Bacillus subtilis* at pH 8.0 and temperature of 32⁰C. Shankar and Isaiarasu, (2011 and Ariffin *et al.* (2006) reported the maximum cellulase activity at pH 6.0 from *Bacillus pumilus* EWBCM1.

Xylanase of *Bacillus agaradhaerens* have pH optima of 5.6. The growth at pH values above 5.5 decreased xylanase production (Poon *et al.*, 2003). Maximum xylanase production was observed at pH between 3.5 and 5.5. Xylanase isolated from *Bacillus* sp. K-1 and *Bacillus* sp. No.C-125 exhibited an optimum pH of 6.0 (Honda *et al.*, 1985). Li *et al.* (2006) optimized the different process parameter i.e. pH, cultivation time and substrate concentration for endoxylanase production. The optimal conditions were: wheat bran 49.3g/l, pH-4.14 and cultivation time 103.7 h.

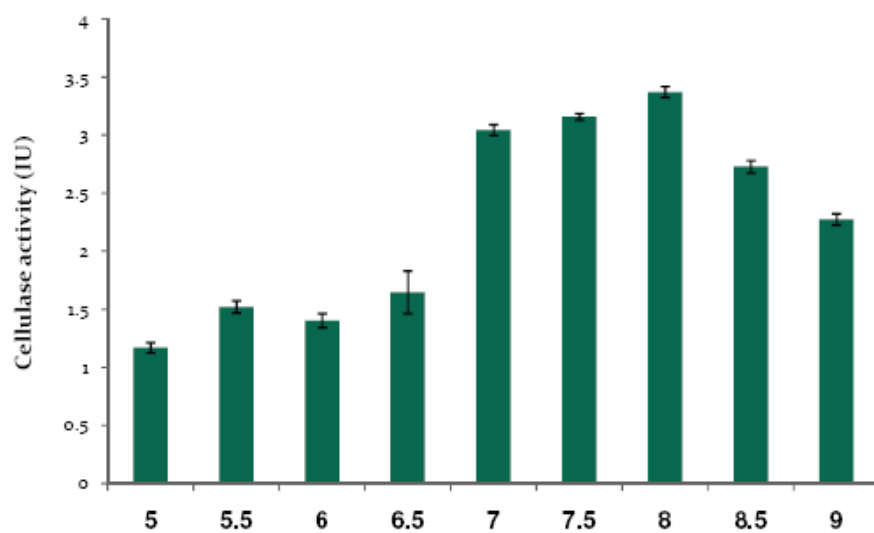


Fig 16. Effect of pH on cellulase production from *B. stratosphericus* N₁₂ (M)

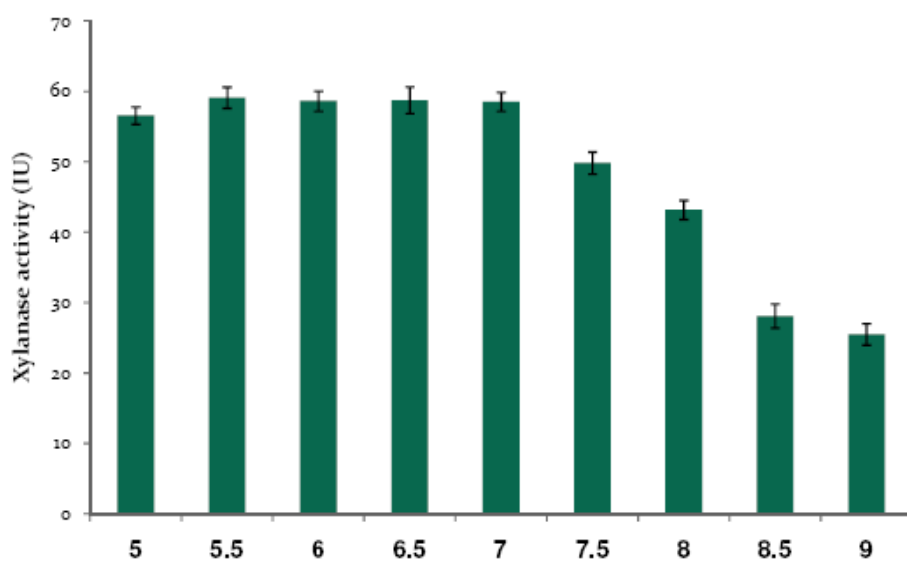


Fig 17. Effect of pH on xylanase production from *B. altitudinis* Kd₁ (M)

Pathania *et al.*, (2012) observed the effect of pH on xylanase production by *Paenibacillus* sp. N1 isolated from hot springs and observed the optimum xylanase production (31.86 IU) at pH 9.0. Maximum yield of xylanase has also been reported at pH 9.0 by *Bacillus* sp. (Anuradha *et al.*, 2007). The effect of initial pH on xylanase production was observed by Bastawde *et al* (1994). Annamalai *et al.*, (2009) observed the optimum pH i.e 9.0 for xylanase production from *Bacillus subtilis*.

4.5.3 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 30°C by *B. stratosphericus* N₁₂ (M) i.e. (3.789 IU) whereas; least cellulase production was found at 45°C (1.082 IU) as shown in Fig.18. Similarly *B. altitudinis* Kd1 (M) also exhibited optimum temperature i.e 30°C (71.33 IU) for maximum xylanase production which is significantly higher than others. Minimum xylanase was produced at 50°C (31.46 IU) as demoed in Fig.19.

Table 13. Effect of different temperature on extracellular cellulase production from *B. stratosphericus* N12 (M) 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	1.87	0.234	0.124	1.235	0.659	0.969	0.517	2.438	1.299
30°C	1.97	0.275	0.139	1.550	0.785	1.964	0.996	3.789	1.919
35°C	2.37	0.206	0.083	1.365	0.576	1.811	0.775	3.383	1.540
40°C	2.87	0.159	0.055	0.381	0.132	1.631	0.567	2.171	0.754
45°C	1.65	0.162	0.097	0.087	0.052	0.832	0.503	1.082	0.652
50°C	2.27	0.143	0.063	0.193	0.084	0.901	0.396	1.238	0.541
CD _{0.05}	0.017	0.161	0.077	0.199	0.091	0.194	0.102	0.494	0.305
S.E.(Mean)	0.008	0.073	0.035	0.091	0.042	0.089	0.046	0.226	0.140

Enzyme activity (IU): μmoles 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. altitudinis* Kd₁ (M) under SmF

Temperature	Protein (mg/ml)	Xylanase activity (I.U.)	Specific activity
25°C	4.217	51.07	12.31
30°C	6.050	71.33	12.12
35°C	5.693	59.62	10.68
40°C	2.750	34.47	13.70
45°C	2.843	33.54	12.80
50°C	4.950	31.46	6.477
C.D. _{0.05}	1.8077	2.8486	5.3616
S.E.(Mean)	0.8296	1.3074	2.4608

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

Incubation temperature is the most important physical factor which affects enzyme production dramatically and their stability. Microorganisms grow slowly at a temperature below or above the normal temperature because of reduced rate of cellular activity. Temperature is a cardinal factor affecting the amount and rate of growth of an organism and increasing temperature has the general effect of increasing enzyme activity but the enzyme begins to suffer thermal inactivation at higher temperature. The reduction in enzyme activity at higher temperature could be due to denaturation of enzymes. Maximum enzyme 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).

Temperature variation is a special feature because it can penetrate physical barrier and can have dramatic effects on the structure of macromolecules and also affects all the levels of biological adaptation (Hickey and Singer, 2004). Similar findings have been reported by Kumar *et al.* (2012) showing increased enzyme activity of 62U/ ml at 30°C and found it as optimum temperature for β -

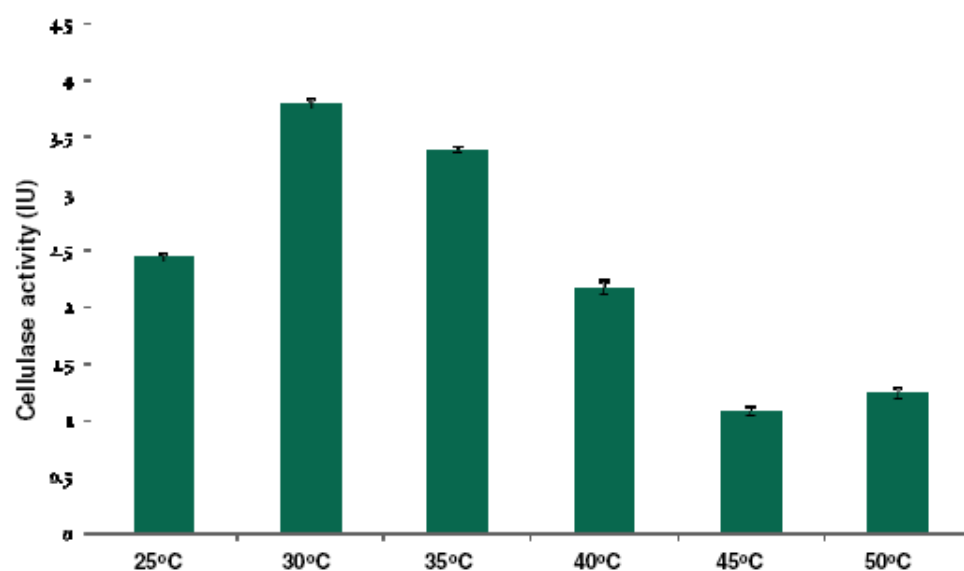


Fig 18. Effect of temperature on cellulase production from *B. stratosphericus* N₁₂ (M)

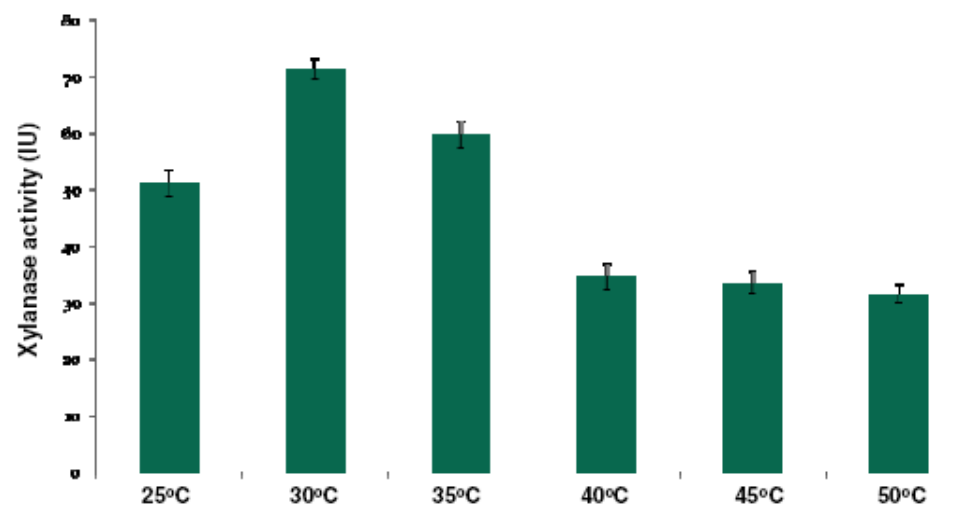


Fig 19. Effect of temperature on xylanase production from *B. altitudinis* Kd₁ (M)

glucosidase production. Sun *et al.* (2011) achieved the maximum cellulase activity (5.86 IU) at 30°C by *Trichoderma viride*.

Optimum temperature recorded for maximum cellulase was 25°C for mutant *Bacillus pumilus* BpCRI6 (Kotchoni *et al.*, 2003), 30°C for *Cellulomonas sp.* and *Bacillus pumilus* (Poorna and Prema, 2007). The effect of temperature on xylanase production by *Fusarium solani* F7 was examined within the temperature range 20- 50°C for 72 h and maximum xylanase production was recorded at the temperature near to the ambient temperature i.e. 30°C with an activity of 58.8 U/ml (Gupta *et al.*, 2009).

4.5.4 Effect of Inoculum Size

Effect of inoculum size on cellulase and xylanase production was evaluated in Table 15 and 16. Different inoculum size used were 2.5%, 5.0 %, 7.5%, 10.0%, 12.5%, 15.0% and 17.5% (V/V). The data revealed that optimum inoculum size found to be 10.0% (V/V) for cellulase production from *B. stratosphericus* N₁₂ (M) revealing 3.808 IU as depicted in Fig 20. Highest xylanase production from *B. altitudinis* Kd₁ (M) i.e. 73.44 IU was obtained at 12.5% of inoculum size having statistically significant differences over other inoculum sizes. The least cellulase and xylanase units were recorded at 15.0% (2.284 IU) and 2.5% inoculum size, respectively. Thus, it is evident from the data that the enzyme titers were significantly affected by different inoculum sizes as shown in Fig 20 and 21.

In the present experiment, inoculum size ranging from 2.5% to 7.5 % probably was 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% (in case of cellulase production) and 12.5% (in case of xylanase production) had improved the titers of cells enzyme reaching to maximum 10.0% (cellulase) and 12.5% (xylanase) showing equilibrium between the number of cells and substrate concentration at this level and enhanced the production of cellulase. Beyond this concentration i.e. @ 12.5% (cellulase) and 15.0% (xylanase) enzymatic activity had dipped due to overload

in the number of cells causing nutrient starvation. It seems that an inoculum size of 2.5% to 7.5% in PYC medium and TGY was under loaded in terms of lesser number of cells leaving most of the nutrients unutilized while size @ 12.5% and 15.0% cause an overload of cell facing nutrient limitation whereas inoculum size of 17.5 % 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% and 12.5% (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.

Similar studies evaluated by various authors revealed that maximum inoculum size 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 cow dung compost and found that 7% inoculum size resulted in maximum enzyme activity $92.747 \mu\text{mg}^{-1} \text{min}^{-1}$.

Table 15. Effect of different inoculum size on extracellular cellulase production from *B. stratosphericus* N₁₂ (M) 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
2.5%	1.93	0.212	0.107	1.184	0.614	1.447	0.752	2.843	1.475
5.0%	2.42	0.255	0.106	1.225	0.578	1.473	0.694	2.954	1.379
7.5%	2.38	0.262	0.109	1.289	0.541	1.504	0.631	3.055	1.281
10.0%	2.35	0.280	0.118	1.567	0.666	1.961	0.834	3.808	1.616
12.5%	1.78	0.264	0.145	1.461	0.816	1.752	0.983	3.477	1.942
15.0%	1.73	0.212	0.119	1.144	0.702	0.927	0.536	2.284	1.313
17.5%	1.70	0.212	0.122	1.188	0.699	1.110	0.653	2.510	1.475
C.D. 0.05	0.70	0.173	0.072	0.103	0.193	0.016	0.218	0.269	0.389
S.E. (Mean)	0.32	0.080	0.033	0.048	0.090	0.007	0.101	0.125	0.181

Enzyme activity (IU): $\mu\text{moles of reducing sugars released/min/ml of enzyme.}$
 Specific activity : enzyme activity/mg of protein

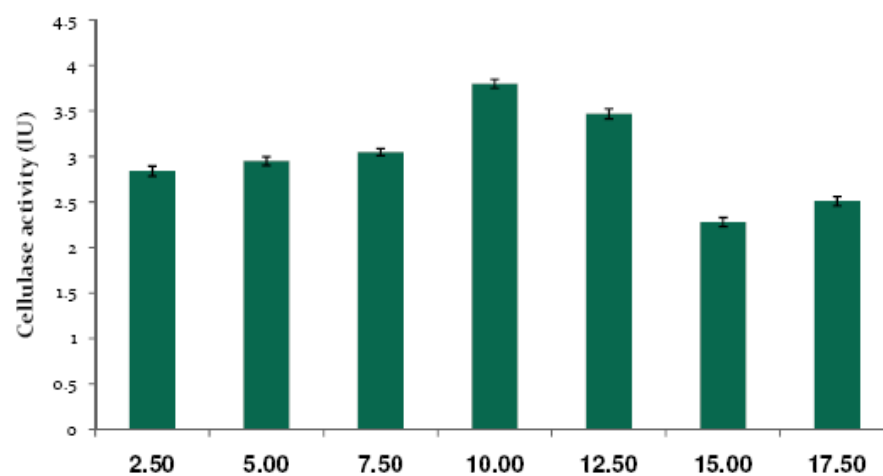


Fig 20. Effect of inoculum size (%) on cellulase production from *B. stratosphericus* N₁₂ (M)

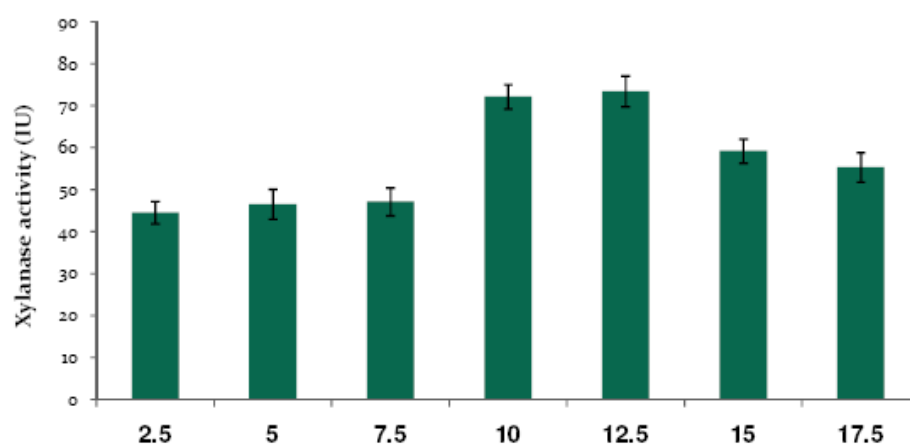


Fig 21. Effect of inoculum size (%) on xylanase production from *B. altitudinis* Kd₁ (M)

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. 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.

Table 16. Effect of different inoculum size on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

Inoculum (%)	Protein(mg/ml)	Xylanase activity (I.U.)	Specific activity
2.5	2.907	44.53	15.32
5.0	4.727	46.55	9.850
7.5	5.447	47.10	8.640
10.0	4.930	72.16	14.630
12.5	5.250	73.44	13.98
15.0	5.103	59.17	11.59
17.5	4.740	55.33	11.67
C.D. _{0.05}	0.1992	1.8976	0.2018
S.E.(Mean)	0.0929	0.8847	0.0941

Enzyme activity (IU): μmoles 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. Effect of inoculum size on xylanase production by *Paenibacillus* sp.N1 was assessed by Pathania *et al.* (2012) and 12.5% was observed as optimum for hyperxylanase (35.85 IU) production. Bacterial strains including *B. licheniformis* A99 and *Bacillus pumilus* ASH 7411 produced highest xylanase when used at 15% inoculum level (Battan *et al.*, 2006).

4.5.5 Effect of Incubation Time

The effect of incubation time on cellulase and xylanase production has been evaluated in Table 17 and 18. Enzyme activity was measured at regular intervals up to periods of 24 to 120h. Highest cellulase activity was measured at 48h (4.259 IU) from *B. stratosphericus* N₁₂ (M) followed by gradual decline on

either side as depicted in Fig 22. Least enzyme production was observed at 24 h (2.34 IU) of fermentation time. Statistically enzyme production was found significantly higher at 48 h than others timings. In case of xylanase, its production was found minimum at 24 h of fermentation time by *B. altitudinis* Kd₁ (M) i.e. 53.51 IU, while maximum xylanase production was noticed at 48 h i.e. 74.45 IU as shown in Fig. 23. At 48 h, the cell population in the culture reaches at its peak which leads to a stable microbial association with the substrate and resulted in maximum enzyme production. The decline in the activity afterwards could be due to various reasons. Cellulase produced by *B. stratosphericus* N₁₂ (M) as well as xylanase produced by *B. altitudinis* Kd₁ (M) were growth associated, reaching to maximum after 48 h at exponential peak and enzyme production decreased upto 96 h.

A decline in the enzyme afterwards may be because of proteolysis or due to depletion of nutrients available to the strain, causing a stressed microbial physiology resulting in an in activation of enzyme (Flores *et al.*, 1997). Another reason could be the product inhibition i.e. the increase in the production of reducing sugars, which after a certain period of growth could exhibit inhibitory effect on enzyme production.

Table 17. Effect of different incubation time on extracellular cellulase production from *B. stratosphericus* N₁₂ (M) under SmF

Incubation time	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
24h	1.86	0.235	0.126	0.805	0.433	1.301	0.700	2.341	1.260
48h	3.82	0.325	0.846	1.614	0.421	2.320	0.605	4.259	1.112
72h	2.95	0.299	0.103	1.578	0.549	2.105	0.734	3.983	1.388
96h	2.36	0.317	0.133	1.555	0.657	2.066	0.874	3.939	1.666
120h	1.28	0.212	0.162	1.203	0.934	1.110	0.908	2.525	1.960
C.D. _{0.05}	0.565	0.076	0.038	0.113	0.087	0.104	0.137	0.272	0.222
S.E.	0.253	0.034	0.017	0.050	0.039	0.047	0.061	0.122	0.100

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

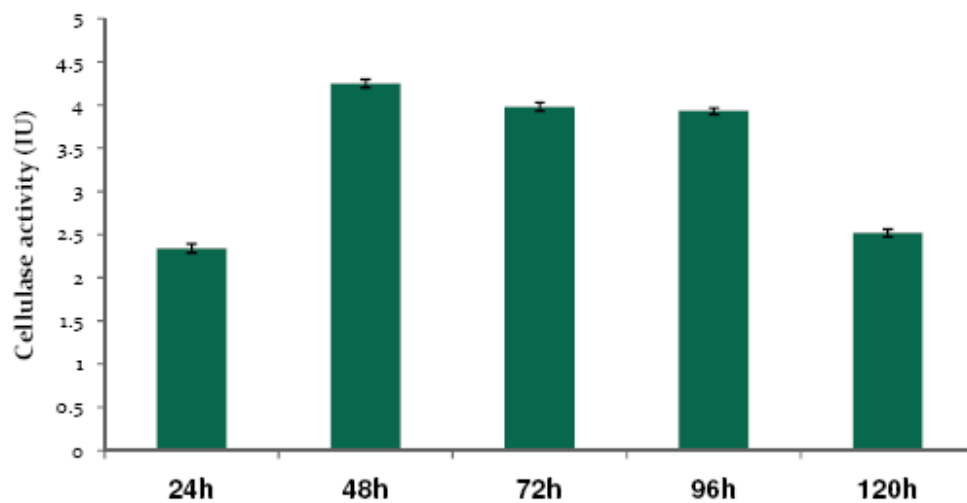


Fig 22. Effect of incubation time on cellulase production from *B. stratosphericus* N₁₂ (M)

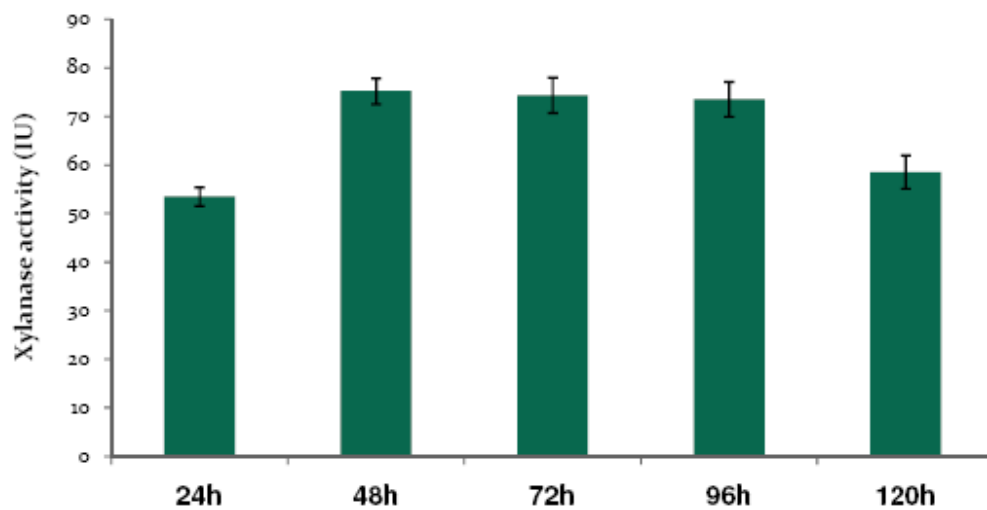


Fig 23. Effect of incubation time on xylanase production from *B. altitudinis* Kd₁ (M)

Shanmugapriya *et al.* (2012) optimized the culture conditions for cellulase production by *Bacillus* sp. and observed the maximum enzyme production at 48 h of incubation, pH-6.0 and temperature of 40°C. Nirmala and Sindhu (2011) recorded maximum cellulase production from *Bacillus* strain at 72 h (5.0 IU/ml) of fermentation time. Shankar and Isaiarasu (2011) reported maximum cellulase production at 72 h (0.5851±0.006 IU/ml) from *Bacillus pumilus* EWBCM1 under varying cultural conditions. 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. While Bai *et al.* (2010) reported optimum production of cellulase at 72 h of fermentation time from *B. subtilis*.

Table 18. Effect of different incubation time on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

Incubation days	Protein (mg/ml)	Xylanase activity (IU)	Specific activity
24h	1.727	53.51	31.04
48h	5.747	74.45	13.07
72h	5.393	74.34	13.69
96h	5.303	73.55	13.87
12h	4.750	58.55	12.31
C.D. _{0.05}	0.1379	2.1487	1.0741
S.E.(Mean)	0.0618	0.9643	0.4820

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

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) from *B. subtilis* using various agricultural wastes. Abou Dobara *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.* (2008) recorded optimum cellulase production after 6 days under submerged fermentation in different substrates from newly isolated *Bacillus* sp. FME.

Pathania *et al.* (2012) optimized the incubation temperature for xylanase by *Paenibacillus* sp.N1. Statistically enzyme produced at 72 h was found significantly higher (29.46 IU) than the others. Maximum production of xylanase is observed by Wahyuntri *et al.* (2009) in a culture incubated at 50⁰C, pH 7.0 at 72 h by *Bacillus* sp. AQ-1. While *Bacillus* SSP-34 produced maximum xylanase activity (38.00 IU) when grown for 96 h (Subramaniyam *et al.*, 2000).

4.5.6 Effect of Carbon Source

Table 19 and 20 depicted the effect of different substrates as carbon source for cellulase production i.e. cellulose, mannose, dextrose, lactose, maltose and starch whereas xylose, maltose, xylan, arabinose, ribose and dextrose was used for hyperxylanase production. Statistical analysis showed significant variation among all the substrates for enzyme titers. The cellulase units varied from 3.130 to 5.515 IU in *B. stratosphericus* N₁₂ (M) whereas, xylanase titer varied from 18.55 to 75.16 IU in *B. altitudinis* Kd₁ (M). Optimal level of cellulase was recorded when lactose was used as a carbon source while maximum xylanase was produced using xylose as carbon source (Fig 24 and 25).

The diverse pattern found in cellulase and xylanase production with the different carbon sources used in the present experiment has reflected a direct linkage of enzyme production to the specific carbon substrates used, showing its direct impact on titers on enzymes. Some of the carbon source used in the medium supports the good growth of microorganisms as well as good enzyme synthesis. Carbon sources are also essential elements for growth and metabolism of microorganisms thus ultimately affect enzyme production.

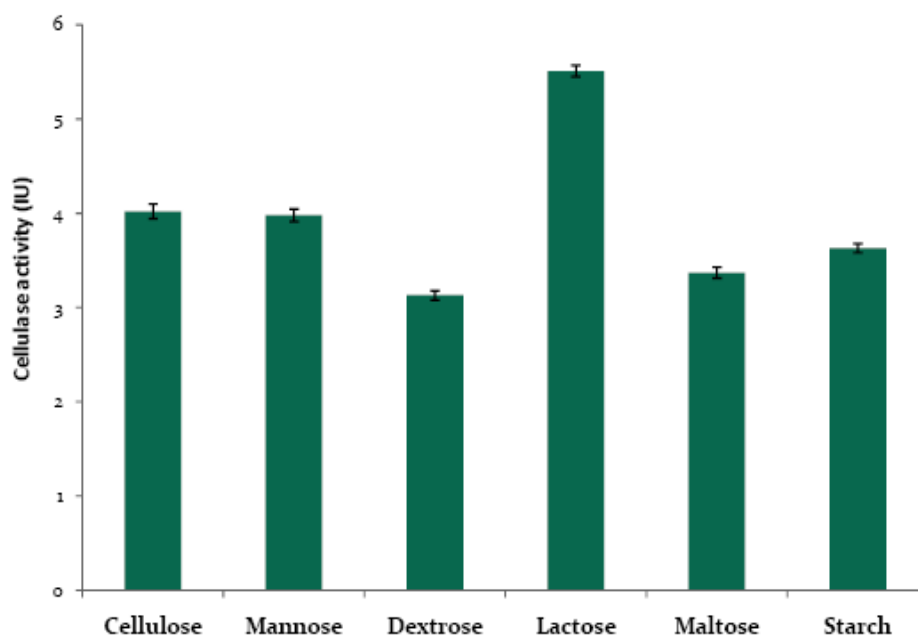


Fig 24. Effect of different carbon sources on cellulase production from *B. stratosphericus* N₁₂ (M)

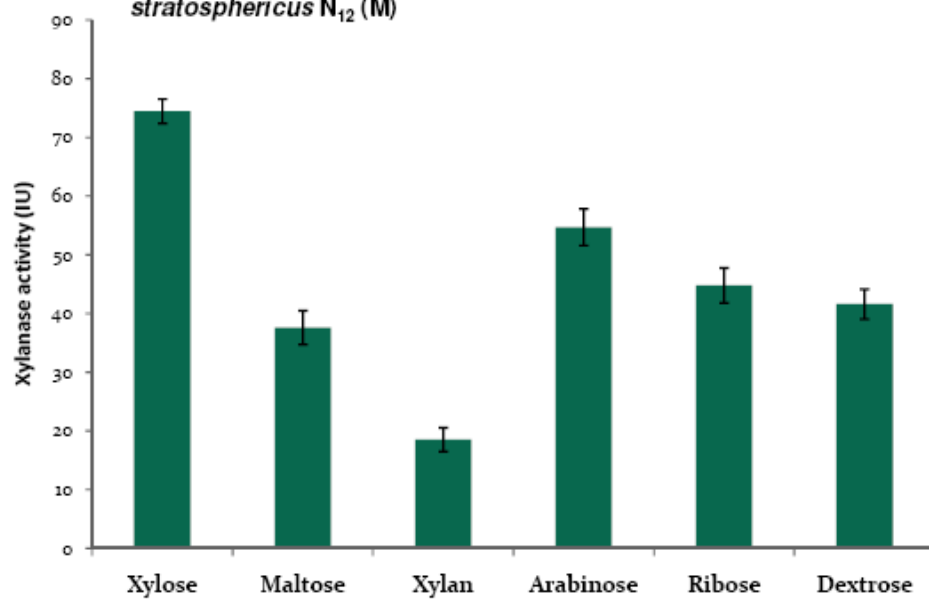


Fig 25. Effect of different carbon sources on xylanase production from *B. altitudinis* Kd₁ (M)

Table 19. Effect of different carbon sources on extracellular cellulase production from *B. stratosphericus* N12 (M) 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	1.96	0.332	0.168	1.606	0.814	2.091	1.061	4.028	2.069
Mannose	2.94	0.807	0.274	1.105	0.375	2.068	0.703	3.980	1.352
Dextrose	3.05	0.356	0.116	0.782	0.256	1.991	0.652	3.130	1.026
Lactose	2.18	1.813	0.831	2.029	0.930	1.673	0.767	5.515	2.526
Maltose	2.69	0.501	0.185	0.847	0.314	2.030	0.754	3.378	1.254
Starch	2.81	0.145	0.051	1.543	0.548	1.948	0.693	3.636	1.292
C.D. 0.05	0.028	0.014	0.003	0.011	0.005	0.052	0.017	0.067	0.038
S.E. (Mean)	0.012	0.006	0.001	0.005	0.002	0.024	0.008	0.031	0.017

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. altitudinis* Kd₁ (M) under SmF

Carbon source	Protein (mg/ml)	Xylanase activity (I.U.)	Specific activity
Xylose	3.27	75.16	22.72
Maltose	2.94	37.60	12.77
Xylan	2.31	18.55	8.023
Arabinose	3.30	54.68	16.57
Ribose	3.46	44.80	12.95
Dextrose	2.76	41.61	15.07
CD _{0.05}	0.1630	1.474	0.617
S.E.(Mean)	0.0748	0.676	0.283

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

The mechanism of lactose induction is believed to be due to the presence of intracellular galactose-1-phosphate levels which is responsible to control the signaling of cellulase production (Seiboth *et al.*, 2007). Cellulase is an inducible enzyme and is induced in the presence of soluble saccharides. Lactose has been found a suitable inducer of cellulase enzyme as compared to other carbon sources viz. glucose, sucrose, fructose and galactose etc (Sadhu *et al.*, 2013). Lactose is a

disaccharide and is readily used by microorganisms to induce cellulases. Kubicek (2009) studied the induction of cellulases using lactose and revealed that the induction of cellulase transcription by this saccharide required simultaneous degradation of the D- galactose moiety of lactose and the alternative reductive D- galactose catabolic pathway.

Fauzi and Makky, (2013) showed the effect of different carbon sources i.e., glucose, lactose, sucrose and starch on Avicelase enzyme production and was observed that lactose sugar exhibited maximum enzyme (1.286 ± 0.074 IU/ml) activity than others. Induction of Avicelase enzyme was reported in the presence of lactose as the carbon source when produced by *Cellulomonas fimi* and *Cellulomonas cellulosa* strains (Kashem *et al.*, 2004).

The influence of supplemented carbon sources to the medium on xylanase production was assessed by Pathania *et al.* (2012). Xylanase production by *Paenibacillus sp.* N1 was significantly higher than others when xylose (40.60 IU) was added as a source of carbon into the medium. Gupta and Kar, (2008) reported stimulation of xylanase production by xylose from thermophilic *Bacillus sp.* under submerged fermentation. Moreover Kapoor *et al.* (2008) observed that maximum xylanase production from *Bacillus pumilus* MK001 in the medium containing xylose as a carbon source.

4.5.7 Effect of Substrate Concentration

Statistical analysis of Table 21 and 22 showed significant variation among different substrate concentrations used for cellulase production ranging from 0.5% to 3.0% from hypercellulolytic *B. stratosphericus* N₁₂ (M) and for xylanase production from hyperxylanolytic *B. altitudinis* Kd₁(M). Optimum level of substrate concentration was found @ 2.0% for *B. stratosphericus* N₁₂ (M) showing significantly higher cellulase activity of 5.983 IU whereas least enzyme activity (3.358 IU) was recorded in 0.5% substrate concentration as presented in Fig 26. Increase in substrate concentration other than optimum level showed a consistent decrease in cellulase production. Whereas effect of substrate concentration on xylanase production from potential bacteria revealed that *B.*

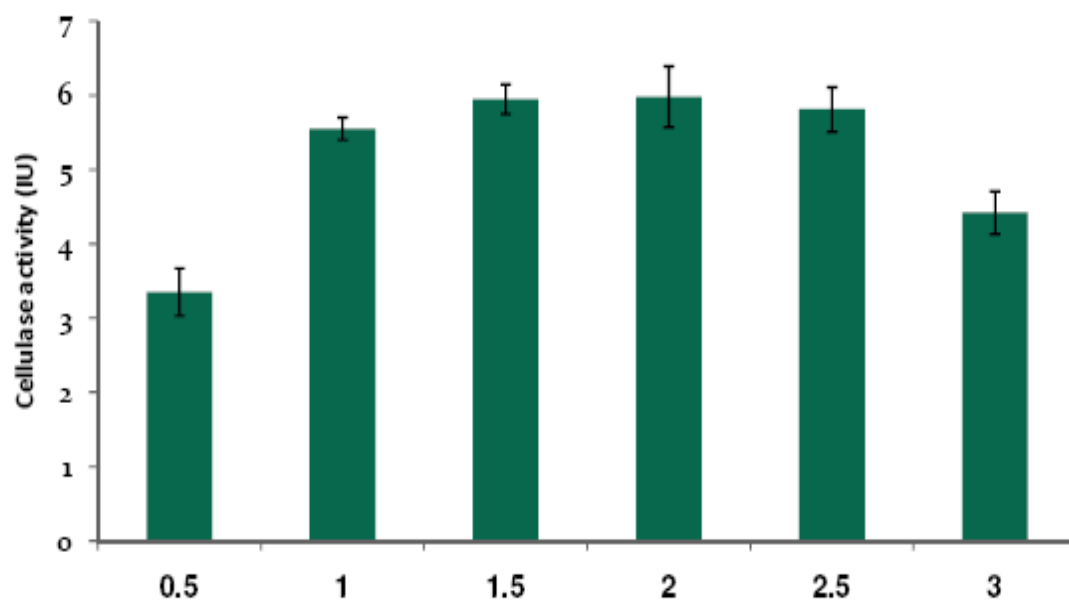


Fig 26. Effect of different concentration of carbon source on cellulase production from *B. stratosphericus* N₁₂ (M)

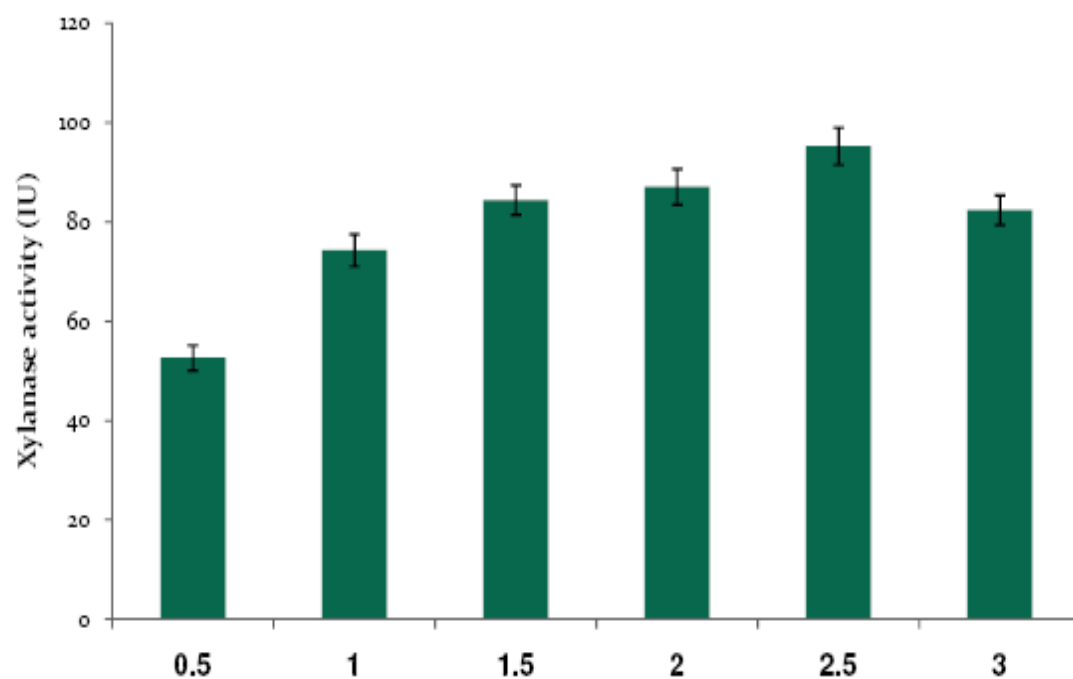


Fig 27. Effect of different concentration of carbon on xylanase production from *B. altitudinis* Kd₁ (M)

altitudinis Kd₁ (M) exhibited statistically significant higher xylanase activity at 2.5% substrate concentration i.e. 95.25 IU as shown in Fig 27. Minimum xylanase production i.e. 52.60 IU was observed at 0.5 % substrate concentration for this hyperxylanolytic bacteria.

Table 21. Effect of different substrate concentration on extracellular cellulase production from *B. stratosphericus* N₁₂ (M) under SmF

% age of carbon source	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
0.5%	1.11	0.842	0.763	0.950	0.855	1.565	1.418	3.358	3.038
1.0%	2.17	1.825	0.842	2.036	0.939	1.690	0.779	5.551	2.561
1.5%	1.98	1.829	0.924	2.078	1.050	2.044	1.033	5.951	3.009
2.0%	2.03	1.852	0.912	2.202	1.084	1.929	0.950	5.983	2.947
2.5%	2.14	1.872	0.874	2.260	1.056	1.685	0.786	5.817	2.718
3.0%	2.45	1.373	0.560	1.431	0.584	1.619	0.659	4.423	1.805
C.D. _{0.05}	0.151	0.012	0.069	0.086	0.039	0.107	0.119	0.158	0.189
S.E. (Mean)	0.069	0.005	0.031	0.039	0.017	0.049	0.051	0.072	0.087

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

Specific activity : enzyme activity/mg of protein

Substrate concentration is an influencing factor that affects the yield of enzyme production versus 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). The maximum production of xylanase was recorded with the supplementation of xylose at 0.75% (w/w) with 25.40 U/g (Kheng and Ibrahim, 2005). Nath *et al.* (2013) had optimized 2.0% lactose concentration for the production of β- glucosidase by *Bacillus safensis* (JUCHE-1). The maximum

production of xylanase obtained by *Aspergillus niger* USMA11 with the supplementation of xylose at 0.75% (w/w) were 25.40 U/g (Kheng and Ibrahim, 2005).

Table 22. Effect of different substrate concentration on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

% age of Carbon source	Final pH	Protein (mg/ml)	Xylanase activity(I.U.)	Specific activity
0.5%	6.2	2.540	52.60	20.69
1.0%	6.0	2.760	74.28	26.92
1.5%	6.4	2.760	84.38	30.59
2.0%	6.4	2.807	87.10	31.05
2.5%	6.5	2.977	95.25	31.99
3.0%	6.6	2.977	82.37	27.67
CD _{0.05}		0.193	0.747	1.840
S.E.(Mean)		0.088	0.342	0.844

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

Specific activity : enzyme activity/mg of protein

As the fig 28 and 29 revealed overall percent increase after optimization of different process parameters i.e media, pH, temperature, inoculum size, incubation time, carbon source and different concentrations of carbon source. Firstly when media was optimized for hypercellulase from *B. stratosphericus* N₁₂ (M) and xylanase production from *B. altitudinis* Kd₁ (M) under submerged fermentation (SmF), 3.230 IU of cellulase were observed in PYC media and 56.83 IU of xylanase in TGY media. Optimization of different process parameters after media optimization, cellulase activity increased from 3.230 to 5.983 IU i.e. 1.85 fold. As far the percent increase is concerned, 85.23 percent increase in cellulase activity was achieved (fig28).

In case of xylanase production it was increased from 51.32 IU to 95.25 IU (1.67 fold) and 85.60 percent increase was noticed in xylanase production after optimizing different process parameters as shown in fig 29. Finally all these optimized conditions were employed for the further study.

