PURIFICATION AND CHARACTERIZATION OF AMYLASE PRODUCED FROM POTENTIAL EXTREMOPHILES FOR INDUSTRIAL APPLICATION

Thesis

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GITANJALI

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This is to certify that the thesis entitled, **"Purification and characterization of amylase produced from potential extremophiles for industrial application"** submitted in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) is a record of bonafide research work carried out by **Ms. Gitanjali (F-2011-12-D)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

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

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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Purification and characterization of amylase produced from potential extremophiles for industrial application"** submitted to Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP) by **Ms. Gitanjali (F-2011-12-D)** in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY.**

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ABBREVIATIONS

%	:	Percent
α	:	Alpha
β	:	Beta
°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
CFU	:	Colony forming units
cm	:	Centimeter
DNSA	:	Dinitrosalicylic acid
DNA	:	Deoxyribonucleic acid
dNTPs	:	deoxyribonucleotide triphosphates
E.C.	:	Enzyme Commission
EtBr	:	Ethidium bromide
Fig.	:	Figure
GH	:	Glycosyl hydrolase
g	:	Gram
g/l	:	Gram per litre
h	:	hour
HPLC	:	High performance liquid chromatography
IU	:	International units
kDa	:	Kilodalton
mg	:	Miligram
mg/ml	:	Miligram per millilitre
min	:	Minute
ml	:	Millilitre
mM	:	Milimolar
MW	:	Molecular weight
N	:	Normal
nm	:	Nano meter
O.D.	:	Optical density
PCR	:	Polymerase Chain Reaction
ppm	:	Parts per million
psi	:	Per square inch
RNase	:	Ribonuclease
rpm	:	Revolution per minute
SmF	:	Submerged Fermentation
sp.	:	Species
SSF	:	Simultaneous saccharification and Fermentation
SHF	:	Separate hydrolysis and fermentation
UV	:	Ultra violet
v/v	:	Volume per volume
v/w	:	Volume per weight
vvm	:	Volume by volume per meter

Chapter-1

INTRODUCTION

Enzymes are substances present in the cells of living organisms in minute amounts which are capable of speeding up chemical reactions (associated with life processes), without themselves being altered after the reaction. They accelerate the velocity of the reaction without necessarily initiating it. Compared with chemical catalysts, enzymes have many advantages such as a high specificity, a high catalytic efficiency, and an adjustable activity, which greatly promote the use of enzymes in pharmaceutical, chemical, and food industries (Pires-Cabral *et al.*, 2010; Yucel *et al.*, 2011). Industrial enzymes are specific catalysts that are utilized to catalyze and accelerate a chemical reaction that does not occur naturally. Due to these novel features, the demand for industrial enzymes has catapulted to the new heights which call for a constant research and development, to optimize their production and minimize resource costs (MarketsandMarkets, 2015). According to the 'Industrial Enzymes Market-Global Trends & Forecasts to 2018' (2015), the world market for industrial enzymes is estimated to grow US$ 6,000 by 2018.

Although the enzymes utilized today are also found in animals (chimosin, pepsin, trypsin and pancreatin) and plants (ficin, papain) however, a bulk of commercial supply is of microbial origin (pectinases, glucoamylase, α-amylase). Microorganisms possess high growing capability, huge amounts of enzyme can be produced in a short period of time as well as the strains can be genetically manipulated, to obtain enzymes with desired characteristics (Sundarram and Murthy, 2014; Tiwari *et al.*, 2014). Microbial enzymes thus are plentifully supplied, well standardized and marketed by several competing companies.

Glycoside hydrolases are a large category of enzymes which are able to metabolize a large variety of saccharides. They have been divided into classes based on their mode of action and families as well as on their well-defined amino acid sequence similarities. Most of the starch converting enzymes belongs to GH-

13 family. Amylases are among the most widely used enzyme in starch processing industries. Based on their mode of action, they are further classified into three categories: α-amylases, β-amylases, and glucoamylases. All amylases are glycoside hydrolyses and act on α-1, 4 glycosidic bonds (Maton *et al.,* 1993). Among the types of amylases, industrially, α-amylase is the most widely used enzyme particularly in starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal, and oil drilling. Since α-amylases are active over a broad pH (5–9) and temperature (35–105 ^{o}C) ranges, they are worldwide centre of attraction for researchers (Tiwari *et al.,* 2014). α-amylases (endo-1,4-*a*-D-glucan glucohydrolase, EC 3.2.1.1) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-*O*-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability.

β-Amylase (*a*-1,4-glucan maltohydrolase, EC 3.2.1.2) is usually of plant origin, but a few microbial strains are also known to produce it. It is an exo-acting enzyme that cleaves non-reducing chain ends of amylose, amylopectin and glycogen molecules. It hydrolyses alternate glycosidic linkages, yielding maltose. Since β-amylase is unable to by-pass *a*-1,6-glycosidic linkages in amylopectin, it results in incomplete degradation of the molecule, yielding 50–60% maltose and a β-limit dextrin(Pandey *et al.,* 2000a). Glucoamylase (exo-1,4-*a*-D-glucan glucanohydrolase, EC 3.2.1.3) hydrolyses single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner. Unlike *a*-amylase, most glucoamylases are also able to hydrolyse the 1,6-*a*-linkages at the branching points of amylopectin, although at a lower rate than 1,4- linkages. Thus glucose, maltose and limit dextrins are the end products of GA action (Pandey *et al.,* 2000a).

But due to the wider industrial application spectra of novel α-amylase, there is an ever increasing demand for these enzymes worldwide. It is desirable that α-amylases should be active at high temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) to economize processes, therefore there has

been a need for more thermophilic and thermostable α-amylases (Sindhu *et al.*, 1997). With the availability of thermostable enzymes, a number of new possibilities for industrial processes have emerged (Haki and Rakshit, 2003). In view of this, researchers have diverted their attention for isolation and characterization of enzymes from extremophiles (Kiran and Chandra, 2008), with their cost effective production techniques. The production of α-amylase has been thoroughly investigated and has been reported to be affected by a variety of physicochemical factors (Elias *et al.*, 2014; Hu *et al.*, 2014; Avwioroko *et al.*, 2015). Most notable among them are the composition of the growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source and nitrogen source (Singh *et al.*, 2009; Andualem, 2014; Singh *et al.*, 2010).

Enzymes generally occur in the cell in association with other proteins, nucleic acids, polysaccharides and lipids. The activity of the enzyme in relation to the total protein present (specific activity) can be determined (Gupta *et al.*, 2003; Sivaramakrishnan *et al.*, 2006). A variety of methods are used to remove contaminating material so the enzyme is purified and its specific activity increases. Thus, it is significant to develop economic processes for their purification to obtain chemically pure enzymes with maximum specific activity. Traditionally the purification of amylases from fermentation media has been done in several steps, using different purification techniques (Pandey, 2000a).