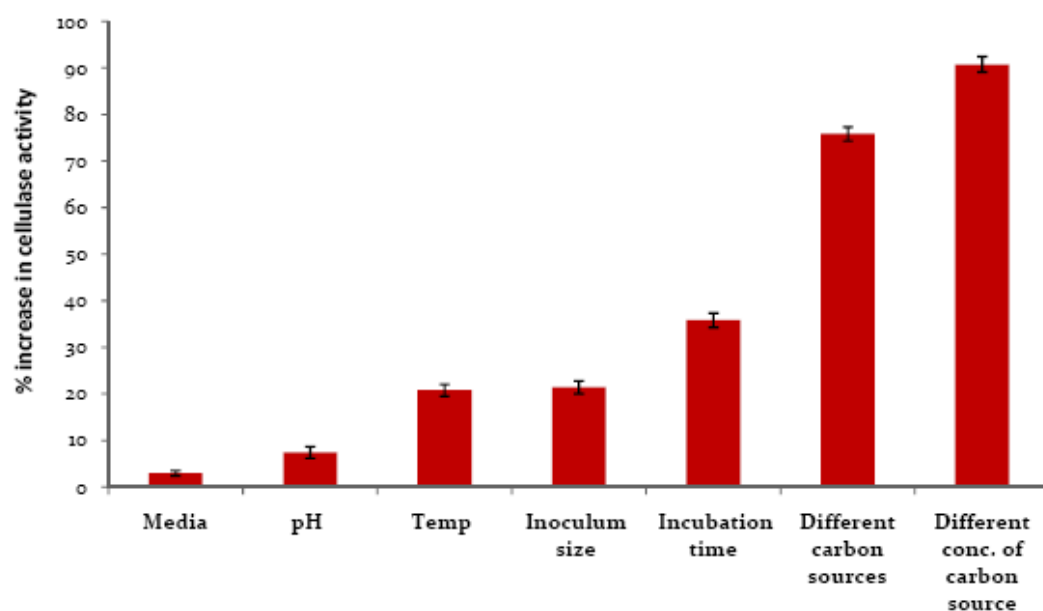


Fig 28. An overview of percent increase in cellulase activity from *B. stratosphericus* N12 (M) after optimization of different parameters under SmF

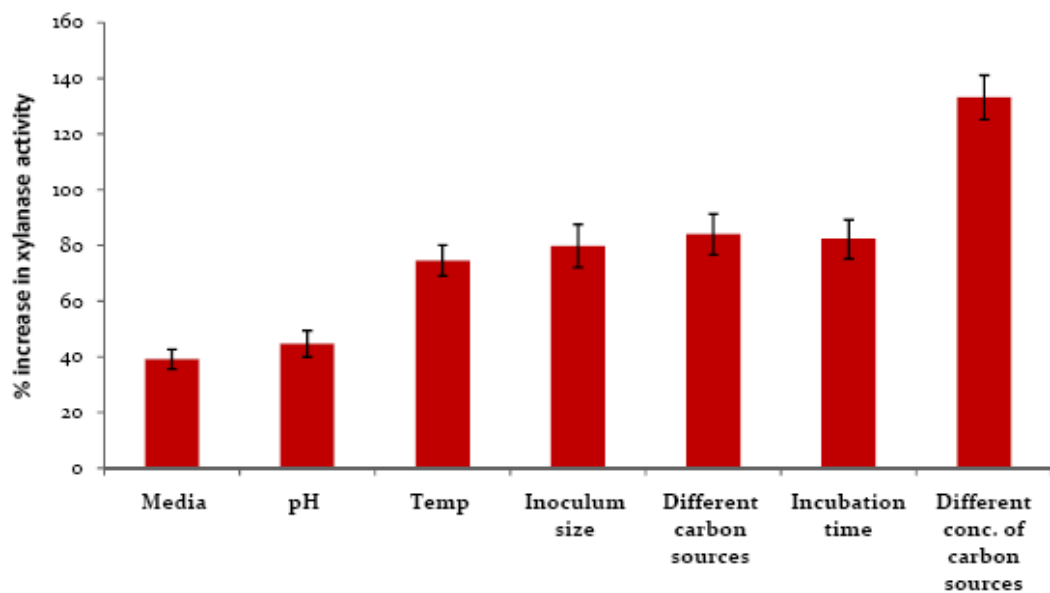


Fig 29. An overview of percent increase in xylanase activity from *B. altitudinis* Kd1 (M) after optimization of different parameters under SmF

Classical approach for one factor at a time (OFAT) used in the present enzyme optimization study has resulted in an impressive increase in the production of cellulase as well as xylanase proving the direct utility of this technique in increasing enzyme titers.

4.6.1 ANALYTICAL STUDIES FOR ESTIMATION OF HOLOCELLULOSE (CELLULOSE + HEMICELLULOSE) AND LIGNIN IN THE UNTREATED AND PRETREATED LIGNOCELLULOSIC FOREST WASTE BY TAPPI (TECHNICAL ASSOCIATION OF PAPER AND PULP INDUSTRY) METHOD

Lignocellulose is an abundant material created from solar energy and renewable resources on earth which makes them attractive feedstocks for their bioconversion into ethanol. It is composed of three main fractions like cellulose (~45% of dry weight), hemicellulose (~ 30% of dry weight) and lignin (~ 25% of dry weight).

In the present study analytical studies for estimation of holocellulose (cellulose + hemicellulose), lignin and extractives were performed by using TAPPI (Technical Association of Paper and Pulp Industry) method.

Table 23 exhibited the percentage of holocellulose (cellulose+ hemicellulose), lignin and extractives in different untreated and pretreated hardwood and softwood biomass. Extractives estimated in the present study vary between 0.2% to 3.5%. Extractives mainly contained alkaloids, proteins, simple and complex phenolics, pectin, gums, resins, terpenes, essential oils etc. The amount of holocellulose was more in hardwoods i.e 78.0% in *D. strictus*, 77.6% in *Eucalyptus* sp. and 75.8% in *P. deltoides* wood in the untreated biomass as compared to softwood biomass. The softwood contains higher content of lignin i.e. 29.5% in *P. wallichiana*, 29.0% in *C. deodara* and 26.00% in *Pinus roxburghii* wood in untreated biomass as compared to hardwoods i.e 20.5% in *D. strictus*, 20.5% in *Eucalyptus* sp. and 22.5% in *P. deltoides* wood.

Different physico-chemical pretreatments were given to the hardwood and softwood biomass. Among the all pretreated lignocellulosic biomass with the main aim to extract maximum lignin out of biomass and simplify the complex structure of cellulose and hemicellulose present in them. Maximum holocellulose content (74.0%) and minimum lignin content i.e. 17.5% were estimated in NaOH + H₂O₂ pretreated *P. deltoides* wood, while 1.4% of extractives were also present. On the other hand minimum holocellulose 68.2 % in NaOH + H₂O₂ pretreated *D. strictus* wood and the maximum lignin i.e. 29.0 % was found in steam pretreated *P. wallichiana* wood.

In lignocellulosic waste products, cellulose and hemicelluloses are closely associated with lignin in the plant cell wall. Cellulose the most abundant polymer on earth is composed of thousands of molecules of anhydroglucose linked by β (1,4) glycosidic bonds. Hemicellulose is a complex carbohydrate structure that consists of different polymers like pentoses (like xylose and arabinose), hexoses (like mannose, glucose and galactose) and sugar acids (Hendriks and Zeeman, 2009). Hemicellulose makes hydrogen bonds to cellulose micro-fibrils, forming a network that provides the structural backbone to the material (Mosier *et al.*, 2005). Softwood and hardwood differ in composition and structure of the hemicelluloses. Softwood hemicelluloses have a higher proportion of mannose and glucose units than hardwood hemicelluloses, which usually contain higher amount of xylose units (Palmquist and Hagerdal, 2006). In hardwood the main hemicelluloses is O-acety-4-O-methylglucuronxylan in which xylan chain is linked at irregular intervals with acetic acid and 4-O-methyl glucuronic acid units.

Lignin concentration was maximum in case of the native soft and hardwood biomass which had been reduced to a varying range significantly after pretreatments due to delignification of biomass. Though some of the holocellulose had also washed out with lignin. But pretreated holocellulose had

Table 23. Analytical studies for estimation of Holocellulose (cellulose +hemicellulose) and lignin in lignocellulosic (hardwood and softwood) forest waste

PRETREATMENTS	BIOMASSES																	
	<i>D. strictus</i>			<i>Eucalyptus</i> spp.			<i>P. deltoides</i>			<i>C. deodara</i>			<i>P. roxburghii</i>			<i>P. wallichiana</i>		
	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)
2.5% H_2SO_4	72.8	19.33	1.4	69.6	19.5	3.5	72.5	20.0	1.5	69.0	26.0	0.4	69.2	23.5	0.7	68.8	28.0	0.5
2.5% HCl	72.8	18.50	1.0	68.4	18.5	2.7	71.4	20.0	1.2	69.2	26.0	0.2	70.4	24.0	2.8	68.6	28.5	0.2
$\text{NaOH}+\text{H}_2\text{O}_2$	68.2	19.50	0.5	69.2	19.0	0.5	74.0	17.5	1.4	68.8	27.0	0.5	72.0	22.5	0.2	68.8	28.0	0.2
5% NH_3	72.0	19.0	0.5	70.0	18.5	1.1	69.2	20.5	0.5	69.2	26.5	0.2	71.4	23.0	1.7	68.6	28.5	1.1
Steam	72.2	19.5	1.7	71.5	20.5	1.6	69.4	21.0	0.5	69.4	28.0	0.6	72.33	24.50	1.4	69.0	29.0	2.1
Untreated	78.0	20.5	1.5	77.6	20.5	1.0	75.8	22.5	0.7	70.4	29.0	0.2	72.0	26.0	1.2	69.2	29.5	1.0
$\text{C.D}_{.0.05}$	1.148	0.202	0.096	1.129	0.202	0.003	1.139	0.197	0.100	1.105	0.171	0.171	1.126	0.182	0.246	1.100	1.128	0.144
S.E.	0.527	0.092	0.044	0.518	0.092	0.141	0.522	0.090	0.046	0.507	0.078	0.078	0.517	0.083	0.113	0.505	0.517	0.066

H.C: Holocellulose; L: Lignin; E: Extractives in percent

become more accessible for enzymatic hydrolysis because of breaking of lignin shield, complex inter and intra bonds and crystalline structure of cellulose. Heck *et al.* (2002) reported that cellulose, hemicellulose and lignin in forest and agricultural residue were present in an average ratio of 4:3:3.

When chemical analysis (cellulose, hemicellulose and lignin) of bamboo sawdust were carried out based on TAPPI Standard Methods, the value of holocellulose was decreased by 2.1 to 10.7% after heating pretreatment. The cellulose content of heat-treated bamboo ranged between 47.4 to 55.2% after 2 to 14% reduction in their level. Lignin content reduced approximately to 1 to 5%.

4.6.2 COST EFFECTIVE PRODUCTIONS OF CELLULASE AND XYLANASE UNDER SUBMERGED FERMENTATION (SmF) AND SOLIDSTATE FERMENTATION (SSF)

Extracellular cellulase and xylanase production from hypercellulolytic *B. stratosphericus* N₁₂ (M) and hyperxylanolytic Kd₁ (M), respectively were evaluated under submerged fermentation (SmF) and solid state fermentation (SSF) by *M. thermophila* SH1 utilizing lignocellulosic forest waste (untreated and pretreated) as carbon source.

Forest residue used in the present study include sawdust of *Dandracalamus strictus*, *Populus deltoides*, *Eucalyptus* sp., *Cedrus deodara*, *Pinus roxburghii* and *Pinus wallichiana* depending upon their local abundance, easy availability and rich cellulosic and hemicellulosic components.

As the major impediments to exploit the commercial potential of cellulase and xylanase are the cost of cellulase and xylanase production and yield stability. One effective approach to reduce the cost of enzyme production is to replace conventional carbon source i.e. pure cellulose and xylan by relatively cheaper substrates such as lignocellulosic forest waste. Several pretreatments (physical and chemical) had been tried to make forest waste readily accessible for enzyme production by hypercellulolytic and xylanolytic microorganisms.

Thus keeping in view the above facts, cost effective production of cellulase and xylanase by selected bacteria under SmF and fungus under SSF using untreated/ pretreated inexpensive forest waste had been done as given below.

4.6.2.1 Enzymes production using untreated lignocellulosic waste

The different lignocellulosic wastes namely, *Dandracalamus strictus*, *Populus deltoides*, *Eucalyptus* sp., *Cedrus deodara*, *Pinus roxburghii* and *Pinus wallichiana* woods were used for the cost effective production of enzymes.

Data pertaining to cellulase units viz., CMCase, FPase and β -glucosidase and xylanase production from untreated biomass under submerged fermentation (SmF) had been presented in Table 24, 25, 26 and 27. Maximum cellulase i.e. 31.88 U/g was observed in *P. deltoides* wood by *B. stratosphericus* N₁₂ (M), while minimum cellulase production 5.76 U/g was produced in *C. deodara* wood. Rest of the values for cellulase production from different untreated forest wastes varied between these two extremes. Maximum reducing sugars released during the submerged fermentation (SmF) process was 6.46 mg/g by *P. deltoides* wood. Highest xylanase production of 76.73 U/g and reducing sugars i.e. 6.47 mg/g by *B. altitudinis* Kd₁ (M) using untreated substrate were found in *P. deltoides* wood followed by *Eucalyptus* sp. (58.59 U/g) and minimum xylanase titers i.e. 24.70 U/g were noticed in *P. wallichiana* wood (Table 25).

In case of SSF by *M. thermophila* SH1, highest cellulase titers 26.64 U/g and 7.64 mg/g of reducing sugars were found in *P. deltoides* wood, while the least 6.84 U/g was noticed in *P. wallichiana* wood using untreated lignocellulosic forest waste (Table 26). The maximum xylanase production under SSF was noticed in *C. deodara* wood i.e. 168.00 U/g of xylanase. While the minimum i.e. 29.82 U/g were observed in *P. wallichiana* wood. The maximum reducing sugars i.e. 10.96 mg/g were found in *P. deltoides* wood as shown in Table 27.

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes are being produced using this process. This

Table 24: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using untreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars mg/g
<i>P. deltoides</i>	9.00	1.91 (16.88)	10.24 (1.02)	10.92 (1.09)	10.72 (1.07)	31.88* (3.18)**	6.46*** (0.64)
<i>D. strictus</i>	9.14	1.28 (6.80)	3.36 (0.33)	2.40 (0.24)	2.96 (0.29)	8.72 (0.87)	1.65 (0.16)
<i>Eucalyptus</i> sp.	8.20	0.86 (10.60)	3.36 (0.33)	3.27 (0.32)	2.58 (0.25)	9.21 (0.92)	1.79 (0.17)
<i>P. roxburghii</i>	8.22	6.72 (10.60)	1.25 (0.12)	3.25 (0.32)	2.63 (0.26)	7.13 (0.71)	1.35 (0.13)
<i>P. wallichiana</i>	9.00	1.25 (12.90)	3.88 (0.38)	4.92 (0.49)	7.45 (0.74)	16.25 (1.62)	3.21 (0.32)
<i>C. deodara</i>	9.16	6.40 (0.90)	0.001 (0.01)	0.16 (0.01)	5.60 (0.56)	5.76 (0.57)	1.10 (0.11)
CD_{0.05}		0.01 (1.01)	0.01 (0.16)	0.02 (0.01)	0.72 (0.01)	0.01 (0.02)	1.03 (0.02)
S.E.		0.01 (0.46)	0.07 (0.01)	0.01 (0.08)	0.33 (0.01)	0.07 (0.10)	0.47 (0.01)

#Initial pH

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 25: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using untreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g)	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	6.2	5.07 (11.70)	58.59* (5.85)**	4.89*** (0.48)
<i>D. strictus</i>	6.5	2.89 (20.10)	58.07 (5.80)	4.73 (0.47)
<i>P. deltoides</i>	6.7	7.45 (21.70)	76.73 (7.67)	6.47 (0.64)
<i>P. roxburghii</i>	6.4	1.97 (18.60)	36.73 (3.67)	3.12 (0.31)
<i>P. wallichiana</i>	6.7	1.40 (17.60)	24.70 (2.47)	2.15 (0.21)
<i>C. deodara</i>	6.3	2.43 (14.30)	34.83 (3.48)	2.97 (0.29)
CD_{0.05}		1.45 (0.01)	0.01 (0.06)	0.45 (0.01)
S.E.		0.66 (0.01)	0.01 (0.01)	0.20 (0.01)

#Initial pH

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 26: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using untreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars(mg/g)
<i>Eucalyptus</i> sp.	7.1	6.90 (2.78)	12.80 (0.06)	0.30 (0.01)	16.36 (0.89)	17.94* (0.89)**	5.15*** (0.25)
<i>D. strictus</i>	7.5	0.311 (38.0)	0.68 (0.03)	2.18 (0.10)	8.96 (0.37)	11.82 (0.59)	3.48 (0.17)
<i>P. deltoides</i>	7.2	0.640 (41.60)	2.86 (0.14)	5.60 (0.28)	18.18 (0.90)	26.64 (1.33)	7.64 (0.38)
<i>P. roxburghii</i>	7.0	1.81 (0.52)	6.52 (0.32)	2.90 (0.14)	0.00 (0.00)	9.42 (0.47)	2.62 (0.13)
<i>P. wallichiana</i>	7.4	7.43 (0.92)	1.04 (0.05)	0.90 (0.04)	4.90 (0.24)	6.84 (0.34)	1.90 (0.09)
<i>C. deodara</i>	7.3	1.38 (16.0)	5.74 (0.28)	5.66 (0.28)	10.72 (0.53)	22.12 (1.10)	6.20 (0.31)
CD_{0.05}		1.03 (1.45)	1.45 (0.10)	1.45 (0.10)	1.62 (0.16)	1.77 (0.16)	0.54 (0.04)
S.E.		0.47 (0.66)	0.66 (0.04)	0.66 (0.04)	0.74 (0.07)	0.81 (0.07)	0.02 (0.02)

#Initial pH-6.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU =μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Values in parentheses depicted reducing sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 27: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using untreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g)	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	7.3	1.45 (60.40)	88.10* (4.40)**	6.45*** (0.32)
<i>D. strictus</i>	7.5	2.29 (55.00)	126.48 (6.32)	9.23 (0.46)
<i>P. deltoides</i>	7.8	1.87 (77.80)	145.84 (7.29)	10.96 (0.54)
<i>P. roxburghii</i>	7.2	1.80 (21.40)	38.64 (1.93)	2.90 (0.14)
<i>P. wallichiana</i>	7.9	1.71 (17.40)	29.82 (1.49)	2.34 (0.11)
<i>C. deodara</i>	7.7	5.91 (28.40)	168.00 (8.40)	10.00 (0.50)
C.D_{0.05}		1.02 (7.44)	1.77 (2.24)	0.58 (0.01)
S.E.		0.47 (3.41)	0.81 (0.66)	0.26 (0.01)

#Initial pH-6.0

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g(Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

involves growing carefully the selected micro organisms in the 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. Submerged fermentation (SmF) is generally preferred for enzyme production from bacteria.

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 that is typically retained within 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 for molds to SSF mode.

Overall among untreated biomass, for cellulase and xylanase production hardwood has emerged as better substrate as compared to softwood used in the present study.

4.6.2.2 Enzymes production using acid (dil. H₂SO₄ and dil. HCl) pretreated lignocellulosic waste

The different lignocellulosic wastes namely, *Dandracalamus strictus*, *Populus deltoides*, *Eucalyptus sp.*, *Cedrus deodara*, *Pinus roxburghii* and *Pinus wallichiana* were treated with acid i.e. H₂SO₄ and HCl for assessing the cellulase and xylanase enzymes.

In case of acid (H₂SO₄) pretreatment as the Table 28 depicted, the maximum cellulase units 39.70 U/g and maximum reducing sugars 7.46 mg/g from *B. stratosphericus* N₁₂ (M) were in *P. deltoides* wood followed by *C. deodara* wood (20.26 U/g) in comparison to other substrate. The least cellulase i.e. 9.930 U/g were expressed in *P. roxburghii* wood. The highest percent increase (39.27) was observed in *P. roxburghii* wood while the least percent increase was seen in *P. wallichiana* wood i.e. 7.63 %. An appraisal of table 29

revealed that in case of HCl pretreated substrates the maximum cellulase of 31.93 U/g and reducing sugars i.e. 6.87 mg/g by *B. stratosphericus* N₁₂ (M) were secreted in *P. deltoides* wood. On the other hand the minimum cellulase 10.15 U/g titers were present in *Eucalyptus sp* wood. The highest percent increase of HCl pretreated substrate over untreated was 157.98 in *C. deodara* wood and the lowest percent increase (0.156) was observed in *P. deltoides* wood over untreated substrates. The highest percent increase was noticed in *C. deodara* wood and least percent increase (0.156%) was found in *P. deltoides* wood. A cursory of data in table 30 exhibited significantly highest xylanase production from *B. altitudinis* Kd₁ (M) by H₂SO₄ pretreated substrate in *P. deltoides* wood i.e. 85.16 U/g and reducing sugars i.e. 8.51 mg/g, while the least xylanase production 49.43 U/g in *P. roxburghii* wood. The highest percent increase 102.51 was observed in *P. wallichiana* wood over the untreated while the least percent increase 10.98 was in case of *P. deltoides* wood. On the other hand xylanase production in case of HCl pretreated substrates was revealed in the table 31. The highest xylanase titers 104.10 U/g were exhibited in case of *P. deltoides* wood while the least xylanase titer 39.06 U/g was found in *P. wallichiana* wood. The maximum reducing sugars 8.69 mg/g were also released in *P. deltoides* wood. The highest percent increase 58.13 over the untreated substrate was observed in *P. wallichiana* wood and the least 2.88 was found in *Eucalyptus sp.* wood.

A perusal of data in case of solid state fermentation (SSF) Table 32 revealed that the significant difference in cellulase production as noticed when different lignocellulosic substrates and dil. H₂SO₄ pretreatments were used by *M. thermophila* SH1. Maximum cellulase titers 34.56 U/g and reducing sugars i.e. 11.80 mg/g were estimated in *Eucalyptus sp.* wood, followed by *C. deodara* (32.90 U/g), *P. deltoides* (28.08 U/g), *P. roxburghii* (13.68 U/g) and *D. strictus* (11.96 U/g) wood in descending order. Minimum cellulase i.e 8.86 U/g was observed in *P. wallichiana* wood. Maximum percent increase 92.64 was observed in *Eucalyptus sp.* wood, while least 5.40 observed in *P. deltoides* wood.

Cellulase production by *M. thermophila* SH1 under SSF in case of dil. HCl pretreatment depicted in Table 33. Highest titers 30.98 U/g were found in *C.*

Table 28: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using dil. H₂SO₄ pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β-glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>P. deltoides</i>	8.9	1.79 (22.10)	31.88 (3.188)	11.82 (1.182)	11.75 (1.17)	16.13 (1.61)	39.70* (3.97) **	24.52	7.46*** (0.74)
<i>D. strictus</i>	8.6	0.91 (11.10)	8.72 (0.87)	3.70 (0.37)	2.86 (0.28)	3.59 (0.35)	10.15 (1.01)	16.39	1.69 (0.16)
<i>Eucalyptus sp.</i>	8.2	0.67 (16.70)	9.21 (0.92)	3.53 (0.35)	3.55 (0.35)	4.69 (0.46)	11.77 (1.17)	27.79	2.00 (0.20)
<i>P. roxburghii</i>	8.5	13.22 (0.85)	7.13 (0.71)	2.21 (0.22)	3.89 (0.38)	3.83 (0.38)	9.93 (0.99)	39.27	1.65 (0.16)
<i>P. wallichiana</i>	8.4	0.92 (18.10)	16.25 (1.62)	4.99 (0.49)	6.25 (0.62)	6.25 (0.62)	17.49 (1.74)	7.63	3.17 (0.31)
<i>C. deodara</i>	8.8	4.74 (4.39)	5.76 (0.57)	4.39 (0.43)	0.36 (0.03)	19.15 (1.91)	20.26 (2.02)	25.17	3.68 (0.36)
CD_{0.05}		0.12 (0.17)	0.016 (0.002)	0.17 (0.01)	0.16 (0.12)	0.17 (0.01)	0.162 (0.01)		1.20 (0.01)
S.E.		0.05 (0.08)	0.07 (0.01)	0.08 (0.01)	0.07 (0.05)	0.08 (0.01)	0.07 (0.07)		0.05 (0.01)

#Initial pH- 8.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 29: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using dil. HCl pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β-glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>P. deltoides</i>	8.3	1.376 (23.20)	31.88 (3.18)	10.50 (1.05)	11.00 (1.10)	10.43 (1.04)	31.93* (3.19)**	0.15	6.87*** (0.68)
<i>D. strictus</i>	8.9	1.15 (11.1)	8.72 (0.87)	3.79 (0.37)	2.96 (0.29)	6.12 (0.61)	12.87 (1.28)	47.59	2.66 (0.26)
<i>Eucalyptus sp.</i>	8.6	0.714 (14.20)	9.21 (0.92)	3.53 (0.35)	3.51 (0.35)	3.11 (0.31)	10.15 (1.01)	10.20	2.78 (0.27)
<i>P. roxburghii</i>	8.6	3.25 (4.06)	7.13 (0.71)	2.03 (0.20)	3.52 (0.35)	7.66 (0.76)	13.21 (1.32)	85.27	2.76 (0.27)
<i>P. wallichiana</i>	8.4	1.41 (13.91)	16.25 (1.62)	3.53 (0.35)	7.43 (0.74)	8.71 (0.87)	19.67 (1.96)	21.04	4.34 (0.43)
<i>C. deodara</i>	8.9	3.72 (3.99)	5.76 (0.57)	0.87 (0.08)	0.83 (0.08)	13.16 (1.31)	14.86 (1.48)	157.98	3.15 (0.31)
CD_{0.05}		0.74 (1.62)	0.01 (0.02)	1.25 (0.73)	1.45 (0.74)	1.77 (0.14)	1.77 (0.12)		1.25 (0.01)
S.E.		0.33 (0.74)	0.07 (0.10)	0.57 (0.33)	0.66 (0.33)	0.81 (0.06)	0.81 (0.05)		0.57 (0.01)

#Initial pH- 8.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 30: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using dil. H₂SO₄ pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus sp.</i>	6.4	3.69 (20.70)	58.59* (5.85)**	76.46 (7.64)	30.50	7.45*** (0.74)
<i>D. strictus</i>	6.7	2.68 (28.60)	58.07 (5.80)	76.87 (7.68)	32.37	7.73 (0.77)
<i>P. deltoidea</i>	6.3	2.89 (30.10)	76.73 (7.67)	85.16 (8.51)	10.98	8.51 (0.85)
<i>P. roxburghii</i>	6.0	2.80 (17.60)	36.73 (3.67)	49.43 (4.94)	34.57	4.73 (0.47)
<i>P. wallichiana</i>	6.7	2.74 (18.20)	24.70 (2.47)	50.02 (5.00)	102.51	4.85 (0.48)
<i>C. deodara</i>	6.2	3.99 (15.60)	34.83 (3.48)	62.27 (6.22)	78.78	6.23 (0.62)
CD_{0.05}		1.77 (0.01)	0.01 (0.01)	0.10 (0.72)		2.41 (0.01)
S.E.		0.81 (0.08)	0.08 (0.07)	0.04 (0.33)		1.10 (0.01)

#Initial pH-5.5

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 31: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using dil. HCl pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars(mg/g)
<i>Eucalyptus sp.</i>	6.6	2.89 (20.10)	58.59 (5.85)	60.28* (5.82)**	2.88	5.02*** (0.50)
<i>D. strictus</i>	6.7	3.15 (24.50)	58.07 (5.80)	77.36 (7.73)	33.21	6.35 (0.63)
<i>P. deltooides</i>	6.3	5.24 (19.70)	76.73 (7.67)	104.10 (10.41)	35.67	8.69 (0.86)
<i>P. roxburghii</i>	6.4	2.67 (18.20)	36.73 (3.67)	48.63 (4.86)	32.39	4.12 (0.41)
<i>P. wallichiana</i>	6.5	2.75 (14.20)	24.70 (2.47)	39.06 (3.90)	58.13	3.10 (0.31)
<i>C. deodara</i>	6.7	3.50 (15.20)	34.83 (3.48)	53.25 (5.32)	52.88	4.31 (0.43)
SE		1.77 (0.01)	0.01 (0.01)	0.01 (0.01)		0.57 (0.01)
CD _{0.050}		0.81 (0.08)	0.08 (0.07)	0.08 (0.05)		1.25 (0.02)

#Initial pH-5.5

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 32: Cost effective production of extracellular cellulase production by *M. thermophila SH1* using dil. H₂SO₄ pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β-glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars(mg/g)
<i>Eucalyptus sp.</i>	7.5	1.28 (26.80)	17.94 (0.89)	2.52 (0.12)	2.360 (0.11)	29.68 (1.48)	34.56* (1.72)**	92.64	11.80*** (0.59)
<i>D. strictus</i>	7.3	0.174 (43.5)	11.82 (0.59)	2.30 (0.11)	5.84 (0.29)	3.82 (0.19)	11.96 (0.59)	11.84	4.17 (0.20)
<i>P. deltoides</i>	7.2	0.61 (46.00)	26.64 (1.33)	4.88 (0.44)	6.96 (0.49)	15.24 (0.91)	28.08 (0.95)	5.40	9.60 (0.48)
<i>P. roxburghii</i>	7.5	3.48 (3.92)	9.42 (0.47)	6.52 (0.32)	3.34 (0.16)	3.82 (0.19)	13.68 (0.68)	45.22	4.56 (0.22)
<i>P. wallichiana</i>	7.3	0.66 (13.40)	6.84 (0.34)	2.44 (0.12)	2.68 (0.13)	3.74 (0.18)	8.86 (0.44)	29.53	3.04 (0.15)
<i>C. deodara</i>	7.1	0.92 (35.80)	22.12 (1.10)	7.14 (0.35)	7.78 (0.38)	28.04 (1.40)	32.96 (2.14)	49.00	10.10 (0.50)
CD_{0.05}		0.74 (2.51)	1.77 (0.16)	1.77 (0.13)	1.78 (0.12)	1.77 (0.07)	1.77 (0.74)		1.25 (0.05)
S.E.		0.33 (1.15)	0.81 (0.07)	0.81 (0.06)	0.82 (0.05)	0.81 (0.03)	0.81 (0.31)		0.57 (0.02)

#Initial pH-6.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 33: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using dil. HCl pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β -glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus sp.</i>	7.4	0.84 (32.00)	17.94 (0.89)	2.80 (0.14)	2.28 (0.11)	21.82 (1.09)	26.90* (1.34)**	49.94	8.48*** (0.42)
<i>D. strictus</i>	7.2	0.62 (44.20)	11.82 (0.59)	2.68 (0.13)	7.76 (0.38)	17.32 (0.86)	27.76 (1.38)	130.62	8.68 (0.43)
<i>P. deltoidea</i>	7.8	0.58 (46.40)	26.64 (1.33)	4.74 (0.33)	6.88 (0.39)	12.36 (0.91)	27.15 (1.14)	1.910	8.56 (0.42)
<i>P. roxburghii</i>	7.9	3.91 (2.84)	9.42 (0.47)	6.30 (0.31)	3.68 (0.18)	1.14 (0.05)	11.12 (0.55)	18.04	3.49 (0.17)
<i>P. wallichiana</i>	7.0	5.35 (2.90)	6.84 (0.34)	3.74 (0.18)	3.75 (0.17)	8.30 (0.41)	15.54 (0.77)	127.19	4.79 (0.23)
<i>C. deodara</i>	7.2	1.09 (28.40)	22.12 (1.10)	6.72 (0.33)	7.22 (0.36)	17.04 (0.85)	30.98 (1.54)	40.05	6.20 (0.31)
CD_{0.05}		0.72 (1.77)	1.77 (0.16)	1.62 (0.12)	1.77 (0.12)	1.62 (0.14)	1.70 (0.16)		0.10 (0.05)
S.E.		0.33 (0.81)	0.81 (0.07)	0.74 (0.05)	0.81 (0.05)	0.74 (0.06)	0.78 (0.07)		0.04 (0.02)

#Initial pH-6.0

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 34: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using dil. H₂SO₄ pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus sp.</i>	7.3	2.68 53.00	88.10 (4.40)	142.22* (7.11)**	61.43	8.97*** (0.44)
<i>D. strictus</i>	7.1	3.79 39.60	126.48 (6.32)	150.42 (7.52)	18.92	9.20 (0.46)
<i>P. deltooides</i>	7.6	3.74 51.40	145.84 (7.29)	192.52 (9.62)	32.00	12.04 (0.60)
<i>P. roxburghii</i>	7.4	2.35 24.00	38.64 (1.93)	56.46 (2.82)	46.11	3.76 (0.18)
<i>P. wallichiana</i>	7.8	2.18 19.96	29.82 (1.49)	43.64 (2.18)	46.34	2.72 (0.13)
<i>C. deodara</i>	7.7	9.94 17.94	168.00 (8.40)	178.44 (8.92)	6.21	10.50 (0.52)
CD_{0.05}		1.62 1.77	1.77 (2.24)	1.77 (1.77)		1.25 (0.04)
S.E.		0.74 0.81	0.81 (0.66)	0.81 (0.81)		0.57 (0.01)

#Initial pH-6.0

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 35: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using dil. HCl pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	7.5	2.83 (48.00)	88.10 (4.40)	136.12* (6.80)**	54.50	9.50*** (0.47)
<i>D. strictus</i>	7.3	3.90 (45.00)	126.48 (6.32)	175.80 (8.79)	38.99	12.01 (0.60)
<i>P. deltoides</i>	7.7	3.80 (51.40)	145.84 (7.29)	195.58 (9.77)	34.10	13.84 (0.69)
<i>P. roxburghii</i>	7.4	2.13 (19.00)	38.64 (1.93)	40.50 (2.02)	4.81	3.56 (0.17)
<i>P. wallichiana</i>	7.3	1.71 (17.40)	29.82 (1.49)	29.92 (1.50)	0.33	3.07 (0.15)
<i>C. deodara</i>	7.7	5.79 (19.60)	168.00 (8.40)	173.64 (9.68)	3.35	10.18 (0.50)
CD_{0.05}		1.62 (1.77)	1.779 (2.24)	1.779 (1.45)		1.79 (0.05)
S.E.		0.74 (0.81)	0.81 (0.66)	0.81 (0.66)		0.82 (0.02)

#Initial pH-6.0

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

deodara wood, which was followed by *D. strictus* (27.76 U/g), *P. deltooides* (27.15 U/g), *Eucalyptus sp.* (26.90 U/g), *P. wallichiana* (15.54U/g) wood in descending order. The minimum cellulase titers 11.12 U/g was observed in *P. roxburghii* wood. Maximum reducing sugars 8.68 mg/g were noticed in *D. strictus* wood. The highest percent increase 134.85 was observed in *D. strictus* wood and the least 1.91 was observed in *P. deltooides* wood. An appraisal of data in Table 34 showed that the significant differences in xylanase titers were observed when dil. H₂SO₄ pretreated lignocellulosic biomass used. Highest xylanase 192.52 U/g production was observed in *P. deltooides* wood, followed by *C. deodara* wood (178.44 U/g). While the least xylanase 43.64 U/g was observed in *P. wallichiana* wood. As the maximum reducing sugars 12.04 mg/g were observed in *P. deltooides* wood. The highest (61.43) percent increase was observed in *Eucalyptus sp.* wood, while the minimum (6.21) was in *C. deodara* wood.

In case of dil. HCl pretreatment the differences in cellulase production was noticed when different lignocellulosic substrates and dil. HCl pretreatment was used for study in *M. thermophila* SH1 under SSF (Table 35). The maximum xylanase units 195.58 U/g and maximum reducing sugars 13.84 mg/g were noticed in *P. deltooides* wood, which was followed by *D. strictus* wood 175.80 U/g and *C. deodara* wood 173.64 U/g. The least xylanase titers 29.92 U/g were found in *P. wallichiana* wood. The highest percent increase of 54.50 was observed in *Eucalyptus sp.* wood, while the least % increase (0.33) was observed in *P. wallichiana* wood.

Acid pretreatment involves the use of concentrated and diluted acids to break the rigid structure of the lignocellulosic material. The most commonly used acid is dilute sulphuric acid, which has been commercially used to pretreat a variety of lignocellulosic biomass. The action mode of dilute acid is to solubilize hemicellulose and remains lignin and cellulose intact so that the enzymatic digestibility of cellulose enhanced.

Marzialesi and coworkers (2008) studied the acid hydrolysis of pine wood using a number of different acids (Tri-fluoro acetic acid, Hydrochloric acid and sulphuric acid). The effect of type of acid, pH, reaction temperature and reaction time on hydrolysis products such as monosaccharides and subsequent degradation products, HMF and furfurals is reported. Tri-fluoro acetic acid is found to yield the highest amount of overall soluble monosaccharides.

Sun and Cheng (2005) studied burmudagrass and rye straw. After 48 h of enzymatic hydrolysis of pretreated burmudagrass and rye straw with 1.5 % sulphuric acid, the total reducing sugars were found to be 197.1 mg/g and 229.3 mg/g of dry biomass. Other studies were done by Saha and co-workers (2005) showed that the maximum sugar yield of wheat straw was 565 ± 10 mg/g and that of rice hull was 287 ± 3 mg/g. Under these conditions, no furfural and hydroxymethyl furfural were produced.

Mosier *et al.* (2005) reported higher hydrolysis yield from lignocellulose pretreated with diluted H_2SO_4 compared to other acids. A saccharification yield of 74% was obtained from wheat straw when subjected to 0.75% v/v of H_2SO_4 at 121°C or 1 h. Cara *et al.* (2007) reported the maximum hemicellulose recovery (83%) of olive tree biomass to be obtained at 170°C and 1% sulfuric acid concentration, but the enzyme accessibility of the corresponding pretreated solid was not very high. The maximum enzymatic hydrolysis yield (76.5%) was obtained when pretreated at 210°C with 1.4% acid concentration. The maximum total sugars, 75% of all sugars present in olive tree biomass, were obtained when the feedstock was pretreated by dilute acid at 180°C with 1% sulphuric acid concentration. This indicates that the highest overall sugars, higher hemicellulose recovery and higher enzymatic hydrolysis yield can be achieved under respectively different conditions.

Fengel and Wegener (1984), investigated that the acidic hydrolysis of hemicellulosic polysaccharides in wood is mainly due to breaking of the glucosidic bonds (1 $\rightarrow$ 4) between monosaccharide units of the chain, the

liberation of ester-bonded side groups such O-acetyl and the breaking of other ether-bonded side groups such as α -D-galactopyranose. This will lower the molecular size such as reduce the degree of polymerization for the polysaccharide and fragments of low molecular by products such as mono- and disaccharides will be solubilized. Rubio *et al.* (1994), reported that cellulose degradation reactions requires more vigorous treatment and higher heat i.e. started at above 210-220°C.

4.6.2.3 Enzymes production using alkaline peroxide pretreated lignocellulosic substrate

Data pertaining to cellulase titers viz., CMCase, FPase and β - glucosidase and xylanase are presented in Table 36, 37, 38 and 39.

A perusal of Table 36 revealed that the significant difference in cellulase production was observed when different lignocellulosic substrates and pretreatments were used by *B. stratosphericus* N₁₂ (M) under submerged fermentation (SmF). Maximum cellulase titers 41.73U/g and reducing sugars i.e. 7.46 mg/g were estimated in *P. deltoides* wood, followed by *P. wallichiana* wood (22.86 U/g). The minimum cellulase titers 10.09 U/g was observed in *P. roxburghii* wood. Maximum percent increase (193.22) was observed in *C. deodara* wood over untreated, while minimum (30.73) was observed in *D. strictus* wood.

An appraisal of data in Table 37 the significant differences in xylanase titers were observed when different lignocellulosic substrate and pretreatment by *B. altitudinis* Kd₁ (M) was used for the study. Highest xylanase titers 111.98 U/g and maximum reducing sugars 9.52 mg/g were observed in *P. deltoides* wood which was followed by *Eucalyptus sp.* wood (87.97 U/g). While the minimum xylanase production 38.40 U/g was observed in *P. wallichiana* wood. As depicted in the same table maximum percent increase (55.46) was observed in *P. wallichiana* wood which was followed by *Eucalyptus sp.* wood (50.14) while least percent increase was found in case of *D. strictus* wood (25.91).

The significant differences in cellulase production were observed when different lignocellulosic substrates and NaOH + H₂O₂ pretreatment was used for study of cellulase production from *M. thermophila SH1* under SSF (Table 38). Maximum cellulase titers 35.32 U/g were observed in *P. deltoides* wood, followed by *Eucalyptus sp.* (30.40 U/g), while the minimum cellulase titer 6.86 U/g was noticed in *P. wallichiana* wood. In case of reducing sugars released the maximum were noticed in *P. deltoides* wood. The maximum percent increase 93.06 was observed in *D. strictus* wood, while the least percent increase 0.29 was observed in *P. wallichiana* wood.

Table 39 revealed the data, the maximum xylanase titers 203.20 U/g and maximum reducing sugars 13.30 mg/g were observed in *P. deltoides* wood which was closely followed by *D. strictus* wood (197.74 U/g), while the least xylanase titer 40.50 U/g was noticed in *P. wallichiana* wood. The maximum percent increase (73.71) was observed in *Eucalyptus sp.* wood and the minimum (10.32) was observed in *C. deodara* wood.

Alkaline peroxide is an effective method for pretreatment of biomass. It is basically a delignification process, in which a significant amount of hemicellulose is solubilized as well. In this method, the lignocelluloses are soaked in pH-adjusted water (e.g. to pH 11-12 using NaOH) containing H₂O₂ at room temperatures for a period of time (e.g. 2-3 h). The process can improve the enzymatic hydrolysis by delignification. The additional advantages of alkaline hydrogen peroxide pretreatments are the use of reagents with low environmental impact (Banerjee *et al.*, 2011). Alkaline peroxide pre-treatments which belong to chemical methods are effective processes for pretreating lignocellulose materials (Zhang, *et al.*, 2011). Zhao *et al.* (2007) reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose compared with H₂SO₄ pretreatment. Compared with acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers Gasper *et al.* (2007).