The application of α-amylase in industrial reactions depends on its unique characteristics, such as its action pattern, substrate specificity, major reaction products, optimal temperature, and optimal pH (Berg, 2003). Enzymes have the ability to catalyze reactions under very mild conditions with a very high degree of substrate specificity, thus decreasing the formation of by-products. Among the reactions catalyzed are a number of very complex chemical transformations between biological macromolecules, which are not accessible to ordinary methods of organic chemistry (Creighton, 1984). However, its poor stability, reusability, and high cost of single use limit its use in industrial production. In recent years, enzyme immobilization technology provides an effective method to

circumvent these issues, which not only improves catalytic properties and operational stability but also facilitates enzymes multiple reuse, separation, and continuous automatic operation in industrial production (Tischer and Wedekind, 1999). There is experimental evidence that the immobilized state might be the most common state for enzymes in their natural environment. The attachment of enzymes to the appropriate surface ensures that they stay at the site where their activity is required (Brena and Viera, 2006). Immobilization thus enhances the concentration at the proper location and it may also protect the enzyme from being destroyed. Several studies indicated that immobilized enzyme has shown increased catalytic activity, increased stability of enzyme (El-Batal *et al.*, 2005), easy recovery of enzyme, easy separation from product minimizing or eliminating protein contamination of product, repeated or continuous use of a single batch of enzyme (Varavinit *et al.*, 2002). To a large extent this procedure prevents enzyme losses due to washout and at the same time maintains biocatalyst at high concentrations (Baldino *et al.*, 2001) and also protects enzyme from microbial contamination.

But with certain limitations of simpler immobilization, recently researchers are improving current technologies and developing new ones that utilize enzymes immobilized on nanoparticles. Iron oxide nano particles are gaining wide attention due to the availability of higher surface area for binding of a larger amount of enzymes firmly (Sohn *et al.*, 2008) moreover the immobilized enzymes can be selectively separated with the help of magnetic field.

Since the late 19th century, the mean temperature on earth has increased with 0.8°C and the major part of this increase is likely due to anthropogenic emissions of greenhouse gases. Carbon dioxide is the type of greenhouse gas with largest emission and this originates from the combustion of fossil fuels as coal, oil and natural gas (Sun and Cheng, 2002). India is the fourth (energy data) largest global contributor to carbon emissions; GOI transport policy has targeted EURO-III and IV vehicle emission norms for vehicles, which in turn require adoption of clean and green fuel. In the past decade there has been renewed interest in the biomass as a renewable energy source worldwide. Ethanol-blended gasoline have the potential to contribute significantly to these emissions

reductions promising and allow a cost-effective use of energy crops e.g. production of methanol and hydrogen by means of gasification processes.

Traditionally first and second generation biofuels have been produced from substrates such as food and oil crops and the lignocellulosic biomass. However, the liquid fuel consumption and requirement is increasing day by day besides the first and second generation biofuels have limitations to meet the ever increasing demand. The most common concern related to the current first generation biofuels is that as production capacities increase, so does their competition with agriculture for arable land used for food production which leads to severe food shortage. The advent of second generation biofuels is intended to produce fuels from lignocellulosic biomass, the woody part of plants that do not compete with food production. Sources include agricultural residues, forest harvesting residues or wood processing waste such as leaves, straw or wood chips as well as the non-edible components of corn or sugarcane. However, converting the woody biomass into fermentable sugars requires costly technologies involving pre-treatment with special enzymes, meaning that second generation biofuels have limitations to be produced economically on a large scale (Brennan and Owende, 2010).

Therefore, 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 (Chisti, 2007; Nigam and Singh, 2010). Microalgae are able to produce 15–300 times more biofuel 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). Microalgae, unicellular algae thus have emerged as a potential source for bioethanol production owing to its ability to fix CO_2 and accumulate a high content of the storage polysaccharide, starch. Presence of certain distinct notable characteristics such as lack of lignin and lack of specific functional parts such as leaves and roots render them a superior alternative over the woody terrestrial biomass. The polysaccharide contained in algal cell wall can

be easily hydrolyzed to simple sugars which can further be fermented into bioethanol (Eshaq *et al.*, 2011).

Since algae mainly are composed of starch along with some of cellulose and pectin in their cell wall, therefore amylases are among the most widely used enzymes for their degradation along with cellulase and pectinase (Emmanuel *et al.*, 2000; Kim *et al.*, 2014). Since alkalothermophilic amylase is in high demand for feasible hydrolysis and bioconversion of algal biomass into ethanol, amylase produced from extremophilic microorganisms, can become the first choice in the process. Microbial communities capable of growing at extremes of temperature, alkalinity/ acidity are the most sought after extremophiles in this field. (Kiran and Chandra, 2008). Keeping in view the importance and industrial application of amylase, the present study was thus undertaken with the following objectives:

1. Isolation of amylase producing microorganisms from different sources
2. Screening and identification of hyperamylase producing microorganisms
3. Purification and characterization of amylase

Chapter-2

REVIEW OF LITERATURE

Microbial life does not seem to be limited to specific environments. During the past few decades it has become clear that microbial communities can be found in the most diverse conditions, including extremes of temperature, pressure, salinity and pH (Kumar *et al.,* 2011). These microorganisms, called extremophiles, produce biocatalysts that are functional under extreme conditions. Consequently, the unique properties of these biocatalysts have resulted in several novel applications of enzymes in industrial processes. At present, only a minor fraction of the microorganisms on earth have been exploited. Novel developments in the cultivation and production of extremophiles related to the cloning and expression of their genes in heterologous hosts will increase the number of enzyme-driven transformations in chemical, food, pharmaceutical and other industrial applications (Berg, 2003).

There are a wide variety of different conditions to which microorganisms have adapted. The biocatalysts obtained from these extremophiles could be applicable in similarly diverse conditions (Table 1). These enzymes/ biocatalysts active at and resistant to high temperatures are used for the degradation of polymers such as starch, chitin or cellulose. Under these extreme conditions the solubility and, consequently, the accessibility of the substrate is improved (Sellek and Chaudhuri, 1999; Marhuenda *et al.*, 2002). This diversity of environments to which different extremophiles have adapted offers many exciting opportunities for a variety of applications.

Microorganisms, like all living things, adapt to the conditions in which they have to live and survive. Thermophiles are reported to contain proteins which are thermostable and resist denaturation and proteolysis (Kumar and Nussinov, 2011). Chaperonins, a group of specialized proteins are produced by these organisms, which help, after their denaturation to refold the proteins to their native form and restore their functions (Everly and Alberto, 2000). The cell

membrane of thermophiles is made up of saturated fatty acids. These fatty acids provide hydrophobic environment for the cell and keep the cell rigid enough to live at elevated temperatures (Herbert and Sharp, 1992). The DNA of these thermophiles contains a reverse DNA gyrase which produces positive super coils in the DNA (Lopez, 1999).

Table1. Classification of extremophiles and examples of applications of some of their enzymes (Berg, 2003)

Type	Growth characteristics	Enzymes	Applications
Thermophiles	Temp> 80 ^{0}C (hyperthermophile) and 60-80 ^{0}C (thermophile)	Amylases Proteases Glycosyl hydrolases (eg. amylases, pullulanase, glucoamylase,glucosidases, cellulases, xylanases) Chitninase Xylanases Lipases, Esterases DNA polymerases Dehydrogenases	Starch processing Detergents, hydrolysis in food and feed, brewing, baking Starch, cellulose, chitin, pectin processing, textiles Chitin modification for food and health products Paper bleaching Detergents, stereo specific reactions (trans-esterification, organic biosynthesis) Molecular biology (PCR) Oxidation reaction
Psychrophiles	Temp <15 ^{0}C	Amylases Proteases Cellulases Dehydrogenases Lipases	Detergent and bakery Detergents, food applications (dairy products) Detergents, feed and textiles Biosensors Detergents, food and cosmetics
Halophiles	High salt (2-5 M NaCl)	Amylases Proteases Dehydrogenases	Sewage treatment Peptide synthesis Biocatalysis in organic media
Alkaliphiles	pH>9	Amylases Proteases, cellulases	Textiles and detergent Detergents, food and feed
Acidophiles	pH< 2-3	Amylases, glucoamylases Proteases, cellulases Oxidases	Starch processing Feed component Desulfurization of coal
Piezophiles	Pressure loving; up to 130 MPa		Food processing and antibiotic production

Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes (Leuschner and Antranikan, 1995; Fredrich and Antrakian, 1996; Diane *et al.,* 1997; Zeikus *et al.,* 1998). One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles.

Other benefits of elevated process temperatures include higher reaction rates, higher process yield and products and favorable equilibrium displacement in endothermic reactions (Mozhaev, 1993; Krahe *et al.,* 1996; Kumar and Swati, 2001). These enzymes can also be used as models for understanding thermostability and thermo-activity useful for protein engineering.

In industrial processes, high temperatures impact such benefits as increased substrate solubility, decreased viscosity of the medium and lower risk of microbial contamination or higher rates of concurrent non-enzymatic reactions (Asgher, 2007). The successful implementation of enzymes as industrial biocatalysts requires the availability of suitable enzymes with high activity, specificity and stability under process conditions. However, naturally occurring enzymes are often not optimized to fulfil these requirements. Within this context, directed evolution is very effective in closing these functional discrepancies. Thermostable enzymes or thermozymes have found a number of commercial applications because of their over all inherent stability (Demirijan *et al.,* 2001). These thermozymes, displaying unique characteristics such as temperature, chemical and pH stability can be used in several industrial processes, in which they replace chemicals or mesophilic enzymes (Bruins*et al.,* 2001).Among other industrial enzymes, amylases constitute a class of industrial enzymes representing approximately 30% of the world enzyme production (Calik and Ozdamar, 2001). Demand for novel amylases worldwide is increasing day by day, as these enzyme applications spectra are spreading in various industrial sectors. In view of this, researchers have diverted their attention for isolation and characterization of amylases from extremophiles (Kiran and Chandra, 2008).

2.1 AMYLASES

α-amylase was the first enzyme to be commercially produced and marketed in 1894. Dr. J. Takamine established the first industrial production of α-amylase from *Aspergillus oryzae* known as Taka diastase, which was used as a digestive aid (Janarthanan *et al.*, 2014). Since then amylases have found diverse applications in a wide variety of industries. Amylases also find use in processes related with the degradation of environmental pollutants, conversion of starch to desired product by many microorganisms (Veille and Zeikus, 2001), infiltration of waste containing starch and production of biochemical material utilizing starch as substrate. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medicinal and analytical chemistry (Dehkordi and Javan, 2013).

Amylases are a class of enzymes that are capable of digesting glycosidic linkages found in starches. These enzymes are classified as endoamylases and exoamylases according to the manner in which the glycosidic bond is attacked. The α-amylase family, *i.e*. the clan GH-H of glycoside hydrolyses, is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities.

Endoamylases are able to cleave α, 1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. α-amylase (EC 3.2.1.1) is a well-known, classical calcium containing 1,4-α-D-glucan glucanohydrolase endo amylase which catalyzes hydrolysis of starch and related carbohydrates by randomly cleaving internal α-D-(1,4) glycosidic linkage, yielding glucose, maltose, maltotriose, and other oligosaccharides. It belongs to family thirteen in the classification of glycoside hydrolases. This family is the most varied of all glycoside hydrolase families, containing many enzymes able to catalyze various reactions, such as hydrolysis, transglycosylation, condensation and cyclization (Senthilkumar *et al.*, 2012).

Enzymes belonging to the second group, the exoamylases, either exclusively cleave α,1-4 glycosidic bonds such as β-amylase (EC 3.2.1.2) or cleave both α,1-4 and α,1-6 glycosidic bonds like amyloglucosidase or

glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α-glucosidase), or maltose and β-limit dextrin. Glucoamylase and α-glucosidase differ in their substrate preference: α-glucosidase acts best on short maltooligosaccharides and liberates glucose with α-configuration while glucoamylase hydrolyzes long-chain polysaccharides best. β-amylases and glucoamylases have also been found in a large variety of microorganisms (Pandey *et al.,* 2000a).

The members of amylase family share a number of common characteristic properties (vander Maarel *et al.,* 2002). Some of them are:

- **Hydrolases activity**- In this activity the enzymes act on the α-glycosidic bonds and hydrolyze this bond to produce α-anomeric monosaccharides or oligosaccharides.
- **Transglycosylation activity**- In this activity α -1-4 or 1-6 glycosidic linkages is formed.
- Enzymes that hydrolyze α -1,4 bond and bypass α-1,6 linkages e.g. α -amylase (endoacting amylases).
- Enzymes that hydrolyze α -1,4 bond and cannot bypass α -1,6 linkages e.g. beta amylase (exoacting amylases producing maltose as a major end product).
- Enzymes that hydrolyze α-1,4 and α-1,6 linkages e.g. amyloglucosidase (glucoamylase) and exoacting amylase.
- Enzymes that hydrolyze only α-1,6 linkage e.g. pullulanase and other debranching enzymes.
- Enzymes that hydrolyze prefentially α-1,4 linkages in short chain oligosaccharides produced by the action of other enzymes on amylose and amylopectin e.g. alpha-glucosidases.
- Enzymes that hydrolyze starch to a series of non-reducing cyclic D-glucosyl polymers called cyclodextrins or Sachardinger dextrins e.g. *Bacillus macerans* amylase (cyclodextrin producing enzyme).

The α-glycosidic bond is very stable having a spontaneous rate of hydrolysis at room temperature (Wolfenden *et al.,* 1998). The catalytic mechanism of the α-amylase family is that of the alpha-retaining double displacement (van der Maarel *et al.,* 2002) and this α-retaining mechanism is the characteristic feature of the α-amylase family. They vary widely in their reaction specificities. The attachment of different domains to the catalytic site, or to extra sugar binding sub sites around the catalytic site, is the prime reason for these differences (van der Maarel *et al.,* 2002).

The starch industry is one of the largest users of enzymes for the hydrolysis and modification of this useful raw material. The starch polymer, like other such polymers, requires a combination of enzymes for its complete hydrolysis. These include α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases (Pandey *et al.,* 2000a).