Table 36: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using NaOH + H₂O₂ pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β-glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>P. deltoides</i>	8.6	1.57 (26.50)	31.88 (3.18)	11.99 (1.19)	12.03 (1.20)	17.71 (1.77)	41.73* (4.17)**	30.89	7.46*** (0.74)
<i>D. strictus</i>	8.5	1.02 (11.1)	8.72 (0.87)	3.59 (0.35)	2.74 (0.27)	5.07 (0.50)	11.40 (1.14)	30.73	2.12 (0.21)
<i>Eucalyptus</i> sp.	8.5	0.72 (19.9)	9.21 (0.92)	3.79 (0.37)	4.17 (0.41)	6.46 (0.64)	14.42 (1.44)	56.56	2.45 (0.24)
<i>P. roxburghii</i>	8.7	3.66 (2.75)	7.13 (0.71)	2.03 (0.20)	3.80 (0.38)	4.26 (0.42)	10.09 (1.00)	41.51	1.79 (0.17)
<i>P. wallichiana</i>	8.5	1.14 (19.90)	16.25 (1.62)	4.95 (0.49)	7.48 (0.74)	10.43 (1.04)	22.86 (2.28)	40.67	4.16 (0.41)
<i>C. deodara</i>	8.9	4.50 (3.75)	5.76 (0.57)	1.02 (0.10)	0.79 (0.07)	15.08 (1.50)	16.89 (1.68)	193.22	3.10 (0.31)
CD_{0.05}		0.16 (1.26)	0.016 (0.02)	0.17 (0.01)	0.17 (0.07)	0.177 (0.01)	0.74 (0.07)		1.67 (0.01)
S.E.		(0.07) (0.58)	0.07 (0.01)	0.08 (0.08)	0.08 (0.03)	0.081 (0.01)	0.34 (0.03)		0.76 (0.01)

#Initial pH- 8.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU =μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 37: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using NaOH + H₂O₂ pretreated lignocellulosic forest waste under submerged fermentation (SmF)

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars(mg/g)
<i>Eucalyptus</i> sp.	6.6	4.46 (19.70)	58.59 (5.85)	87.97* (8.79)**	50.14	7.35*** (0.73)
<i>D. strictus</i>	6.4	3.58 (20.40)	58.07 (5.80)	73.12 (7.31)	25.91	6.15 (0.61)
<i>P. deltoides</i>	6.7	5.98 (18.70)	76.73 (7.67)	111.98 (11.19)	45.94	9.52 (0.95)
<i>P. roxburghii</i>	6.4	3.03 (17.00)	36.73 (3.67)	51.58 (5.15)	40.43	4.35 (0.43)
<i>P. wallichiana</i>	6.6	2.08 (18.40)	24.70 (2.47)	38.40 (3.84)	55.46	3.23 (0.32)
<i>C. deodara</i>	6.3	2.37 (20.10)	34.83 (3.48)	47.77 (4.77)	37.15	4.06 (0.40)
SE		1.77 (0.73)	0.01 (0.01)	0.01 (0.07)		0.74 (0.01)
CD _{0.050}		0.81 (0.33)	0.08 (0.07)	0.08 (0.03)		1.62 (0.02)

#Initial pH-5.5

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 38: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using NaOH + H₂O₂ pretreated lignocellulosic forest waste under solidstate fermentation

Biomass	#Final pH	Protein mg/g (mg/ml)	Total enzyme (U/g) Untreated	CMCase	FPase	β- glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars(mg/g)
<i>Eucalyptus</i> sp.	7.8	0.81 (37.20)	17.94 (0.89)	4.02 (0.20)	3.22 (0.16)	23.16 (1.15)	30.40* (1.52)**	69.45	9.12*** (0.45)
<i>D. strictus</i>	7.2	0.49 (46.3)	11.82 (0.59)	3.12 (0.15)	6.88 (0.34)	12.82 (0.64)	22.82 (1.41)	93.06	6.34 (0.31)
<i>P. deltoides</i>	7.9	0.75 (47.00)	26.64 (1.33)	1.68 (0.08)	6.36 (0.31)	27.28 (1.36)	35.32 (1.76)	32.58	10.60 (0.53)
<i>P. roxburghii</i>	7.0	18.38 (0.62)	9.42 (0.47)	5.06 (0.25)	3.48 (0.17)	0.86 (0.04)	9.50 (0.57)	0.84	2.95 (0.14)
<i>P. wallichiana</i>	7.4	2.97 (2.14)	6.84 (0.34)	2.84 (0.14)	2.44 (0.12)	1.08 (0.05)	6.86 (0.34)	0.29	2.10 (0.10)
<i>C. deodara</i>	7.6	1.44 (18.60)	22.12 (1.10)	6.70 (0.33)	6.70 (0.33)	13.40 (0.67)	26.80 (1.34)	21.15	7.90 (0.39)
CD_{0.05}		1.62 (1.62)	1.77 (0.16)	1.62 (0.12)	1.77 (0.12)	1.45 (0.12)	1.77 (0.16)		1.02 (0.15)
S.E.		0.74 (0.74)	0.81 (0.07)	0.74 (0.05)	0.81 (0.05)	0.66 (0.05)	0.81 (0.07)		0.47 (0.01)

#Initial pH-6.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 39: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using NaOH + H₂O₂ pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars(mg/g)
<i>Eucalyptus</i> sp.	7.9	3.16 (48.40)	88.10 (4.40)	153.04* (7.65)**	73.71	10.12*** (0.50)
<i>D. strictus</i>	7.0	4.37 (45.20)	126.48 (6.32)	197.74 (9.88)	56.34	13.06 (0.65)
<i>P. deltoides</i>	7.5	4.00 (50.80)	145.84 (7.29)	203.20 (10.16)	39.33	13.30 (0.66)
<i>P. roxburghii</i>	7.4	2.42 (20.00)	38.64 (1.93)	48.56 (2.42)	25.67	3.96 (0.19)
<i>P. wallichiana</i>	7.2	2.70 (15.00)	29.82 (1.49)	40.50 (2.02)	35.81	3.43 (0.17)
<i>C. deodara</i>	7.1	4.35 (19.60)	168.00 (8.40)	185.34 (9.26)	10.32	12.06 (0.60)
CD_{0.05}		1.77 (1.77)	1.779 (2.24)	1.77 (1.77)		1.25 (0.05)
S.E.		0.81 (0.81)	0.81 (0.66)	0.81 (0.81)		0.57 (0.02)

#Initial pH-6.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU =μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

In the literature it is described that not only does alkaline hydrogen peroxide pre-treatment causes selective removal of lignin and xylan without having a large effect on cellulose, but it also decreases cellulose crystallinity and swelling of biomass, thus it decreases the recalcitrance of lignocelluloses and enhances the enzymatic digestibility of cellulose. Apart from that, it has been reported that no furfural or HMF (inhibitory sugar degradation products) were detected after this pre-treatment (Taherzadeh and Karimi, 2008). In this process the pH is one of the most important parameters for efficient application of peroxide. Depending on pH adopted during lignin oxidation, no significant changes in chemical structure might be observed since the oxidizing agent acts only in the aliphatic part of the macromolecule. It is generally accepted that the hydro peroxide anion formed in alkaline media, is the principal active species in hydrogen peroxide bleaching systems. On the contrary, hydrogen peroxide is unstable in alkaline conditions and readily decomposes particularly in the presence of certain transition metals such as manganese, iron, and copper. The decomposition products such as hydroxyl radicals and superoxide anion radicals are thought to cause the oxidation of lignin structures, which leads to the introduction of hydrophilic (carboxyl) groups, cleavage of some interunit bonds and eventually, the dissolution of lignin and hemicelluloses.

Sun *et al.* (2005) studied a two-stage process based on steam explosion pretreatment followed by alkaline peroxide post-treatment. Wheat straw was first steamed at 200-220 °C and 15-22 bar. The washed fiber was then delignified by 2% H₂O₂ at 50 °C for 5 h under pH 11.5. When compared with the steam explosion pretreatment, steam resulted in a significant loss in hemicelluloses, and about 11-12% lignin removal, while the alkaline peroxide post-treatment resulted in 81-88% removal of the original lignin, which altogether removed 92-99% of the original lignin from wheat straw. They also showed that the basic structure remaining is of lignin nature only, which is important for those who plan biomass strategies especially in terms of chemical uses of lignin. Curreli *et al.* (1997) suggested two steps, mild alkaline/oxidative pretreatment at low temperature (25-40 °C) and low concentration of chemicals. Alkaline pretreatment (1% NaOH for 24 h) in the first step solubilizes hemicellulose and a second alkaline/oxidative

step (1% NaOH and 0.3% H₂O₂ for 24 h) in order to solubilize and oxidize lignin. The pretreatment is also useful in removing waxes, silica, and the waterproof cutins that coat plant tissue.

Alkaline peroxide pre-treatment was evaluated by Diaz *et al.* (1992) for the conversion of rice hulls to simple sugars throughout enzyme hydrolysis for the subsequent ethanol production. For this purpose, the effects of peroxide concentration and reaction time were studied. Peroxide concentration showed a positive effect on degradation of rice hulls lignocellulosic structure providing a greater access to hydrolytic enzymes. Thus, better global yields (sum of the pre-treatment and hydrolysis yields) were attained when higher peroxide concentrations were used. Among the conditions tested, the highest global yield of $86.48 \pm 3.07\%$ was attained with a peroxide concentration of 7.5%, at pH 11.5, 90°C and 1h. With the proposed pre-treatment, a global yield around 70% can be achieved in 15 min of incubation.

4.6.2.4 Enzymes production using ammonia (NH₃) pretreated lignocellulosic waste

Table 40 revealed the data on cellulase production utilizing ammonia (NH₃) treated substrate from *B. stratosphericus* N₁₂ (M) under SmF. Among all the six forest waste used, maximum cellulase units 34.75 U/g and 6.94 mg/g of reducing sugars had been depicted in *P. deltoides* wood, while the minimum cellulase i.e. 6.110 U/g was noticed in *C. deodara* wood and other substrates showed cellulase production between these two ranges. The maximum percent increase 66.66 was noticed in *Eucalyptus sp.* wood, while minimum was observed in *C. deodara* wood i.e. 6.07.

The scrutiny of data in Table 41 revealed highest xylanase titers 102.50 U/g which was significantly higher than the others and maximum reducing sugars i.e. 8.16 mg/g by *B. altitudinis* Kd₁ (M) using *P. deltoides* wood followed by *Eucalyptus sp.* wood (77.60 U/g) and *D. strictus* wood (75.03 U/g), while the least xylanase units were observed in *P. wallichiana* wood i.e. 40.65 U/g. The maximum percent increase 64.57 was found in case of *P. wallichiana* wood over

untreated substrate. While the minimum was observed in *D. strictus* wood (29.20) and other substrates showed percent increase between these two ranges.

Table 42 showed the results in terms of cellulase activity from NH₃ pretreated forest wastes by *M. thermophila* SH1 under solidstate fermentation. The results revealed that *P. deltooides* wood which was subjected to ammonia pretreatment gave maximum 31.06 U/g which was followed by *D. strictus* wood (28.82 U/g) and maximum reducing sugars 8.64 mg/g were also released in *P. deltooides* wood. While the least cellulase activity was observed in case of *P. roxburghii* wood (9.56 U/g). As observed the percent increase, the highest percent increase was noticed in *D. strictus* wood i.e. 143.82 and the minimum i.e 1.00% were observed in *Eucalyptus sp.* wood.

The scrutiny of data in Table 43 revealed highest xylanase production 200.00 U/g and reducing sugars i.e. 12.00 mg/g were estimated in *P. deltooides* wood which was followed by *C. deodara* wood (175.80 U/g) and the minimum xylanase units 43.64 U/g were noticed in *P. roxburghii* wood and *P. wallichiana* wood. The maximum percent increase in xylanase production i.e 46.34 was noticed in *P. wallichiana* wood and minimum 4.64 was observed in *C. deodara* wood.

Pretreatment of biomass with aqueous ammonia at elevated temperatures reduces lignin content and removes some hemicellulose while decrystallising cellulose. Ammonia pretreatment techniques include the ammonia fibre explosion-method (AFEX), ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA). Soaking in aqueous ammonia (SAA) at low temperature removes efficiently the lignin in the raw material by minimizing the interaction with hemicellulose. As a result an increase of surface area and pore size of biomass is achieved. Thus, retained hemicellulose and cellulose can be hydrolyzed to fermentable sugars by most xylanase and cellulase enzymes mixtures. Aqueous ammonia was found to not significantly degrade hemicellulose; in addition lignin can react with aqueous ammonia and depolymerized which leads to the cleavage of lignin- carbohydrate linkage in hardwood. Two advantages of ammonia pretreatments are that it does not form inhibitors for

Table 40: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using NH₃ pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β-glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>P. deltoides</i>	8.6	1.57 (22.00)	31.88 (3.18)	11.36 (1.13)	11.42 (1.14)	11.97 (1.19)	34.75* (3.47)**	9.00	6.94*** (0.69)
<i>D. strictus</i>	8.5	2.27 (5.30)	8.72 (0.87)	4.11 (0.41)	3.17 (0.31)	4.78 (0.47)	12.06 (1.20)	38.30	2.23 (0.22)
<i>Eucalyptus</i> sp.	8.9	0.94 (16.30)	9.21 (0.92)	4.07 (0.40)	4.29 (0.42)	6.99 (0.69)	15.35 (1.53)	66.66	3.15 (0.31)
<i>P. roxburghii</i>	8.6	9.25 (0.86)	7.13 (0.71)	2.08 (0.20)	3.49 (0.34)	2.39 (0.23)	7.96 (0.79)	11.64	1.49 (0.14)
<i>P. wallichiana</i>	8.8	0.96 (19.70)	16.25 (1.62)	4.62 (0.46)	6.04 (0.60)	8.42 (0.84)	19.08 (1.90)	17.41	3.89 (0.38)
<i>C. deodara</i>	8.9	1.74 (3.50)	5.76 (0.57)	0.45 (0.04)	0.40 (0.04)	5.26 (0.52)	6.11 (0.61)	6.07	1.26 (0.12)
CD_{0.05}		0.72 (0.81)	0.016 (0.02)	1.45 (0.14)	1.62 (0.16)	0.72 (0.01)	0.01 (0.07)		1.25 (0.01)
S.E.		0.33 (0.37)	0.07 (0.01)	0.66 (0.06)	0.74 (0.07)	0.33 (0.09)	0.07 (0.03)		0.57 (0.01)

#Initial pH- 8.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU =μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 41: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using NH₃ pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars(mg/g)
<i>Eucalyptus</i> sp.	6.7	3.93 (19.70)	58.59 (5.85)	77.60* (7.76)**	32.44	6.01*** (0.60)
<i>D. strictus</i>	6.4	3.50 (21.40)	58.07 (5.80)	75.03 (7.50)	29.20	5.90 (0.50)
<i>P. deltoides</i>	6.6	5.02 (20.40)	76.73 (7.67)	102.50 (10.25)	33.58	8.16 (0.81)
<i>P. roxburghii</i>	6.0	1.46 (30.40)	36.73 (3.67)	49.71 (4.97)	35.33	3.95 (0.39)
<i>P. wallichiana</i>	6.8	2.06 (19.70)	24.70 (2.47)	40.65 (4.06)	64.57	3.31 (0.33)
<i>C. deodara</i>	6.4	2.75 (18.40)	34.83 (3.48)	50.61 (5.06)	45.30	3.89 (0.38)
CD_{0.05}		1.45 (0.01)	0.01 (0.01)	0.01 (0.01)		0.72 (0.01)
S.E.		0.66 (0.08)	0.08 (0.07)	0.08 (0.05)		0.33 (0.01)

#Initial pH-5.5

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 42: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using NH₃ pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β-glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	7.5	0.47 (38.20)	17.94 (0.89)	3.18 (0.15)	2.46 (0.12)	11.48 (0.57)	18.12* (0.85)**	1.00	5.10*** (0.25)
<i>D. strictus</i>	7.7	0.61 (47.00)	11.82 (0.59)	2.40 (0.12)	6.04 (0.30)	20.38 (1.09)	28.82 (1.44)	143.82	8.08 (0.40)
<i>P. deltoidea</i>	7.0	0.69 (44.80)	26.64 (1.33)	5.14 (0.25)	6.40 (0.32)	19.52 (0.97)	31.06 (1.55)	16.59	8.64 (0.43)
<i>P. roxburghii</i>	7.9	4.97 (1.92)	9.42 (0.47)	5.60 (0.28)	2.54 (0.12)	1.42 (0.07)	9.56 (0.47)	1.48	2.60 (0.13)
<i>P. wallichiana</i>	7.4	0.67 (19.00)	6.84 (0.34)	2.48 (0.12)	2.28 (0.11)	8.12 (0.40)	12.88 (0.64)	88.30	3.65 (0.18)
<i>C. deodara</i>	7.3	1.31 (17.40)	22.12 (1.10)	6.76 (0.33)	4.50 (0.32)	10.96 (1.04)	22.82 (1.71)	3.16	6.24 (0.31)
CD_{0.05}		0.74 (1.62)	1.77 (0.16)	1.77 (0.12)	1.77 (0.12)	1.62 (0.12)	1.77 (0.17)		1.77 (0.04)
S.E.		0.34 (0.74)	0.81 (0.07)	0.81 (0.05)	0.81 (0.05)	0.74 (0.05)	0.81 (0.08)		0.81 (0.01)

#Initial pH-6.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU = μmoles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 43: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using NH₃ pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	7.2	3.26 (37.40)	88.10 (4.40)	122.10* (6.10)**	38.59	7.29*** (0.36)
<i>D. strictus</i>	7.5	4.10 (40.40)	126.48 (6.32)	166.02 (8.30)	31.26	9.06 (0.45)
<i>P. deltoides</i>	7.9	4.44 (45.00)	145.84 (7.29)	200.00 (10.00)	37.13	12.00 (0.60)
<i>P. roxburghii</i>	7.4	2.50 (17.40)	38.64 (1.93)	43.64 (2.18)	12.93	3.65 (0.18)
<i>P. wallichiana</i>	7.4	3.51 (12.40)	29.82 (1.49)	43.64 (2.18)	46.34	2.99 (0.14)
<i>C. deodara</i>	7.3	11.41 (15.40)	168.00 (8.40)	175.80 (8.79)	4.64	9.58 (0.47)
CD_{0.05}		1.77 (1.77)	1.77 (2.24)	1.77 (1.77)		1.09 (0.02)
S.E.		0.81 (0.81)	0.81 (0.66)	0.81 (0.81)		0.50 (0.01)

#Initial pH-6.0

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

downstream processes and it does not require reducing particle size (Holtzapple *et al.*, 1990).

Kim *et al.* 2008 evaluated SAA (soaking in aqueous ammonia) as a pretreatment method of biomass. They destarched barley hull was treated with 15-30% aqueous ammonia, at 30-75°C for 12h to 7days with no agitation and a solid-to-liquid ratio 1:12. After soaking, the solids were recovered by filtrating, washed and analyzed. As result, they obtained 66% of lignin solubilization and observed saccharification yields of 83% for glucan and 63% for xylan when treating biomass with 15% aqueous ammonia at 75°C during 48h.

Approximately 96% glucan and 81% xylan conversions were achieved after 168 h of enzymatic hydrolysis at a 1% glucan loading using cellulase and R-glucosidase along with xylanase and Tween-80 supplementation. (Murren *et al.*,2007). Isci *et al.* (2008) reported that 40-50% delignification was achieved when switchgrass was soaked in aqueous ammonium hydroxide (30%) with different liquid/solid ratios (5 and 10 mL/g) for either 5 or 10 days. The hemicellulose content decreased by approximately 50%. The highest delignification (47%) was achieved with soaking in ammonium hydroxide for 10 days at 10 mL/g of biomass. Lee *et al.* (2000) reported approximately 50% lignin removal from corn stover in 4 days with a loading of 12 mL/g of ground corn stover. Kim and co-workers (2005) reported that the ARP process can remove up to 85% of lignin from corn stover.

4.6.2.5 Enzymes production using steam pretreated lignocellulosic waste

The different lignocellulosic substrate namely, *Eucalyptus sp.*, *P. deltoides*, *D. strictus*, *P. roxburghii*, *P. wallichiana* and *C. deodara* 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 44, 45, 46 and 47.

A perusal of Table 44 showed significant differences in cellulase production when different substrate and steam pretreatment was used for the

study in *B. stratosphericus* N₁₂ (M) under SmF. Maximum cellulase titers 33.59 U/g and reducing sugars i.e 6.54 mg/g were produced in *P. deltoides* wood and the least cellulase titers 9.30 U/g were observed in *C. deodara* wood, while other substrates showed cellulase production between these two ranges. Maximum percent increase 84.01 was observed in *P. roxburghii* wood over untreated, while least percent increase found in *Eucalyptus sp.* wood i.e. 5.32.

Table 45 revealed the highest xylanase units of 95.40 U/g and 7.43 mg/g of reducing sugars by *B. altitudinis* Kd₁ (M) was noticed in *P. deltoides* wood followed by *Eucalyptus sp.* wood i.e. 70.28 U/g, while the least xylanase titers 37.83U/g was observed in *C. deodara* wood. Maximum percent increase i.e. 54.04 in xylanase production was observed in *P. wallichiana* wood and the minimum of 1.60 was observed in *D. strictus* wood over untreated substrate.

In case of SSF, Table 46 revealed highest cellulase production 29.00 U/g and reducing sugars i.e. 8.24 mg/g by *M. thermophila* SH1 in *P. deltoides* wood, followed by *Eucalyptus sp.* wood (25.82 U/g) and the minimum was observed in *P. wallichiana* wood (7.28 U/g). Maximum percent increase 43.92 was noticed in *Eucalyptus sp.* wood and the least i.e. 3.16 was found in *C. deodara* wood. On the other hand, the maximum xylanase units 169.60 U/g were found in *C. deodara* wood followed by *P. deltoides* wood (157.56 U/g), while the minimum xylanase production was noticed in *P. wallichiana* wood (38.64 U/g). The maximum reducing sugars 13.54 mg/g were noticed in *P. deltoides* wood and the maximum percent increase 31.50% was noticed in *Eucalyptus sp.* wood, while the least 0.95 was found in *C. deodara* wood.

The steam explosion pretreatment process has been a proven technique for the pretreatment of different biomass feedstocks. It is able to generate complete sugar recovery while utilizing a low capital investment and low environmental impacts concerning the chemicals and conditions being implemented and has a higher potential for optimization and efficiency.

Steam pretreatment involves heating of biomass at high temperature pressure followed by mechanical disruption of the pretreated material either by

Table 44: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using steam pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β-glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugar (mg/g)
<i>P. deltoides</i>	8.6	1.607 (20.09)	31.88 (3.18)	10.45 (1.04)	11.17 (1.11)	11.97 (1.19)	33.59* (3.35)**	5.36	6.54*** (0.65)
<i>D. strictus</i>	8.6	1.81 (6.40)	8.72 (0.87)	3.54 (0.35)	2.68 (0.26)	5.41 (0.54)	11.63 (1.16)	33.37	2.35 (0.23)
<i>Eucalyptus</i> sp.	8.5	0.73 (13.20)	9.21 (0.92)	3.67 (0.36)	3.45 (0.34)	2.58 (0.25)	9.70 (0.97)	5.32	1.78 (0.17)
<i>P. roxburghii</i>	8.9	7.85 (1.67)	7.13 (0.71)	1.94 (0.19)	3.62 (0.36)	7.56 (0.75)	13.12 (1.31)	84.01	2.45 (0.24)
<i>P. wallichiana</i>	8.6	1.16 (16.31)	16.25 (1.62)	5.11 (0.51)	6.70 (0.67)	7.24 (0.72)	19.05 (1.90)	17.23	3.59 (0.35)
<i>C. deodara</i>	8.5	2.66 (3.49)	5.76 (0.57)	0.66 (0.06)	0.31 (0.03)	8.33 (0.83)	9.30 (0.93)	61.45	1.75 (0.17)
CD_{0.05}		1.02 (1.77)	0.01 (0.02)	1.62 (0.12)	1.45 (0.14)	1.77 (0.16)	1.62 (0.72)		1.02 (0.01)
S.E.		0.47 (0.81)	0.07 (0.01)	0.74 (0.05)	0.66 (0.06)	0.81 (0.07)	0.74 (0.33)		0.47 (0.01)

#Initial pH- 8.0

* U/g = μmoles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 45: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using steam pretreated lignocellulosic forest waste under submerged fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	6.8	3.39 (20.70)	58.59 (5.85)	70.28* (7.02)**	19.95	5.31*** (0.53)
<i>D. strictus</i>	6.6	2.99 (19.70)	58.07 (5.80)	59.00 (5.90)	1.60	4.75 (0.47)
<i>P. deltooides</i>	6.7	5.10 (18.70)	76.73 (7.67)	95.40 (9.54)	24.33	7.43 (0.74)
<i>P. roxburghii</i>	6.4	1.86 (20.40)	36.73 (3.67)	38.09 (3.80)	3.70	2.68 (0.26)
<i>P. wallichiana</i>	6.6	1.90 (20.00)	24.70 (2.47)	38.05 (3.80)	54.04	2.92 (0.29)
<i>C. deodara</i>	6.4	1.97 (17.60)	34.83 (3.48)	37.83 (3.48)	8.61	2.94 (0.29)
CD_{0.05}		1.25 (0.72)	0.01 (0.01)	0.72 (0.07)		0.72 (0.01)
S.E.		0.57 (0.33)	0.08 (0.07)	0.33 (0.03)		0.33 (0.01)

.#Initial pH-5.5

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 46: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using steam pretreated lignocellulosic forest waste under solid state fermentation

Biomass	#Final pH	Specific activity	Total enzyme (U/g) Untreated	CMCase	FPase	β -glucosidase	Total enzyme (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	7.9	2.42 (10.64)	17.94 (0.89)	1.80 (0.09)	0.56 (0.02)	23.46 (1.17)	25.82* (1.29)**	43.92	7.20*** (0.36)
<i>D. strictus</i>	7.6	0.60 (27.10)	11.82 (0.59)	1.38 (0.06)	2.00 (0.10)	13.00 (0.92)	16.38 (0.86)	38.57	4.73 (0.23)
<i>P. deltooides</i>	7.4	0.68 (42.60)	26.64 (1.33)	6.46 (0.32)	6.46 (0.32)	16.08 (0.84)	29.00 (1.45)	8.85	8.24 (0.41)
<i>P. roxburghii</i>	7.2	6.04 (1.62)	9.42 (0.47)	5.92 (0.28)	3.88 (0.17)	0.00 (0.00)	9.80 (0.96)	4.03	2.94 (0.14)
<i>P. wallichiana</i>	7.6	2.14 (3.40)	6.84 (0.34)	1.50 (0.07)	1.78 (0.08)	4.00 (0.20)	7.28 (0.36)	6.43	2.15 (0.10)
<i>C. deodara</i>	7.8	0.67 (34.00)	22.12 (1.10)	6.76 (0.33)	4.50 (0.32)	10.96 (1.04)	22.82 (1.71)	3.16	6.32 (0.31)
CD_{0.05}		1.26 (1.62)	1.77 (0.16)	1.26 (0.12)	1.45 (0.10)	1.45 (0.10)	1.77 (0.15)		0.04 (0.02)
S.E.		0.57 (0.74)	0.81 (0.07)	0.58 (0.05)	0.66 (0.04)	0.66 (0.04)	0.81 (0.07)		0.02 (0.01)

#Initial pH-6.0

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 47: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using steam pretreated lignocellulosic forest waste under solidstate fermentation

Biomass	#Final pH	Specific activity	Xylanase (U/g) Untreated	Xylanase (U/g) Treated	% Increase	Reducing sugars (mg/g)
<i>Eucalyptus</i> sp.	7.5	2.79 (41.40)	88.10 (4.40)	115.86* (5.79)**	31.50	9.80*** (0.49)
<i>D. strictus</i>	7.0	3.33 (42.60)	126.48 (6.32)	142.02 (7.10)	12.28	11.02 (0.55)
<i>P. deltoides</i>	7.1	3.26 (48.20)	145.84 (7.29)	157.56 (7.87)	8.03	13.54 (0.67)
<i>P. roxburghii</i>	7.9	1.44 (20.60)	38.64 (1.93)	39.82 (1.99)	3.05	3.90 (0.19)
<i>P. wallichiana</i>	7.9	2.97 (13.00)	29.82 (1.49)	38.64 (1.93)	29.57	3.54 (0.17)
<i>C. deodara</i>	7.7	10.60 (15.80)	168.00 (8.40)	169.60 (8.39)	0.95	13.50 (0.67)
CD_{0.05}		1.62 (7.44)	1.77 (2.24)	1.77 (1.45)		0.91 (0.01)
S.E.		0.74 (3.41)	0.81 (0.66)	0.81 (0.66)		0.41 (0.01)

#Initial pH-6.0

* U/g = μ moles of reducing sugars released / min / g of biomass

** IU = μ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)

***Reducing sugars released mg/g(Value in parentheses depicted sugars mg/ml)

Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

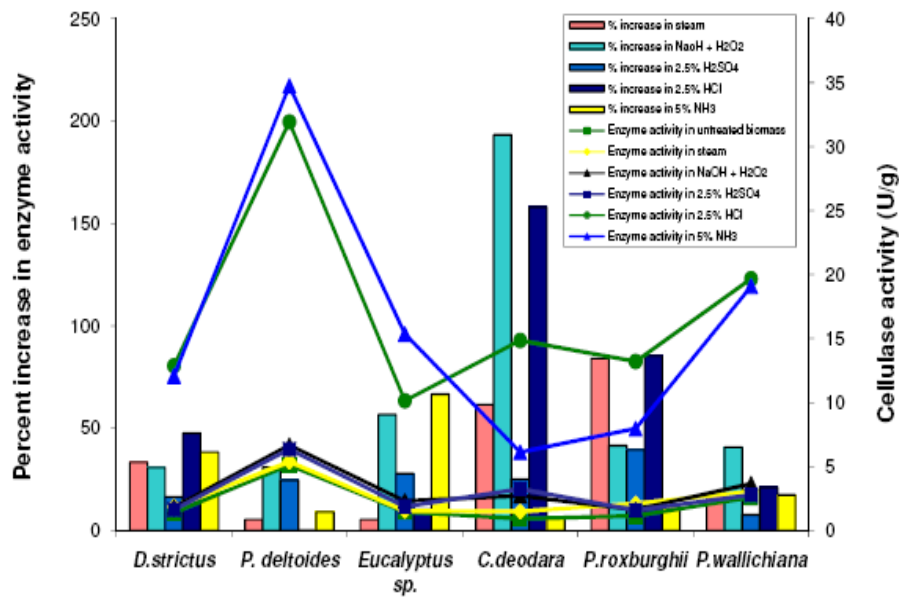


Fig 30. A comparison of percent increase in cellulase activity of pretreated lignocellulosic forest waste over untreated lignocellulosic forest waste by *B. stratosphericus* N₁₂ (M)

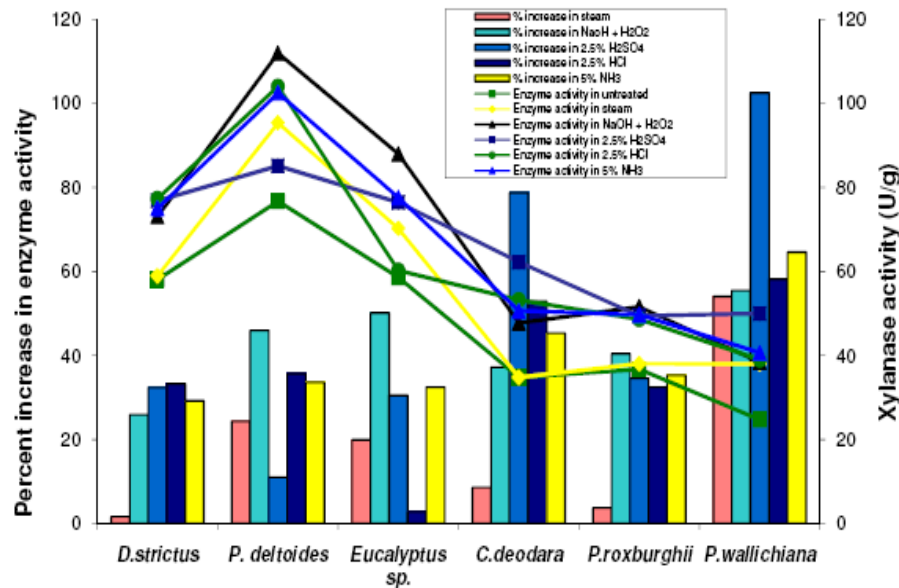


Fig 31. A comparison of percent increase in Xylanase activity of pretreated lignocellulosic forest waste over untreated lignocellulosic forest waste by *B. altitudinis* Kd₁ (M)

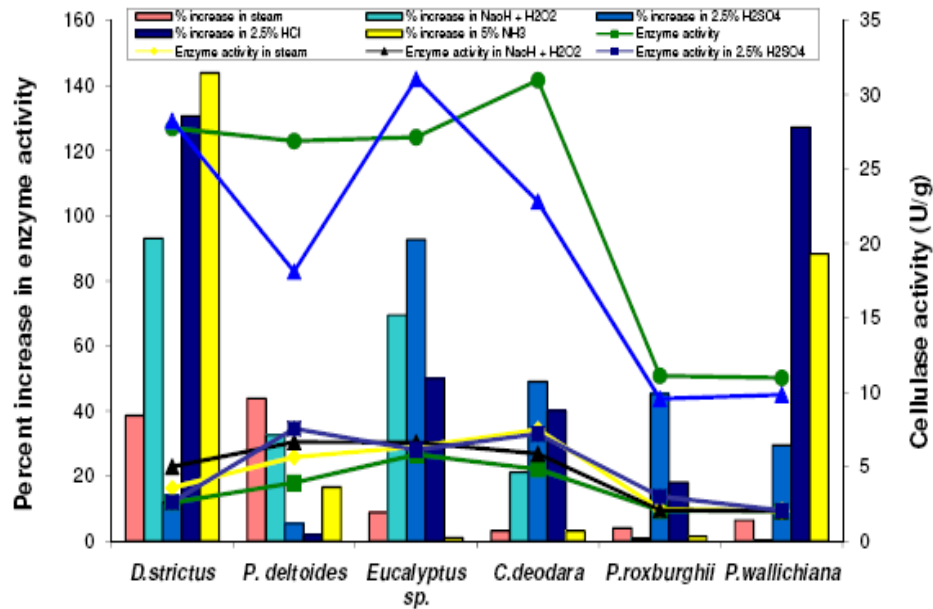


Fig 32. A comparison of percent increase in cellulase activity of pretreated lignocellulosic forest waste over untreated lignocellulosic forest waste by *M. thermophila* SH1

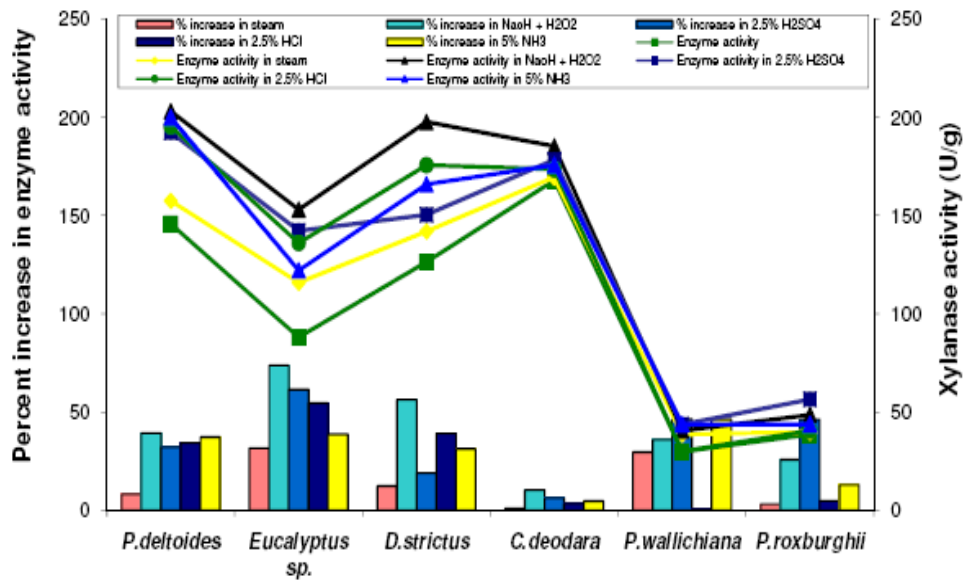


Fig. 33. A comparison of percent increase in xylanase activity of pretreated lignocellulosic forest waste over untreated lignocellulosic forest waste by *M. thermophila* SH1

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. It is one of the most intensively studied pretreatment methods for bioconversion of lignocellulosic materials. The steam explosion process uses high temperature and pressure followed by sudden release to separate individual fibers within the wood. Meanwhile most of hemicellulose is hydrolysed and removed during steam explosion, contributing to the improved susceptibility of the substrate. However, the steam explosion process does not result in net delignification. The lignin content after steam explosion is actually increased due to the removal of hemicellulose (Pan *et al.*, 2010). Steam pretreatment destruct a part of the xylan in biomass, incompletely solubilization of lignin- carbohydrate matrix and generate inhibitory compounds that may affect the growth of microorganisms used in downstream processes.

Ruiz *et al.* (2008) studied steam explosion for pretreatment of sunflower stalks before enzymatic hydrolysis at a temperature in the range of 180–230 °C. The highest glucose yield was obtained in steam-pretreated sunflower stalks at 220 °C, while the highest hemicellulose recovery was obtained at 210 °C pretreatment temperature. Using a steam explosion process for pretreatment of poplar (*Populus nigra*) biomass, at 210 °C and 4 min, resulted in cellulose recovery above 95%, enzymatic hydrolysis yield of about 60%, and 41% xylose recovery in the liquid fraction. Furthermore, the large particles can be used for poplar biomass, since no significant effect of particle size on enzymatic hydrolysis was observed (Negro *et al.*, 2003). Ballesteros *et al.* (2004) applied steam explosion for production of ethanol from several lignocellulosic materials with *Kluyveromyces marxianus*. The Poplar and Eucalyptus chips were treated at 210°C for 4 min; wheat straw at 190 °C for 8 min; *Brassica carinata* residue at 210 °C at 8 min; and sweet sorghum bagasse at 210 °C for 2 min. Steam explosion extensively solubilized the hemicellulosic sugars and decreased 75–90% of xylose content, depending on the substrate. It is possible to combine steaming and mechanical treatment to effectively disrupt the cellulosic structure.

Several combination technologies have been developed (Katzen *et al.*, 1995). Tjeerdsma and Militz (2005), found that hemicellulose is more vulnerable for hydrolysis than cellulose. Dehydration reactions occur naturally during thermal treatments of polysaccharides, causing a decomposition of hydrolysed sugars (Fengel and Wegener, 1984). Pyranosidic ring structures will be dehydrated into hydroxymethylfurfural and furanosidic ring structures in furfural (Bobleter and Binder, 1980). According to Abatzoglou *et al.* (1990), hemicellulose are easy to be hydrolysed at high temperatures between 200 to 230°C because of its structure and characteristics for example non-crystalline, highly disordered and heteropolymeric forms (Ishii and Shimizu, 2001).

Overall, it has been observed that lignocellulosic forest waste can act as a suitable substrate for cellulase and xylanase production, though; different physico-chemical pretreatments are prerequisite to enhance the yield of enzyme. As shown in Fig 30, 31, 32 and 33 all pretreated forest substrate have resulted in more titers of cellulase and xylanase as compared to untreated ones from *B. stratosphericus* N₁₂ (M) and *B. altitudinis* Kd₁ (M) under submerged fermentation (SmF) as well as by *M. thermophila* SH1 under solidstate fermentation (SSF). 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 alkaline hydrogen peroxide pretreatment. Plates 4, 5, 6, 7, 8 and 9 showed the growth of *M. thermophila* SH1 and cellulase as well as xylanase production under solid state fermentation using different (hardwood and softwood biomass) untreated and pretreated lignocellulosic forest biomass.