Starch is a polymer of glucose linked to one another through C1 oxygen by a glycosidic bond. At the end of the polymeric chain, a latent aldehyde group is present. This group is known as the reducing end. Pure starch consists predominantly of α-glucan in the form of amylose and amylopectin. While amylopectin is soluble in water, amylose and the starch granule itself are insoluble in cold water. Amylose is a linear polymer consisting of up to 6000 glucose units with α, 1-4 glycosidic bonds (Fig. 1). The relative content of amylose and amylopectin varies with the source of starch. Average amylose content in most common starches is 20-30% (Marc *et al.,* 2002). Amylopectin consists of short α, 1-4 linked linear chains of 10–60 glucose units and α, 1-6 linked side chains with 15–45 glucose units (Fig. 2). The average number of branching points in amylopectin is 5% (Thompson, 2000). The complete amylopectin molecule contains about 2 000,000 glucose units, thereby being one of the largest molecules in nature (Marc *et al.,* 2002).

Starches are defined as waxy when the ratio of amylose to amylopectin is low (~15%), normal when amylase represents ~16-35% and high-amylose (or amylo-) when amylose exceeds ~36%. Starch depolymerization by amylase is the basis for several industrial processes such as preparation of glucose syrups, bread

making and brewing. Amylases also play a significant role in seed germination and maturation, and are instrumental in starch digestion resulting in the formation of sugars, which are subsequently used in various metabolic activities (Leloup *et al.*, 1994). Enzymes used for the industrial starch hydrolysis are estimated to occupy 10-15 per cent of the total world enzyme market. This industrial sector is the second biggest consumer of enzymes by market sector (Vengadaramana, 2013).

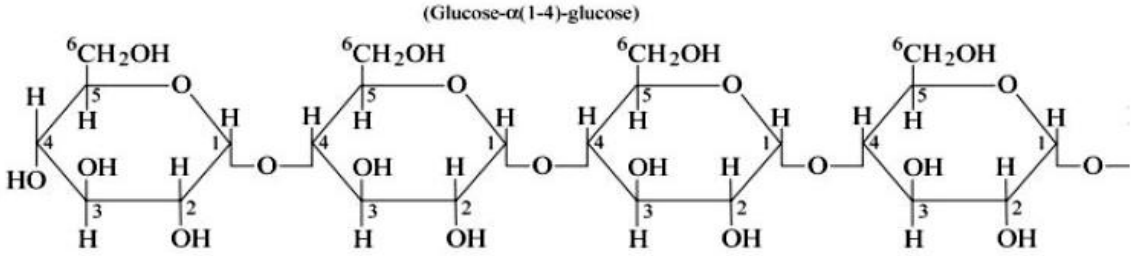


Fig. 1 Structure of amylase

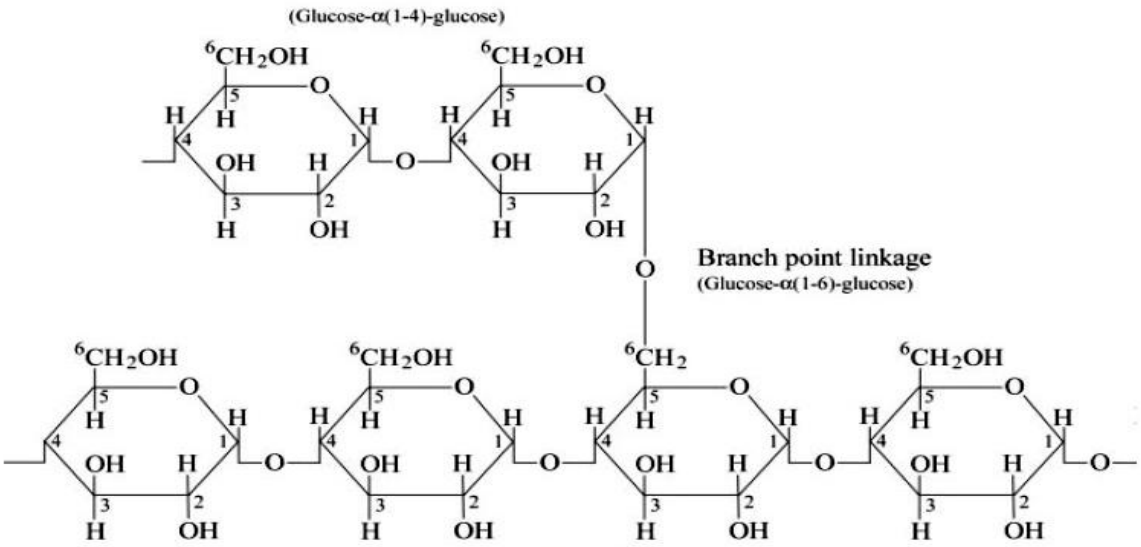


Fig. 2 Structure of amylopectin

2.2 SOURCES OF THERMOSTABLE AMYLASE: ISOLATION AND SCREENING

Advances in the use of microbial amylases in industry have been possible with the isolation of a large number of beneficial thermophilic microorganisms from different exotic ecological zones of earth and the subsequent extraction of useful enzymes from them (Groboillot, 1994; Bharat and Hoondal, 1998; Bauer

et al., 1999; Kohilu *et al.,* 2001). A number of microbial sources exist for the efficient production of this enzyme, but only a few selected strains of bacteria and fungi meet the criteria for commercial production (Pandey *et al*., 2000a). Several reports have been published on the isolation, identification and characterization of α-amylase from a variety of sources (Pederson and Nielson, 2000; Kilic *et al.,* 2005; Muralikrishna and Nirmala, 2005; Goyal *et al.,* 2005; Hernandez *et al.,* 2006; Ashger *et al.,* 2007; Hmidet *et al.*,2008; Bakri *et al.,* 2009; Asad *et al.,* 2010; Iftikhar *et al.,* 2014).

Several workers reported α-amylase production by bacterial species of *Bacillus* (Muralikrishna and Nirmala, 2005; Ashger *et al.,* 2007), *Pseudomonas* (Haq *et al.,* 2002; Shiau and Hung, 2003) and *Clostridium* (Kılıc *et al.,* 2005). For the thermal stabilities of their α-amylase enzymes to be utilized in various fermentation processes, extreme thermophilic bacteria such as *Rhodothermus marinus* and mesophilic bacteria such as *Bacillus megaterium, B. macerans* and *B. coagulans* are generally selected and utilized (Saroja *et al.,* 2000; Gimbi and Kitabatake, 2002). Most thermostable α-amylase utilized in the industry is produced from *B. licheniformis* (Reda, 2007; Hmidet *et al.,* 2008). Highly thermostable α-amylases are also obtained in hyperthermophilic and thermophilic archaea such as *Pyrococcus furiosus, Thermococcus hydrothermalis, T. profundus, Sulfolobus acidocaldarius* and *S. solfataricus* (Goyal *et al.*,2005; Hernandez *et al.,* 2006; Arikan, 2008).

Qualitative screening of amylogenic strains based on their ability to hydrolyse starch on starch agar plates forming a clear halo around the colony has been exploited for isolation of amylolytic strains. Vaseekaran *et al.* (2010) isolated 72 single bacterial colonies on starch-nutrient agar plates which produced clear halos with iodine solution. Three strains BS1, FS1 and GS1 giving maximum halo diameters of 20, 18 and 17 mm respectively were chosen for further studies and DNA analysis for species identification. Similarly, among 30 isolates reported by Kaur *et al.* (2012), 6 isolates hydrolysed starch on starch agar medium. The strains showing maximum inhibition zone by enzyme in starch hydrolysis test were selected and further characterized by morphological and biochemical tests. Study by Zohra and Ahmad (2012) reported 92 strains isolated

from soil. Among them 56 were found to be extracellular amylase producers as they produced clear halos around colonies grown on starch supplemented plates when flooded with iodine solution. Amylogenic cultures were purified and stored in nutrient broth containing 15% glycerol at -20˚C. Highest enzyme producing culture was identified as gram positive, spore forming *Bacillus* sp. according to biochemical and morphological characteristics.

Bacterial species such as *Bacillus subtilis* (Sumrin *et al.*,2011; Rajput and Li, 2012; Rajput *et al.,* 2013), *B. licheniformis* and *Bacillus* sp. are generally preferred for the production of α-amylase because they appear to be very productive (Goyal *et al.,* 2005; Kokab *et al.,* 2007; Reda, 2007; Niazi *et al.*, 2010). Sohail *et al*. (2005) isolated 40 amylogenic isolates out of 117 bacterial strains (34.2%) while 21 out of 130 fungal isolates (16.15%) were found to be amylolytic. As identified by standard microbiological procedures, all the good bacterial amylase producers belonged to the genus *Bacillus*. Similarly, a study by Alkando and Ibrahim (2011) reported 270 bacterial isolates out of which, 20 potential α-amylase producing organisms were selected during primary screening. Secondary screening of these isolates yielded a highly thermostable alkaline α-amylase producing isolate. Of all the species tested, *B. licheniformis* gave maximum α-amylase activity of 0.79 U/mg/ml at pH 8 in iodine method, and (0.02 U/mg/ml) in 3, 5-dinitrosalicylic acid (DNS) method. Among one thousand six hundred and thirty bacterial colonies, two bacterial strains belonging to *Bacillus* sp. producing highest enzyme at a temperature of 55^0C and 45^0C, respectively have been isolated from hot springs of Ethiopia by Hailemariam *et al.* (2013). Similarly, Alrumman *et al*. (2014) identified *B. axarquiensis* as an amylogenic strain among sixty one thermoalkalophilic bacteria, isolated from soil samples in Saudi Arabia.

Saxena *et al.* (2007) screened a total of three hundred bacterial isolates from soil for amylase production on starch agar plates containing (%) soluble starch 1.0, yeast extract 0.4, K_2HPO_4 0.1, and $MgSO_4.7H_2O$ 0.15, pH 7.0 at 37 °C. Amylolytic isolates were selected by flooding the agar plates with Gram's iodine solution. Isolates having a higher ratio of clearing zone to colony size were grown in liquid broth and were declared as amylolytic. A hyperthermostable α-

amylase producing strain, *Geobacillus* sp. IIPTN (MTCC 5319) has been isolated from the hot spring of Uttarakhand's Himalayan region by Dheeran *et al*. (2010). The optimal temperature and pH were 60°C and 6.5, respectively, for growth and enzyme production. Although it was able to grow in temperature ranges from 50 to 80°C and pH 5.5–8.5. Maximum enzyme production was in exponential phase with activity 135 U ml^{-1}at 60°C.

2.3 PRODUCTION OF MICROBIAL α-AMYLASES

The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and easy manipulation of microbes to obtain enzymes of desired characteristics (Ramesh and Lonsane, 1989). Screening for the α-amylase producers is a key step for production. Starch hydrolysis is usually detected directly on plates as clear zones surrounding the colonies. Al-ZaZaee *et al*. (2011) reported *B. cereus* Ms6 as an amylogenic strain based on the zone of hydrolysis formed by the isolate on starch agar medium. Alkando and Ibrahim (2011) isolated bacterial strains from soil samples which were primarily screened for amylase production by starch hydrolysis test. Kaur *et al*. (2012) screened 6 bacterial strains among 30 isolated from soil based on the starch hydrolysis test performed on starch agar medium plates. In general, the diameter of the area of hydrolysis has been related to the potency of the amylase (Dhawale *et al.,* 1982).

2.3.1. Factors affecting production

The production and stability of α-amylase in the medium is affected by a variety of physicochemical factors. It is well documented that extracellular amylase production by microbes is greatly influenced by media components, especially carbon and nitrogen sources, minerals and physical factors such as pH, temperature, agitation, dissolved oxygen and inoculum density (Babu and Satyanarayana, 1993; Dey *et al.,* 2001; Gigras *et al.,* 2002).

2.3.1.1 Effect of carbon source: induction of α-amylase

Carbon source in the form of either monosaccharides or polysaccharides may influence the production of amylase enzyme. Many researchers have shown

that different carbon sources have varied influence on the production of extracellular enzymes especially among amylase producing strains (Saxena *et al.*, 2007; Sumrin *et al.*, 2011; Lily *et al.*, 2012; Vijayabaskar *et al.*, 2012; Deb *et al.*, 2013; Andualem, 2014). *α*-amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose. Apart from maltose, in some strains, other carbon sources as dextrin, wheat bran, dextrin and xylose also served as inducers of α-amylase (Saxena *et al.*, 2007). According to study glucose, galactose, fructose, sucrose and lactose supported growth but amylase production was not induced, suggesting the inducible nature of amylase (Kiran and Chandra, 2008). Similarly Syed *et al.* (2009); Hashemi *et al.* (2010) and Sankaralingam *et al*. (2012) studied the effect of different carbon sources such as sucrose, starch, arabinose, sorbitol, mannitol, raffinose, lactose, xylose, fructose and glucose on α-amylase activity. Among the various carbon sources used, starch at a concentration 1% (w/v) only showed maximum yield of 2,216.6 U/ml. In *Bacillus* sp., Santos and Martins (2003) reported that increasing starch concentration in the medium beyond 1% did not increase enzyme activity.

α-amylase production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes. Synthesis of amylases in most species of the genus *Bacillus* is repressed by readily metabolizable substrates such as glucose and fructose as reported by Haseltine *et al.* (1996); Lin *et al.* (2007); Kiran and Chandra (2008); Premila (2013). However, the role of glucose in the production of α-amylase in certain cases is controversial. Thus, amongst all the variable carbon sources those have been utilized for the production of α-amylase, the use of starch remains promising and ubiquitous. A number of other non-conventional substrates as, casitone, oilseed cakes (Kiran and Chandra, 2008) and starch processing waste water (Jin *et al.*, 2001) have also been used for the production of α-amylase while the agro-processing byproduct, wheat bran has been used for the economic production of α-amylase by SSF (Ramesh and Lonsane, 1989; Kiran and Chandra, 2008). High α-amylase activities from *A. fumigatus* have also been reported using α-methyl-D-glycoside (a synthetic analogue of maltose) as substrate (Goto *et al.*, 1998).