As far as cellulase and xylanase production profile under submerged fermentation (SmF) is concerned, maximum cellulase and xylanase activity was observed in alkaline hydrogen peroxide pretreated *P. deltoides* wood reaching upto 41.73 U/g and 11.98 U/g, respectively. Maximum per cent increase in cellulase activity was observed in dil. H₂SO₄ pretreated *C. deodara* wood by *B. stratosphericus* N₁₂ (M) whereas, maximum per cent increase in xylanase activity was observed in dil. H₂SO₄ pretreated *P. wallichiana* wood by *B. altitudinis* Kd₁

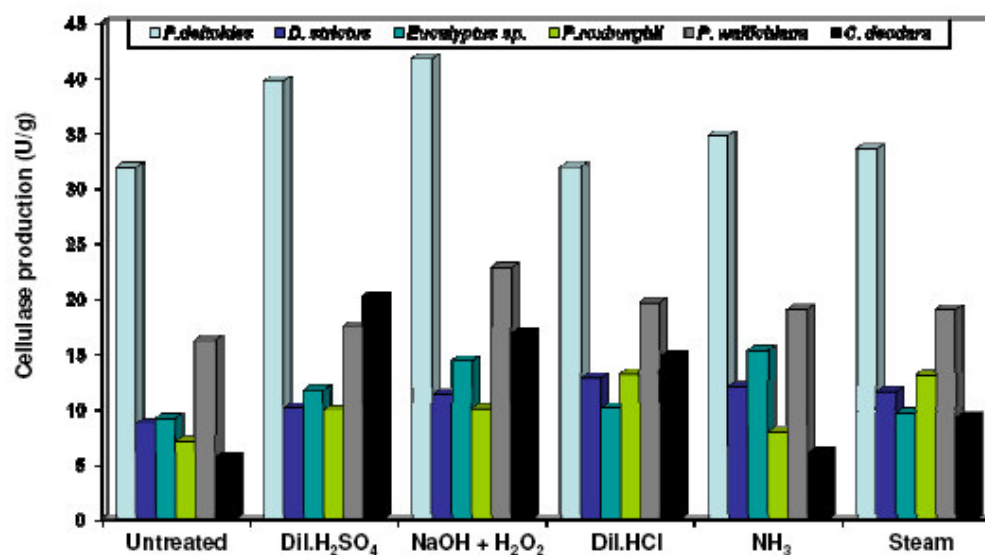


Fig 34 An overview of cellulase production by *B. stratosphericus* N₁₂ (M) using untreated and pretreated lignocellulosic forest wastes

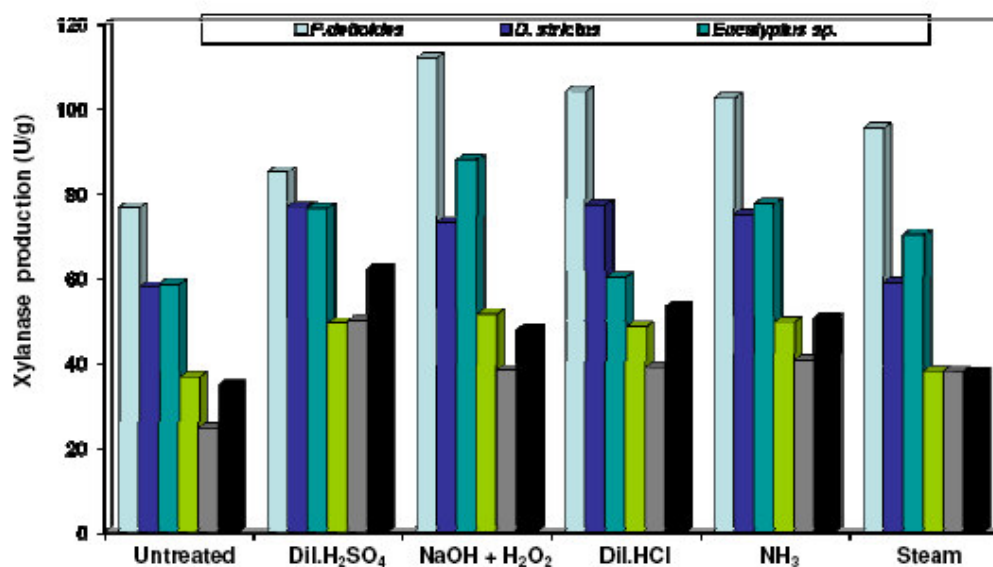


Fig 35. An Overview of xylanase production by *B. altitudinis* Kd₁ (M) using untreated and pretreated lignocellulosic forest wastes

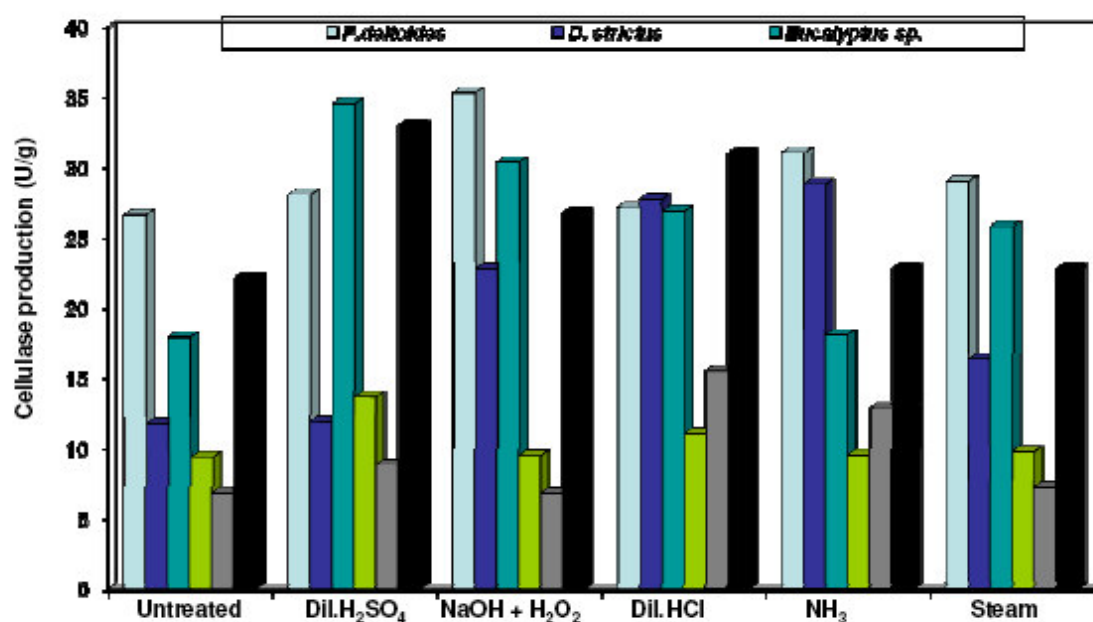


Fig 36. An Overview of cellulase production by *M. thermophila* SH1 (M) using untreated and pretreated lignocellulosic forest wastes

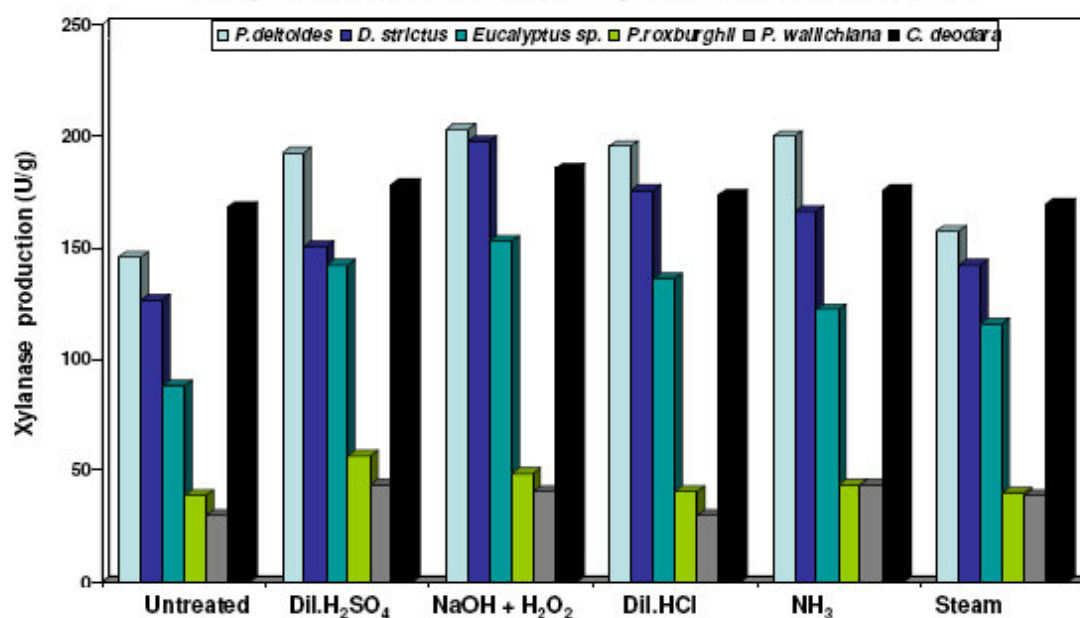


Fig 37. An overview of xylanase production by *M. thermophila* SH1 (M) using untreated and pretreated lignocellulosic forest waste

(M). In case of solidstate fermentation (SSF) highest cellulase and xylanase enzyme activity was found in alkaline hydrogen peroxide pretreated *P. deltoides* wood reaching upto 35.32 U/g and 203.20 U/g respectively. Maximum per cent increase in cellulase was found in ammonia pretreated *D. strictus* wood, while per cent increase in xylanase was found in alkaline hydrogen peroxide pretreated *Eucalyptus* sp. wood as shown in Fig. 30, 31, 32 and 33. Therefore statistically cellulase and xylanase production in untreated and pretreated biomass was significantly different from each other. The overall maximum enzymes production from bacteria and fungi under submerged fermentation (SmF) as well as solid state fermentation (SSF) was shown in fig 34, 35, 36 and 37. Fig 34 showed that the maximum cellulase production by *B. stratosphericus* N₁₂ (M) was observed in NaOH + H₂O₂ pretreated *P. deltoides* wood biomass. Similarly in fig 35, 36 and 37 it was observed that among the all pretreated substrates, NaOH + H₂O₂ pretreated *P. deltoides* wood was observed for maximum cellulase and xylanase production under submerged fermentation (SmF) as well as solid state fermentation (SSF). So *P. deltoides* wood was considered as the best substrate and alkaline hydrogen peroxide as the best pretreatment and were selected for the further study.

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 *B. stratosphericus* N₁₂ (M), *B. altitudinis* Kd₁ (M) and *M. thermophila* SH1 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 a cost effective process (Hu *et al.*, 2008). These screening variables which vary from biomass to biomass play a vital role to assess the effect of pretreatments 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 from wood to wood of forest

species. It strongly proves that cellulase and xylanase production from enzymatic hydrolysis of forest biomass is substrate and pretreatment specific.

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.

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 glucronic acids (Persson *et al.*, 2006; 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 six substrates taken for the enzyme production in the study, *P. deltoides* wood followed by *D. strictus* wood and *Eucalyptus sp.* wood has been rated the best substrates for cellulase and xylanase production by *B. stratosphericus* N₁₂ (M), *B. altitudinis* Kd₁ (M) and *M. thermophila* SH1 under

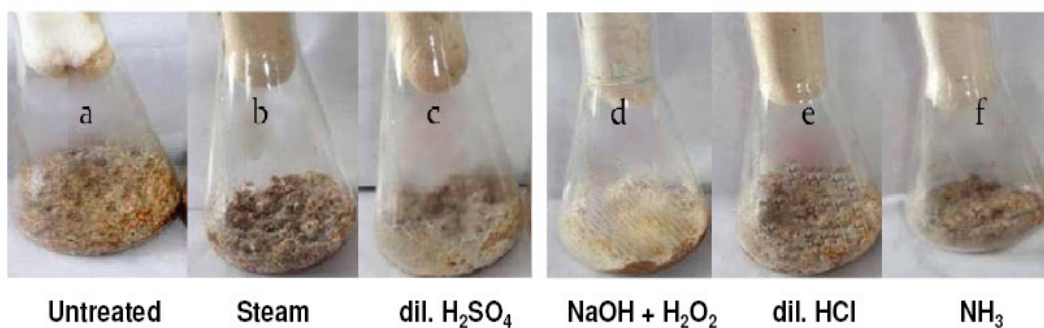


Plate 4 Extracellular cellulase and xylanase production by *M. thermophila* SH₁ under SSF using untreated and pretreated *D. strictus* wood

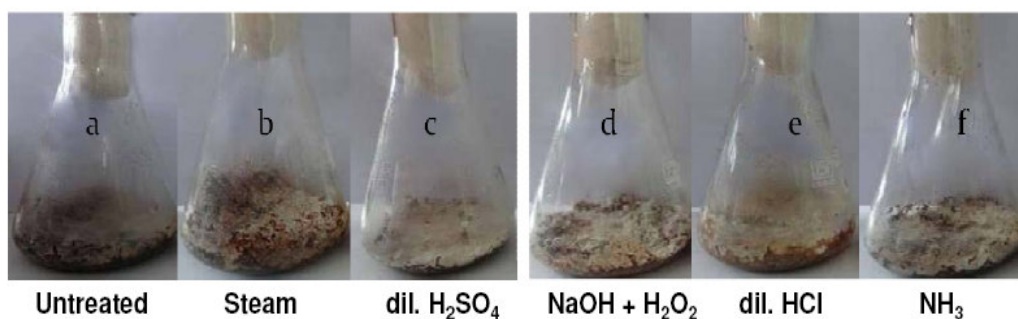


Plate 5. Extracellular cellulase and xylanase production by *M. thermophila* SH₁ under SSF using untreated and pretreated *Eucalyptus* sp wood

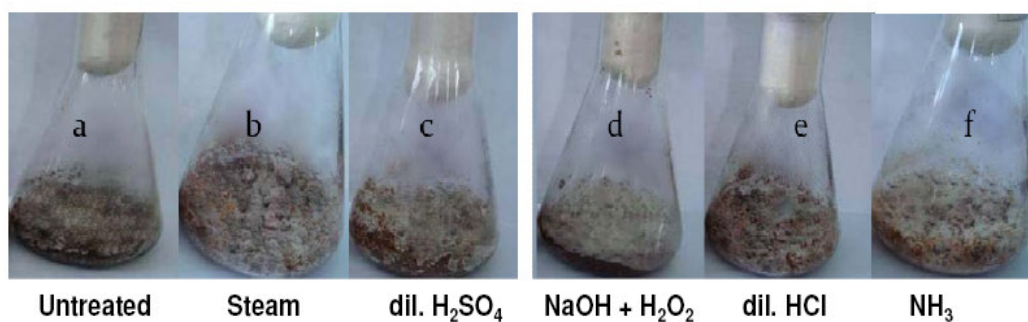


Plate 6. Extracellular cellulase and xylanase production by *M. thermophila* SH₁ under SSF using untreated and pretreated *P. deltoides* wood

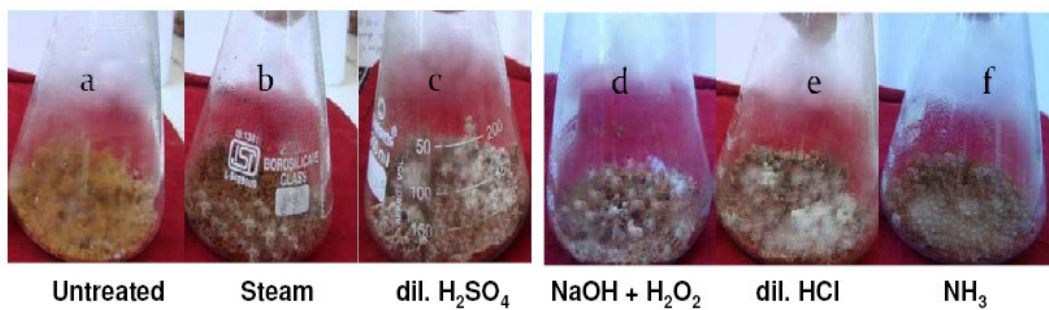


Plate 7. Extracellular cellulase and xylanase production by *M. thermophila* SH1 under SSF using untreated and pretreated *P. roxburghii* wood

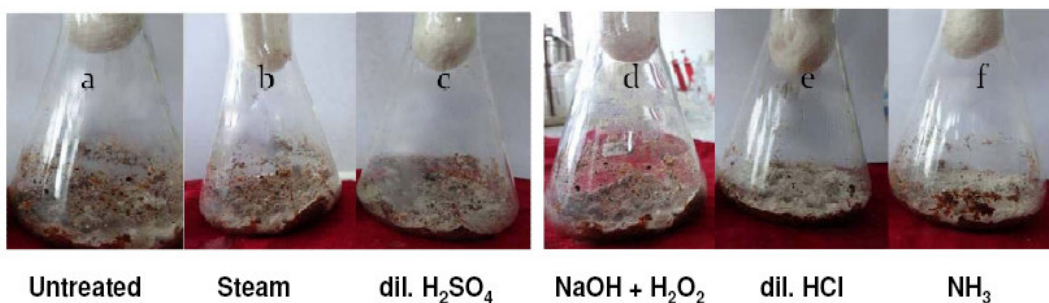


Plate 8. Extracellular cellulase and xylanase production by *M. thermophila* SH1 under SSF using untreated and pretreated *C. deodara* wood

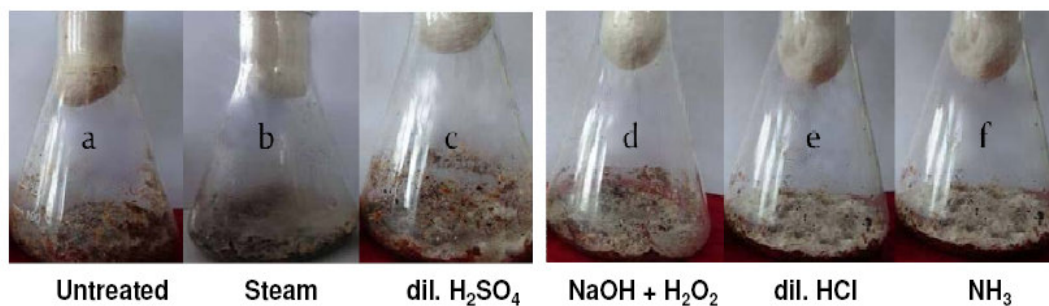


Plate 9. Extracellular cellulase and xylanase production by *M. thermophila* SH1 under SSF using untreated and pretreated *P. wallichiana* wood

SSF. As far as pretreatment is concerned, alkaline peroxide pretreatment has emerged as the most suitable method to enhance the accessibility of biomass for enzymolysis by *B. stratosphericus* N₁₂ (M), *B. altitudinis* Kd₁ (M) and *M. thermophila* SH1. 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 34, 35, 36 and 37 pretreated forest waste has resulted in more titers of cellulase and xylanase as compared to untreated ones. 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 alkaline peroxide pretreatment. As far as cellulase and xylanase production profile is concerned, maximum cellulase and xylanase activity was observed in alkaline peroxide pretreated *P. deltoides* wood reaching upto 41.73 U/g and 111.98 U/g, respectively under submerged fermentation. While under solid state fermentation by *M. thermophila* SH1 these were 35.32 U/g of cellulase units and 203.20 U/g units of xylanase were achieved.

The percent increase as shown in the figs 30, 31, 32 and 33, maximum percent increase (193.22) in cellulase activity was observed in alkaline peroxide pretreated *C. deodara* wood from *B. stratosphericus* N₁₂ (M) (fig 30). Whereas percent increase in xylanase activity was observed in H₂SO₄ pretreated *P. wallichiana* wood by *B. altitudinis* Kd₁ (M) as depicted in fig 31.

In case of solid state fermentation by *M. thermophila* SH1, maximum cellulase i.e. 35.32 U/g as well as xylanase activity i.e. 203.20 U/g were observed in alkaline peroxide pretreated *P. deltoides* wood. As shown in fig 32, overall the highest percent increase in cellulase activity i.e. 143.82 was achieved by ammonia pretreated *D. strictus* wood, while the highest percent increase in xylanase activity (73.71) was found in alkaline peroxide pretreated *Eucalyptus* sp. wood as shown in fig 33. Therefore statistically cellulase and xylanase production in untreated and pretreated biomass was significantly different from each other.

Overall it has been observed that lignocellulosic forest waste can act as a suitable substrate for cellulase and xylanase enzymes production. Though different physico-chemical pretreatments have been found prerequisite to enhance the yield of enzymes.

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 *B. stratosphericus* N₁₂ (M), *B. altitudinis* Kd₁ (M) and *M. thermophila* SH1 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 forest biomass is substrate and pretreatment specific.

In principle, an effective pretreatment method is expected to disrupt multiple barriers so that cellulose and hemicellulose can be hydrolysed easily and more of extracellular cellulase and xylanase enzymes can be secreted.

4.7 PARTIAL PURIFICATION OF CELLULASE AND XYLANASE BY AMMONIUM SULPHATE PRECIPITATION

To strengthen the enzyme activity, the enzymes cellulase and xylanase were partially purified by ammonium sulphate precipitation. Enzymes of bacteria i.e. cellulase of *B. stratosphericus* N₁₂ (M), xylanase of *B. altitudinis* and

cellulase as well as xylanase of fungus *M. thermophila* SH1 were partially purified by using salt precipitation technique.

The titers of crude cellulase of *B. stratosphericus* N12 (M) i.e CMCase: 2.202 IU, FPase: 1.852 IU, β - glucosidase was 1.929 IU were strengthened after ammonium sulphate precipitation as shown in Table 48 and reached to : CMCase: 2.384 IU, FPase: 2.249 IU and β - glucosidase: 2.451 IU within an increase of 1.08, 1.21 and 1.27 fold respectively. The final recovery of partially purified cellulase was 1.605 fold with 73.89% yield and final specific activity of 1.63 was observed.

Partial purification of xylanase from *B. altitudinis* Kd₁ (M) was performed by ammonium sulphate precipitation and it gave the maximum xylanase between 0-70%. The initial units of crude xylanase i.e 69.93 IU were escalated upto 71.49 IU after ammonium sulphate precipitation. Here 1.02 fold of increase in xylanase activity was observed with specific activity of 30.55 (Table 49). The final recovery of ammonium sulphate precipitated xylanase of *B. altitudinis* Kd₁ (M) was 1.428 fold with 71.55% yield.

Similarly partial purification of cellulase by *M. thermophila* SH1 was performed by salt saturation method. The precipitation was attained at 30-60% for CMCase and FPase and 0.30% level of saturation for β -glucosidase. Suspended pellets were collected in sterilized vials. CMCase: 0.084 IU, FPase: 0.318 IU and β - glucosidase of 1.364 IU were noticed in crude enzyme resulted in an increase of 20.85, 3.75 and 1.88 fold respectively after ammonium sulphate precipitation. Finally cellulase yielded as: CMCase (1.752 IU), FPase (1.193 IU) and β - glucosidase (2.571 IU) with the specific activity of 2.78, 1.89 and 4.07 respectively (Table 50), while the final recovery of partially purified cellulase was 3.72 fold with 84.00% yield.

As the Table 51 depicted the results of partial purification of xylanase by *M. thermophila* SH1 under SSF, Crude xylanase units of 10.16 IU were enhanced to 12.59 IU after ammonium sulphate precipitation. The partially purified

xylanase depicted 1.23 fold increases in xylanase activity with the specific activity of 11.44 and 1.712 fold of final recovery with 72.36% yield.

As is well known Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Two distinct effects are observed in the process: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). Since proteins differ markedly in their solubilities at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein (Bollag and Edelstein, 1993).

Bacillus strain M-9 was isolated from decomposing rice bran produced significant amount of endoglucanase after 72 h of fermentation. Endoglucanase was partially purified by ammonium sulphate precipitation (20-50% saturation) and characterized for few properties (Bajaj *et al.*, 2009). Yin *et al.* (2010) obtained endo- β -1,4-glucanase at 60-80 % ammonium sulphate saturation from *Bacillus subtilis* YJ1. The proteins in the crude preparation were precipitated by the addition of solid ammonium sulphate at 40% to 80% saturation from *Bacillus sp.* *Bacillus pumilus* produced xylanase demonstrating maximal activity at 35⁰C and at pH 7.0. Crude extract fractionated by ammonium sulphate precipitation had a specific activity of 0.69 $\mu\text{M min}^{-1} \text{mg}^{-1}$. Enzyme kinetics and properties were studied by DNS assay method. Xylanase had K_m of 4.0 mg ml⁻¹ and V_{max} of $0.068 \times 10^{-4} \text{ mM min}^{-1} \text{mg}^{-1}$. 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).

Table 48. Partial purification of cellulase by *Bacillus stratosphericus* N₁₂ (M) using NaOH +H₂O₂ pretreated *P. deltoides* wood under SmF

Steps	Volume (ml)	Protein (mg/ml)**	CMCase activity		FPase activity		β -glucosidase activity		Total cellulase		Total activity* (IU)	Purification Fold****	Recovery %*****
			CMCase (IU)	Specific activity (U/mg)	FPase (IU)	Specific activity (U/mg)	β -glucosidase (IU)	Specific activity (U/mg)	Total cellulase	Specific activity***			
Crude culture supernatant	100	2.03	2.202	1.084	1.852	0.912	1.929	0.950	5.983	2.94	598.3	1.000	100
(NH ₄) ₂ SO ₄ precipitated	50	1.50	2.384	1.589	2.249	1.499	2.451	1.634	7.084	4.72	354.2	1.605	73.89

*Total activity was determined by multiplication of volume and activity

** Protein concentration was determined by Lowry's method

***Specific activity is the activity unit/ protein

****Purification fold is increase in specific activity

*****Recovery % is remaining protein concentration as % of the initial protein

Table 49. Partial purification of xylanase by *B. altitudinis* Kd₁ (M) using NaOH +H₂O₂ pretreated *P. deltoides* wood under SmF

Steps	Volume (ml)	Xylanase activity (IU)	Protein concentration (mg/ml)**	Specific activity (U/mg)***	Total activity (IU)*	Purification fold****	Recovery %*****
Crude culture supernatant	100	69.93	3.27	21.38	6993.0	1.000	100
(NH ₄) ₂ SO ₄ precipitated	50	71.49	2.34	30.55	3574.5	1.428	71.55

*Same as Table 48

Table 50. Partial purification of cellulase by *M. thermophila* SH1 using NaOH +H₂O₂ pretreated *P. deltoides* wood under SSF

Steps	Volume (ml)	Protein (mg/ml)**	CMCase activity		FPase activity		β-glucosidase activity		Total cellulase		Total activity (IU)*	Purification fold****	Recovery %*****
			CMCase (IU)	Specific activity (U/mg)	FPase (IU)	Specific activity (U/mg)	β-glucosidase (IU)	Specific activity (U/mg)	Total cellulase	Specific activity***			
Crude culture supernatant	100	0.75	0.084	0.112	0.318	0.424	1.364	1.818	1.766	2.35	176.6	1.000	100
(NH ₄) ₂ SO ₄ precipitated	50	0.63	1.752	2.780	1.193	1.893	2.571	4.079	5.516	8.75	275.8	3.72	84.00

*same as Table 48

Table 51. Partial purification of xylanase by *M. thermophila* SH1 using NaOH +H₂O₂ pretreated *P. deltoides* wood under SSF

Steps	Volume (ml)	Xylanase activity (IU)	Protein concentration (mg/ml)**	Specific activity (U/mg)***	Purification fold****	Total activity*	Recovery %*****
Crude culture supernatant	100	10.16	1.52	6.684	1.000	101.60	100
(NH ₄) ₂ SO ₄ precipitated	50	12.59	1.10	11.44	1.712	629.5	72.36

*same as Table 48

Devendra *et al.* (2012), optimized the cellulase production by *Trichoderma harzianum* using mahua (*Bassia latifolia*) pomace as substrate under submerged fermentation (SmF). Maximum production of CMCase and β -glucosidase was achieved on 7th day while FPase on 5th day of incubation. Purification to the extent of 5.05 fold could be achieved by ammonium sulphate (50-70% saturated) precipitation. According to Fadel (2000) partial purification at 20-40% ammonium sulphate saturation was most suitable for cellulase recovery from *Aspergillus niger* using alkali treated wheat straw. The enzyme produced by *Trichoderma viride*-IR05 in solid state fermentation was purified by ammonium sulphate fractionation followed by dialysis. The enzyme was fractionated with different percentage of ammonium sulphate. Results indicated that maximum enzyme activity was observed with 60% ammonium sulphate precipitation with specific activity of 77.2 U/mg. The precipitated enzyme was further dialyzed against the same buffer showing purification fold of 1.6 with specific activity of 112.5 U/mg and 68.9% of enzyme yield (Irfan and Syad, 2012).

4.8 SCALE UP OF CELLULASE AND XYLANASE ENZYMES IN A STIRRED TANK BIOREACTOR

The optimized conditions of cellulase and xylanase production were shifted from shake flask studies to a batch in the bioreactor. The bioreactor was equipped with accessories and automatic control system for DO, pH, antifoam, impeller speed, aeration rate and temperature.

4.8.1 Scale up of cellulase production by *B. stratosphericus* N₁₂ (M)

In the present study, batch cultivation for the production of cellulase by *B. stratosphericus* N₁₂ (M) was carried out in a 7.5 L stirred tank bioreactor with a 3.0 L working volume at 30⁰C and pH 8.0. Carbon and nitrogen sources; for batch fermentations were 1.0% grinded wood biomass of *P. deltoides*, K₂HPO₄ – 1.0 g/L, NaCl – 5.0 g/L, peptone – 5.0 g/L, yeast extract – 5.0 g/L, MgSO₄7H₂O – 0.2 g/L. Foaming was controlled with addition of 2-3 drops of polypropylene glycol. The medium was sterilized in situ for 20 min at 121⁰C. The bioreactor was inoculated with 10% inoculum of 24 h old culture under aseptic conditions. The

agitation speed of 200 rpm and agitation rate of 1.0 vvm were used for batch cultivation in the bioreactor. The sample from fermentor was withdrawn regularly at different intervals ranging from 2, 4.....10 h.

As the Table 52 depicted, the maximum cellulase titers of 2.443 IU were attained after 8 h of fermentation time followed by 10 h i.e 1.942 IU. The minimum cellulase production was observed in 2 h of fermentation time i.e. 0.674 IU. Fig 39 showing the physiological behaviour of *B. stratosphericus* N₁₂ (M) during cellulase production in a stirred tank bioreactor.

The amount of cellulase production is affected by the composition of the nutrient medium and environmental conditions, which can be inducers or repressors of cell density and growth rate (Umikalsom *et al.*, 1998). In addition, cellulase production is also influenced by the hydrodynamic conditions in bioreactor (Mariano *et al.*, 2006).

Cellulase was produced in 2L stirred tank bioreactor by *Bacillus pumilus* EB3 with the productivity of 0.53, 3.08 and 1.78 U/l/h and the maximum enzyme activity of 0.011, 0.079 and 0.038 U/ml for FPase, CMCase and β - glucosidase respectively in 24 h of fermentation (Ariffin *et al.*, 2006). 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.

Cellulase production by *Trichoderma reesei* was carried out using high concentration of cellulose to substitute glucose with the aim to improve cellulase

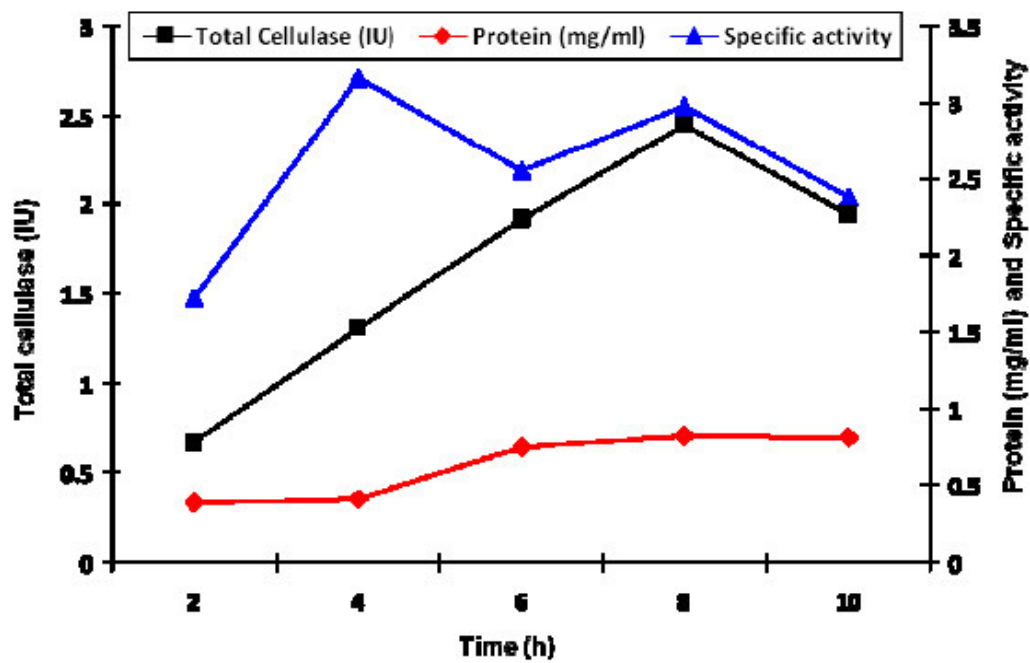


Fig. 38. Scale up of cellulase production by *B. stratosphericus* N12 (M) in a stirred Tank Bioreactor

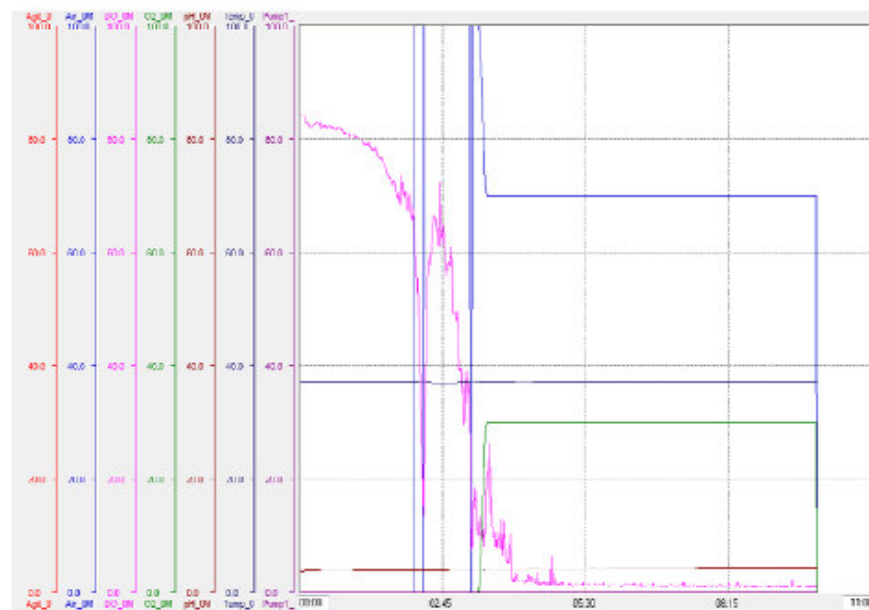


Fig 39. Physiological behaviour of *B. stratosphericus* N12 (M) during cellulase production in a stirred tank bioreactor

production and to reduce production costs in a 7 L stirred tank bioreactor. A mixture of lactose and lactobionic acid was added into the bioreactor as cellulase inducers. It yielded the highest enzyme and cell production with a volumetric enzyme activity of 69.8 U/l/h, a filter paper activity of 5.02 U/ml, a CMCase activity of 4.2 U/ml and a fungal biomass of 14.7g/l (Ahmed *et al.*, 2009).

Table 52. Cellulase production by *B. stratosphericus* N₁₂ (M) in a stirred tank Bioreactor

Time (h)	CMCase	FPase	β -glucosidase	Total Cellulase(IU)	Protein (mg/ml)	Specific activity
2	0.114	0.058	0.502	0.674	0.393	1.715
4	0.180	0.117	1.010	1.307	0.413	3.164
6	0.276	0.151	1.484	1.911	0.747	2.558
8	0.277	0.199	1.967	2.443	0.822	2.972
10	0.271	0.163	1.508	1.942	0.816	2.379

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

Specific activity : enzyme activity/mg of protein.

The effect of different levels of dissolved oxygen tension (DOT) on the production of three main components of extracellular cellulases (CMCase, FPase and β - glucosidase) at a fixed agitation speed by *Aspergillus terreus* was investigated by Shahriarinnour and Wahab, (2011). The results revealed that the cell growth and cellulase production were approximately two- fold higher in stirred tank bioreactor compared with shake flask culture. At a DOT of 55% air saturation, cell growth and cellulase production were higher than at low DOT (40% air saturation) or high DOT (80% air saturation). The highest activities of FPase (2.33 U/ml), CMCase (51.10 U/ml) and β -glucosidase (16.18 U/ml) were obtained at a DOT of 55% air saturation, yielding overall productivities of 19.40, 425.00 and 67.40 U/h respectively. 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 Oil Mill effluent as substrate. Maximum cell biomass (3.16 gL⁻¹) and cellulase activity (0.1748 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). Jo *et al.* (2008) reported the optimal conditions for pilot-scale production of the carboxymethylcellulase (CMCase) by

Bacillus amyloliquifaciens DL-3. 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.

4.8.2 Scale up of xylanase production by *B. altitudinis* Kd₁ (M)

Production of xylanase by *B. altitudinis* Kd₁ (M) was carried out in a 7.5 L stirred tank bioreactor with a 3.0 L working volume at 30°C at pH 5.5. Carbon and nitrogen sources for fermentations were 1.0 % *P. deltooides* wood, Foaming was controlled with addition of 2-3 drops of polypropylene glycol. 12.5% inoculum was added to the bioreactor under sterilized conditions. The agitation speed 200rpm and agitation rate 1.0vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4.....10h.

As the table 53 showed the maximum xylanase units of 11.10 IU after 8 h of fermentation time followed by 10 h i.e. 9.540 IU. The minimum xylanase production 2.953 IU was found in 2 h of fermentation time. Fig 41 depicted the physiological behaviour of *B. altitudinis* Kd₁ (M) in a stirred tank bioreactor during xylanase production.

Table 53. Xylanase production by *B. altitudinis* Kd₁ (M) in a stirred tank Bioreactor

Time (h)	Xylanase (IU)	Protein (mg/ml)	Specific activity
2	2.952	0.954	3.094
4	3.368	1.120	3.007
6	7.436	1.343	5.536
8	11.10	1.926	5.763
10	9.540	1.794	5.317

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

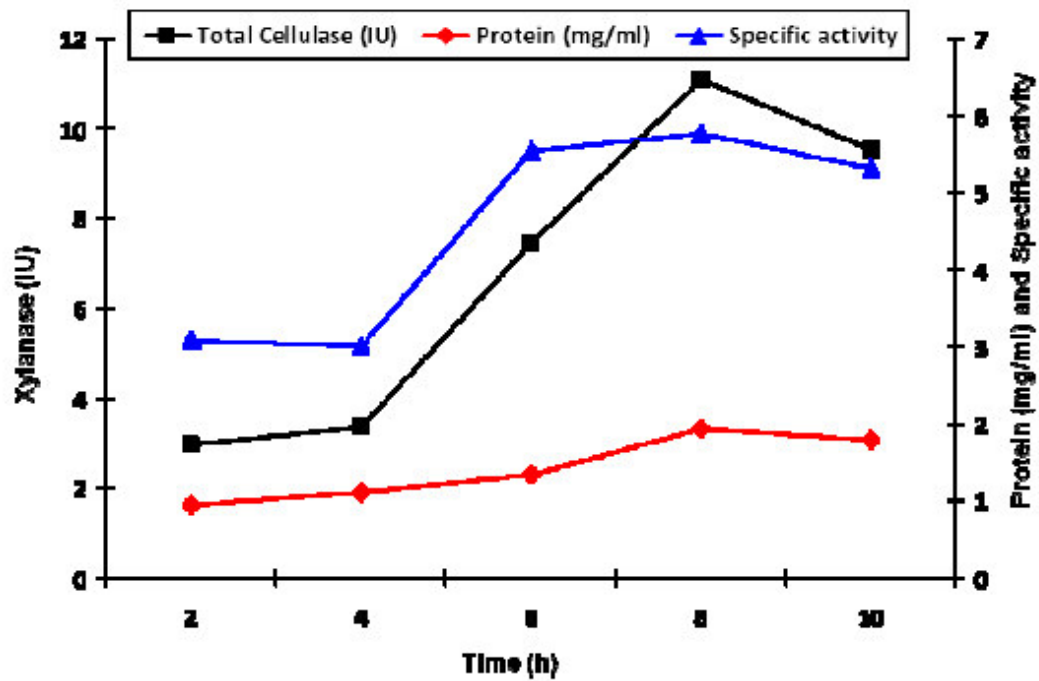


Fig. 40. Scale up of Xylanase production by *B. altitudinis* Kd1 (M) in a stirred Tank Bioreactor

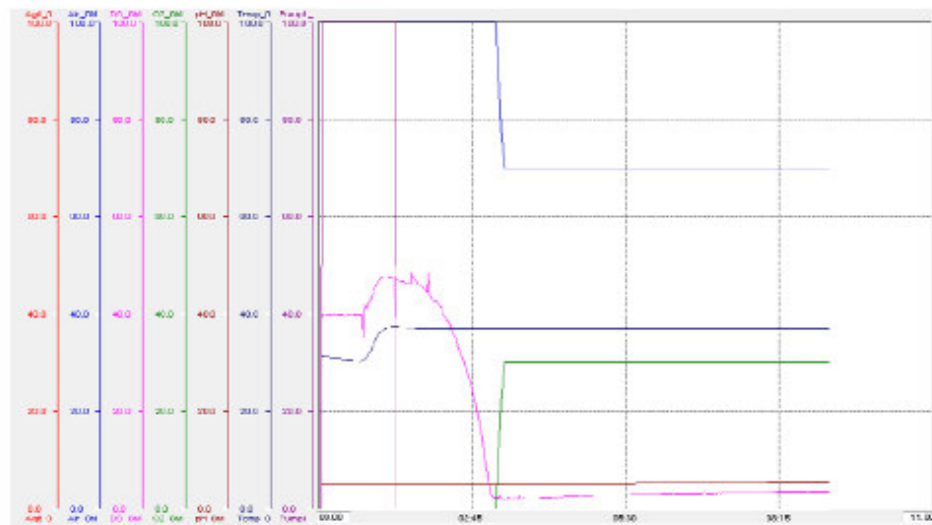


Fig 41. Physiological behaviour of *B. altitudinis* Kd1 (M) during xylanase production in a stirred tank bioreactor

Similarly, the production of xylanase from agro industrial by product such as wheat straw has been optimized by Gupta *et al.* (2012). The statistical approach of Response Surface Methodology has been employed for the optimization of different nitrogen sources. Optimization of other factors such as inoculum age, inoculum size, pH, growth temperature and agitation significantly enhanced xylanase activity to about 425 ± 5 IU/ ml in shake flask using optimized fermentation media and conditions. The scale up of fermentation process upto 14 L bioreactor yield a maximum xylanase activity of about 520 IU/ml in 36 h of fermentation which is 1.22 fold higher than obtained in shake flask level.

A similar study was conducted by Basar *et al.* (2010) for enhancement of thermophilic xylanase production by recombinant *Escherichia coli* DH5 α through suitable medium formulation initially using shake flask cultures. 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. Xylanase production was enhanced at DOT level controlled at 20% saturation, though growth was not significantly improved. Substantially high xylanase production (1784.57U/ml) was obtained in fermentation optimal medium composition and DOT level. These results indicate that efficient process control strategy is important for the mass production of xylanase enzyme by *E. coli* DH5 α . 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.* (2010). 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. Thermostable cellulase free xylanase produced by *Streptomyces* sp. Ab 106 using agricultural waste, sugarcane bagasse, 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.

4.9 BIOCONVERSION OF CELLULOSIC WASTE INTO BIOETHANOL

Lignocellulosic materials are renewable, largely unused and abundantly available sources of raw materials for the production of ethanol fuel. Utilization of lignocellulosic wastes may act as a potential source for future low cost ethanol production. These materials contain sugars polymerized in the form of cellulose and hemicellulose, which can be liberated after enzymatic hydrolysis and subsequently fermented to ethanol by microorganisms.

In the present study, the lignocellulosic forest wastes viz. *Eucalyptus* sp., *P. deltooides*, *D. strictus*, *P. roxburghii*, *P. wallichiana* and *C. deodara* were used after subjecting them to different physical and chemical pretreatments. On the basis of highest reducing sugars produced, NaOH + H₂O₂ pretreated *P. deltooides* wood was selected for ethanol bioconversion studies under different modes/ permutations and combinations of fermentation.

4.9.1 Bioconversion of NaOH + H₂O₂ pretreated *P. deltooides* wood under Separate hydrolysis and Fermentation (SHF) mode

4.9.1.1 Separate Hydrolysis and Fermentation (SHF) using cocktail of inhouse enzymes [cellulase of *B. stratosphericus* N₁₂ (M) + *M. thermophila* SH1 and xylanase of *B. altitudinis* Kd₁ (M) + *M. thermophila* SH1]

A sequential process where the hydrolysis of cellulose + hemicellulose and the fermentation of lignocellulosic biomass is carried out as two separate units, this configuration is known as separate hydrolysis and fermentation (SHF). In the present study, different methods of separate hydrolysis and fermentation (SHF) have been devised in order to maximize the final yield of bioethanol

production. Table 54 depicted bioethanol production by using different ethanologenic microorganisms (monoculture and co-culture) i.e. *S. cerevisiae* I, *S. cerevisiae* II, *P. stipitis*, *C. shehatae*, *Z. mobilis*, *S. cerevisiae* I + *P. stipitis*, *S. cerevisiae* I + *C. shehatae*, *S. cerevisiae* II + *P. stipitis* and *S. cerevisiae* II + *C. shehatae* under SHF using alkali pretreated *P. deltoides* wood as carbon source. The hydrolytic enzymes produced during this study i.e cellulase from *B. stratosphericus* N₁₂ (M) + *M. thermophila* SH1 and xylanase from *B. altitudinis* Kd₁ (M) + *M. thermophila* SH1 were used as a cocktail for enzymatic degradation of wood into fermentable sugars. The inhouse enzymes with their total dose @ 5ml/5g, where cellulases and xylanases are mixed in the ratio of 4:1 i.e. 2 ml of cellulase from *M. thermophila* SH1 (with CMCase: 1.752 IU, FPase: 1.193 IU, β -glucosidase: 2.571 IU + 2 ml of cellulase from *B. stratosphericus* N₁₂ (M) (with CMCase: 2.384 IU, FPase: 2.249 IU, β - glucosidase: 2.451 IU) and 0.5 ml of xylanase from *B. altitudinis* Kd₁ (M) (71.49 IU) + 0.5 ml of xylanase from *M. thermophila* SH1 (12.69 IU) with an aim of complete hydrolysis of alkali pretreated wood.

As the results presented in table 54.1, in case of the first method where pooling of supernatant of alkali pretreatment with wood hydrolysate was done (without detoxification), the maximum ethanol i.e 7.93 g/l was fermented by co-culture of *S. cerevisiae* II + *C. shehatae* closely followed by 7.85 g/l by *S. cerevisiae* II + *P. stipitis*, while the minimum ethanol production i.e 3.48 g/l was noticed in monoculture of *P. stipitis*. The maximum fermentation efficiency (31.10%) was obtained in co-culture of *S. cerevisiae* II and *C. shehatae*.

Efficiency of inhouse enzymes for saccharifying pretreated *P. deltoides* wood and subsequently their fermentation into ethanol had been compared with control i.e commercial enzymes - Cellulclast1.5L, Novozyme188 and Pantopan Mono BG. NaOH + H₂O₂ pretreatment in the present study was employed with an apparent aim of delignification by breaking the ester bonds cross linking lignin and xylan, thus exposing most of cellulose and xylan for enzymatic degradation and also increasing the porosity of biomass as well causing swelling and thus

leading to increase in internal surface area (Sun and Cheng, 2002; Ratnam *et al.*, 2005).