2.3.1.2 Effect of nitrogen sources

Nitrogen sources are the most important secondary energy compounds for the growth and metabolism of microorganisms. The nature of these compounds and the concentration used may stimulate or down regulate the production of enzymes (Sharma *et al.,* 2012). Amylase synthesis by several microorganisms has been correlated to the presence or absence of different nitrogen sources and various amino acids in the growth medium. Organic sources like yeast extract, peptone usually have stimulating effects (Hamilton *et al.,* 1999; Hewitt and Solomons, 1996). Organic as well as inorganic nitrogen sources have been utilized for the production of α-amylase. Various organic nitrogen sources utilized were tyrptone, gelatin, casein, soya meal, beef extract, peptone, urea and yeast extract (Saxena *et al.,* 2007; Bozic *et al.,* 2011; Lily *et al.,* 2012; Sankaralingam *et al.,* 2012; Deb *et al.,* 2013) and inorganic nitrogen sources utilized were ammonium nitrate, ammonium chloride, sodium nitrate, ammonium sulphate, L-asparagine and potassium nitrate etc. (Nguyen *et al.,* 2000; Saxena *et al.,* 2007; Sankaralingam *et al.,* 2012).

Among the nitrogen sources tested yeast extract amended media showed the maximum production of α-amylase probably due to its high content in minerals, vitamins, coenzymes and nitrogen components (Asgher *et al.,* 2007; Ashwini *et al.,* 2011; Vijayabaskar *et al.,* 2012). Zohra and Ahmed (2012) and Deb *et al*. (2013) reported that tryptone (0.2%) was the best nitrogen source for amylase production. Urea however has been found to inhibit α-amylase production (Swain *et al.,* 2006; Deb *et al.,* 2013). Qader *et al*. (2009); Gupta *et al*. (2008); Demirkan *et al*. (2011) and Lily *et al*. (2012) reported peptone to be the best nitrogen source for amylase production. It has been observed that inorganic sources gave comparatively higher enzyme yields than organic nitrogen sources (Sankaralingam *et al.,* 2012). However, supplementation of medium with more than one nitrogen source gave better results. Teodoro and Martins (2000) and Saxena *et al*. (2007) supplemented peptone with 0.3% yeast extract and got better results. With soy meal, peptone and meat extract as the nitrogen source, resulted in the highest α-amylase activity (2804 U/l) after 48 h (Haeshmi *et al.,* 2011)

Amino acids in conjunction with vitamins have also been reported to affect α-amylase production. However, no conclusion can be drawn about the role of amino acids and vitamins in enhancing the α-amylase production in different microorganisms as the reports are highly variable. α-amylase production by *Bacillus amyloliquefaciens* ATCC 23350 increased by a factor of 300 in the presence of glycine (Zhang *et al.*, 2013). p-Alanine, DL-nor valine and D-methionine were effective for the production of alkaline amylase by *Bacillus* sp. A-40-2. However, the role of amino compounds was considered to be neither as nitrogen nor as a carbon source, but as stimulators of amylase synthesis and excretion (Ikura and Horikoshi, 1987.). It has been reported that only asparagine gave good enzyme yields (Nguyen *et al.*, 2000) while the importance of arginine for α-amylase production from *B. subtilis* has also been well documented (Lee and Parulekar, 1993).

2.3.1.3 Effect of pH

Enzymes are pH sensitive thus pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta *et al.*, 2003). pH stability is among the important criteria in enzymes studies. Most of the *Bacillus* strains used commercially for the production of α-amylases by SmF have an optimum pH between 6.0 and 9.0 for growth and enzyme production (Burhan *et al.*, 2003; Asgher *et al.*, 2007). Further increase or decrease in the pH resulted decrease in the activity of amylase. Majority of the bacteria produce their extracellular amylase at neutral pH only (Zohra and Ahmed, 2012; Premila, 2013). In consonance with these findings, Rasooli *et al.* (2008) and Prabakaran and Hewitt (2009) reported pH 7.0 to be the best for efficient production of α-amylase by *Bacillus* sp. and *B. cereus*, *B. subtilis* respectively. A study by Singh *et al*. (2009) reported maximum amylase activity at pH 7.0. Further increase in the pH resulted decrease in the activity of amylase.

Requirement of slight alkaline pH by the bacteria for amylase production has also been reported by several workers. Qader *et al*. (2006) reported amylase

production by *Bacillus* sp. AS-1 at pH 7.5. The pH range examined by Saxena *et al*. (2007) for extracellular α-amylase production by *Bacillus* sp. was 4.0-12.0 and the ranges of pH stability were found to be 5.0 to 10.5. Rao and Satyanarayana (2007) evaluated different pH for improving amylolytic enzyme production by the amylolytic hyperthermophile *Geobacillus thermoleovorans* and pH 8.0 was found to be optimum. Kiran and Chandra (2008) reported maximum amylase production at pH 8.0 with 60% residual activity at pH 10.5. Jayaramu *et al*. (2011) reported the growth of *B. aquimaris* VITP4 over a pH range of 7.0 to 9.0. However the optimum pH for the maximum production of enzyme was obsereved at pH 8.0. Lily *et al.* (2012) reported a steady increase in amylase production in pH range of 4.0-8.0 achieving maxima at pH 8.0, thereafter it declined sharply. Andualem (2014) studied the effect of pH on amylase production from *Bacillus* sp. in a range of 4.0 to 9.0 and found 8.0 to be the optimum.

On the contrary, acidic pH has also been reported optimum by certain workers for maximum amylase production. Li *et al*. (2007) observed the effect of pH between pH 3.0 to 10.0 on amylase activity and found maximum activity at pH 4.5. Fooladi and Sajjadian (2010) found pH 6.0 to be the best while studying the effect of pH ranging from 5.0-8.0. Deb *et al*. (2013) studied the effect of pH on amylase production from 5.5-8.0 and found 6.5 to be the optimum. He also observed lowered amylase activity at alkaline pH. Sani *et al*. (2014) reported maximum α-amylase from *B. subtilis* to be at pH 6.0. According to Singh *et al*. (2014), the optimum pH for α-amylase activity from *Streptomyces* sp. MSC702 ranged from pH 3.0 to 7.0 (retained>91% activity) with a maximum activity at pH 5.0. A complete loss in the enzyme activity was observed above pH 9.8.

2.3.1.4 Effect of temperature

Temperature is one of the most important physical parameters which affect enzyme production. After optimum temperature, as temperature increases, amylase activity decresed due to the breakage of secondary, tertiary and quaternary bonds that maintain the three dimensional structure of enzymes at high temperature leading to conformational changes in the enzyme active site. It

is known that enzyme activity increases with increasing temperature up to the optimum temperature as result of increasing kinetic energy, which can favour rate of collisions between substrate and enzyme during hydrolysis process (Andualem, 2014).