Table 54. Bioconversion of NaOH + H₂O₂ pretreated *P. deltoides* wood using cocktail of inhouse hydrolytic enzymes of bacterial and fungal origin under Separate hydrolysis and fermentation (SHF)

Table 54.1 (Method-I): SHF of alkaline hydrogen peroxide pretreated *P. deltoides* wood by pooling supernatant + solid residue without detoxification

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
<i>Saccharomyces cerevisiae-I</i>	0.82	6.50	0.13	25.49	6.40
<i>Saccharomyces cerevisiae-II</i>	0.92	7.28	0.143	28.61	6.40
<i>Pichia stipitis</i>	0.44	3.48	0.06	13.52	6.40
<i>Candida shehatae</i>	0.52	4.12	0.08	16.07	6.40
<i>Zymomonas mobilis</i>	0.82	6.50	0.13	25.49	6.40
<i>S. cerevisiae-I</i> + <i>P. stipitis</i>	0.80	6.34	0.12	24.70	6.40
<i>S. cerevisiae-I</i> + <i>C. shehatae</i>	0.80	6.34	0.12	24.70	6.40
<i>S. cerevisiae-II</i> + <i>P. stipitis</i>	0.99	7.85	0.15	30.78	6.40
<i>S. cerevisiae-II</i> + <i>C. shehatae</i>	1.00	7.93	0.15	31.10	6.40
S.E..	0.04	0.27	0.01	1.30	
C. D. _{0.05}	0.03	0.18	0.01	0.89	

The different ethanologens were used in the present study in monoculture as well as co-culture combinations for fermenting sugars produced from enzymatic saccharification of lignocellulosic wood. Co- culture of *S. cerevisiae* II + *C. shehatae* and *S. cerevisiae* II+ *P. stipitis* produced highest ethanol as compared to other co-culture and monoculture. Since *S. cerevisiae* is widely known for hexose utilization whereas *P. stipitis* can ferment pentose sugars thus this co-culture combination would have utilized most of fermentable sugars formed in the wood hydrolysate to yield highest ethanol concentration.

Table 54.2 exhibited the results of second modified method in which pooling of supernatant after NaOH + H₂O₂ pretreatment was done with enzyme hydrolysate along with detoxification using Ca (OH)₂ and the highest ethanol production of 18.24 g/l was noticed in co-culture *S. cerevisiae* II + *P. stipitis* followed by *S. cerevisiae* I + *P. stipitis* i.e. 17.44 g/l while the least ethanol yield was formed in monoculture of *P. stipitis* (8.72g/l).

The maximum fermentation efficiency of co-culture *S. cerevisiae* II + *P. stipitis* was 71.53%. Since during the pretreatment several inhibitors like furfurals, 5-hydroxy methyl furfurals, acetic acid and levulinic acid and vanillin are generated due to sugar and lignin degradation that hinder the overall process of ethanol fermentation by adsorbing binding sugars and also by exerting lethal effect on fermenting yeasts. Therefore, detoxification or removal of inhibitors from hydrolysate becomes very important for efficient fermentation of sugars formed from lignocellulosic biomass and thus eventually increasing the yield of ethanol.

Table 54.2. (Method-II): SHF of alkaline hydrogen peroxide pretreated *P. deltoides* wood by pooling supernatant + solid residue followed by detoxification with calcium hydroxide

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
<i>Saccharomyces cerevisiae-I</i>	1.29	10.23	0.204	40.00	7.81
<i>Saccharomyces cerevisiae-II</i>	1.30	10.31	0.206	40.43	7.81
<i>Pichia stipitis</i>	1.10	8.72	0.174	34.11	7.81
<i>Candida shehatae</i>	1.19	9.43	0.188	37.01	7.81
<i>Zymomonas mobilis</i>	1.23	9.75	0.195	38.25	7.81
<i>S. cerevisiae-I</i> + <i>P. stipitis</i>	2.20	17.44	0.348	68.42	7.81
<i>S. cerevisiae-I</i> + <i>C. shehatae</i>	1.31	10.38	0.207	40.58	7.81
<i>S. cerevisiae-II</i> + <i>P. stipitis</i>	2.30	18.24	0.364	71.53	7.81
<i>S. cerevisiae-II</i> + <i>C. shehatae</i>	1.40	11.10	0.222	43.52	7.81
S. E.	0.137	0.182	0.022	1.610	
C.D-0.05	0.094	0.125	0.015	1.108	

Higher ethanol production after detoxification of pooled liquor is due to removal of most of the inhibitors formed during pretreatment and hydrolysis, thus facilitating the fermentation of sugars smoothly by ethanologenic microorganisms which had also been proved in experiment 4.10 and figs 59, 60 and 61 where 5-HMF estimated through HPLC in the hydrolysates had shown considerable reduction in their concentration.

Over-liming with a combination of high pH and temperature has for a long time been considered as a promising detoxification method for pretreated hydrolysate of lignocellulosic biomass (Chandel *et al.*, 2007). This process has been demonstrated to help in the removal of volatile inhibitory compounds such

as furfurals and hydroxyl methyl furfurals from the hydrolysate (Chandel, 2011). The only disadvantage of this method is that it not only affects inhibitors, but to an extent also the sugars, which affect the ethanol yield (Nilvebrant *et al.*, 2003).

Saha *et al.* (2005) measured the yield of ethanol (per liter) from acid pretreated enzyme saccharified wheat straw (78.3g) hydrolysate by recombinant *Escherichia coli* strain FBR5 was 19 ± 1 g with a yield of 0.24 g/g DS. Detoxification of acid and enzyme treated wheat straw hydrolysate by overliming reduced the fermentation time from 18 to 39 h in case of SHF (35°C, pH 6.5) and increased the ethanol yield from 13 ± 2 to 17 ± 0 g/l.

In another study ethanol production was evaluated from wheat straw (WS) hemicellulose acid hydrolysate using an adapted and parent strain of *Pichia stipitis* NRRL Y-7124. The treatment by boiling and overliming with $\text{Ca}(\text{OH})_2$ significantly improved the fermentability of the hydrolysate. Straw was hydrolysed at 90°C with 1.85% sulphuric acid for 18 h. The suspension was then centrifuged to remove the unhydrolysed residue. The residue was washed with water at $80 \pm 0.5^\circ\text{C}$ to extract the sugars. The centrifugate and washing were pooled together. The hydrolysate was boiled at 100°C for 15 min and overlimed with $\text{Ca}(\text{OH})_2$ upto pH 10. Ethanol yield and productivity were increased 2.4 ± 0.10 and 5.7 ± 0.24 folds, respectively (Nigam, 2001). Two stage acid hydrolysis was carried out with concentrates of perchloric acid using wheat straw. The powdered wheat straw was treated with perchloric acid at 100°C for 20 min. The mixture was filtered and residual wheat straw was kept for second stage hydrolysis and was hydrolysed with perchloric acid (35%) at 100°C for 40 min. The filtrates of first and second stage were pooled together and overlimed with CaO (calcium oxide) to decrease the fermentation inhibitors. The ethanol yields obtained from hydrolysate with the co-culture of *S. cerevisiae* and *P. stipitis* were higher than the monoculture of *S. cerevisiae*. The ethanol concentration was 11.42 g/l in 42 h with a yield of 0.475 g/g, productivity of 0.272 g/l/h and fermentation efficiency of 92.95% using a co-culture of *S. cerevisiae* and *P. stipitis*.

Results in case of third method in which supernatant of the alkali pretreated slurry was discarded and only solid mass after thorough washing was subjected to enzymatic hydrolysis without any detoxification. The results presented in Table 54.3 showed that the maximum ethanol of 7.63 g/l was produced by co-culture of *S. cerevisiae* II + *P. stipitis* with highest fermentation efficiency of 29.80%, while the least ethanol of 3.33 g/l was found by monoculture of *P. stipitis*.

The alkaline hydrogen peroxide pretreatment of *P. deltooides* causes delignification of biomass thus most of lignin along with lignin degrading products viz. Vanillin, levulinic acid, furfurals, 5 HMF etc. are removed with supernatant in turn minimizing the total load of inhibitors in the wood hydrolysate. Therefore with an idea to minimize the inhibitors from the hydrolysate in the present experiment, the supernatant after pretreatment had been discarded, solid wood biomass was subjected to enzymatic saccharification and it was used as sugary substrate for fermentation with different monoculture and co-culture combinations without any overliming. In method-III, the co-culture *S. cerevisiae* + *P. stipitis* was observed the best for ethanol production.

Table 54.3. (Method-III): SHF of solid residue of *P. deltooides* wood after alkaline hydrogen peroxide pretreatment by discarding supernatant without detoxification

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
<i>Saccharomyces cerevisiae-I</i>	0.90	7.13	0.142	27.84	11.26
<i>Saccharomyces cerevisiae-II</i>	0.89	7.05	0.141	27.64	11.26
<i>Pichia stipitis</i>	0.42	3.33	0.066	13.06	11.26
<i>Candida shehatae</i>	0.51	4.04	0.082	15.86	11.26
<i>Zymomonas mobilis</i>	0.78	6.18	0.123	24.25	11.26
<i>S. cerevisiae-I</i> + <i>P. stipitis</i>	0.79	6.26	0.125	24.50	11.26
<i>S. cerevisiae-I</i> + <i>C. shehatae</i>	0.83	6.58	0.131	25.68	11.26
<i>S. cerevisiae-II</i> + <i>P. stipitis</i>	0.96	7.63	0.152	29.80	11.26
<i>S. cerevisiae-II</i> + <i>C. shehatae</i>	0.86	6.82	0.136	26.66	11.26
S.E.	0.016	0.222	0.002	0.405	
C.D. _{0.05}	0.011	0.153	0.001	0.279	

Table 54.4 (Method-IV) SHF of solid residue of *P. deltoides* wood after alkaline hydrogen peroxide pretreatment by discarding supernatant followed by detoxification with calcium hydroxide

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
<i>Saccharomyces cerevisiae-I</i>	1.39	11.02	0.220	43.13	12.45
<i>Saccharomyces cerevisiae-II</i>	1.40	11.10	0.225	43.54	12.45
<i>Pichia stipitis</i>	1.23	9.75	0.195	38.23	12.45
<i>Candida shehatae</i>	1.20	9.51	0.190	37.25	12.45
<i>Zymomonas mobilis</i>	1.29	10.23	0.204	40.12	12.45
<i>S. cerevisiae-I</i> + <i>P. stipitis</i>	2.30	18.24	0.364	71.53	12.45
<i>S. cerevisiae-I</i> + <i>C. shehatae</i>	1.40	11.10	0.222	43.54	12.45
<i>S. cerevisiae-II</i> + <i>P. stipitis</i>	2.33	18.47	0.369	72.46	12.45
<i>S. cerevisiae-II</i> + <i>C. shehatae</i>	1.48	11.73	0.234	46.03	12.45
S.E.	0.109	0.317	0.024	0.981	
C.D. _{0.05}	0.075	0.218	0.017	0.675	

Yah *et al.* (2010) compared two methods used for production of bioethanol i.e. acid hydrolysis and enzyme hydrolysis. The dried corncobs were delignified by treating with 0.3 M HCl solution at 121°C for 1 h. The pretreated cellulosic residue was then washed with distilled water to remove the residual acid. This cellulosic residue to convert into fermentable sugars was subjected to enzymatic hydrolysis (50 ml water + 300µl of cellulase). Acid hydrolysis of corncobs gave higher amount of sugars than enzymatic saccharification. The maximum bioethanol produced at 25°C was 11.99 mg/ml by *S. cerevisiae* and *P. stipitis*. But in the present study maximum bioethanol was produced by enzyme hydrolysis in case of *S. cerevisiae* and *P. stipitis*. The non-detoxified dilute acid hydrolysate was used to produce ethanol by co-culture *S. cerevisiae* Y5 + *P. stipitis* CBS6054. The co-culture consumed glucose along with furfurals and HMF completely in 12 h and all xylose within 96 h, resulting in a final ethanol concentration of 24.4 g/l and ethanol yield of 0.43 g ethanol/ g sugar corresponding to 85.1% of the maximal theoretical value (Wan *et al.*, 2012).

As the results revealed in case of fourth method (Table 54.4) in which supernatant obtained after alkaline hydrogen peroxide pretreatment was discarded and detoxification of woody mass with calcium hydroxide was done followed by

enzymatic hydrolysis with inhouse enzymes. Highest yield of 18.47 g/l was observed in co-culture *S. cerevisiae* II + *P. stipitis* followed by *S. cerevisiae* I + *P. stipitis* i.e. 18.24 g/l. The least ethanol of 9.51 g/l was observed in monoculture *C. shehatae*. The maximum fermentation efficiency of *S. cerevisiae* II + *P. stipitis* was observed as 72.46 %. Here, the supernatant of the hydrolysate was decanted off after delignification to remove lignin and its degraded compounds and the overliming was done additionally in this scheme with an apparent aim to eliminate most of the toxic inhibitors generated during enzymatic hydrolysis from hydrolysate, thus resulting in the highest yield of ethanol.

Fig 42 showed a comparative bioethanol yield by different methods i.e. I, II, III and IV of SHF using inhouse enzymes for hydrolysis of *P. deltooides* wood. Among all these methods, the highest ethanol yield was obtained in case of method IV. Among different ethanologenic strains used as monoculture and co-culture combinations, *S. cerevisiae* II + *P. stipitis* and *S. cerevisiae* I + *P. stipitis* had emerged as the best co-culture combinations for improved bioethanol production. This combination seem to have an edge over others probably due to conversion of both glucose and xylose in fermentation liquor to ethanol as well as degrading inhibitors in the hydrolysate effectively, thus increasing their fermentation efficiency (Wan *et al.*, 2012). Furfurals have been shown to decrease the ethanol productivity of yeast considerably Larsson *et al.* (1999) and Modig *et al.* (2002) suggested that furfural inhibits several glycolytic enzymes. But adaptation of some of fermenting microorganisms on high furfural concentration has been found a successful option to decrease the furfural effect on growth to an extent. It may be due to the synthesis of new enzymes or co-enzymes by fermenting microorganisms for furfural reduction.

The commercial enzymes i.e. Celluclast 1.5L, Novozyme188 and Pantopan Mono BG have been used as control to compare the degree of robustness of inhouse enzymes prepared as part of this study for lignocellulosic biomass hydrolysis.

The reducing sugars formed during saccharification with commercial enzymes were 531.50 mg/g yielding 21.41 g/l of ethanol and fermentation

efficiency of 83.97% *S. cerevisiae* II + *P. stipitis*, while the minimum ethanol production was 15.86 g/l (62.15% of its fermentation efficiency) with mono-culture of *P. stipitis* (Table 55).

The variation in ethanol yield is dependent upon sugars formed from biomass. The variation in the concentration of sugars hydrolysed with enzymes is due to several factors viz. substrate concentration, type of substrate, enzyme dose, titers and inhibitors which can influence the enzymatic hydrolysis of cellulose. A low substrate concentration would result in a low overall glucose yield. An increase in the substrate concentration would lead to an increased glucose yield as well as an increased rate of reaction (Hamelinck *et al.*, 2005). However, a high substrate concentration can cause substrate inhibition, which would substantially decrease the rate of the hydrolysis, and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme. A high cellulase dosage would also significantly raise process costs (Prasad *et al.*, 2007). The susceptibility of cellulosic substrates to enzymatic hydrolysis is another important factor.

Table 55. Bioconversion of alkaline hydrogen peroxide pretreated *P. deltoides* wood using commercial enzymes under Separate hydrolysis and fermentation (SHF)

Strains used	Sugars (mg/g)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
<i>Saccharomyces cerevisiae-I</i>	531.50	2.3	18.24	0.364	71.37
<i>Saccharomyces cerevisiae-II</i>	531.50	2.4	19.03	0.380	74.50
<i>Pichia stipitis</i>	531.50	2.0	15.86	0.317	62.15
<i>Candida shehatae</i>	531.50	2.1	16.65	0.333	65.29
<i>Zymomonas mobilis</i>	531.50	2.5	19.82	0.396	77.64
<i>S. cerevisiae-I</i> + <i>P. stipitis</i>	531.50	2.3	18.24	0.364	71.37
<i>S. cerevisiae-I</i> + <i>C. shehatae</i>	531.50	2.5	19.82	0.396	77.64
<i>S. cerevisiae-II</i> + <i>P. stipitis</i>	531.50	2.7	21.41	0.428	83.97
<i>S. cerevisiae-II</i> + <i>C. shehatae</i>	531.50	2.6	20.62	0.412	80.70
<i>S. cerevisiae-I</i> + <i>C. shehatae</i> + <i>P. stipitis</i>	531.50	2.1	16.65	0.333	65.29
<i>S. cerevisiae-II</i> + <i>C. shehatae</i> + <i>P. stipitis</i>	531.50	2.3	18.24	0.364	71.37
S.E.		0.85	4.38	0.069	2.62
C.D. _{0.05}		1.77	9.09	0.144	5.45

As is shown in fig 43, an attempt to use inhouse enzymes for hydrolysis of pretreated *P. deltoides* wood to ferment to ethanol with co-culture of *S. cerevisiae* II and *P. stipitis* was appreciable as compared to control, thus proving their efficiency. This strongly advocates the use of inhouse enzymes prepared during this study to replace commercial enzymes for hydrolysis of lignocellulosic

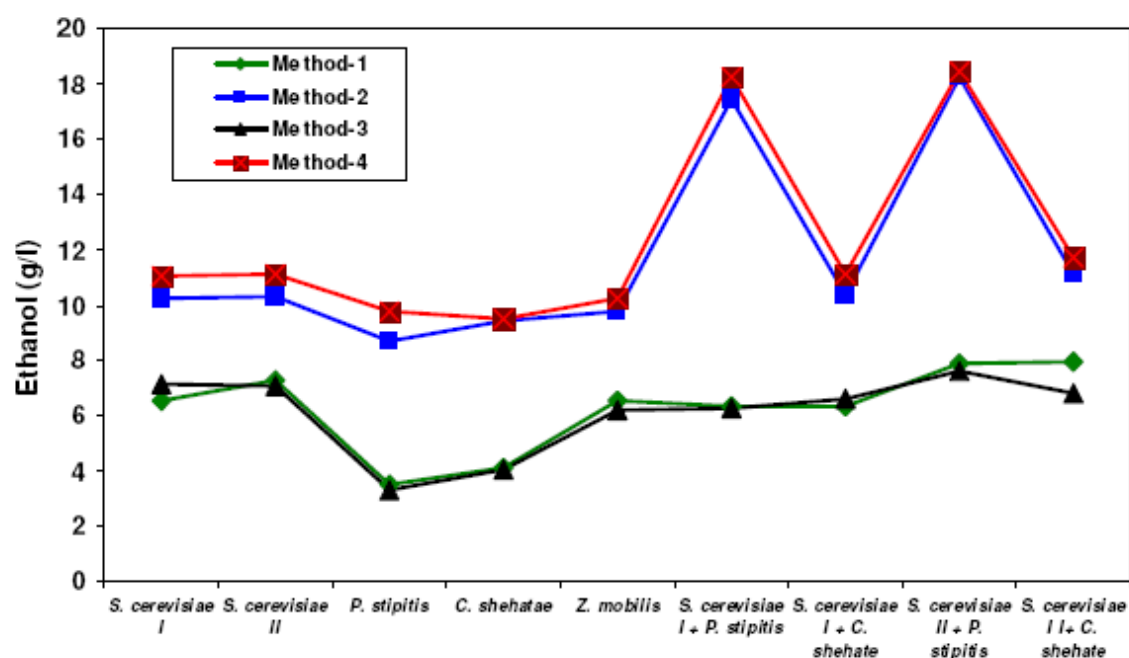


Fig. 42. Overall comparison of bioethanol production from *P. deltoides* wood by four methods of SHF using inhouse cocktail of enzymes

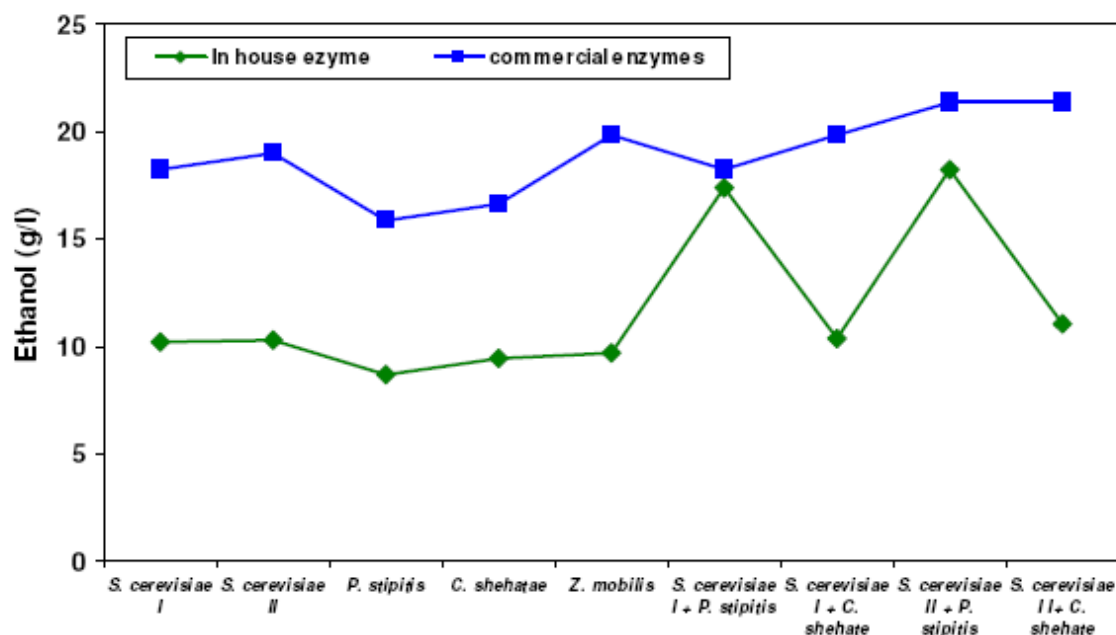


Fig. 43. Comparison of ethanol production from *P. deltoides* wood by using inhouse enzyme hydrolysate (method-IV) with commercial enzymes hydrolysates under SHF

substrates turning these bioconversion to ethanol as a cost effective process as compared to highly expensive commercial enzymes.

Vaithanomsat *et al.* (2011) reported that coconut husk was treated with 20, 25 and 30% sodium hydroxide solution at 100°C for 2-3 h. The commercial enzymes, Cellulclast 1.5 L and Novozyme 188 were used for cellulose hydrolysis and yeast *S. cerevisiae* was used for fermentation under Simultaneous Saccharification and Fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF) for ethanol efficiency. The results showed high ethanol productivity of the coconut husk cellulose from the SHF and SSF processes at 21.21 and 20.67%, respectively. The SHF process gave the highest ethanol content as compared to SSF.

Ethanol production from alfalfa fiber using separate hydrolysis and fermentation (SHF) with and without liquid hot water (LHW) pretreatment. Commercial enzyme preparations of pectinase (SP-249) was procured from Novo (Franklinton, NC). Cellulase (Multifect B) was procured from Genencore (Rochester, NY). *Candida shehatae* FPL-702 produced 5 g/l ethanol with a yield of 0.25 g ethanol/g sugar respectively by SHF from alfalfa fiber without pretreatment. With LHW pretreatment using SHF, it produced 9.6 g/l ethanol, a yield of 0.47 g ethanol/g sugar from raffinate. These results show that untreated alfalfa fiber produced a lower ethanol yield. Although LHW pretreatment can increase ethanol production from raffinate fiber fractions, it does not increase production from the hemicellulosic and pectin fractions (Sreenath *et al.*, 2001).

Cassava stalks were successfully converted to ethanol by fermentation using *Saccharomyces cerevisiae* TISTR5048, *S. cerevisiae* KM1195, *S. cerevisiae* KM7253 and co-culture of *S. cerevisiae* TISTR5048 and *Candida tropicalis* TISTR5045. The sugar hydrolysate was bioconverted to ethanol with separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The highest ethanol yields of 98.43% and 95.29% were obtained in SHF and SSF, respectively by *S. cerevisiae* KM1195. The fermentation time of SSF process was 24–32 h shorter than that of the SHF (≈56

h), but the ethanol productions (5.42-6.22 g/L for SSF; 5.9-6.23 g/L for SHF) were not significantly different (Sovoravet and Kongkiattikajorn, 2012)

In SHF, hydrolysis and fermentation are carried out as completely separate steps, whereby enzymes are added and saccharification is executed to completion, only after which, yeasts are added. The main advantage of this process that is it allows for each step to be performed at its optimum conditions for temperature and pH i.e. hydrolysis at 50⁰C and pH 4.8 where cellulase enzymes are maximally active, where as for fermentation 30⁰C, pH 6.0 for fermenting microbes is provided. However, a major disadvantage of SHF is that the accumulation of glucose and cellobiose during hydrolysis may lead to end-product inhibition (Galbe and Zacchi, 2002).

4.9.2 Bioconversion of NaOH + H₂O₂ pretreated *P. deltoides* wood under Simultaneous Saccharification and Fermentation (SSF) mode

Simultaneous Saccharification and Fermentation (SSF) using cocktail of inhouse enzymes [cellulase from *M. thermophila* SH1 + *B. stratosphericus* N12 (M) and xylanase from *B. altitudinis* Kd₁ (M) + *M. thermophila* SH1]

The simultaneous saccharification and fermentation (SSF) process entails inoculating the enzyme- producing microorganism and ethanol producing microorganisms at the same time so that the produced sugars are simultaneously converted to ethanol. Because the produced sugars are immediately converted to ethanol, the problem of feedback inhibition is avoided (Sharma *et al.*, 2012).

In the present study, Simultaneous Saccharification and Fermentation (SSF) was performed in which saccharification of lignocellulosic biomass was done by using inhouse enzymes i.e cellulase from *M. thermophila* SH1 + *B. stratosphericus* N₁₂ (M) and xylanase of *B. altitudinis* Kd₁ (M) + *M. thermophila* SH1 and results were compared with commercial enzymes used as control (i.e. cellulclast1.5L, Novozyme188 and Pantopan Mono BG). The highest ethanol yield of 11.26 g/l was observed in co-culture of *S. cerevisiae* II + *P. stipitis* with inhouse enzymes (fermentation efficiency of 44.16%) followed by mono-culture of *S. cerevisiae* II and co-culture of *S. cerevisiae* I + *C. shehatae* i.e 10.31 g/l of

ethanol (40.39% of fermentation efficiency). On the other hand least ethanol production of 5.65 g/l was noticed in co- culture of three strains i.e *S. cerevisiae* II + *C. shehatae* + *P. stipitis* having fermentation efficiency of 22.08% (Table 56).

On the other hand, in case of control using commercial enzymes maximum ethanol yield of 16.65 g/l was received in co- culture of *S. cerevisiae* II + *C. shehatae* and fermentation efficiency of 65.31%. It was closely followed by co-culture of *S. cerevisiae* I + *C. shehatae* i.e 16.02 g/l having 62.85% of fermentation efficiency. While the least ethanol concentration i.e. 11.10 g/l was noticed in co- culture of *S. cerevisiae* II + *P. stipitis* + *C. shehatae* with the least fermentation efficiency of 43.54%. Fig 44 showed the comparison of bioethanol production using inhouse and commercial enzymes for hydrolysis.

Though as cited in the literature simultaneous saccharification and fermentation (SSF) process has been identified as economically viable for the conversion of substrates to fermentation products (Cao *et al.*, 2009). The advantage of SSF is that a multistage process for the production of ethanol is carried out in one vessel and glucose produced during saccharification is simultaneously fermented to ethanol by yeast cells. Besides, being relatively cheaper as compared to the process involving addition of enzymes, this process also avoids catabolite repression of the enzymes produced during saccharification step due to higher glucose concentration. But in the present study, SSF has shown lesser ethanol production as compared to SHF. The possible reason for it may be the process conditions that are used in combination for hydrolysis and fermentation. In case of SSF reaction was performed at $32\pm 2^{\circ}\text{C}$ temperature and pH 6.0 which may not appropriate for enzyme action. Because most of the enzymes are found active at 50°C and yeasts are noticed for highest ethanol production at $25\pm 2^{\circ}\text{C}$. Since SSF were operated at a compromised set of temperature and pH resulting in lesser ethanol yield. Further because in SSF, slurry in total and thus in the hydrolysate was used after pretreatment sugars as well as inhibitors remained as, thus affecting the rate and yield of fermentation by affecting fermenting microorganism. While in case of SHF, detoxification was

Table 56. Bioconversion of alkaline hydrogen peroxide pretreated *P. deltoides* wood under Simultaneous saccharification and fermentation (SSF) by using inhouse enzyme and commercial enzymes

Strains used	Inhouse enzymes				Commercial enzymes			
	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
<i>Saccharomyces cerevisiae-I</i>	1.10	8.72	0.174	34.21	1.60	12.68	0.253	49.76
<i>Saccharomyces cerevisiae-II</i>	1.30	10.31	0.206	40.39	1.58	12.53	0.250	49.14
<i>Zymomonas mobilis</i>	0.79	6.32	0.125	24.57	1.50	11.89	0.237	46.65
<i>S. cerevisiae-I</i> + <i>P. stipitis</i>	1.20	9.51	0.190	37.32	1.84	14.59	0.291	57.22
<i>S. cerevisiae-I</i> + <i>C. shehatae</i>	1.30	10.31	0.200	40.43	2.0	16.02	0.320	62.82
<i>S. cerevisiae-II</i> + <i>P. stipitis</i>	1.42	11.26	0.225	44.16	1.92	15.22	0.304	59.71
<i>S. cerevisiae-II</i> + <i>C. shehatae</i>	1.26	9.99	0.19	39.18	2.10	16.65	0.333	65.31
<i>S. cerevisiae-I</i> + <i>C. shehatae</i> + <i>P. stipitis</i>	0.79	6.32	0.125	24.57	1.29	10.23	0.204	40.12
<i>S. cerevisiae-II</i> + <i>C. shehatae</i> + <i>P. stipitis</i>	0.71	5.65	0.112	22.08	1.40	11.10	0.222	43.54
S.E.	0.04	1.38	0.021	0.27	0.273	4.383	0.696	2.629
C.D. 0.05	0.10	2.90	0.044	0.58	0.566	9.090	0.144	5.452

Inhouse enzymes: [cellulase of *M. thermophila* SH1 and *B. stratosphericus* N12 (M) + xylanase of *M. thermophila* SH1 + *B. altitudinis* Kd1 (M)]

Commercial enzymes: [Celluclast 1.5 + Novozyme188 + Pantopan Mono BG]

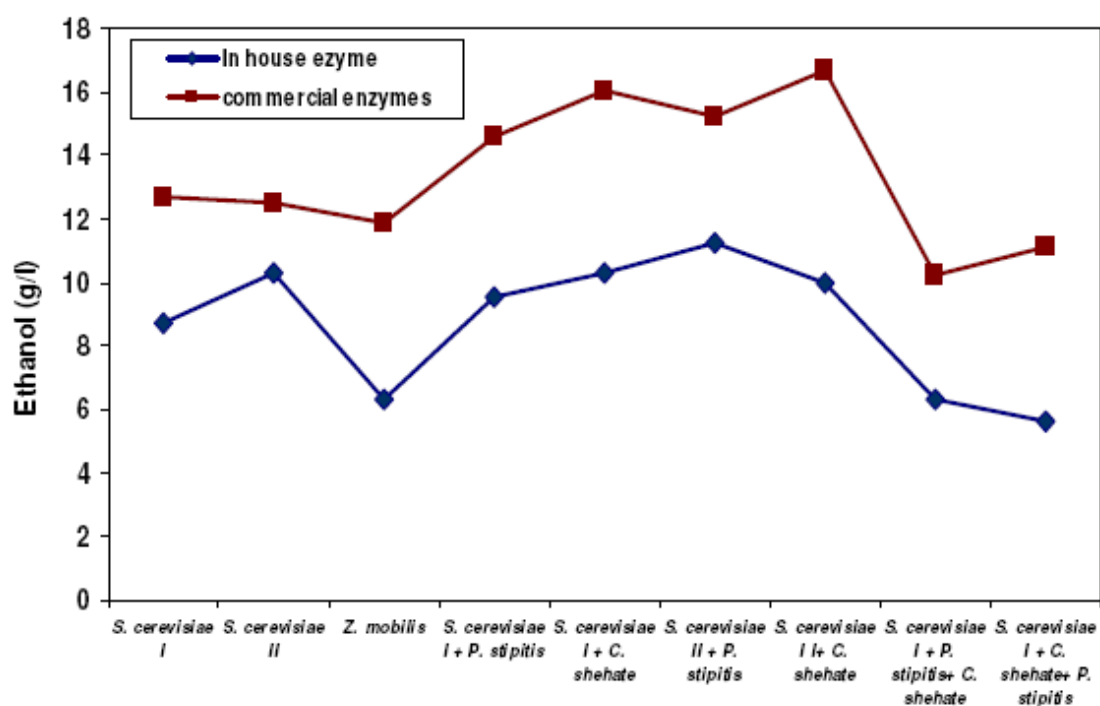


Fig 44. Comparison of bioethanol production by inhouse enzyme hydrolysate with commercial enzymes hydrolysate under simultaneous saccharification and fermentation (SSF)

done as well as precise optimum conditions i.e. temperature and pH were provided separately for hydrolysis and fermentation at each step.

Conversion of glucose and xylose to ethanol by co-culture has been successfully obtained by Taniguchi *et al.* (1997) using a respiratory deficient mutant of *S. cerevisiae* and *P. stipitis*. *P. stipitis* strain ferment xylose at a high capacity of 57 g/l than any other yeast, provided the pH is maintained at between 4.5 and 6 and temperature of 25-26°C (Jeffries *et al.*, 2007). According to Jeffries *et al.* (2007), maximum yield of ethanol is obtained when a mixture of *S. cerevisiae* and *P. stipitis* are introduced into a medium containing both glucose and xylose. The amount of bioethanol produced therefore, depends on the optimal temperature which, invariably influence sugar utilization by yeast cells.

Krishna *et al.* (1998) carried out ethanol production by simultaneous saccharification and fermentation (SSF) of pretreated sugarcane leaves (with alkaline H₂O₂), using cellulytic enzyme complex from *Trichoderma reesei* (QM9414) and *Saccharomyces cerevisiae* (NRRL-Y-132). They observed 92 per cent conversion of 2.5 per cent substrate to sugar and increase in ethanol yield with addition of extra β -glucosidase.

Ethanol production by co-culture was studied by Verma *et al.* (2000) using *Saccharomyces diastaticus* and *S. cerevisiae* 21 in raw unhydrolysed starch which yielded ethanol of 48 per cent higher (24.8 g/l) than that obtained with monoculture of *S. diastaticus* (16.8 g/l). More than 40 yeast strains were screened by Sreenath *et al.* (2001). Among them of *Pichia stipitis* and *Candida shehatae* to determine their fermentation rates on mixed sugars. All the tested strains fermented both glucose and xylose and attained ethanol concentration of 34.8 $\pm$ 2.42 g/l with *Pichia stipitis* and 34.0 $\pm$ 1.67 g/l with *Candida shehatae*.

4.9.3 Bioethanol production under Simultaneous Saccharification and Co-Fermentation (SSCF) mode

In the present study, bioethanol production from NaOH + H₂O₂ pretreated *P. deltoides* wood waste was done under Simultaneous Saccharification and Co-

Fermentation (SSCF) by *Clostridium thermocellum* strain DSM 1313 [Accession No. JCP002416] (Table 57).

Clostridium thermocellum a recombinant strain used in the present study for converting cellulosic biomass into ethanol through consolidated bioprocessing. Consolidated bioprocessing is an alternative processing strategy in which cellulase production, substrate hydrolysis and fermentation are accomplished in a single step and in one vessel (Cardona and Sanchez, 2007).

Table 57. Bioconversion of alkaline hydrogen peroxide pretreated *P. deltoides* wood waste under Simultaneous saccharification and co-fermentation (SSCF) by a recombinant strain of *Clostridium thermocellum* DSM 1313

Strain	<i>Clostridium thermocellum</i>
Ethanol (%)	0.30 ± 0.005
Ethanol (g/g)	0.04 ± 0.001
Ethanol (g/l)	2.37 ± 0.050
Fermentation efficiency (%)	7.84 ± 0.100

In the present study, it gave 2.37 g/l of ethanol yield and fermentation efficiency was noticed as 7.84% which was meager as compared to ethanol produced in Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). One of the possible reason for less yield may be due to the reason that during the fermentation of glucose along with main product other compounds such as lactate and acetate are also produced. In addition to that *C. thermocellum* also lacks the ability to convert pentose sugars efficiently to ethanol.

A recombinant strain of *Saccharomyces cerevisiae* TMB 3400 was used in simultaneous saccharification and fermentation (SSF). The TMB 3400 co-fermented glucose and xylose with relatively high ethanol yields (Ohgren *et al.*, 2006). *Zymomonas Mobilis* was genetically improved for pentose utilization to increase the final ethanol concentration. It showed good fermentation ability on both soluble sugar mixture and lignocellulose. Simultaneous saccharification and co- fermentation (SSCF) of NaOH pretreated corncob was then carried out in a

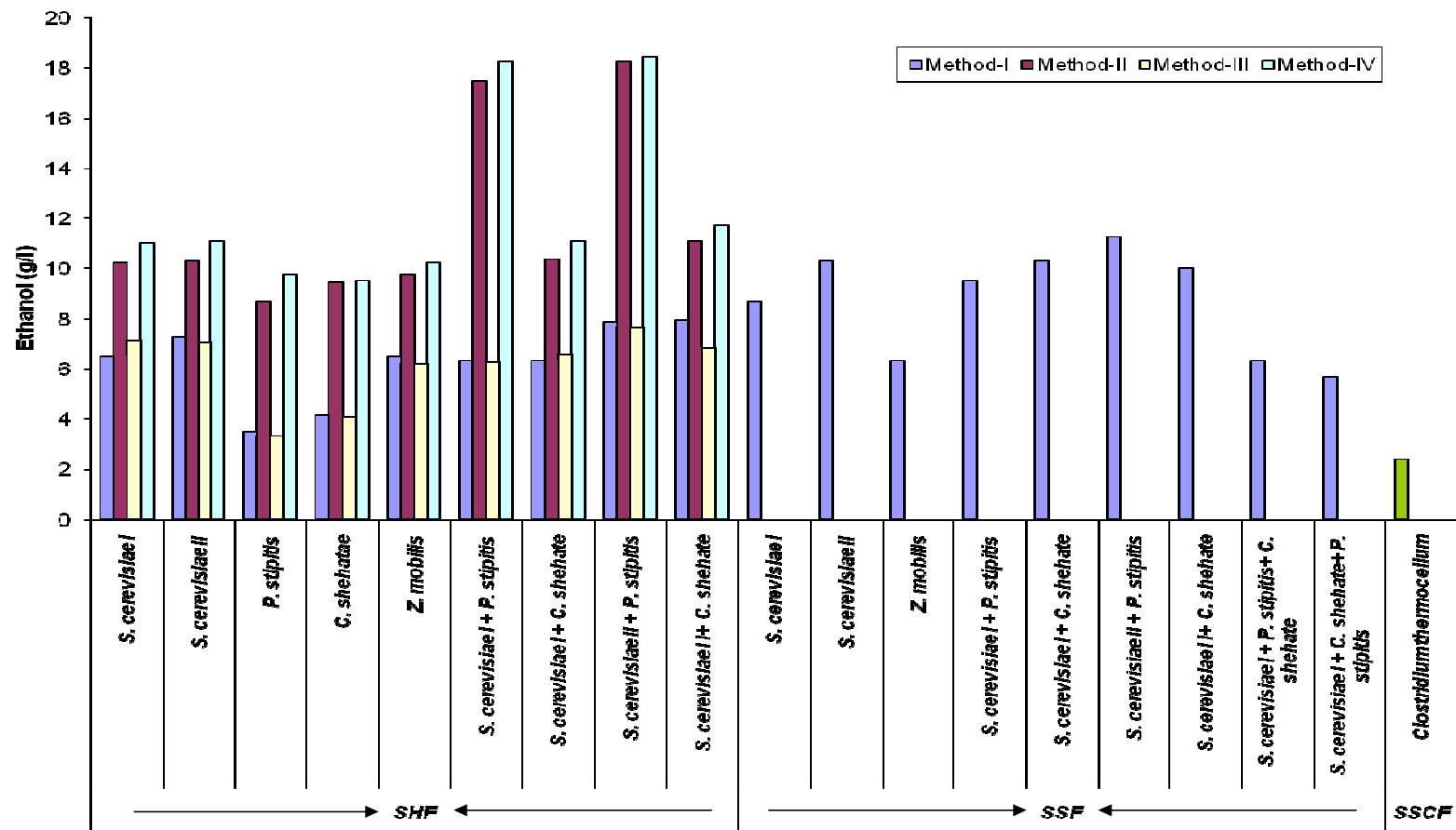


Fig. 45. Overview of bioethanol production from NaOH + H₂O₂ pre-treated *P. deltoides* wood under different modes i.e. SHF, SSF and SSCF of fermentation

high dry matter loading of 15-25 w/v %. The suitable operating conditions were determined i.e. *Z. mobilis* loading of 0.30g dry weight / L at 30°C (pH 5.5), under which the ethanol concentration reached 49.2 g/l (Su *et al.*, 2012).

In a study conducted by Chen *et al* (2011), Poplar wood pretreated by steam explosion was used as the feedstock of simultaneous saccharification and co-fermentation (SSCF) process for ethanol production. For co- fermentation, the mixed culture of recombinant *Escherichia coli* (KO11) and *S. cerevisiae* resulted in superior ethanol production than its single culture counterparts. The maximum ethanol production was 8.97 g/l and 13.98 g/l in 5% and 10% substrate, respectively.

In our study, the overall ethanol production by three modes of fermentation i.e. SHF, SSF and SSCF have been compared (Fig 45), out of which the maximum ethanol yield as well as fermentation efficiency was observed in separate hydrolysis and fermentation (SHF). Thus it has been recommended as the best mode of fermentation for improved ethanol yield.

4.10 ESTIMATION OF INHIBITORS AT DIFFERENT BIOETHANOL PRODUCTION STEPS

4.10.1 Qualitative estimation of inhibitors

Presence of inhibitors i.e. furfurals and 5- HMF was checked by performing Molish's Test for the samples collected at different steps bioethanol production. A purple coloured ring was observed for the presence of inhibitors. Large quantity of inhibitors was detected by a thick purple coloured ring at the bottom of the test tube, in case of small amount of inhibitors a thin coloured ring, while in case of minor inhibitors a faint coloured ring was observed (Table 58.1)

4.10.2 Quantitative estimation of inhibitors (5-hydroxy methyl furfurals) using High Performance Liquid Chromatography (HPLC) during bioethanol production steps

The detection of most of inhibitors generated during hydrolysis can be accomplished through high-performance liquid chromatography (HPLC), but with different combinations of detector wavelength. 5- Hydroxymethyl furfurals

(5-HMF) is one of the important inhibitors generated during the degradation of sugars resulting in the toxicity of fermenting liquor and thus limiting the fermentation.