Bacterial amylases are produced at a much wider range of temperature i.e. 35–80^{o}C. (Castro *et al.*,1999; Lin *et al.*, 1998; Burhan *et al.*, 2003; Gangadharan *et al*., 2008; Shafique *et al*., 2009). *Bacillus amyloliquefaciens, B. subtilis, B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce α-amylase at temperatures between 37-60°C (Mishra *et al.*, 2005; Mendu *et al.*, 2005). Asgher *et al.* (2007) studied enzyme synthesis at temperatures between 30-50^{o}C by *B. subtilis.* The bacterium could grow satisfactorily at all temperatures tested but the maximal α-amylase activity in the growth medium was achieved at 50^{0}C. A reduction in enzyme activity was observed at temperatures above 50^{o}C. The influence of temperature on amylase production is related to the growth of the organism. Saxena *et al*. (2007) examined temperature ranging from 25-70°C for the production of amylases from *Bacillus sp.* PN5. Aygan *et al.* (2008) reported the activity of amylase by *Bacillus* sp. AB68 in the temperature range of 20 to 90^{o}C with an optimum activity at 50 ^{0}C. Zohra and Ahmad (2012) reported highest enzyme production within a range of 50-55^{o}C however, the organism survived up to 62^{o}C. Sankaralingam *et al.* (2012) reported maximum enzyme production at 40^{o}C by *B. licheniformis* which survives upto 80^{o}C. Deb *et al*. (2013) studied the effect of temperature on amylase production by *B. amyloliquefaciens* ranging from 35-70^{o}C and observed maximum amylase activity at 60^{o}C. Above 60^{o}C, there was a decrease in amylase production.

Andualem (2014) analyzed amylase production from *Bacillus* sp. at a temperature range of 40-80°C. Amylase activity of isolate relatively increased from 40^{o}C up to 55^{o}C and sharply reached optimum reaction activity at 60^{o}C. Maximum α–amylase activity from *B. subtilis* isolated from soils in Nigeria was found at 60^{o}C by Sani *et al.* (2014). The optimum temperature according to Singh *et al*. (2014) for the α-amylase activity from *Streptomyces* sp. MSC702 was in a wide range of 50–75^{o}C (retained >74% relative activity at the temperature upto

75^{o}C.), with maximum activity at 55^{o}C. Certain hyperthermophiles such as *Thermococcus profundus* and *Thermatogae maritima* have been reported to produce α-amylase at 80°C (Vieille, 2011). Rao and Satyanarayana (2007) found 100°C to be an optimum temperature for α-amylase and glucoamylase production by the amylolytic hyperthermophile *Geobacillus thermoleovorans*. The optimum temperature for alpha-amylase production by a thermophile was found to be 70°C (Raharjo *et al.,* 2010).

A cold active α-amylase from Antarctic psychrophile *Alteromonas haloplanktis* was reported to exhibit maximum α-amylase production at 4°C (Feller *et al.,* 1998).

2.3.1.5 Effect of divalent metal ions, additives and inhibitors

The variety of ways by which metal ions affect enzyme catalysis is by modifying the electron flow in the enzyme substrate reaction or by changing the orientation of the substrate with reference to the functional group at active site. Metal ions accept or donate electrons and act as electrophiles, mask nucleophiles to prevent unwanted side reactions, bind enzyme and substrate by coordinate bonds, hold the reacting groups in the required 3D orientation, and simply stabilize a catalytically active conformation of the enzyme (Palmer, 2001).

Most of the bacterial amylases are known to be metalloenzymes, dependent upon divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, etc. (Pandey *et al.,* 2000a; De Azeredo *et al.,* 2004). α-amylase is known to be a calcium metalloenzyme having at least one calcium ion associated with its molecule (Kiran and Chandra, 2008; Deb *et al.,* 2013). The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus, causing the adoption of a compact structure (Goyal *et al.,* 2005). However, Ca-independent enzymes have also been reported (Uyar *et al.,* 2003; Ghollasi *et al.,* 2013; Hashemi *et al.,* 2011; Singh *et al.,* 2014). Rao and Satyanarayana (2007) while working on amylolytic hyperthermophile *Geobacillus thermoleovorans* showed that the activity of the enzyme was enhanced strongly by Co^{2+} and Fe^{2+} while enhanced slightly by Ba^{2+}, Mn^{2+}, Ni^{2+} and Mg^{2+}. Ni^{2+} has also been reported to enhance amylase activity by

Premila, (2013). Chavan and Wadatkar (2014) reported stimulation of enzyme activity by Mo^{2+}, K^{+}, Co^{2+}, Mg^{2+} and Ca^{2+} ions.

Certain divalent ions on the other hand have inhibitory effect on enzyme production. Ni^{2+}, Cd^{2+}, Zn^{2+} and Hg^{2+} ions strongly inhibited the enzymatic activity by 82, 91, 100, and 100%, respectively (De Azeredo *et al.*, 2004). According to a study by Asgher *et al*. (2007), Ni^{2+} and Cd^{2+} slightly inhibited amylase activity in *Bacillus sp*. TS-23. Rao and Satyanarayana (2007) reported a strong inhibition by Sn^{2+}, Hg^{2+} and Pb^{2+} while it was unaffected by the presence or absence of Ca^{2+}. Among the different divalent ions studied by Premila (2013), Se^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} showed an inhibitory effect. According to a study by Singh *et al*. (2014) of the various metal salts and chemical reagents tested, it was found that the α-amylase activity was almost completely inhibited by (5mM) Pb^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, Hg^{2+}, Sn^{2+}, Cr^{3+}, and Al^{3+} metal ions. Ag^{+} and Fe^{2+} inhibited α-amylase activity up to 40.27% and 50.96%, respectively.

Zn^{2+} has varied effect on different amylases. Zn^{2+} was reported to inhibit thermostable α-amylases from a thermophilic *Bacillus* sp. suggesting that the inhibition with the ion determined the thermostability of enzyme. The presence of Zn^{2+} decreased the activity of ANT-6 enzyme indicating its high thermostability (Burhan *et al.*, 2003).

In order to have applications in detergent industries, amylase must be stable to various detergent ingredients, such as surfactants. With respect to the effect of surfactants, α-amylase showed 40.92%, 47.37%, 34.21% and 36.52% relative activity in presence of Triton X-100 (1% v/v), Tween 80 (1% v/v), SLS (5mM), and glycerol (1% v/v), respectively (Singh *et al.*, 2014). Chelating agent EDTA completely inhibited α-amylase activity, as reported by Rao and Satyanarayana (2007); Chakraborty *et al*. (2009) and Chavan and Wadatkar (2014). The inhibitory effect of EDTA provides evidence that the enzyme's activity is metal dependent. The denaturation of the α-amylase activity in the presence of urea showed that the enzyme consists of hydrophobic amino acid composition (Arikan, 2008). The differences in nutritional requirements of

various α-amylase producing organisms could be attributed to the differences in their genetics (Rasooli *et al.*, 2008).

2.4 OPTIMIZATION OF PARAMETERS FOR ENHANCED AMYLASE PRODUCTION

Microbial amylases with great success have replaced the chemical hydrolysis of starch in the starch processing industry. It is because of the fact that use of microorganisms is not only economical but also eco-friendly. Microbial amylases are the most produced and used in industry, due to their productivity, efficiency and thermostability (Burhan *et al.,* 2003). Industrially important enzymes including amylases have traditionally been obtained from submerged cultures because of an ease in handling and control of environmental and nutritional factors. The most frequently used operation in biotechnology is to improve the fermentation conditions for maximizing cell density, high level of desired metabolic product or enzyme levels in microbial system (Mukhopadhyay *et al.*, 2008).