Table 58.1 Qualitative estimation of inhibitors by Molish's Test

Sr. No.	Name of sample	Type of ring formed (purple)
1.	S1	Thin
2.	S2	Thick
3.	S3	Thick
4.	S4	Thin
5.	S5	Thin
6.	S6	Thin
7.	S7	Faint
8.	S8	Faint
9.	S9	Thin
10.	S10	Thick
11.	S11	Thick
12.	S12	Thick

S1: Supernatant after pretreatment

S2: Enzymatic hydrolysate (with inhouse enzymes)

S3: Supernatant + enzymatic hydrolysate

S4: Supernatant + enzymatic hydrolysate of inhouse enzymes with detoxification

S5: Enzymatic hydrolysate of inhouse enzymes with detoxification

S6: Fermented liquor of S3

S7: Fermented liquor of S4,

S8: Fermented liquor of S5

S9: Fermented liquor of S2,

S10: Enzymatic hydrolysate (with commercial enzymes)

S11: Enzymatic hydrolysate of commercial enzyme with detoxification

S12: Fermented liquor of S11

In the present study, concentration of 5-hydroxy methyl furfurals (HMF) of different samples collected at different steps during the process was analysed to find out the effect of these inhibitors on ethanol production. The column used for the study was Ultra C18 Restek Corp., 250 mm × 4.6 mm, 5µm. Mobile phases were 90:10 water: methanol, 10mM ammonium formate (mobile phase A)

and 10:90 water: methanol, 10mM ammonium formate (mobile phase B) respectively. Injection volume used for loading of sample was 10 μ L and flow rate standardized as 0.5ml/min and it was run at ambient temperature. UV detector used at 280nm of wavelength. The representative chromatogram obtained from standard solution i.e 500 ppm of 5- hydroxyl methyl furfural is given in Fig 46 showing chromatographic peak in 14.22 min of retention time depicting the peak of 5- hydroxyl methyl furfurals. The quantitative analysis of HMF of different 15 samples ranging from alkali pretreatment, hydrolysis and bioethanol production have been compared as given in Table 58.2.

Fig. 47 showed the chromatogram of sample 1 (S1) which was collected after the alkaline hydrogen peroxide pretreatment of *P. deltoides* wood referring to step 1 of bioconversion study. As the Table 58.2 showed that S1 has 2.25 ppm of HMF in retention time of 13.62 min. Some other components like oligosaccharides i.e glucose, xylose, furfurals, levulinic acid, acetic acid etc. seemed to be present in the hydrolysates as indicated in the HPLC chromatogram with different retention time.

Sample 2 (S2) represented second step of the study i.e. after enzymatic saccharification showing the chromatographic peak of 5- hydroxyl methyl furfurals in 12.51 min of retention time (Fig 48) and 40.92 ppm of HMF were detected as shown in Table 58.2. The enzymatic saccharification had been performed by using inhouse enzymes [cellulase from *M. thermophila* SH1 + *B. stratosphericus* N₁₂ (M) and xylanase from *B. altitudinis* Kd₁ (M) + *M. thermophila* SH1]. The enzymatic hydrolysate thus formed was pooled with the supernatant of first step and designated as sample 3 (S3). Pooled sample S3 was further treated with Ca (OH)₂ for detoxification and detoxified sample was named as sample (S4).

As indicated by HPLC chromatogram (Fig 49 and 50) in S3 and S4 the concentration of HMF was found 42.57 ppm and 33.18 ppm respectively, while their chromatographic peaks were noticed in 12.56 min and 12.85 min of retention time.

Table 58.2. Estimation of concentration of inhibitors (5-hydroxy methyl furfurals) from NaOH + H₂O₂ pretreated *P. deltoides* wood during bioethanol production using High performance Liquid Chromatography (HPLC)

Sr. No.	Sample	Quantity of HMF (ppm or mg/L)			
		NDT*		DT**	
		Hydrolysate	Fermented liquor	Hydrolysate	Fermented liquor
1.	S1	2.25	-	-	-
2.	S2 ,S9	40.92	22.39	-	-
3.	S3 ,S6	42.57	32.10	-	-
4.	S4 ,S7	-	-	33.18	27.01
5.	S5 ,S8	-	-	30.70	10.80
6.	S10 ,S11, S12	68.59	-	42.07	38.60

NDT*: Non detoxified

DT**: Detoxified

S1: Supernatant after pretreatment

S3: Supernatant + enzymatic hydrolysate

S5: Enzymatic hydrolysate of inhouse enzymes with detoxification

S7: Fermented liquor of S4,

S9: Fermented liquor of S2,

S11: Enzymatic hydrolysate of commercial enzyme with detoxification

S2: Enzymatic hydrolysate (with inhouse enzymes)

S4: Supernatant + enzymatic hydrolysate of inhouse enzymes with detoxification

S6: Fermented liquor of S3

S8: Fermented liquor of S5

S10: Enzymatic hydrolysate (with commercial enzymes)

S12: Fermented liquor of S11

In sample 5 (S5), the enzymatic hydrolysate formed in the second step detoxified with $\text{Ca}(\text{OH})_2$ was analysed for the presence of 5- hydroxymethyl furfurals. The chromatographic peak of S5 was observed in 12.91 min of retention time and the concentration of HMF was 30.70 ppm (Fig 51).

After fermentation with *S. cerevisiae* II + *P. stipitis* these fermented samples were further subjected to assess the quantity of 5-hydroxymethyl furfurals in them. The retention time as shown in figs 52, 53, 54 and 55 for S6, S7, S8 and S9 was 12.55 min, 12.85 min, 12.71 min and 12.54 min respectively. The concentration of HMF was observed as 32.10 ppm, 27.01 ppm, 10.80 ppm and 22.39 ppm respectively (Table 58.2).

Detoxification followed by fermentation for samples S10, S11 and S12 using commercial enzymes for hydrolysis of pretreated wood had been referred as control and related concentration of HMF had been given in Table 58.2 and fig 56, 57 and 58. S10 collected after enzymatic saccharification showed chromatographic peak in 12.55 min of retention time with 68.59 ppm of HMF (Fig 56). After detoxification (S11) it decreased to 42.07 ppm (fig 57) with the retention time of 12.79 min. While in fermented liquor 38.60 ppm of 5-HMF was found (Fig 58).

Fig 59 and 60 showed the comparison of concentration of 5- HMF present in detoxified and non-detoxified hydrolysate in both the cases i.e. by pooling hydrolysate with supernatant (Fig 59) as well as hydrolysate without pooling (Fig 60). Estimation of 5- HMF was also done in the hydrolysate prepared by commercial enzymes. Fig 61 showed concentration of 5-HMF in non- detoxified, detoxified and fermented liquor of the hydrolysate prepared by commercial enzymes.

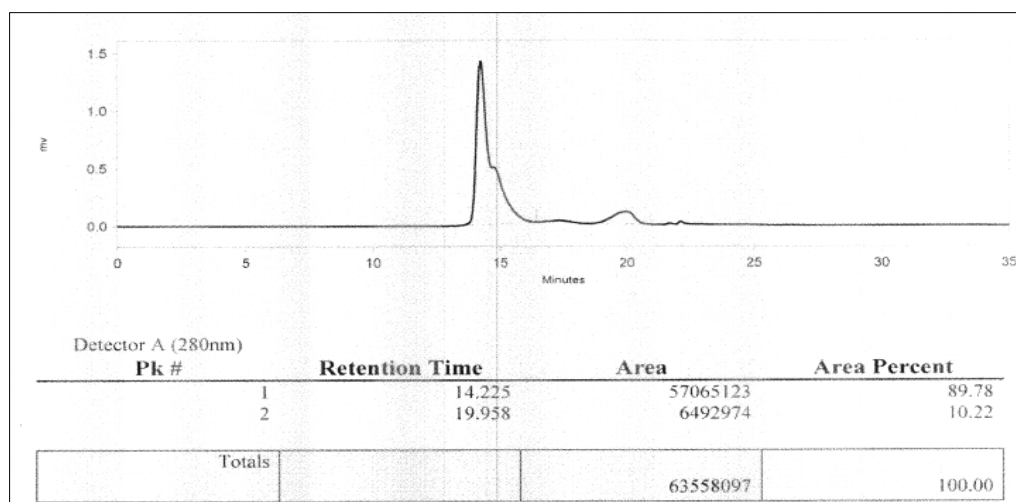


Fig 46. Standard of 5-HMF for detection of inhibitors (5-HMF)

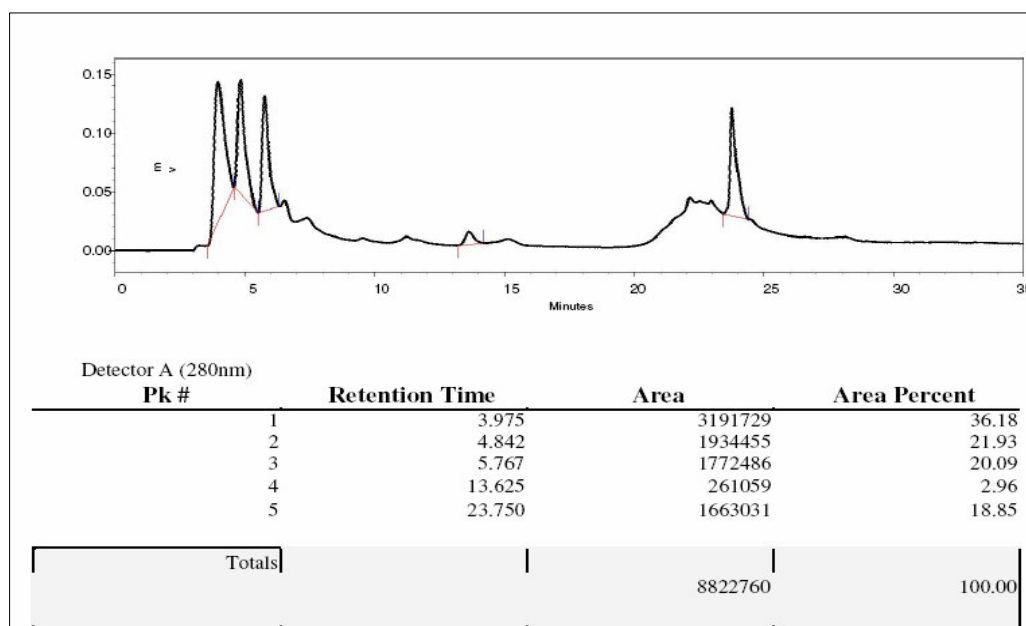


Fig 47. Estimation of concentration of 5-HMF in S1 i.e. after NaOH + H₂O₂ pretreatment of *P. deltoides* wood

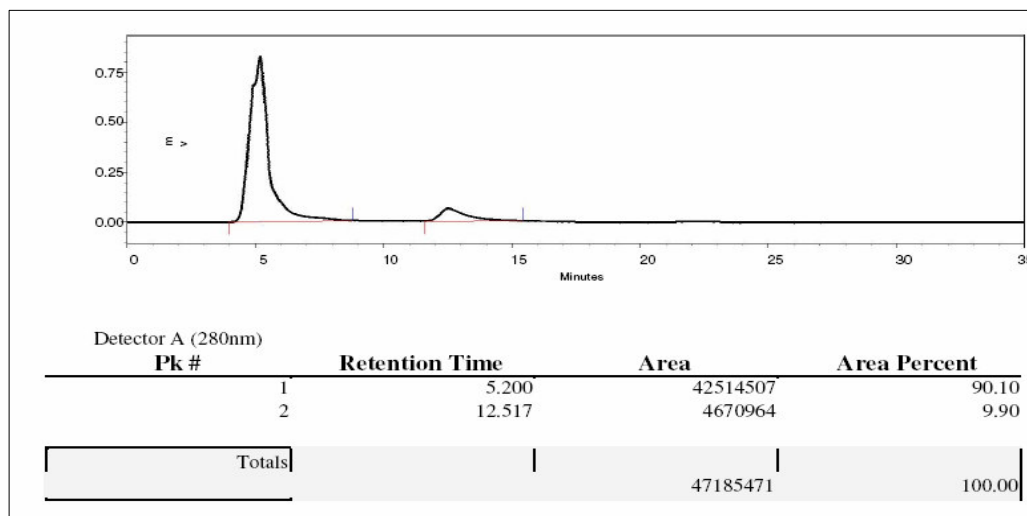


Fig 48. Estimation of concentration of 5-HMF in S2 i.e. enzymatic hydrolysate without detoxification

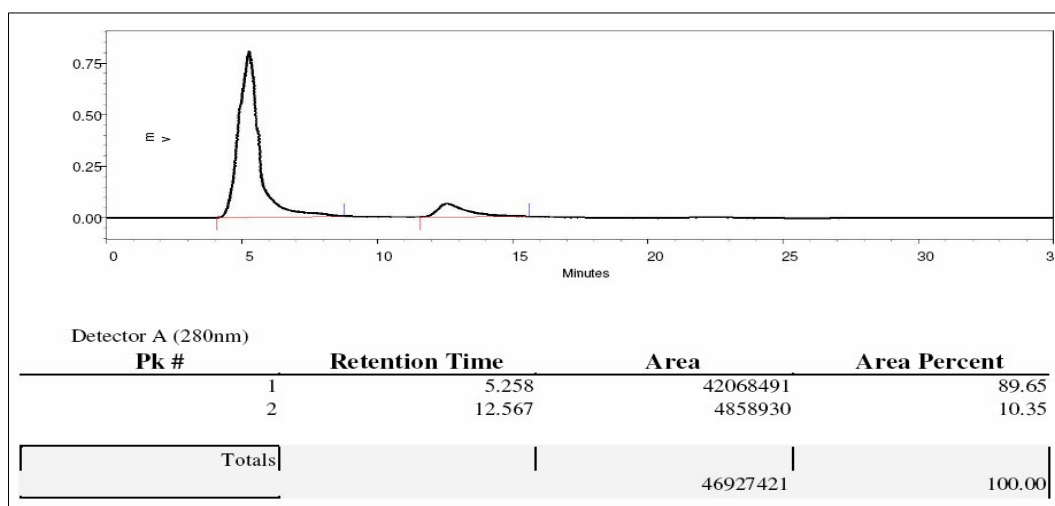


Fig 49. Estimation of concentration of 5-HMF in S3 i.e. supernatant + enzymatic hydrolysate (pooled) without detoxification

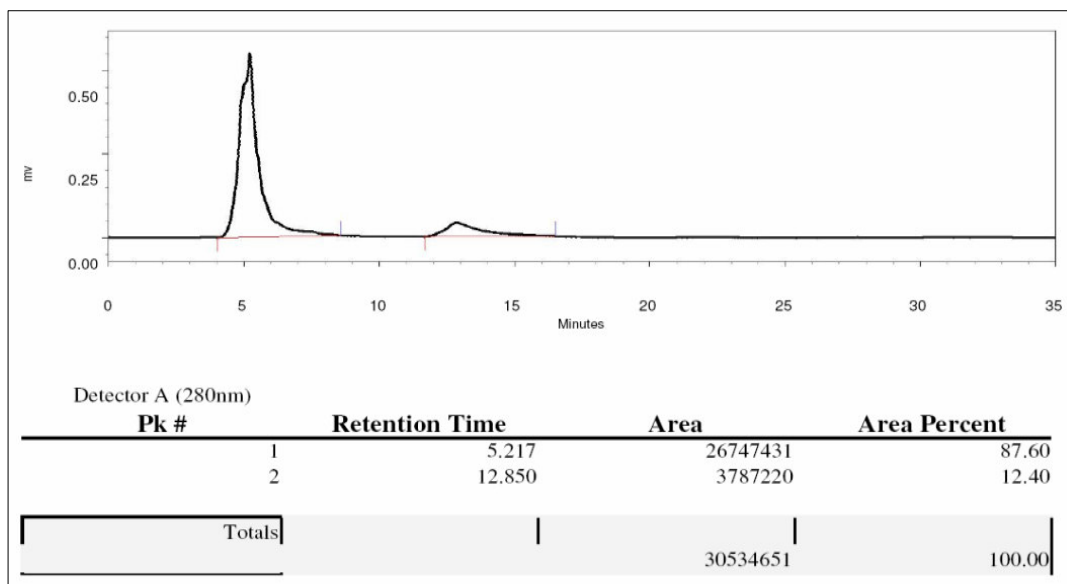


Fig 50. Estimation of concentration of 5-HMF in S4 i.e. supernatant + enzymatic hydrolysate (pooled) with detoxification

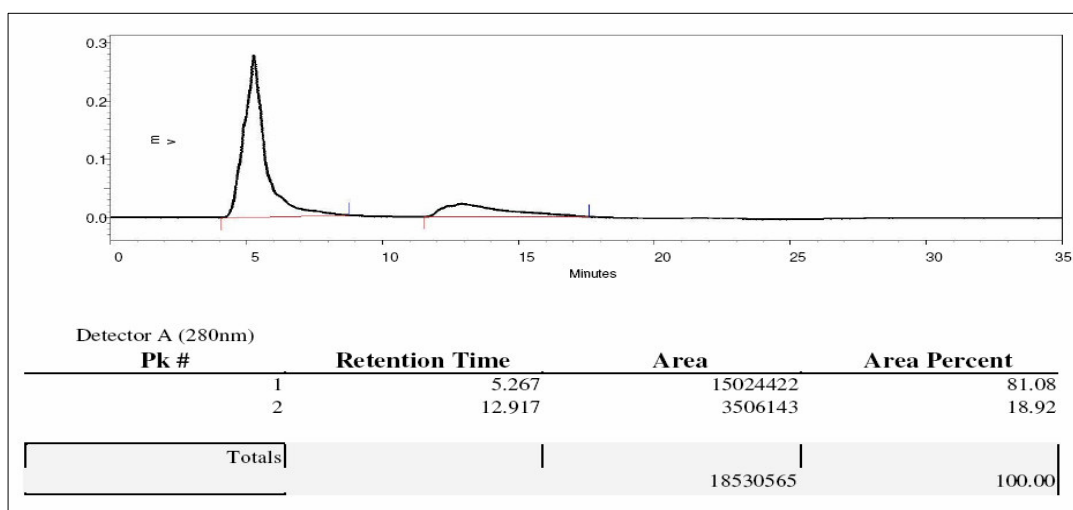


Fig 51. Estimation of concentration of 5-HMF in S5 i.e. enzymatic hydrolysate with detoxification

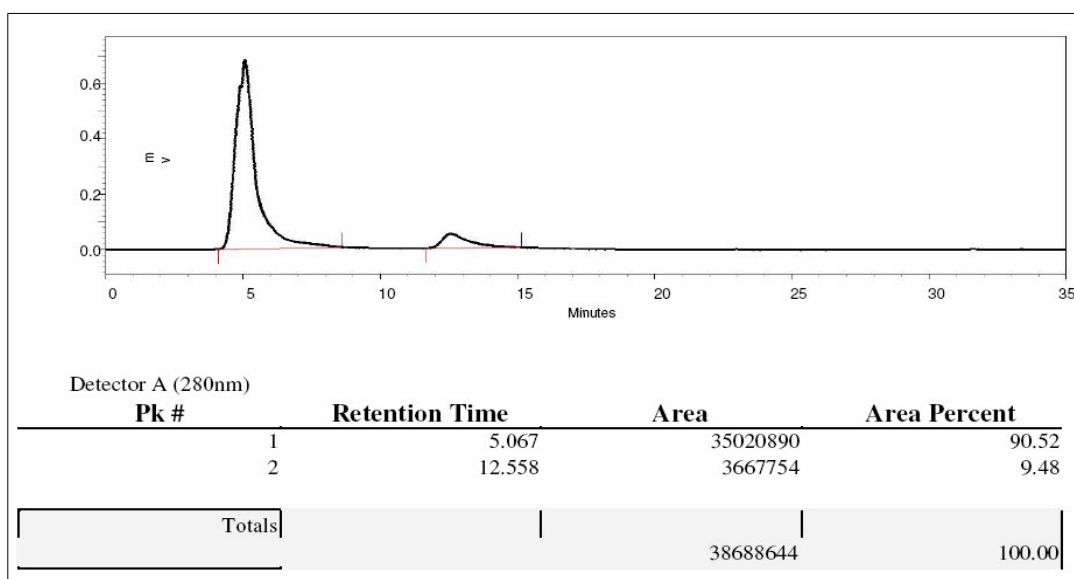


Fig 52. Estimation of concentration of 5-HMF in S6 i.e. Fermented liquor of S3

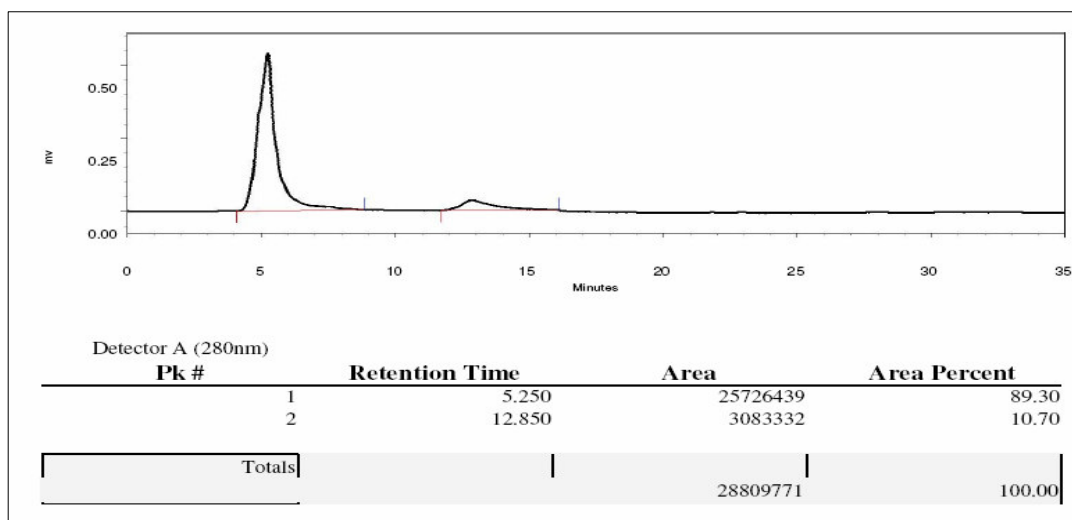


Fig 53 Estimation of concentration of 5-HMF in S7 i.e. Fermented liquor of S4

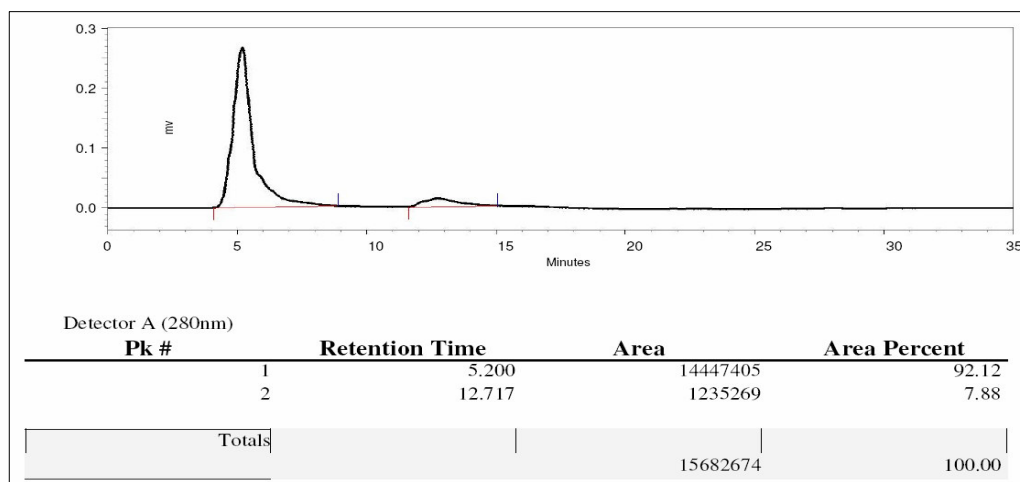


Fig 54. Estimation of concentration of 5-HMF in S8 i.e. Fermented liquor of S5

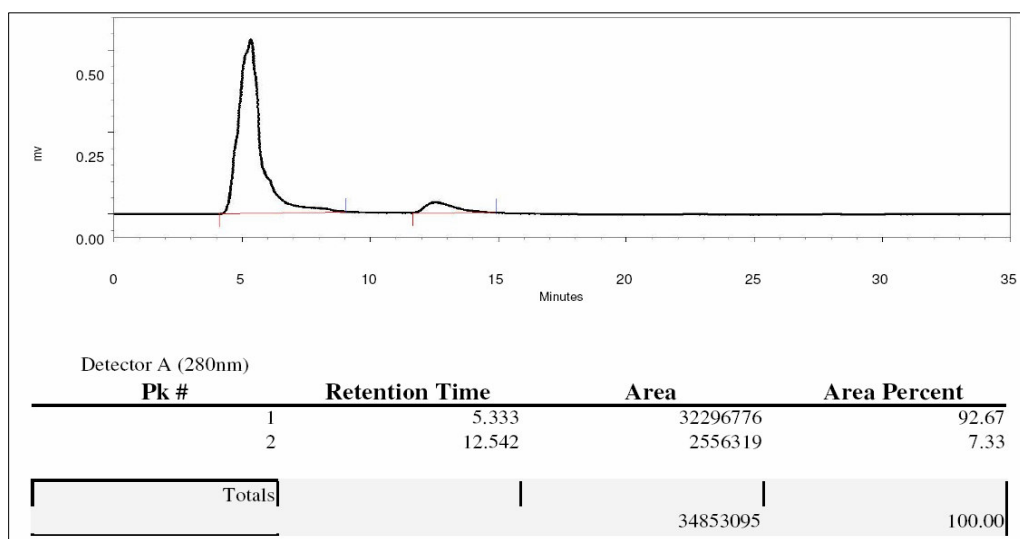


Fig 55. Estimation of concentration of 5-HMF in S9 i.e. Fermented liquor of S2

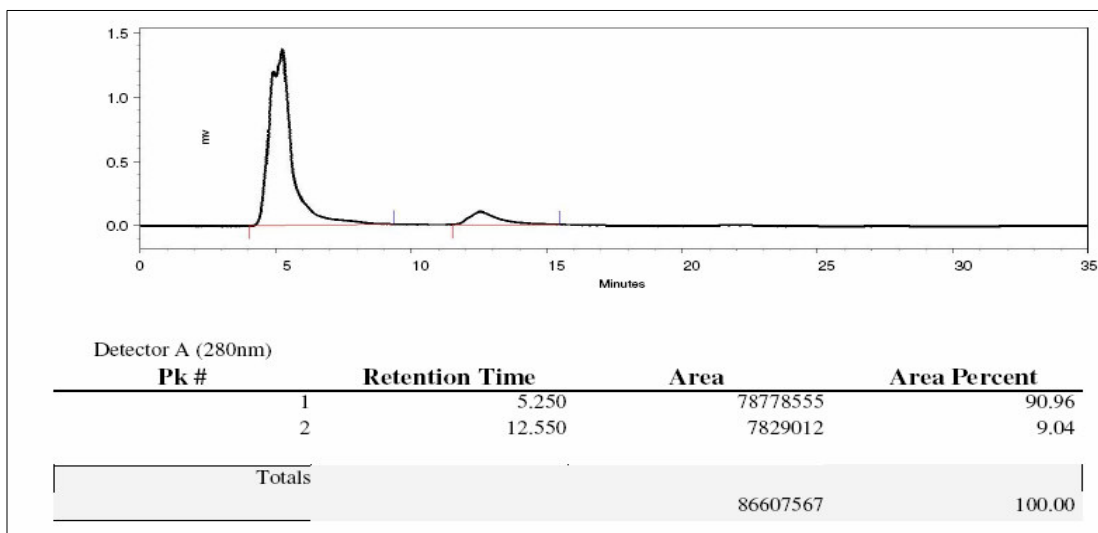


Fig 56 Estimation of concentration of 5-HMF in S10 i.e. Enzymatic hydrolysate with commercial enzymes

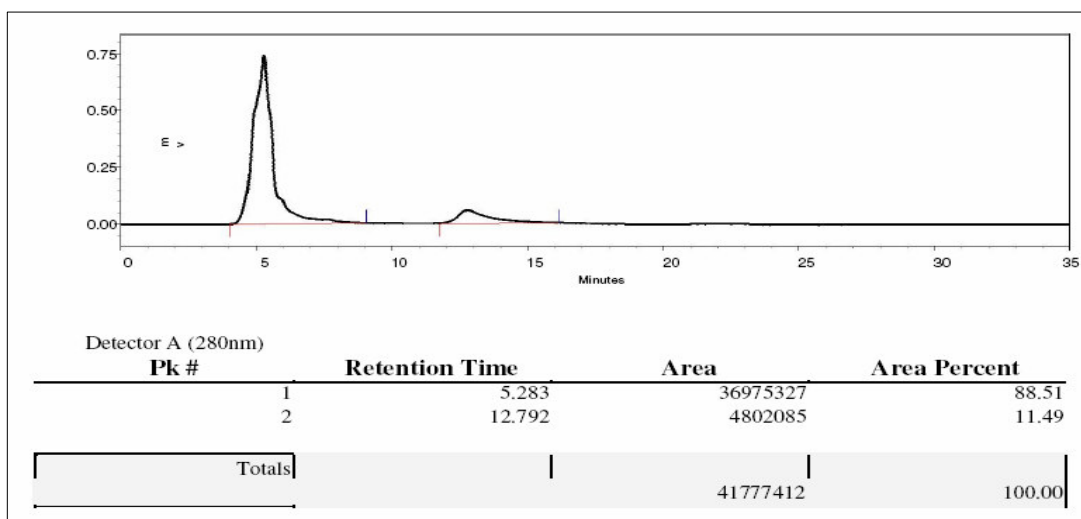


Fig 57. Estimation of concentration of 5-HMF in S11 i.e. after detoxification of S10

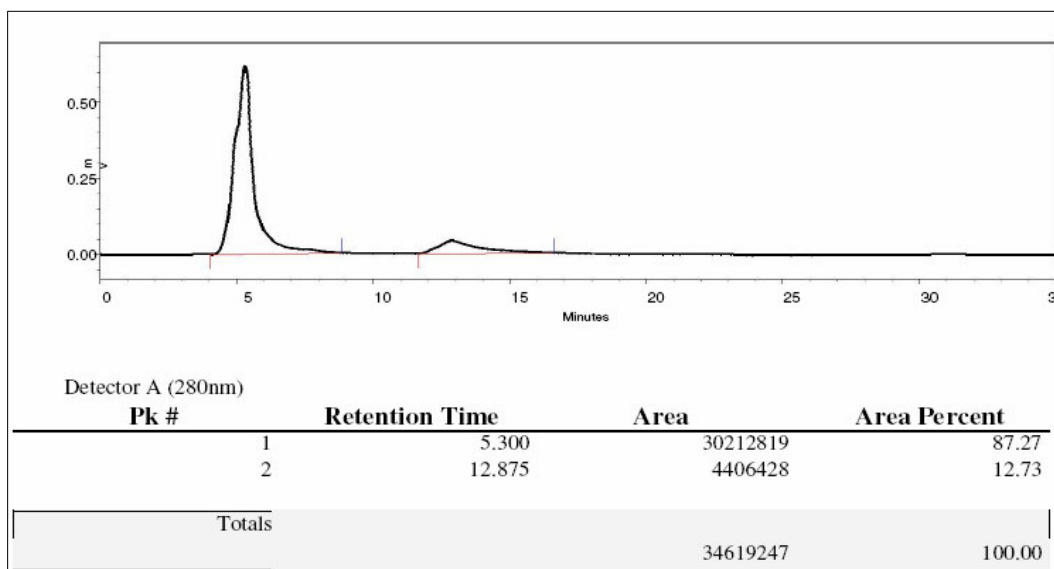


Fig 58. Estimation of concentration of 5-HMF in S12 i.e. Fermented liquor of S11

The hydrolysis of lignocellulosic biomass does not only have positive effects on the fermentation process but an inevitable part is the formation of inhibitory compounds. These inhibitors may have negative effect on the growth of the fermenting microbes and tend to decrease ethanol yields (Palmqvist & Hahn-Hagerdal, 2000a). The amount and type of toxic compounds formed depend on what kind of feedstock is used as well as pretreatment and other operational conditions employed for hydrolysis viz. temperature, time, pressure and pH (Taherzadeh *et al.*, 2000). These toxic compounds that are formed during lignocellulosic pretreatment can be divided into four groups: sugar degradation products, lignin degradation products, compounds derived from lignocellulose structure, and heavy metal ions (Olsson & Hahn-Hagerdal, 1996). When glucose and xylose are placed under harsh conditions viz. high pressure and elevated temperatures etc during pretreatment can be degraded into furfural and hydroxymethylfurfural (5-HMF) compounds that can further be degraded into other toxic compounds. These compounds negatively affect the cells specific growth rate and cell-mass yield of microorganisms.

Among sugar derived inhibitors, furfurals have been found to inactivate the cell replication that reduces the growth rate and cell mass yield on ATP, volumetric growth rate and specific productivities (Taherzadeh *et al.*, 1999a). It is

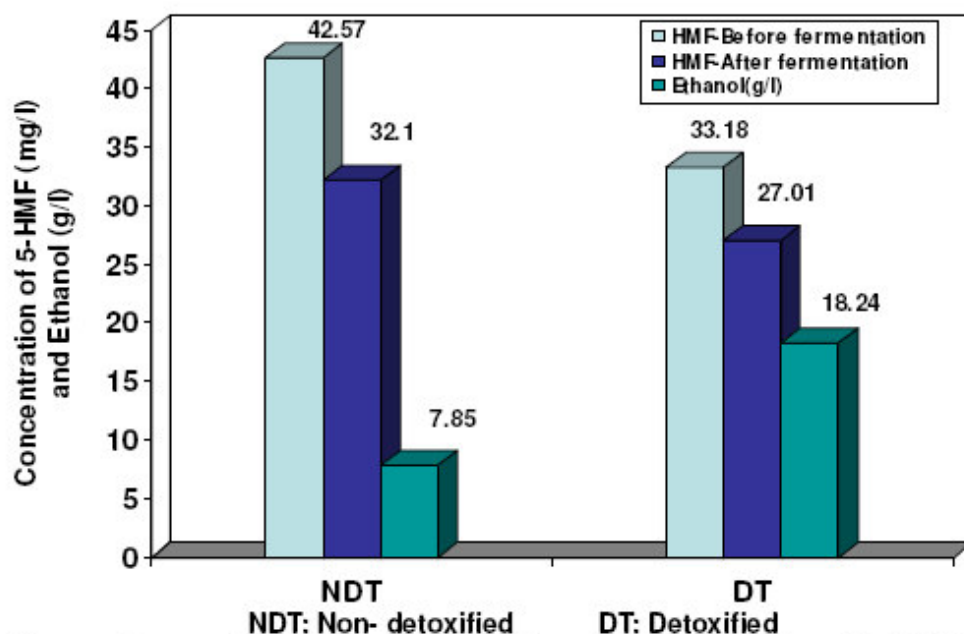


Fig 59. Comparison of ethanol production and concentration of 5-HMF in non- detoxified and detoxified supernatant + in house enzymatic hydrolysate of alkaline hydrogen peroxide pretreated *P. deltoides* wood

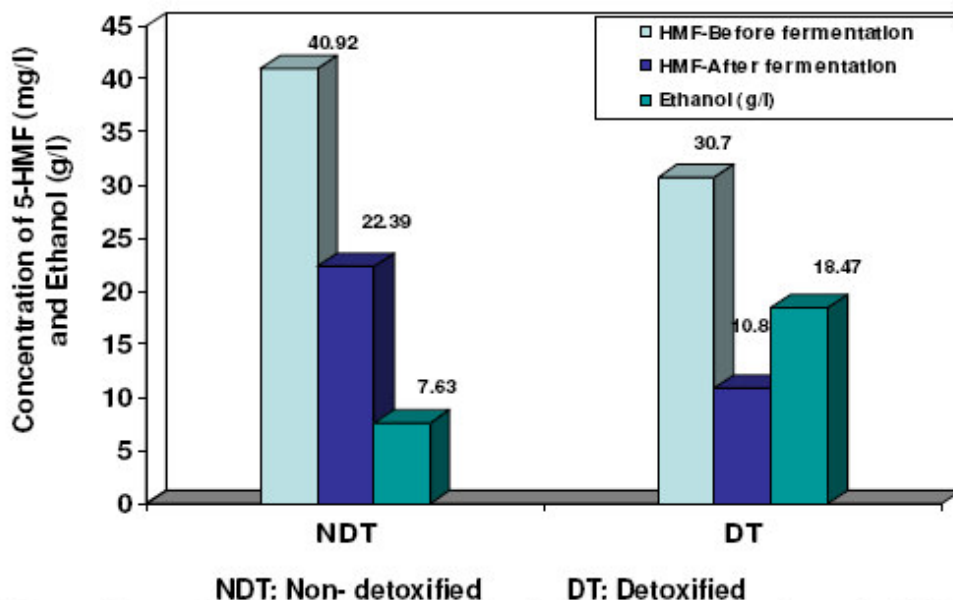


Fig 60. Comparison of ethanol production and concentration of 5-HMF in non-detoxified and detoxified in house enzymatic hydrolysate

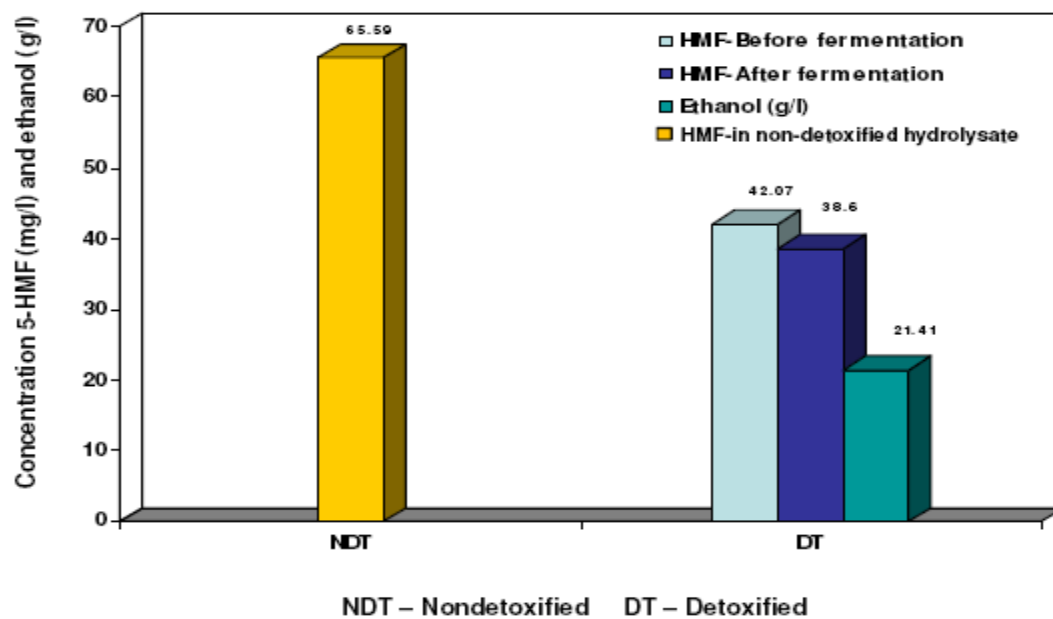


Fig. 61. A comparison of ethanol production and 5-HMF using commercial enzymes hydrolysate

reported that furfurals prevent formation of glycerol which is necessary to regenerate the excess NADH during fermentation and to maintain the intracellular redox balance. These findings were in accordance with those of Oliva *et al.* (2006), who pointed out that the presence of furfural and HMF at the experimental concentration affected the respiration and oxidative phosphorylation and slowed down the electron transport system. As a result, ATP production in the cells decreases and cell growth ceased, thus furfurals had been suggested to be highly sensitive to cell growth. During anaerobic fermentation, furfural is reduced to furfuryl alcohol, while furoic acid is produced from oxidation of furfural during aerobic cultivation. HMF has the similar inhibitory effect as furfural, except that it has a lower conversion rate that it can be because of its lower membrane permeability. An addition of 4g/L of HMF decrease the CO₂ evolution rate about 32%, ethanol production rate 40%, specific growth rate 70 %.

During lignocellulosic hydrolysis, a large variety of polyaromatic and phenolic compounds are released due to lignin and sugar degradation. They are toxic to fermenting microorganisms even more than furan limiting the microbial growth. They cause a partition and disturb the membranes in turn affecting their ability to serve as selective barriers and enzyme matrices affecting the cell growth and sugar assimilation (Palmqvist and Hahn- Hagerdaal, 2000a). Delgenes *et al.* (1996) reported the inhibitory effect of lignin derived compounds on the sugar utilization efficiency of *C. shehatae*, *P. stipitis*, *S. cerevisiae* and *Z. mobilis*. Vanillin was found to be the strongest inhibitor of growth and ethanol production in both pentose and hexose fermenting yeast. Hu *et al.* (2009) observed the p-hydroxy benzaldehyde and vanillin at 10mM concentration inhibits the growth of *Rhodospiridium toruloides*. The furfural concentration above 1g/L was found to decrease significantly the CO₂ evolution rate and the cell multiplication also the total viable cell number in the early phase of fermentation (Azhar *et al.*, 1992).

The toxicity of inhibitors to affect the bioconversion of lignocellulosic biomass into ethanol mainly depends upon the concentration, type of

fermentative organisms, the mode of cultivation and cultivation conditions (i.e pH, inoculum, dissolved oxygen and temperature). The toxic component may lead to stop the growth of microorganism by affecting the rate of sugar uptake with simultaneous decay in product formation. These inhibitors also affect the cellular physiology by disturbing the function of biological membrane, resulting poor microbial growth lingering towards extended incubation time with poor metabolite production.

Thus different methods of detoxification viz. overliming with $\text{Ca}(\text{OH})_2$ and activated charcoal have been employed in literature to remove most of the toxic compounds from hydrolysate improving the ethanol yield. These methods have been effective in the removal of furans and phenolic compounds from lignocellulosic hydrolysate (Todhanakasem *et al.*, 2012).

In the present study, overliming has been done to detoxify the hydrolysate with an aim to eliminate most of the inhibitors generated during process. 5- HMF one of the most important inhibitor generated due to the degradation of hexoses and pentoses sugars has been estimated in the present study using HPLC. Based upon the above mentioned results of significant reduction in 5-HMF with overliming, it has been hypothesized that most of the other inhibitory compounds present in the hydrolysate would have also been removed leading to the improved ethanol production.

Overliming with $\text{Ca}(\text{OH})_2$ and lignin removal have been found to be a cost effective processes for the detoxification of lignocellulosic hydrolysate to remove the volatile and non-volatile inhibitors such as furans and phenolic compounds. A study was conducted to analyze on the availability of toxic compounds and the amount of glucose obtained from two different pretreatments using sulfuric acid and mixed cellulase enzymes (without and with delignification/ activated charcoal) using rice bran as a substrate. The concentration of furfural, 5- hydroxymethyl furfural (5-HMF), levulinic acid, vanillin, syringaldehyde and 4-hydroxybenzaldehyde (4-HB) and the percent acetic acid were found to be 0.0517 ± 0.049 mg/L, 0.032 ± 0.06 mg/L, 21074 ± 1685.62 mg/L, 126.265 ± 6.005 mg/L, 2.89 ± 0.30 mg/L, 0.37 ± 0.031 mg/L and

0.72% under the pretreatment process without delignification/ activated charcoal treatment and 384.47 ± 99.02 g/L, 0.068 mg/L, 142107.62 ± 8664.6 mg/L, 0.19 mg/L, 5.43 ± 3.29 mg/L, 4.80 ± 0.76 mg/L and 0.254% under the pretreatment process with delignification/ activated charcoal treatment respectively (Todhanakasem *et al.*, 2012).

It has been reported in the literature that many co-culture combinations of yeasts have ability to metabolize furfural and HMF completely. In a study conducted by Wan *et al.* (2012) an effective co-culture of *S. cerevisiae* Y5 + *P. stipitis* CBS6054 fermented lignocellulosic hydrolysate initially. The glucose was used up and furfurals and 5-HMF were completely metabolized within 12 h, xylose was used up in 96 h with ethanol concentration of 27.4 g/l ethanol. These co-culture studies match with our findings i.e. *S. cerevisiae* and *P. stipitis* had shown the maximum ethanol yield.

4.11 SCALE UP OF BIOETHANOL PRODUCTION FROM NaOH + H₂O₂ PRETREATED *P. deltoides* WOOD USING CO-CULTURE OF *S. cerevisiae* II + *P. stipitis* IN A STIRRED TANK BIOREACTOR UNDER SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

Optimization of batch conversion of lignocellulosic hydrolysate to ethanol was carried out by co-culture of *S. cerevisiae* and *P. stipitis* which was selected from the previous study on the basis of maximum ethanol yield. The medium optimized from shale flask studies was added in a 7.5 L stirred tank bioreactor with a 3.0 L working volume at $32 \pm 0^{\circ}\text{C}$ and pH 5.5. Carbon and nitrogen sources; for batch fermentations were 50 g of *P. deltoides* wood biomass, peptone – 0.5%, yeast extract – 0.5%. 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 bioreactor was inoculated with 10% inoculum of 24 h old culture of yeasts (co-culture of *S. cerevisiae* II + *P. stipitis*) and 50 ml of enzyme solution i.e. 20 ml of cellulase (CMCase: 2.384 IU, FPase: 2.249 IU and β - glucosidase: 2.451 IU) of *B. stratosphericus* N₁₂ (M), 20 ml of cellulase (CMCase: 1.752 IU, FPase: 1.193 IU and β - glucosidase: 2.571 IU) from *M. thermophila* SH1, 5 ml of xylanase (71.49 IU) from *B. altitudinis* Kd1 (M) and 5 ml of xylanase (12.59 IU) from *M. thermophila* SH1 under aseptic conditions. The agitation speed of 200 rpm and

agitation rate of 1.0 vvm were used for batch cultivation in the bioreactor. The sample from fermentor was withdrawn regularly at different intervals ranging from 2, 4, 6.....12 h and ethanol estimation was done. Table 59 revealed the ethanol yield produced by the co-culture at different timings under anaerobic conditions. The maximum ethanol yield was observed in 10 h i.e. 12.68 g/l with the fermentation efficiency of 49.51%, which was constant upto 12 h and ethanol yield was reduced to 9.99 g/l after 24 h. The yield of ethanol is found to be proportional to fermentation time. It increases with the increase in time upto 10-12h and then declines. The time of fermentation was also reduced dramatically to 10 h for maximum ethanol yield as compared to shake flask (72 h)

Table 59. Scale up of bioethanol production from NaOH + H₂O₂ pretreated *P. deltooides* in a stirred tank bioreactor using co-culture of *S. cerevisiae* + *P. stipitis* under simultaneous saccharification and fermentation (SSF)

Time (h)	O.D.	Ethanol (%)	Ethanol (g/g)	Ethanol (g/l)	Fermentation efficiency (%)
2	0.032	0.42	0.066	3.33	12.91
4	0.041	0.51	0.080	4.04	15.65
6	0.100	1.20	0.190	9.51	37.18
8	0.110	1.31	0.206	10.31	40.31
10	0.146	1.60	0.253	12.68	49.51
12	0.146	1.60	0.253	12.68	49.51
24	0.106	1.26	0.199	9.99	38.94

The environmental conditions optimized at shake flask level have initially been adapted in the bioreactor. Since a first step to improve the large scale process should be mimic of the optimal lab scale process. The results of bioreactor when compared with shake flask experiment, an improved yield with shorter fermentation time have emerged as the remarkable achievement of scale up process. The precisely controlled process parameters in a bioreactor might be the possible reason for it. Bioethanol production in a stirred tank bioreactor maintains operating conditions such as temperature, pH, agitation speed, type of substrates/sugars for ethanol production and growth of microorganisms which play an important role in process scale up. Fig 62 showed the physiological behaviour of co-culture of *S. cerevisiae* II+ *P. stipitis* in the stirred tank bioreactor during bioethanol production.

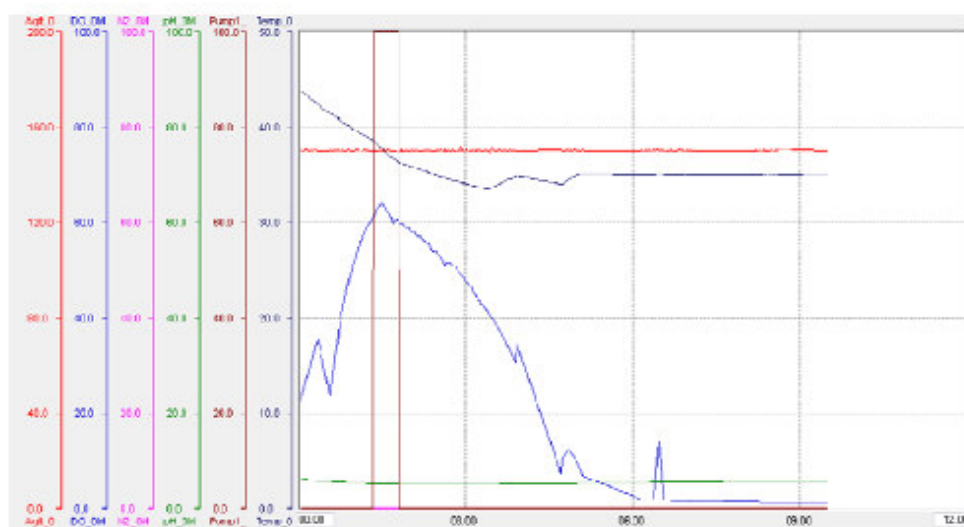


Fig 62: Physiological behaviour of co-culture of *S. cerevisiae* and *P. stipitis* under simultaneous saccharification and fermentation (SSF) in a stirred tank bioreactor

Thus this process has been considered viable for bioconversion of cellulosic substrates to fermentation products. In simultaneous saccharification and fermentation (SSF) process both cellulose hydrolysis and fermentation of glucose are carried out in presence of fermentative microorganisms in a single step and the process optimally operates at 37°C to 38°C. This technique reduces the number of steps in the process and is a promising way for converting cellulose to ethanol (Lynd *et al.*, 2005).

Conversion of glucose and xylose to ethanol by co-yeast strains has been successfully obtained by Taniguchi *et al.* (1997) using a respiratory deficient mutant of *S. cerevisiae* and *P. stipitis*. *P. stipitis* strains ferment xylose at a high capacity of 57g/l than any other yeast, provided the pH is maintained between 4.5 and 6.0 and temperature of 25°C- 26°C as these conditions invariably influence sugar utilization by yeast cells. According to the Jefferies *et al.* (2007), maximum yield of ethanol is obtained when the mixture of *S. cerevisiae* and *P. stipitis* are introduced into a medium containing both glucose and xylose.

According to Galbe and Zacchi (2002), a temperature of 35°C for simultaneous saccharification and fermentation (SSF) is a compromise, but the development of a thermotolerant yeast strain is expected to improve the performance of SSF. The SSF of corn cobs into ethanol by yeast was carried out at 37°C in a shaker at 150 rpm (Latif and Rajoka, 2001). Verma *et al.* (2011) reported that the optimum temperature for maximum ethanol production using starch in co-cultures of amylolytic yeasts and *S. cerevisiae* was 30°C, and there was no remarkable loss in ethanol yield up to 40°C. Yeasts, under anaerobic conditions, metabolize glucose to ethanol primarily by the Embden-Meyerhof pathway. Generally, studies comparing SSF and SHF have illustrated that determination of the better method is dependent on a number of factors. Several studies have confirmed that SHF produces higher overall yields, while SSF requires less time (Alfani *et al.* 2000; Cantarella *et al.* 2004). An ideal operating process would be flexible to allow for either method to be used in accordance to feedstock availability and the desired products.

Chapter-5

SUMMARY AND CONCLUSION

In the present investigation entitled “**Bioconversion of cellulosic waste into bioethanol as biofuel**”, an attempt had been made to isolate most efficient cellulolytic and xylanolytic bacteria and fungi from soil collected from mid hills of Himachal Pradesh located in sub temperate zone of northern Himalayas, their primary screening to select hyperenzyme producing strains, secondary screening by physical and chemical mutation, identification of selected bacterial strains, optimization of various growth parameters, partial purification of cellulase and xylanase, scale up of cellulase and xylanase enzyme in a bioreactor, bioconversion of cellulosic waste into bioethanol, scale up of bioethanol in a stirred tank bioreactor and quantitative estimation of inhibitors formed during bioethanol production by High Performance Liquid Chromatography (HPLC) was done.

In total, 89 isolates including 84 bacteria (36% from Solan, 25% from Shimla and 39% from Kangra district) and 5 fungi (3 from Solan, 1 from Shimla and 1 from Kangra district) had been isolated from the soil collected from three districts of Himachal Pradesh viz. Solan, Shimla and Kangra. Different physiochemical and biochemical tests were performed with these bacterial isolates for their tentative identification. Out of 84 bacterial isolates 61% were Gram positive, 59% were rod shaped and 41% were spherical in shape. Where as 73 % were found catalase positive, 62% were H₂S producers, 58% were found positive for citrate utilization, 67% were MR positive, 33% were VP positive, 30% for casein and 24% were found positive for carbohydrate utilization. All the isolates were gelatin positive but indole and urease negative.

The screening of the isolates was done on the basis of their cellulase and xylanase activities under submerged (SmF) fermentation. Among all, 7 extracellular cellulase producing bacterial strain viz. N₅, N₁₂, N₁₃, NS₁, S₁, S₇, S₁₂

and 3 hyperxylanase producers i.e. Kd₁, SL₈ and N₁₁ gave the hypercellulase and xylanase activity. Hypercellulase producers i.e. N₅, N₁₂, N₁₃, NS₁, S₁, S₇, S₁₂ showed cellulase activities i.e 1.503 IU, 1.476 IU, 2.138 IU, 1.689 IU, 1.639 IU, 1.135 IU and 1.220 IU respectively, while hyperxylanase producers i.e. N₁₁, Kd₁ and SL₈ gave xylanase activities of 29.64 IU, 31.72 IU and 61.54 IU respectively. These 10 bacterial (7 for cellulase and 3 for xylanase) were selected for secondary screening.

Secondary screening was performed by inducing mutation with physical and chemical mutagens i.e UV radiations and ethidium bromide (EtBr). Out of 10 bacterial isolates, mutants of N₁₂ (EtBr treated) and Kd₁ (UV irradiated) exhibited appreciable increase in cellulase and xylanase production respectively and hence were selected for further study.

Identification of selected strains N₁₂ and Kd₁ and their mutants was done by using 16S rRNA technique and were identified as *Bacillus stratosphericus* N₁₂ and *Bacillus altitudinis* Kd₁ respectively. They had been registered with NCBI vide accession no. i.e. *Bacillus stratosphericus* N₁₂ (W) [KC995116], *Bacillus stratosphericus* N₁₂ (M) [KC995118], *Bacillus altitudinis* Kd₁ (W) [KC995115] and *Bacillus altitudinis* Kd₁ (M) [KC995117] respectively.

The different process parameters i.e. media, pH, temperature, incubation time, inoculum size, different carbon sources, concentration of carbon source for cellulase and xylanase production from mutant strains of *B. stratosphericus* N₁₂ (M) and *B. altitudinis* Kd₁ (M), respectively were optimized using Classical approach One Factor at a Time (OFAT). After optimization of different process parameters, PYC media, 8.0 pH, 72 h of incubation days, 10% inoculum size and lactose as carbon source @ 2.0% was found the best. Cellulase titers were increased from 3.230 IU to 5.983 IU after optimization, attaining 85.23% increase in its production.

For xylanase production, TGY media, 5.5 pH, 72 h of incubation days, 12.5% inoculum size, xylose as carbon source @ 2.5% were found the best.

During optimization of these process parameters enzyme titers increased from 56.83 IU to 96.25 IU exhibiting 85.60% increase in xylanase production.

Cost effective production of cellulase and xylanase was done by replacing expensive conventional carbon source with inexpensive lignocellulosic forest waste to make the process economically viable. Lignocellulosic forest biomass of different hardwood and softwood species was selected on abundance basis and different pretreatments were given to them to evaluate the increase in their susceptibility for enzymes production. The hardwood and softwood biomass selected were *Eucalyptus sp.*, *D. strictus*, *P. deltoides*, *P. roxburghii*, *P. wallichiana* and *C. deodara*. To the selected lignocellulosic biomass different physiochemical pretreatments were given i.e Chipping, grinding, steam, 2.5% H₂SO₄, 2.5% HCl, 2% NaOH + H₂O₂ (9:1) and 5% NH₃ to make it easily accessible for enzymatic hydrolysis.

To reduce the production cost of cellulase and xylanase enzymes from *B. stratosphericus* N₁₂ (M), *B. altitudinis* Kd₁ (M) and *M. thermophila* SH1, cheap untreated and pretreated lignocellulosic forest biomass was used as a substrate under submerged fermentation. Maximum cellulase and xylanase i.e. 41.73 U/g and 111.98 U/g was noticed by *B. stratosphericus* N₁₂ (M) and *B. altitudinis* Kd₁ (M) respectively using NaOH+ H₂O₂ pretreated *P. deltoides* wood under SmF. In case of a potential inhouse *M. thermophila* SH1, SSF mode of fermentation was used for cellulase as well as xylanase using alkaline hydrogen peroxide pretreated *P. deltoides* wood as cultivation medium secreting maximum cellulase and xylanase i.e. 35.32 U/g and 203.20 U/g respectively. Alongwith enzymes, highest reducing sugars i.e. 13.30 mg/g were also produced by NaOH + H₂O₂ pretreated wood. Based upon maximum reducing sugars produced from NaOH + H₂O₂ pretreated *P. deltoides* wood during enzymes production, it had been selected for ethanol bioconversion studies.

To strengthen the enzymes concentration, partial purification of cellulase and xylanase was done by ammonium sulphate precipitation. After ammonium sulphate precipitation, between 30-60% of saturation level maximum CMCase and FPase activity was recovered, where as β - glucosidase activity was found in

the range of 0-30%, while the maximum xylanase was noticed between 0-70% of saturation level.

Cellulase production by *B. stratosphericus* N₁₂ (M) and xylanase by *B. altitudinis* Kd₁ (M) had been scaled up in a 7.5 L stirred tank bioreactor with 3.0 L working volume at 30°C (pH- 8.0 and PYC) for cellulase and TGY medium (pH-5.5) for xylanase at 200rpm with agitation rate of 1.0 vvm and finally obtaining enhanced 2.443 IU of cellulase and 11.10 IU of xylanase after reduced i.e. only 8 h of fermentation.

Bioethanol production from *P. deltooides* biomass was studied under different fermentation processes i.e. Separate hydrolysis and fermentation (SHF), Simultaneous saccharification and fermentation (SSF) and Simultaneous saccharification and co-fermentation (SSCF) in order to have a complete enzyme cocktail for hydrolysis by preparing a cocktail of different bacterial and fungal enzymes in a fixed proportion i.e. 5mg/5g of biomass where cellulases and xylanases were mixed in the ratio of 4:1 i.e. 2 ml of cellulase from *M. thermophila* SH1 + 2 ml of cellulase from *B. stratosphericus* N₁₂ (M) and 0.5 ml of xylanase from *B. altitudinis* Kd₁ (M) + 0.5 ml of xylanase from *M. thermophila* SH1. The different ethanologens in monoculture and co-culture combinations were used for fermentation i.e. *S. cerevisiae* I, *S. cerevisiae* II, *P. stipitis*, *C. shehatae*, *Z. mobilis*, *S. cerevisiae* I + *P. stipitis*, *S. cerevisiae* II + *P. stipitis*, *S. cerevisiae* I + *C. shehatae*, *S. cerevisiae* II + *C. shehatae* and a recombinant *C. thermocellum* from IMTECH-Chandigarh, NCIM- Pune (India) and DSM- Germany.

During bioconversion study, different methods has been designed to delimit the constraints that act as bottlenecks for successful ethanol production process. Separate hydrolysis and fermentation (SHF) was evaluated by modifying four different sub-processes i.e. method-I, II, III and IV of non-detoxification and detoxification as well pooling and non-pooling of pretreated liquor of wood hydrolysate which was further subjected to fermentation step. The maximum ethanol yield was achieved in method-IV of SHF i.e. 18.47 g/l with co-culture of *S. cerevisiae* II + *P. stipitis* with fermentation efficiency of 72.46%. The degree

of robustness of inhouse enzymes prepared as a part of this study for lignocellulosic biomass hydrolysis was compared with commercial enzymes i.e. Celluclast 1.5L, Novozyme188 and Pantopan Mono BG.

In simultaneous saccharification and fermentation (SSF) process, the maximum ethanol concentration of 11.26 g/l was observed in co-culture of *S. cerevisiae* II + *P. stipitis* with inhouse enzymes (fermentation efficiency of 44.16%). Similarly, Simultaneous saccharification and co-fermentation (SSCF) was performed by a recombinant strain of *Clostridium thermocellum* DSM 1313 and achieved 2.37 g/l of ethanol with fermentation efficiency of 7.85% which was quite low as compared to ethanol fermented in other two processes i.e SHF and SSF.

Among all the three processes of fermentation evaluated in the present study, Separate hydrolysis and fermentation (SHF) was found the best in terms of highest ethanol productivity. On the other hand, in case of strains used as monoculture as well as in co-culture *S. cerevisiae* II + *P. stipitis* was observed the best combination.

Thus in the present study, development of a technology for effectively converting alkaline hydrogen peroxide pretreated *P. deltoides* wood to simple sugars by potential inhouse enzymes produced from isolated microorganisms and intern fermenting them to appreciable concentration of ethanol fulfills the main aim of our study, thus envisaging sustainable energy production and improved environmental quality.

Chapter-6

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Name of Major Advisor : Dr. (Mrs.) Nivedita Sharma
Major field : Microbiology
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ABSTRACT

The present investigation was carried out to isolate, screen and identify the most efficient cellulolytic and xylanolytic microorganisms from soil. Mutation of hypercellulase and xylanase producers, enzyme production, optimization, partial purification, bioconversion of cellulosic waste into bioethanol and scale up studies were performed with selected strains to recommend their use for industries. In total 89 microorganisms including 84 bacteria and 5 fungi were isolated. Among them, ten hypercellulase and xylanase producing bacteria were subjected to mutation for enhanced enzyme production. N₁₂ (M) and Kd₁ (M) were screened for cellulase and xylanase enzyme production studies. The wild and mutant bacterial isolates were identified as *B. stratosphericus* N₁₂ (W), *B. stratosphericus* N₁₂ (M), *B. altitudinis* Kd₁ (W) and *B. altitudinis* Kd₁ (M) respectively by 16S rRNA PCR technique and registered with NCBI under accession no. |KC995116|, |KC995118|, |KC995115| and |KC995117|. Cellulase and xylanase enzymes were optimized through classical approach one factor at a time (OFAT) under submerged fermentation varying medium, pH, temperature, inoculum size, incubation time, carbon source and substrate concentration. The percent increase in enzyme activity obtained after optimization of different process parameters was 85.23% for cellulase of *B. stratosphericus* N₁₂ (M) and 85.60% for xylanase of *B. altitudinis* Kd₁ (M). To reduce the production cost of enzymes, cheap untreated and pretreated lignocellulosic forest biomass i.e. hardwood and softwood were used as a substrate under SmF by *B. stratosphericus* N₁₂ (M) and *B. altitudinis* Kd₁ (M), SSF by *M. thermophila* SH1 and among them alkaline hydrogen peroxide pretreated *P. deltoides* wood was found the best for hypercellulase and xylanase production under SmF as well as SSF. The partial purification of hydrolytic enzymes was done by ammonium sulphate precipitation. Scale up of cellulase from *B. stratosphericus* N₁₂ (M) as well as xylanase from *B. altitudinis* Kd₁ (M) was performed in 7.5 L bioreactor at 200 rpm, 1 vvm and 30°C, achieving 2.443 IU cellulase and 11.10 IU xylanase respectively, after only 8 h of fermentation. Bioconversion of alkaline hydrogen peroxide pretreated *P. deltoides* wood to ethanol was studied under three different fermentation processes i.e. separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF). Different strategies had been designed to delimit the constraints of fermentation process. SHF was evaluated by modifying four different sub-processes of detoxification and non-detoxification as well as pooling and nonpooling of pretreated liquor. Maximum ethanol was achieved in method –IV of SHF i.e. 18.47g/l by co-culture of *S. cerevisiae* II and *P. stipitis* with the fermentation efficiency of 72.46%. Among all the three processes of fermentation evaluated in the present study, SHF was found to be the best and in case of strains used for fermentation, co-culture of *S. cerevisiae* II and *P. stipitis* was observed the best combination for highest bioethanol production.

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APPENDIX-I

Table 1: Secondary screening of hypercellulase producing bacterial isolates using UV irradiation as mutagen

Table 1a. Screening of hypercellulase producing bacterial isolate N₁₂

UV exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β-glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.5	1.274	0.093	-	1.367	2.72	0.502
15	7.9	0.635	0.118	0.584	1.337	1.16	1.152
30	7.9	0.135	0.020	-	0.155	1.15	0.134
45	7.8	0.121	0.043	-	0.164	0.79	0.207
60	7.2	0.171	0.012	-	0.183	1.13	0.161
75	7.2	0.120	0.054	-	0.174	1.18	0.147
CD _{0.05}		0.102	0.001	-	0.110	0.730	0.102
S.E.		0.047	0.0008	-	0.050	0.335	0.047

Initial pH - 6.8

Table1b. Screening of hypercellulase producing bacterial isolate S₁₂

UV exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β-glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.5	0.012	0.086	1.122	1.220	1.34	0.910
15	7.8	0.079	0.050	-	0.129	1.12	0.115
30	7.9	0.114	0.013	0.047	0.174	1.43	0.121
45	7.4	0.079	0.004	0.095	0.178	1.02	0.174
60	7.1	0.160	0.042	-	0.202	0.95	0.212
75	7.5	0.077	0.084	-	0.161	0.63	0.255
CD _{0.05}		0.0017	0.0017	0.0012	0.072	0.017	0.008
S.E.		0.0008	0.0008	0.0005	0.033	0.008	0.003

Initial pH - 6.8

Table1c. Screening of hypercellulase producing bacterial isolate N₅

UV exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β-glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.2	0.740	0.126	0.637	1.503	1.83	0.821
15	7.8	0.231	0.100	0.837	1.168	1.87	0.624
30	7.6	0.228	0.079	0.550	0.857	1.44	0.595
45	7.4	0.118	0.067	0.047	0.232	0.58	0.400
60	7.7	0.081	0.007	-	0.088	0.41	0.214
75	7.4	0.058	0.003	-	0.061	0.39	0.156
CD _{0.05}		0.125	0.0016	0.125	0.125	0.017	0.162
S.E.		0.057	0.0007	0.057	0.057	0.008	0.074

Initial pH - 6.8

Table1d. Screening of hypercellulase producing bacterial isolate S₇

UV exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β-glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.4	0.002	0.053	1.080	1.135	1.76	0.644
15	7.5	0.059	0.027	1.335	1.421	1.11	1.280
30	7.8	0.026	0.004	1.326	1.356	1.09	1.244
45	7.6	0.003	-	0.071	0.074	0.85	0.087
60	7.9	0.013	-	1.067	1.080	0.93	1.161
75	7.3	-	0.004	1.144	1.148	0.69	1.663
CD _{0.05}		0.001	0.0014	0.102	0.102	0.017	0.145
S.E.		0.0007	0.0006	0.047	0.047	0.008	0.066

Initial pH - 6.8

Table1e: Screening of hypercellulase producing bacterial isolate N₁₃

UV exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.0	1.577	0.088	0.473	2.138	3.160	0.676
15	7.5	0.214	0.029	1.268	1.511	1.160	1.302
30	7.9	0.154	0.043	0.799	0.996	0.802	1.241
45	7.5	0.036	0.051	0.560	0.647	0.606	1.067
60	7.3	0.056	0.014	-	0.070	0.700	0.100
75	7.2	0.035	0.002	-	0.037	0.638	0.058
CD _{0.05}		0.102	0.0017	0.145	0.431	0.740	0.125
S.E.		0.047	0.0008	0.066	0.198	0.339	0.057

Initial pH - 6.8

Table1f. Screening of hypercellulase producing bacterial isolate NS₁

UV exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.3	1.200	0.068	0.421	1.689	2.920	0.578
15	7.5	0.059	0.036	1.484	1.579	1.740	0.907
30	7.6	0.077	0.013	1.532	1.622	1.470	1.103
45	7.9	0.053	0.006	1.029	1.088	1.770	0.614
60	7.5	0.006	0.002	1.278	1.286	1.150	1.110
75	7.3	-	0.014	0.560	0.574	0.790	0.726
CD _{0.05}		0.072	0.0017	0.162	0.162	0.740	0.126
S.E.		0.033	0.0008	0.074	0.074	0.339	0.057

Initial pH - 6.8

Table 1g: Screening of hypercellulase producing bacterial isolate S₁

UV exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.1	1.096	0.148	0.395	1.639	1.80	0.910
15	7.5	0.674	0.132	0.981	1.787	1.68	1.063
30	7.6	0.297	0.166	1.029	1.492	1.60	0.932
45	7.6	0.472	0.191	1.053	1.716	1.51	1.136
60	7.3	0.430	0.142	0.951	1.529	1.53	0.999
75	7.0	0.248	0.242	0.885	1.375	0.99	1.388
CD _{0.05}		0.162	0.072	0.102	0.178	0.074	0.102
S.E.		0.074	0.033	0.047	0.081	0.034	0.047

Initial pH - 6.8

Table 2: Secondary screening of hypercellulase producing bacterial isolates using EtBr as mutagen**Table 2a. Secondary screening of hypercellulase producing bacterial isolate N₁₂**

EtBr exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.2	1.274	0.093	-	1.367	2.72	0.502
30	7.4	0.917	0.151	0.847	1.915	1.48	1.29
60	7.7	1.090	0.158	1.134	2.382	1.54	1.54
90	7.5	1.133	0.160	1.843	3.136	1.88	1.66
120	7.3	1.133	0.065	1.900	3.098	1.96	1.58
CD _{0.05}		0.115	0.0018	0.140	1.156	0.813	0.167
S.E.		0.051	0.0008	0.063	0.518	0.365	0.075

Initial pH - 6.8

Table 2b. Secondary screening of hypercellulase producing bacterial isolate S₁₂

EtBr exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.4	0.012	0.086	1.122	1.220	1.34	0.910
30	7.6	0.134	0.006	1.039	1.179	0.79	1.490
60	7.7	0.120	0.006	1.436	1.562	1.15	1.359
90	7.4	0.194	0.031	0.320	0.545	1.39	0.390
120	7.2	0.111	0.032	-	0.143	1.78	0.080
CD _{0.05}		0.0018	0.0018	0.115	0.140	0.018	0.082
S.E.		0.0008	0.0008	0.051	0.063	0.008	0.036

Initial pH - 6.8

Table 2c. Secondary screening of hypercellulase producing bacterial isolate N₅

EtBr exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.2	0.740	0.126	0.637	1.503	1.83	0.82
30	7.3	0.070	0.065	-	0.135	0.54	0.25
60	7.6	0.062	0.093	-	0.155	0.49	0.31
90	7.4	0.066	0.074	-	0.140	0.76	0.18
120	7.1	0.018	0.082	0.335	0.435	0.74	0.58
CD _{0.05}		0.082	0.0018	0.081	0.115	0.018	0.082
S.E.		0.036	0.0008	0.036	0.051	0.008	0.037

Initial pH - 6.8

Table 2d. Secondary screening of hypercellulase producing bacterial isolate S₇

EtBr exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.6	0.002	0.053	1.080	1.135	1.76	0.644
30	7.7	0.050	0.033	0.952	1.035	1.15	0.900
60	7.4	0.043	0.015	1.134	1.192	1.09	1.093
90	7.4	0.017	0.013	1.651	1.681	0.86	1.954
120	7.2	0.004	0.007	2.513	2.524	0.69	3.65
CD _{0.05}		0.008	0.0018	0.821	0.817	0.018	0.821
S.E.		0.003	0.0008	0.368	0.366	0.008	0.368

Initial pH - 6.8

Table 2e. Secondary screening of hypercellulase producing bacterial isolate N₁₃

EtBr exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.5	1.577	0.088	0.473	2.138	3.16	0.676
30	7.6	0.149	0.036	0.790	0.975	1.15	0.847
60	7.8	0.143	0.013	0.320	0.476	1.34	0.355
90	7.4	0.142	0.004	-	0.146	0.89	0.164
120	7.3	0.104	-	-	0.104	0.55	0.189
CD _{0.05}		0.081	0.0016	0.140	0.821	0.813	0.140
S.E.		0.036	0.0007	0.063	0.368	0.365	0.063

Initial pH - 6.8

Table 2f. Secondary screening of hypercellulase producing bacterial isolate NS₁

EtBr exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.3	1.200	0.068	0.421	1.689	2.92	0.578
30	7.5	0.114	0.069	1.565	1.748	2.65	0.659
60	7.6	0.104	0.084	2.513	2.701	2.72	0.993
90	7.4	0.056	0.065	1.556	1.677	2.94	0.570
120	7.2	0.037	0.068	1.484	1.589	2.23	0.712
CD _{0.05}		0.0813	0.0018	0.829	0.829	1.819	0.162
S.E.		0.036	0.0008	0.372	0.372	0.816	0.073

Initial pH - 6.8

Table 2g. Secondary screening of hypercellulase producing bacterial isolate S₁

EtBr exposure time (min)	Final pH	CMCase (I.U.)	FPase (I.U.)	β -glucosidase (I.U.)	Total Cellulase (I.U.)	Protein (mg/ml)	Specific activity
0	7.6	1.096	0.148	0.395	1.639	1.80	0.910
30	7.4	0.533	0.109	1.005	1.645	1.64	1.003
60	7.4	0.588	0.130	1.221	1.939	1.78	1.089
90	7.3	0.605	0.194	1.221	2.020	2.29	0.882
120	7.2	0.613	0.272	1.005	1.890	1.38	1.369
CD _{0.05}		0.115	0.0817	0.140	0.829	0.825	0.115
S.E.		0.051	0.036	0.063	0.372	0.370	0.051

Initial pH - 6.8

Table 3a: Secondary screening of hyperxylanase producing bacterial isolates using UV irradiation as mutagen

UV exposure time (min)	Kd ₁				SL ₈				N ₁₁			
	Final pH	Xylanase (I.U.)	Protein (mg/ml)	Specific activity	Final pH	Xylanase (I.U.)	Protein (mg/ml)	Specific activity	Final pH	Xylanase (I.U.)	Protein (mg/ml)	Specific activity
0	7.6	31.72	2.50	12.68	7.4	61.54	3.45	17.83	7.2	29.05	1.75	16.60
15	7.7	36.03	2.87	12.55	7.7	31.77	1.97	16.12	7.3	27.18	1.43	19.00
30	7.5	51.32	3.10	16.55	7.6	4.75	0.97	4.89	7.0	30.09	1.98	15.19
45	7.4	32.58	2.42	13.46	7.3	6.43	0.99	6.49	7.4	31.30	2.87	10.90
60	7.4	27.05	1.91	14.16	7.3	7.19	0.56	12.83	7.4	23.15	1.10	21.04
75	7.2	23.24	1.50	15.49	7.1	8.55	1.00	8.55	7.6	22.62	1.03	21.96
C.D _{0.05}		1.779	1.454			1.779	0.726			1.779	0.726	1.779
S.E.		0.816	0.667			0.816	0.333			0.816	0.333	0.816

Initial pH - 6.8

Table 3b: Secondary screening of hyperxylanase producing bacterial isolates using EtBr as mutagen

EtBr exposure time (min)	Kd ₁				SL ₈				N ₁₁			
	Final pH	Xylanase (I.U.)	Protein (mg/ml)	Specific activity	Final pH	Xylanase (I.U.)	Protein (mg/ml)	Specific activity	Final pH	Xylanase (I.U.)	Protein (mg/ml)	Specific activity
0	7.5	35.98	3.25	11.07	7.6	61.54	3.04	20.24	7.4	29.64	2.54	11.66
30	7.4	29.18	2.05	14.23	7.4	29.14	2.95	9.87	7.5	1.255	0.60	2.09
60	7.3	32.10	2.87	11.18	7.3	25.32	2.32	10.91	7.4	3.920	0.80	4.90
90	7.2	40.84	3.32	12.30	7.6	24.63	2.04	12.07	7.6	41.76	2.60	16.06
120	7.1	33.80	2.95	11.45	7.4	25.32	2.16	11.72	7.4	30.00	2.57	11.67
C.D _{0.05}		1.819	1.819			1.819	1.819			1.629	1.629	1.819
S.E.		0.816	0.816			0.816	0.816			0.731	0.731	0.816

-

Initial pH - 6.8

Table 4: Percent survival of hypercellulase producers after exposure with ultraviolet rays (UV) at different time interval

Time of exposure (min)	Survival percent after exposure													
	N ₁₂		S ₁₂		N ₅		S ₇		N ₁₃		NS ₁		S ₁	
	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)
0	98×10 ⁷	100	65×10 ⁷	100	69×10 ⁷	100	75×10 ⁷	100	90×10 ⁷	100	87×10 ⁷	100	94×10 ⁶	100
15	28×10 ⁷	28.57	20×10 ⁷	30.76	20×10 ⁷	28.98	22×10 ⁷	29.33	35×10 ⁷	38.88	26×10 ⁷	29.88	74×10 ⁵	7.87
30	55×10 ⁶	5.61	32×10 ⁶	4.92	60×10 ⁶	8.69	35×10 ⁶	4.66	65×10 ⁶	6.63	35×10 ⁶	4.02	55×10 ⁵	5.85
45	35×10 ⁶	3.57	28×10 ⁶	4.30	40×10 ⁶	5.79	20×10 ⁶	2.66	40×10 ⁶	4.44	26×10 ⁶	2.98	40×10 ⁵	4.25
60	20×10 ⁶	2.04	16×10 ⁶	2.46	26×10 ⁶	3.76	12×10 ⁶	1.60	29×10 ⁶	3.22	14×10 ⁶	1.60	26×10 ⁵	2.76
75	8×10 ⁶	0.816	6×10 ⁶	0.92	10×10 ⁶	1.44	7×10 ⁶	0.93	12×10 ⁶	1.33	5×10 ⁶	0.57	10×10 ⁵	1.06

Table 5: Percent survival of hypercellulase producers after treatment with different concentration of EtBr at 60 min exposure

Concentration of EtBr (mg/ml)	Survival percent at 60 min of exposure													
	N ₁₂		S ₁₂		N ₅		S ₇		N ₁₃		NS ₁		S ₁	
	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)
0	96×10 ⁶	100	87×10 ⁶	100	89×10 ⁶	100	90×10 ⁶	100	92×10 ⁶	100	82×10 ⁶	100	88×10 ⁶	100
0.5	20×10 ⁶	20.83	12×10 ⁶	13.79	75×10 ⁵	8.42	26×10 ⁶	28.88	34×10 ⁶	36.95	21×10 ⁶	25.60	36×10 ⁶	40.90
1.0	35×10 ⁵	3.64	40×10 ⁵	4.59	56×10 ⁵	6.29	45×10 ⁵	5.00	56×10 ⁵	6.08	46×10 ⁵	5.60	57×10 ⁵	6.47
1.5	29×10 ⁵	3.02	32×10 ⁵	3.67	40×10 ⁵	4.49	30×10 ⁵	3.33	41×10 ⁵	4.45	31×10 ⁵	3.78	40×10 ⁵	4.54
2.0	18×10 ⁵	1.87	26×10 ⁵	2.98	31×10 ⁵	3.48	19×10 ⁵	2.11	28×10 ⁵	3.04	19×10 ⁵	2.31	28×10 ⁵	3.18
2.5	10×10 ⁵	1.04	12×10 ⁵	1.37	22×10 ⁵	2.47	9×10 ⁵	1.00	12×10 ⁵	1.30	7×10 ⁵	0.85	12×10 ⁵	1.36
3.0	-	-	2×10 ⁵	0.22	6×10 ⁵	0.67	-	-	3×10 ⁵	0.32	-	-	2×10 ⁵	0.22

}

-

No survival

Table 6: Percent survival of hypercellulase producers after treatment with ethidium bromide (EtBr) (2.5 mg/ml) at different time interval

Time of exposure (min)	Survival percent after exposure													
	N ₁₂		S ₁₂		N ₅		S ₇		N ₁₃		NS ₁		S ₁	
	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)
0	65×10 ⁷	100	75×10 ⁶	100	75×10 ⁷	100	65×10 ⁷	100	79×10 ⁷	100	95×10 ⁷	100	84×10 ⁷	100
30	75×10 ⁶	11.53	87×10 ⁶	11.60	86×10 ⁶	11.46	79×10 ⁶	12.15	86×10 ⁶	10.88	87×10 ⁶	9.15	75×10 ⁶	8.92
60	56×10 ⁶	8.61	63×10 ⁵	8.40	52×10 ⁶	6.93	45×10 ⁶	6.92	40×10 ⁶	5.06	43×10 ⁶	4.52	32×10 ⁶	3.80
90	28×10 ⁶	4.30	40×10 ⁵	5.33	28×10 ⁶	3.73	21×10 ⁶	3.23	19×10 ⁶	2.40	22×10 ⁶	2.31	14×10 ⁶	1.66
120	12×10 ⁶	1.84	17×10 ⁵	2.26	9×10 ⁶	1.20	10×10 ⁶	1.53	0×10 ⁶	-	3×10 ⁶	0.31	2×10 ⁶	0.23
150	0×10 ⁶	-	2×10 ⁵	0.26	2×10 ⁶	0.26	0×10 ⁶	-	0×10 ⁶	-	0×10 ⁶	-	0×10 ⁶	-

- }

No survival

Table 7: Percent survival of hyperxylanase producers after exposure with ultraviolet (UV) rays at different time interval

Time of exposure (min)	Survival percent after UV exposure					
	Kd ₁		SL ₈		N ₁₁	
	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)
0	87×10 ⁷	100	65×10 ⁷	100	90×10 ⁷	100
15	20×10 ⁷	22.98	15×10 ⁷	23.07	26×10 ⁷	28.88
30	45×10 ⁶	5.17	75×10 ⁶	11.53	57×10 ⁶	6.33
45	24×10 ⁶	2.75	46×10 ⁶	7.07	32×10 ⁶	3.55
60	7×10 ⁶	0.80	19×10 ⁶	2.92	14×10 ⁶	1.55
75	0×10 ⁶	-	5×10 ⁶	0.76	2×10 ⁶	0.22

- }

No survival

Table 8: Percent survival of hyperxylanase producers after treatment with ethidium bromide (EtBr) with different concentration at 60 min exposure

Concentration of EtBr (mg/ml)	Survival percent after exposure					
	Kd ₁		SL ₈		N ₁₁	
	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)
0	84×10 ⁶	100	79×10 ⁶	100	59×10 ⁶	100
0.5	75×10 ⁵	8.92	56×10 ⁵	7.08	70×10 ⁵	11.86
1.0	49×10 ⁵	5.83	43×10 ⁵	5.44	56×10 ⁵	9.49
1.5	28×10 ⁵	3.33	24×10 ⁵	3.03	29×10 ⁵	4.91
2.0	11×10 ⁵	1.30	9×10 ⁵	1.13	16×10 ⁵	2.71
2.5	2×10 ⁵	0.23	0×10 ⁵	-	3×10 ⁵	0.50
3.0	0×10 ⁵	-	0×10 ⁵	-	0×10 ⁵	-

—

No survival

Table 9: Percent survival of hyperxylanase producers after treatment with ethidium bromide (EtBr) (2.0 mg/ml) at different time interval

Concentration of EtBr (mg/ml)	Survival percent after exposure					
	Kd ₁		SL ₈		N ₁₁	
	CFU/ml	Survival (%)	CFU/ml	Survival (%)	CFU/ml	Survival (%)
0	97×10 ⁶	100	86×10 ⁶	100	69×10 ⁶	100
30	59×10 ⁵	6.08	74×10 ⁵	8.60	70×10 ⁵	10.14
60	32×10 ⁵	3.29	50×10 ⁵	5.81	32×10 ⁵	4.63
90	16×10 ⁵	1.64	19×10 ⁵	2.20	6×10 ⁵	0.86
120	2×10 ⁵	0.20	1×10 ⁵	0.11	0×10 ⁵	-

—

No survival

APPENDIX-II

ANOVA for Table 9. Effect of different media on extracellular cellulase production from *Bacillus stratosphericus* N12 (M) under SmF

	CMCase				FPase				β -glucosidase				Total cellulase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	0.915	0.183	1221.18	5	0.057	0.011	197.96	5	0.231	0.046	10.98	5	1.796	0.339	56.74	5	3.097	0.619	2064.70
Error	12	0.001	0.0001		12	0.0006	0.00005		12	0.0506	0.0042		12	0.076	0.0063		12	0.0036	0.0030	
Total	17				17	0.057			17	0.282			17	1.872			17	3.100		

ANOVA for Table 10. Effect of different media on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	3571.8	714.36	162.68	5	23.57	4.715	775.88	5	180.21	36.04	141.12
Error	12	52.69	4.391		12	7.293	6.077		12	3.064	0.255	
Total	17	3624.5			17	23.65			17	183.27		

ANOVA for Table 11. Effect of different pH on extracellular cellulase production from *Bacillus stratosphericus* N12 (M) under SmF

	CMCase				FPase				β -glucosidase				Total cellulase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	6.149	0.768	80.60	8	0.020	0.0025	16.42	8	2.989	0.373	17.99	8	17.154	2.144	40.19	8	5.410	0.676	1193.44
Error	18	0.171	0.0095		18	0.002	0.00015		18	0.373	0.202		18	0.960	0.0533		18	0.010	0.0005	
Total	26	6.320			26	0.023			26	3.362			26	18.115			26	5.420		

ANOVA for Table 12. Effect of different pH on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	4384.3	548.03	178.69	8	31.91	3.989	20.53	8	76.97	9.621	20.46
Error	18	55.20	3.066		18	3.496	0.194		18	8.462	0.470	
Total	26	4439.5			26	35.41			26	85.43		

ANOVA for Table 13 Effect of different temperature on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

	CMCase				FPase				β -glucosidase				Total cellulase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	6.365	1.273	101.09	5	0.039	0.0078	0.96	5	3.484	0.769	64.25	5	18.06	3.613	46.78	5	2.821	0.564	5642.00
Error	12	0.151	0.0125		12	0.098	0.0081		12	0.143	0.0119		12	0.926	0.0772		12	0.0012	0.0001	
Total	17	6.517			17	0.137			17	3.992			17	18.992			17	4.176		

ANOVA for Table 14. Effect of different temperature on extracellular cellulase production from *B. stratosphericus* N12 (M) under SmF

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	4042.7	808.54	315.35	5	29.62	5.925	5.74	5	100.05	20.01	2.20
Error	12	30.76	2.563		12	12.39	1.032		12	109	9.083	
Total	17	4073.5			17	42.01			17	209.05		

Table 15. Effect of different inoculum size on extracellular cellulase production from *B. stratosphericus* N₁₂ (M) under SmF

	CMCase				FPase				β -glucosidase				Total cellulase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	6	0.459	0.0766	21.96	6	0.015	0.0025	0.26	6	2.231	0.371	4308.77	6	4.985	0.830	35.04	6	1.919	0.319	1.98
Error	14	0.048	0.0034		14	0.136	0.0097		14	0.0012	0.00008		14	0.332	0.023		14	2.257	0.161	
Total	20	0.508			20	0.152			20	2.233			20	5.317			20	4.176		

ANOVA for Table 16. Effect of different inoculum size on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	6	2610.9	435.15	370.61	6	12.864	2.144	165.59	6	112.93	18.822	1416.23
Error	14	16.438	1.174		14	0.1812	0.0129		14	0.1860	0.0132	
Total	20	2627.4			20	13.045			20	113.12		

ANOVA for Table 17 Effect of different incubation time on extracellular cellulase production from *B. stratosphericus* N₁₂ (M) under SmF

	CMCase				FPase				β -glucosidase				Total cellulase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	4	1.445	0.361	92.68	4	0.031	0.007	4.40	4	3.474	0.868	261.98	4	9.764	2.441	108.43	4	11.572	2.892	29.95
Error	10	0.039	0.0039		10	0.017	0.001		10	0.033	0.003		10	0.225	0.022		10	0.966	0.096	
Total	14	1.484			14	0.049			14	3.507			14	9.990			14	12.538		

ANOVA for Table 18 Effect of different incubation time on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	4	1250.5	312.62	224.12	4	32.148	8.037	1398.57	5	29.62	5.925	5.74
Error	10	13.949	1.394		10	5.746	0.0057		12	12.39	1.032	
Total	14	1264.4			14	32.20			17	42.01		

ANOVA for Table 19 Effect of different carbon sources on extracellular cellulase production from *B. stratosphericus* N₁₂ (M) under SmF

	CMCase				FPase				β -glucosidase				Total cellulase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	3.579	0.715	17344.72	5	5.523	1.104	17786.32	5	0.351	0.070	80.39	5	10.66	2.132	1479.17	5	2.868	0.573	2294.76
Error	12	0.0004	0.00004		12	0.0007	0.00006		12	0.0104	0.0008		12	0.017	0.0014		12	0.0030	0.0002	
Total	17	3.580			17	5.524			17	0.3615			17	10.679			17	2.871		

ANOVA for Table 20 Effect of different carbon source on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	5178.6	1035.7	1507.75	5	2.739	0.547	65.21	5	357.98	71.59	593.75
Error	12	8.243	0.686		12	0.100	0.008		12	1.447	0.120	
Total	17	5186.8			17	2.839			17	359.43		

ANOVA for Table 21 Effect of different substrate concentration on extracellular cellulase production from *B. stratosphericus* N₁₂ (M) under SmF

	CMCase				FPase				β -glucosidase				Total cellulase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	4.082	0.816	346.54	5	2.597	0.519	10623.33	5	0.532	0.106	29.26	5	17.03	3.406	428.25	5	3.126	0.625	86.43
Error	12	0.028	0.0023		12	0.00058	0.00004		12	0.0437	0.0036		12	0.095	0.0079		12	0.086	0.0072	
Total	17	4.110			17	2.597			17	0.576			17	17.128			17	3.212		

ANOVA for Table 22 . Effect of different substrate concentration on xylanase production from *B. altitudinis* Kd₁ (M) under SmF

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	3265.4	653.08	3702.06	5	3.996	0.079	6.77	5	259.48	51.89	48.47
Error	12	2.116	0.176		12	1.416	0.011		12	12.84	1.070	
Total	17	3267.5			17	5.412			17	272.33		

Table 23. Analytical studies for estimation of Holocellulose (cellulose +hemicellulose) and lignin in lignocellulosic (hardwood and softwood) forest waste

(i) *D. strictus*

	Holocellulose				Lignin				Extractives			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	31829.95	3978.743	5968.115	8	2262.057	282.7571	424.1357	8	11.28	1.41	2.115
Error	18	12	0.666667		18	12	0.666667		18	12	0.666667	
Total	26	31841.95			26	2274.057	87.46373		26	23.28	0.895385	

(ii) *Eucalyptus sp.*

	Holocellulose				Lignin				Extractives			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	30458.88	3807.36	5711.04	8	2274.667	284.3333	426.5	8	37.62667	4.703333	7.055
Error	18	12	0.666667		18	12	0.666667		18	12	0.666667	
Total	26	30470.88	1171.957		26	2286.667	87.94872		26	49.62667	1.908718	

(iii) *P. deltoides*

	Holocellulose				Lignin				Extractives			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	31248.12	3906.015	5859.023	8	2500.5	312.5625	468.8438	8	8.706667	1.088333	1.6325
Error	18	12	0.666667		18	12	0.666667		18	12	0.666667	
Total	26	31260.12	1202.312		26	2512.5	96.63462		26	20.70667	0.79641	

(iv) *C. deodara*

	Holocellulose				Lignin				Extractives			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	30456.86	3807.107	5710.661	8	3455.167	431.8958	647.8438	8	22.64667	2.830833	4.24625
Error	18	12	0.666667		18	12	0.666667		18	12	0.666667	
Total	26	30468.86	1171.879		26	3467.167	133.3526		26	34.64667	1.332564	

(v) *P. roxburghii*

	Holocellulose				Lignin				Extractives			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	31248.12	3906.015	5859.023	8	2500.5	312.5625	468.8438	8	8.706667	1.088333	1.6325
Error	18	12	0.666667		18	12	0.666667		18	12	0.666667	
Total	26	31260.12	1202.312		26	2512.5	96.63462		26	20.70667	0.79641	

(vi) *P. wallichiana*

	Holocellulose				Lignin				Extractives			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	8	28428.99	3553.623	5330.435	8	4907.167	613.3958	920.0938	8	12.18	1.5225	2.28375
Error	18	12	0.666667		18	12	0.666667		18	12	0.666667	
Total	26	28440.99	1093.884									

ANOVA for Table 24: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using untreated lignocellulosic forest waste under submerged fermentation

	CMCase				FPase			β glucosidase			Total cellulase			Sp. activity			Reducing sugars		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	188.15	37.631	450670.57	200.98	40.196	200978.20	162.27	32.453	194.62	1459.30	291.85	3495258.56	111.37	22.274	22274324.02	123.46	24.692	246917.30
Error	12	0.0010	0.00008		0.0024	0.0002		2.00	0.1667		0.0010	0.00008		0.00001	0.000001		0.0012	0.0001	
Total	17	188.16			200.98			164.27			1459.30			111.37			123.46		

ANOVA for Table 25: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using untreated lignocellulosic forest waste under submerged fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	5645.0	1129.0	11290008.79	5	24.411	4.882	7.30	5	51.74	10.34	103479.30
Error	12	0.0012	0.0001		12	8.020	0.668		12	0.001	0.0001	
Total	17	5645.0			17	32.431			17	51.741		

ANOVA for Table 26: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using untreated lignocellulosic forest waste under solid state fermentation

	CMCase				FPase				β -glucosidase				Total cellulase				Specific activity				Reducing Sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	307.80	61.560	92.11	5	78.54	15.71	23.45	5	704.53	140.41	169.09	5	896.56	179.31	179.31	5	154.84	30.96	91.08	5	145.64	29.12	34.88
Error	12	8.020	0.668		12	8.040	0.670		12	10.00	0.833		12	12.00	1.00		12	4.080	0.340		12	10.02	0.835	
Total	17	315.82			17	86.58			17	714.53			17	908.56			17	158.92			17			

ANOVA for Table 27: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using untreated lignocellulosic forest waste under solid state fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	48770.0	9754.1	9754.06	5	42.85	8.571	25.34	5	216.73	43.346	43.35
Error	12	12.000	1.000		12	4.060	0.338		12	12.00	1.000	
Total	17	48782			17	46.91			17	228.73		

ANOVA for Table 28: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using dil. H₂SO₄ pretreated lignocellulosic forest waste under submerged fermentation

		CMCase			FPase			β glucosidase			Total cellulase			Sp. activity			Reducing sugars		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	224.13	44.82	4482.60	228.81	45.762	5480.45	705.40	141.08	14107.90	1933.7	386.73	46315.47	275.90	55.181	10926.87	101.11	20.22	2022.21
Error	12	0.1200	0.0100		0.100	0.0083		0.120	0.0100		0.1002	0.0083		0.060	0.0050		0.120	0.010	
Total	17	224.25			228.91			705.52			1933.8			245.96			101.23		

ANOVA for Table 29: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using dil. HCl pretreated lignocellulosic forest waste under submerged fermentation

		CMCase			FPase			β glucosidase			Total cellulase			Sp. activity			Reducing sugars		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	169.21	33.842	67.68	203.32	40.663	60.99	181.08	36.216	36.22	938.63	187.73	187.73	22.85	4.570	26.37	97.72	19.545	112.39
Error	12	6.006	0.500		8.004	0.666		12.00	1.00		12.00	1.00		2.080	0.173		2.086	0.173	
Total	17	175.21			211.32			193.08			950.63			24.93			99.813		

ANOVA for Table 30: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using dil. H₂SO₄ pretreated lignocellulosic forest waste under submerged fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	3406.70	681.33	200392.64	5	4.836	0.967	0.97	5	65.56	13.112	19.67
Error	12	0.040	0.003		12	12.00	1.000		12	8.00	0.666	
Total	17	3406.7			17	16.837			17	73.56		

ANOVA for Table 31: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using dil. HCl pretreated lignocellulosic forest waste under submerged fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	8373.20	1674.60	16746484.08	5	14.489	2.897	2.90	5	45.936	9.187	91871.70
Error	12	0.001	0.0001		12	12.00	1.000		12	0.001	0.0001	
Total	17	8373.20			17	26.489			17	45.937		

ANOVA for Table 32: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using dil. H₂SO₄ pretreated lignocellulosic forest waste under solid state fermentation

	CMCase				FPase				B-glucosidase				Total cellulase				Specific activity				Reducing Sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	71.87	14.37	14.38	5	91.35	16.27	16.11	5	2271.1	454.22	454.22	5	1970.8	394.15	394.15	5	20.46	4.093	23.61	5	237.73	47.54	56.94
Error	12	12.00	1.00		12	12.12	1.010		12	12.00	1.000		12	12.00	1.00		12	2.080	0.173		12	10.02	0.835	
Total	17	83.87			17	93.47			17	2283.1			17	1982.80			17	22.54			17	247.75		

ANOVA for Table 33: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using dil. HCl pretreated lignocellulosic forest waste under solid state fermentation

	CMCase				FPase				B-glucosidase				Total cellulase				Specific activity				Reducing Sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	45.01	9.003	10.80	5	79.11	15.82	15.82	5	827.81	165.56	198.67	5	950.41	190.08	206.61	5	62.77	12.55	75.48	5	135.04	27.00	32.34
Error	12	10.00	0.833		12	12.00	1.000		12	10.00	0.833		12	11.04	0.920		12	1.996	0.166		12	10.02	0.835	
Total	17	55.01			17	91.11			17	837.81			17	961.45			17	64.77			17	145.06		

ANOVA for Table 34: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using dil. H₂SO₄ pretreated lignocellulosic forest waste under solid state fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	58930	11786	11786.03	5	129.20	25.839	31.01	5	291.05	58.21	58.21
Error	12	12.00	1.000		12	10.000	0.8333		12	12.00	1.000	
Total	17	58942			17	139.20			17	303.05		

ANOVA for Table 35: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using dil. HCl pretreated lignocellulosic forest waste under solid state fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	70330	14066	14065.92	5	32.690	6.537	7.83	5	130.51	26.101	31.32
Error	12	12.00	1.000		12	10.020	0.835		12	10.00	0.8333	
Total	17	70342			17	42.710			17	140.51		

ANOVA for Table 36: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using NaOH + H₂O₂ pretreated lignocellulosic forest waste under submerged fermentation

					FPase			β glucosidase			Total cellulase			Sp. activity			Reducing sugars		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	227.57	45.51	4489.01	245.23	49.04	4904.55	462.33	92.46	9246.69	2076.6	415.33	2373.30	37.36	7.473	895.03	113.43	22.685	2268.54
Error	12	0.1216	0.010		0.120	0.010		0.120	0.0100		2.100	0.1750		0.100	0.008		0.120	0.010	
Total	17	227.69			245.35			462.45			2078.7			37.469			113.55		

ANOVA for Table 37: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using NaOH + H₂O₂ pretreated lignocellulosic forest waste under submerged fermentation (SmF)

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	11739	2347.8	23477757.53	5	289.06	57.812	578120.00	5	84.610	16.922	169220.90
Error	12	0.0012	0.0001		12	0.0012	0.0001		12	0.0012	0.0001	
Total	17	11739			17	289.06			17	84.612		

ANOVA for Table 38: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using NaOH + H₂O₂ pretreated lignocellulosic forest waste under solidstate fermentation

	CMCase				FPase				B-glucosidase				Total cellulase				Specific activity				Reducing Sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	47.58	9.516	11.42	5	60.49	12.09	12.10	5	1790.20	358.02	535.72	5	2017.30	403.47	403.47	5	648.77	129.75	155.39	5	2643.70	528.74	789.16
Error	12	10.00	0.833		12	12.00	1.00		12	8.020	0.668		12	12.00	1.00		12	10.02	0.835		12	8.040	0.670	
Total	17	57.58			17	72.49			17	1798.20			17	2029.30			17	658.79			17	2651.7		

ANOVA for Table 39: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using NaOH + H₂O₂ pretreated lignocellulosic forest waste under solid state fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	80013	16003	16002.55	5	10.903	2.180	2.18	5	122.10	24.41	24.42
Error	12	12.00	1.00		12	12.00	1.000		12	12.00	1.000	
Total	17	80025			17	22.903			17	134.10		

ANOVA for Table 40: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using NH₃ pretreated lignocellulosic forest waste under submerged fermentation

	CMCase				FPase			β glucosidase			Total cellulase			Sp. activity			Reducing sugars		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	219.09	43.81	65.72	208.07	41.613	49.84	165.83	33.07	198.37	1618.1	323.62	3875707.90	154.16	30.831	184.95	130.96	26.19	261920.10
Error	12	8.004	6.667		10.020	0.835		2.00	0.166		0.0010	0.00008		2.0	0.166		0.0012	0.0001	
Total	17	227.09			218.09			167.38			1618.1			156.16			130.96		

ANOVA for Table 41: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using NH₃ pretreated lignocellulosic forest waste under submerged fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	8079.6	1615.90	16159127.60	5	23.659	4.731	7.100	5	59.35	11.871	118705.70
Error	12	0.0012	0.0001		12	8.004	0.666		12	0.0012	0.0001	
Total	17	8079.60			17	31.659			17	59.35		

ANOVA for Table 42: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using NH₃ pretreated lignocellulosic forest waste under solid state fermentation

	CMCase				FPase				B-glucosidase				Total cellulase				Specific activity				Reducing Sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	0.124	0.024	4.98	5	49.84	9.96	9.97	5	765.35	153.07	183.32	5	1108.60	221.71	221.71	5	45.89	9.177	52.45	5	714.63	142.93	284.88
Error	12	0.060	0.005		12	12.00	1.00		12	10.02	0.835		12	12.00	1.00		12	2.100	0.175		12	6.020	0.501	
Total	17	0.184			17	61.84			17	775.37			17	1120.60			17	47.990			17	720.65		

ANOVA for Table 43: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using NH₃ pretreated lignocellulosic forest waste under solid state fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	69406	13881	13881.21	5	160.82	32.165	32.16	5	673.24	134.65	134.65
Error	12	12.00	1.000		12	12.00	1.000		12	12.00	1.000	
Total	17	69418			17	172.82			17	685.24		

ANOVA for Table 44: Cost effective production of extracellular cellulase by *B. stratosphericus* N₁₂ (M) using steam pretreated lignocellulosic forest waste under submerged fermentation

	CMCase				FPase			β glucosidase			Total cellulase			Sp. activity			Reducing sugars		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	174.72	34.94	41.83	215.79	43.158	64.57	146.12	29.22	29.22	1292.0	258.39	309.45	104.20	20.841	62.20	115.66	23.13	24.70
Error	12	10.024	0.835		8.020	0.668		12.0	1.0		10.20	0.835		4.0207	0.335		8.004	0.666	
Total	17	184.75			223.81			158.12			1302.00			108.22			123.66		

ANOVA for Table 45: Cost effective production of extracellular xylanase by *B. altitudinis* Kd₁ (M) using steam pretreated lignocellulosic forest waste under submerged fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	8569.2	1713.80	10277.89	5	24.08	4.817	9.63	5	62.08	12.418	124175.30
Error	12	2.001	0.166		12	6.000	0.500		12	0.0012	0.0001	
Total	17	8571.20			17	30.087			17	62.089		

ANOVA for Table 46: Cost effective production of extracellular cellulase production by *M. thermophila* SH1 using steam pretreated lignocellulosic forest waste under solid state fermentation

	CMCase				FPase				B-glucosidase				Total cellulase				Specific activity				Reducing Sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	105.41	21.18	41.95	5	69.93	13.98	20.89	5	1064.10	212.81	319.21	5	1165.70	233.14	233.14	5	65.78	13.15	26.14	5	168.43	33.68	50.40
Error	12	6.060	0.505		12	8.034	0.669		12	8.002	0.666		12	12.00	1.00		12	6.040	0.503		12	8.020	0.668	
Total	17	111.97			17	77.97			17	1072.10			17	1177.70			17	71.82			17	176.45		

ANOVA for Table 47: Cost effective production of extracellular xylanase production by *M. thermophila* SH1 using steam pretreated lignocellulosic forest waste under solidstate fermentation

	Xylanase				Specific activity				Reducing sugar			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	54445	10889	10889.02	5	160.83	32.16	38.60	5	498.59	99.718	99.72
Error	12	12.00	1.000		12	10.00	0.833		12	12.00	1.000	
Total	17	54457			17	170.83			17	510.59		

ANOVA for Table 54.1 (Method-1): SHF of alkaline hydrogen peroxide pretreated *P. deltoides* wood by pooling supernatant + solid residue without detoxification

	Ethanol (%)				Ethanol (g/l)			Ethanol (g/g)			Fermentation efficiency (%)		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	8	0.769	0.096	3.1051	56.061	7.0076	7.0060	0.0222	0.00277	24.7834	880.931	110.116	4.7876
Error	18	0.557	0.0309		18.004	1.0002		0.00201	0.00011		414.001	23	
Total	26	1.327	0.0510		74.065	2.8487		0.02422	0.00093		1294.932	49.8050	

ANOVA for Table 54.2 (Method-2): SHF of alkaline hydrogen peroxide pretreated *P. deltoides* wood by pooling supernatant + solid residue followed by detoxification with calcium hydroxide

	Ethanol (%)				Ethanol (g/l)			Ethanol (g/g)			Fermentation efficiency (%)		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	8	4.756	0.5946	2.32659	299.221	37.4026	83.4632	0.11906	0.01488	2.1487	4616.70	577.087	16.4866
Error	18	4.6002	0.25567		8.0664	0.4481		0.12467	0.0069		630.060	35.003	
Total	26	9.357	0.35988		307.287	11.8187		0.2437	0.00937		5246.76	201.798	

ANOVA for Table 54.3 (Method-3): SHF of solid residue of *P. deltoides* wood after alkaline hydrogen peroxide pretreatment by discarding supernatant without detoxification

	Ethanol (%)				Ethanol (g/l)			Ethanol (g/g)			Fermentation efficiency (%)		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	8	0.8087	0.1010	28.944	51	6.375	9.51161	0.01965	0.002457	25.6921	773.317	96.6646	43.4755
Error	18	0.66286	0.0034		12.064	0.6702		0.00172	0.000095		40.0216	2.2234	
Total	26	0.8715	0.0335		63.064	2.4255		0.21377	0.00082		813.339	31.282	

ANOVA for Table 54.4 (Method-4) SHF of solid residue of *P. deltoides* wood after alkaline hydrogen peroxide pretreatment by discarding supernatant followed by detoxification with calcium hydroxide

	Ethanol (%)				Ethanol (g/l)			Ethanol (g/g)			Fermentation efficiency (%)		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	8	2.7544	0.34430	2.12143	289.104	36.1380	26.5805	0.11514	0.01439	1.737709	4472.44	559.055	43.0042
Error	18	2.9214	0.1623		24.4722	1.3595		0.14909	0.008283		234	13.00	
Total	26	5.6758	0.2183		313.577	12.0606		0.26423	0.01063		4706.44	181.016	

ANOVA for Table 55. Bioconversion of alkaline hydrogen peroxide pretreated *P. deltooides* wood using commercial enzymes under Separate hydrolysis and fermentation (SHF)

	Ethanol (%)				Ethanol (g/l)			Ethanol (g/g)			Fermentation efficiency (%)		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	8	1.246667	0.155833	0.155833	78.37447	9.796808	2.449202	0.03129	0.003911	0.097781	1202.856	150.357	31.06549
Error	18	18	1		72	4		0.72	0.04		87.12	4.84	
Total	26	19.24667	0.740256		150.3745	5.783633		0.75129	0.028896		1289.976	49.61446	

ANOVA for Table 56. Bioconversion of alkaline hydrogen peroxide pretreated *P. deltooides* wood under Simultaneous saccharification and fermentation (SSF) by using inhouse enzyme and commercial enzymes

(i) Inhouse enzymes

	Ethanol (%)				Ethanol (g/l)			Ethanol (g/g)			Fermentation Efficiency (%)		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	8	1.6866	0.210825	5.270625	104.0664	13.0083	13.0083	0.040257	0.005032	3.145052	1629.437	203.6796	12.72998
Error	18	0.72	0.04		18	1		0.0288	0.0016		288	16	
Total	26	2.4066	0.092562		122.0664	4.694862		0.069057	0.002656		1917.437	73.74758	

(ii) For commercial enzymes

	Ethanol (%)				Ethanol (g/l)			Ethanol (g/g)			Fermentation Efficiency (%)		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	8	1.957867	0.244733	6.118333	123.1171	15.38963	0.961852	0.049427	0.006178	3.861458	1893.236	236.6545	14.7909
Error	18	0.72	0.04		288	16		0.0288	0.0016		288	16	
Total	26	2.677867	0.102995		411.1171	15.81219		0.078227	0.003009		2181.236	83.89368	

ANOVA FOR APPENDIX-I

ANOVA for Table1a. Screening of hypercellulase producing bacterial isolate N₁₂ (UV)

	CMCase				FPase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	3.292	0.658	197.48	0.025	0.0051	5169.20	5.552	1.1105	285.92	7.031	1.406	8.35	2.410	0.482	143.88
Error	12	0.040	0.0033		0.00001	0.00001		0.0466	0.0038		2.020	0.168		0.0402	0.0033	
Total	17	3.332			0.0258			5.599			9.052			2.450		

ANOVA for Table1b. Screening of hypercellulase producing bacterial isolate S₁₂ (UV)

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	0.035	0.007	7146.50	0.017	0.0035	3556.50	3.0115	-10 ⁷	204602.40	2.771	0.5542	332.37	1.244	0.248	2488.10	1.391	0.278	13256.65
Error	12	0.00001	0.00001		0.00001			0.000006	0.00000005		0.020	0.00166		0.0012	0.0001		0.0002	0.00002	
Total	17	0.035			0.017			3.0115			2.791			1.245			1.392		

ANOVA for Table1c. Screening of hypercellulase producing bacterial isolate N₅ (UV)

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	0.970	0.194	38.81	0.0370	0.0074	8887.20	2.088	0.417	83.54	5.628	1.125	225.13	7.472	1.494	14945.60	0.994	0.1989	23.82
Error	12	0.060	0.0005		0.00001	0.0000008		0.060	0.005		0.060	0.0050		0.0012	0.0001		0.100	0.008	
Total	17	1.030			0.0370			2.148						7.474			1.0949		

ANOVA for Table1d. Screening of hypercellulase producing bacterial isolate S_{7(UV)}

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	0.007	0.0015	1851.00	0.0068	0.00136	2051.40	3.338	0.667	201.28	3.601	0.720	216.02	3.601	0.720	216.02	2.058	0.4116	4116.80
Error	12	0.00001	0.0000008		0.000008	0.0000066		0.039	0.0033		0.0400	0.0033		0.040	0.0033		0.0012	0.0001	
Total	17	0.0077			0.0068			3.378			3.6411			3.641			2.059		

ANOVA for Table1e: Screening of hypercellulase producing bacterial isolate N₁₃

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	5.539	1.107	332.32	0.0139	0.0027	2788.10	3.455	0.709	106.37	8.040	1.608	27.31	14.75	2.950	17.02	4.657	0.9315	186.30
Error	12	0.040	0.003		0.00001	0.000001		0.080	0.0066		0.7066	0.0588		2.080	0.1733		0.0600	0.0050	
Total	17	5.579			0.0139			3.625			8.740			16.83			4.717		

ANOVA for Table1f. Screening of hypercellulase producing bacterial isolate NS₁

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	3.383	0.676	405.89	0.0093	0.0018	1862.90	3.326	0.665	79.84	2.714	0.542	65.15	7.970	1.594	9.20	0.837	0.1675	33.40
Error	12	0.0200	0.0016		0.00001	0.000001		0.100	0.008		0.1000	0.0083		2.080	0.1733		0.0602	0.0050	
Total	17	3.403			0.0093			3.426			2.814			10.051			0.898		

ANOVA for Table1 g: Screening of hypercellulase producing bacterial isolate S₁

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	5	1.464	0.292	35.14	0.0250	0.0050	3.01	0.9077	0.1815	54.46	0.351	0.0702	6.99	1.174	0.234	134.22	0.465	0.0931	27.93
Error	12	0.1000	0.008		0.020	0.00166		0.040	0.0033		0.1206	0.0100		0.0210	0.0017		0.0400	0.0033	
Total	17	1.564			0.0450			0.947			0.471			1.195			0.5056		

ANOVA for Table2a. Secondary screening of hypercellulase producing bacterial isolate N₁₂ (EtBr)

	CMCase				FPase			β glucosidase			Total cellulose			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	4	0.1968	0.04920	12.30	0.0228	0.0057	5709.90	7.371	1.8429	307.14	6.988	1.747	4.32	2.943	0.735	3.68	2.704	0.6761	80.11
Error	10	0.0400	0.00400		0.00001	0.000001		0.0600	0.0060		4.040	0.404		2.000	0.200		0.0844	0.0084	
Total	14	0.236			0.0228			7.4315			11.028			4.944			2.788		

ANOVA for Table2b. Secondary screening of hypercellulase producing bacterial isolate S₁₂

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	4	0.0517	0.0129	12936.60	0.0128	0.0032	3201.60	4.303	1.075	268.91	3.939	0.984	164.13	1.566	0.3916	3916.50	4.402	1.1006	539.42
Error	10	0.00001	0.50		0.00001			0.0400	0.004		0.0600	0.0060		0.0010	0.0001		0.0204	0.0020	
Total	14	0.0517			0.0128			4.343			3.999			1.567			4.423		

ANOVA for Table2c. Secondary screening of hypercellulase producing bacterial isolate N₅

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	4	1.134	0.283	138.74	0.0066	0.0016	1672.50	0.9871	0.2467	123.38	4.165	1.041	260.32	3.611	0.902	9029.10	0.851	0.2129	102.36
Error	10	0.0204	0.002		0.00001	0.000001		0.0200	0.0020		0.0400	0.0040		0.0010	0.0001		0.00208	0.00208	
Total	14	1.155			0.0067			1.0071			4.205			3.612			0.8724		

ANOVA for Table 2d. Secondary screening of hypercellulase producing bacterial isolate S₇

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. Activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	4	0.0052	0.001	57.78	0.004	0.00105	1059.60	4.961	1.240	6.08	4.574	1.143	5.66	1.990	0.497	4975.50	17.932	4.482	21.97
Error	10	0.0002			0.00001	0.000001		2.040	0.204		2.020	0.202		0.0010	0.0001		2.050	0.204	
Total	14	0.005			0.0042			7.001			6.594			1.991			19.972		

ANOVA for Table2e. Secondary screening of hypercellulase producing bacterial isolate N₁₃

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	4	4.997	1.249	618.35	0.0157	0.0039	4920.75	1.347	0.336	56.13	8.497	2.124	10.41	12.43	3.108	15.54	1.102	0.275	45.94
Error	10	0.0202	0.0020		0.000008	0.0000008		0.0600	0.0060		2.040	0.204		2.000	0.2000		0.0600	0.0060	
Total	14	5.0179			0.0157			1.4072			10.538			14.435			1.162		

ANOVA for Table2f. Secondary screening of hypercellulase producing bacterial isolate NS₁

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. Activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	4	3.035	0.758	379.23	0.0006	0.00017	170.10	6.593	1.648	7.92	2.561	0.640	3.08	0.988	0.2471	0.25	0.358	0.0895	11.20
Error	10	0.0200	0.002		0.00001	0.000001		2.080	0.208		2.080	0.208		10.00	1.000		0.0800	0.0080	
Total	14	3.055			0.0006			8.673			4.641			10.980			0.438		

ANOVA for Table2g. Secondary screening of hypercellulase producing bacterial isolate S₁

	CMCase				FPase			β glucosidase			Total cellulase			Protein			Sp. Activity		
Source	DF	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Treatment	4	0.638	0.159	39.93	0.0503	0.0125	6.23	1.377	0.344	57.38	0.3666	0.0916	0.44	1.320	0.330	1.60	0.459	0.114	28.74
Error	10	0.0400	0.004		0.0202	0.0020		0.060	0.0060		2.080	0.208		2.060	0.206		0.040	0.0040	
Total	14	0.679			0.0705			1.437			2.446			3.380			0.4999		

ANOVA for Table3 a: Secondary screening of hyperxylanase producing bacterial isolates using UV irradiation as mutagen

For kd1

	Xylanase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	1424.10	284.81	284.81	5	5.309	1.061	1.59
Error	12	12.00	1.00		12	8.020	0.668	
Total	17	1436.10			17	13.32		

For SL8

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	7728.0	1545.60	1545.60	5	17.092	3.418	20.50	5	253.09	50.61	50.62
Error	12	12.00	1.000		12	2.008	0.1667		12	12.00	1.000	
Total	17	7740.0			17	19.093			17	265.09		

For N11

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	5	197.87	39.57	39.57	5	6.994	1.398	8.39	5	253.09	50.61	50.62
Error	12	12.00	1.00		12	2.001	0.1667		12	12.00	1.000	
Total	17	209.87			17	8.995			17	265.09		

ANOVA for Table3 b: Secondary screening of hyperxylanase producing bacterial isolates using EtBr as mutagen For Kd1

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	4	230.60	57.65	57.65	4	3.072	0.768	0.77	4	3.072	0.768	0.77
Error	10	10.00	1.000		10	10.00	1.000		10	10.000	1.000	
Total	14	240.60			14	13.07			14	13.072		

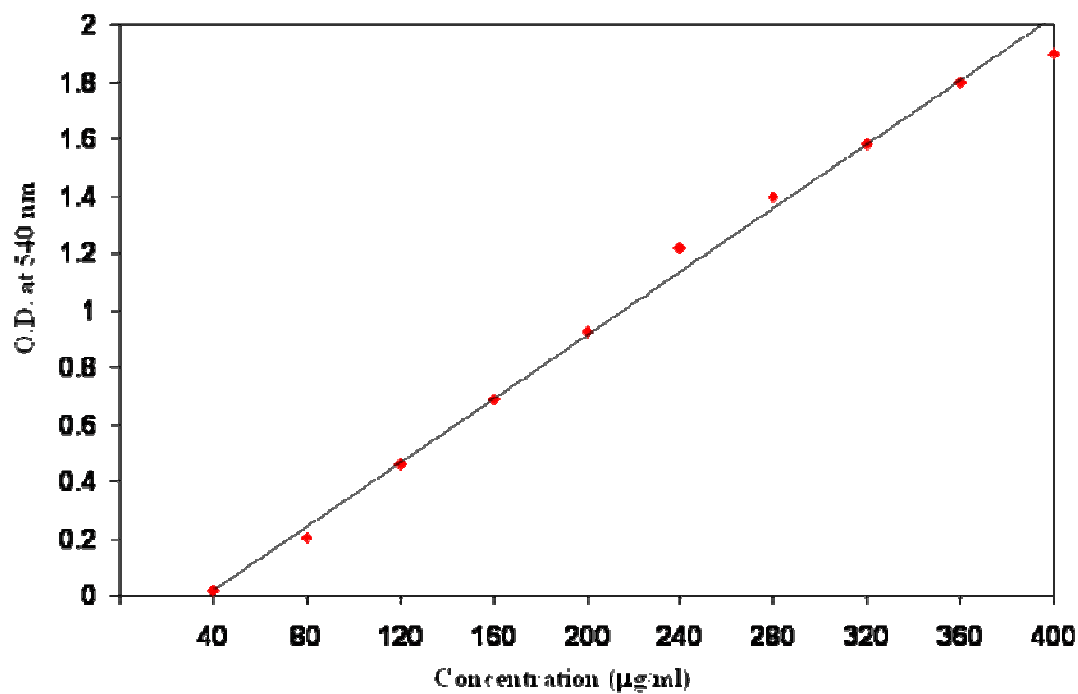
For SL8

	Xylanase				Protein			
Source	DF	SS	MS	F	DF	SS	MS	F
Treatment	4	3051.80	762.95	762.95	4	2.561	0.640	0.64
Error	10	10.00	1.000		10	10.000	1.000	
Total	14	3061.8			14	12.561		

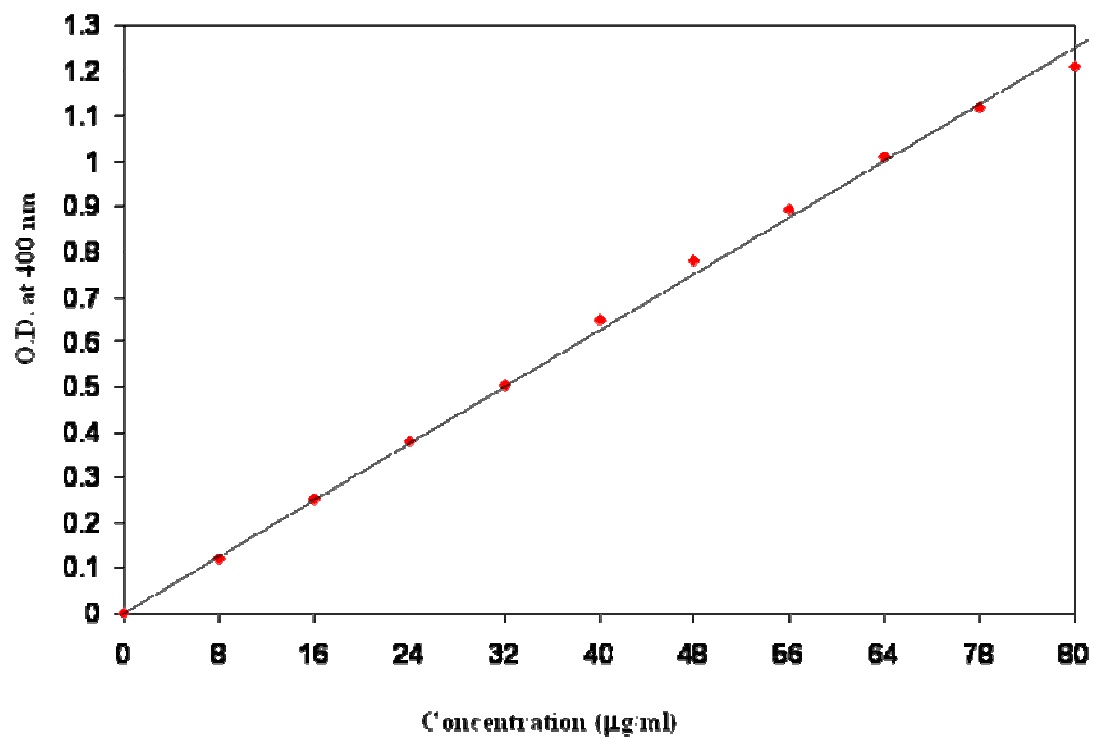
For N11

	Xylanase				Protein				Specific activity			
Source	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Treatment	4	3803.2	950.79	1185.53	4	12.654	3.163	5.24	4	384.68	96.169	96.17
Error	10	8.0200	0.802		10	6.040	0.604		10	10.000	1.000	
Total	14	3811.2			14	18.694			14	394.68		

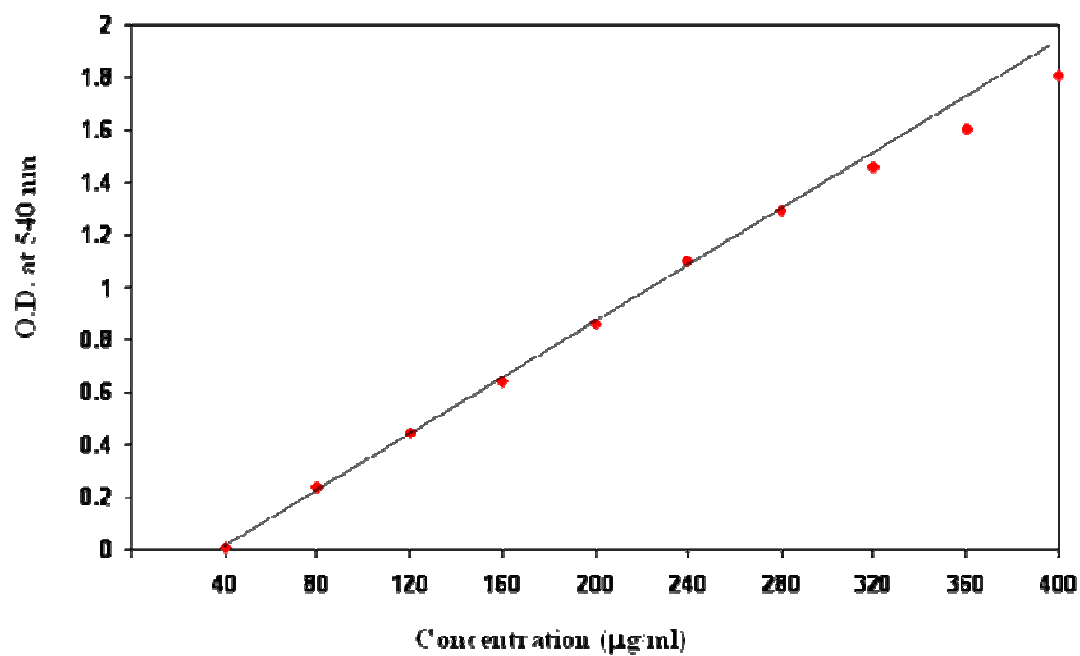
APPENDIX-III



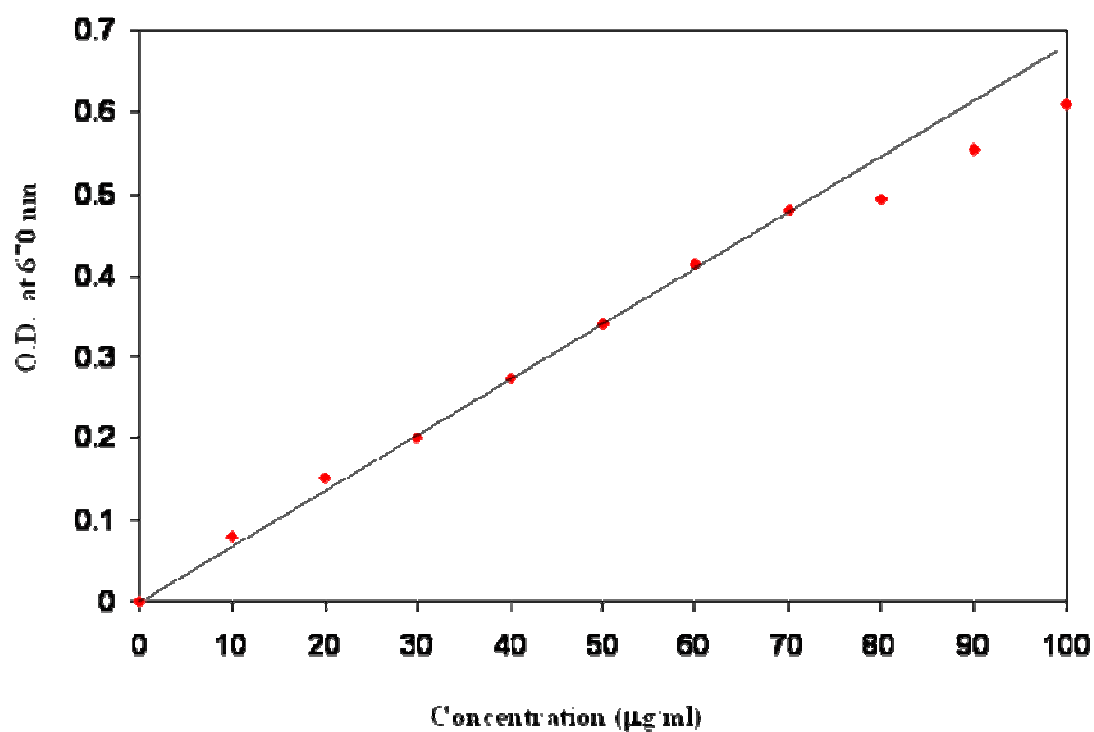
Standard curve of glucose



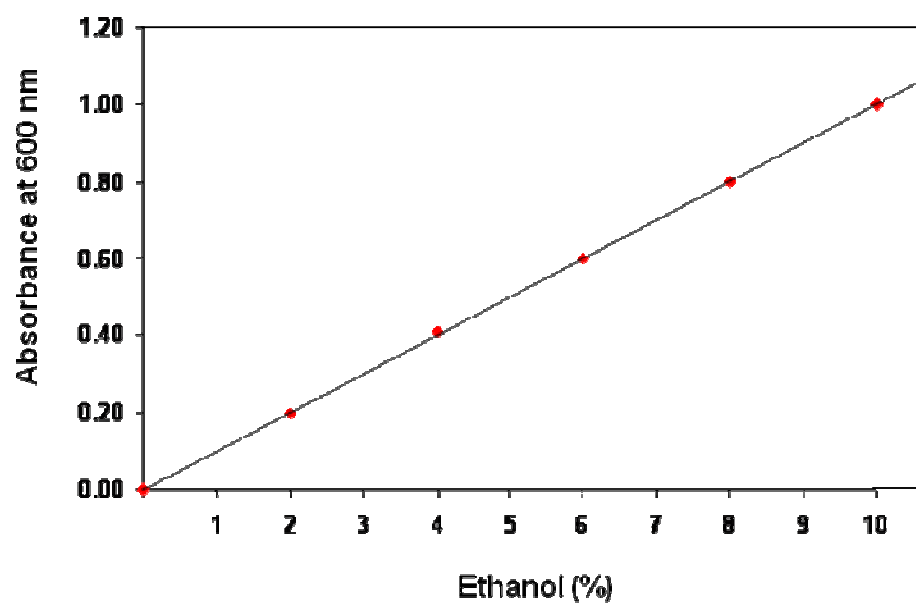
Standard curve of p-nitrophenol



Standard curve of xylose



Standard curve of Bovine Serum Albumin



Standard curve for Ethanol estimation

CURRICULUM VITAE

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