Enzyme overproduction can be achieved by both genetic manipulations and media engineering. Recombinants to increase enzyme production may not be stable. As excretion of products of metabolism is a part of survival strategy of microbes in certain environments, overproduction of enzymes by media manipulation may be considered a better strategy (Dey *et al.,* 2001). Culture conditions play a significant role on growth and production of amylase. Amylase production is affected by a variety of factors such as pH, temperature, incubation time, substrate concentration etc. The medium optimization is an important aspect to be considered in the development of fermentation technology.

2.4.1 Optimization by using One Variable at a Time (OVAT) Approach

Optimization of medium by the classical method involves changing one independent variable keeping the other factors constant.

Singh *et al*. (2010) described the production of α-amylase from agricultural byproducts by *B. cereus* MTCC 1305 in solid state fermentation (SSF) using wheat bran as a substrate. Effects of process variables, namely

incubation time, temperature, initial moisture content, pH of medium, supplementary carbon source, supplementary nitrogen source, and inoculum level on production of α-amylase were studied, and accordingly optimum conditions were determined. It was found that α-amylase production was the highest at 80^{th} hour of incubation, 55°C incubation temperatures, and substrate: moisture ratio 1:1, pH of 5.0 and 10% inoculum level. Glucose (0.05 g/g) has been found the best supplementary carbon source. Supplementation of different nitrogen sources (0.02 g/g) showed decline in enzyme production. Allah *et al*. (2011) reported the production of α-amylase from *B. coagulans* isolated from soil. Conditions for production of enzyme were optimized utilizing OVAT approach. Maximum amylase activity of 31.67 U/ml was obtained at 30 ^{0}C and pH 5 with a substrate concentration of 0.75% (w/w).

Hashemi *et al.* (2011) investigated the production of crude Ca^{++} independent and low pH active α-amylase by *Bacillus* sp. KR-8104 in submerged fermentation (SmF) and solid-state fermentation (SSF) systems. Different parameters were evaluated in each system using one factor at a time (OFAT) approach to improve the production of enzyme. The results showed that in SmF the maximum enzyme production was achieved in culture medium that contained dextrin as a carbon source, as well as yeast extract and meat extract as nitrogen sources incubated at 37ºC and 180 rpm for 48 h. While SSF of *Bacillus* sp. KR-8104 using wheat bran (WB) as a substrate showed that using tap water or distilled water as a moistening agent, a substrate-water ratio of 1:1.5 (w/v) and incubation at 37ºC for 48 h gave the maximum α-amylase production.

Different fermentation parameters for extracellular amylase production from *Aspergillus versicolor* were evaluated by employing submerged fermentation method (Jayaramu *et al.*, 2011). The amylase producers were detected by a clear zone around the colony by simple plate assay method. There was a considerable increase from 3.89 IU to 6.21 IU in amylase production was observed after the optimization of fermentation parameters i.e. the optimum pH 4.0, temperature 30^0C and inoculum size 1.0 ml.

Soniyamby *et al*. (2011) reported twelve amylolytic bacterial strains isolated from marine water. Among them isolate KUMB07 exhibiting highest amylase activity was identified as *Bacillus* sp. A further optimization study was done by solid state fermentation using wheat bran as a substrate. 35^0C temperature, 7.0 pH and 48 h of incubation period was found to be the best for highest amylase activity of 62.81 U/g. To exploit the potential of thermotolerant bacteria for the production of starch degrading enzymes, soil samples were explored by Zohra and Ahmad (2012). Physical and biochemical parameters of the highest enzyme producing strain, affecting enzyme production were optimized. The strain was identified as *Bacillus* sp. showing highest amylase activity at 55^oC and pH 7.0. Starch and tryptone were adjudged as best carbon and nitrogen sources respectively for amylase production with yeast extract, $MgSO_4$ and K_2HPO_4 as media additives.

2.4.2 Optimization by using Response Surface Methodology (RSM)

The conventional methods for multifactor experimental design are time-consuming and incapable of detecting the true optimum, due especially to the interactions among the factors. In fermentation process, the operational variables interact and influence thereby affecting the response. The optimization method should be such that it accounts for interactions among variables so that a set of optimal experimental condition can be determined (Silva and Roberto, 2001). This limitation of a single factor optimization process can be eliminated by different techniques. A collection of experimental strategies, mathematical methods and statistical inference is used to explain the combined effects of all the factors in a fermentation process.

One of the most used statistical techniques Response Surface Methodology (RSM) includes factorial design and regression analysis which helps in evaluating the effective factors and their interaction and to find out the optimum conditions of variables for a desirable response (Tunga *et al.,* 1999; Coninck *et al.,* 2000; Reddy *et al.,* 2003; Kunamneni *et al.,* 2005; Gangadharan *et al.,* 2008). Response Surface Methodology is a collection of statistical techniques for designing an experiment, building models, evaluating the effects of factors

and searching for optimum conditions for desirable responses. For optimization of process parameters, RSM is more satisfactory than classical methods such as one-variable-at-a-time (OVAT) or mathematical models because it can study many variables simultaneously with low number of observations, saving time and costs. Central composite design (CCD) is one of the response surface methodologies usually utilized to obtain data that fits a full second-order polynomial equation. The graphical representation of the model equation results in response surface plots that represent the individual and interactive effects of test variables on the response.

Response Surface Method (RSM), as an experimental strategy for seeking the optimum conditions of a multivariable system, is a much more efficient methodology for optimization (Tanyildizi *et al.,* 2005). Recently, a number of statistical experimental designs with response surface methodology have been employed for optimizing enzyme production from microorganisms (Koteswara *et al.,* 2006; Thys *et al.,* 2006; Zambare, 2011; Mohandas *et al.,* 2010). However, 3D and counter plots for response surfaces can provide a good way for visualizing the parameter interaction. Therefore, statistical technique is often used for predicting optimum process conditions for microbial enzyme production (Mullai *et al.,* 2010). It is well known that extracellular enzyme production in microorganisms is greatly influenced by nutritional factors like carbon sources, nitrogen sources and mineral salts (Dey *et al.,* 2001; Adinarayana and Elliaiah, 2002; Chauhan and Gupta, 2004). Enhancement in extracellular amylase production from *Bacillus* sp. by environmental factor optimization has not been attempted so far (Zambare, 2011).

Tanyildizi *et al.* (2005) studied the combined effects of macronutrients of media on α-amylase production by *Bacillus* sp. using response surface methodology. This procedure limited the number of actual experiments performed while allowing for possible interactions between four components. A 22 full-factorial central composite design was chosen to explain four medium constituents, viz. starch, glycerine, yeast extract and peptone. The optimal combinations of media constituents for maximum α-amylase production were

determined as 17.58 g/l starch, 12.37% (v/v) glycerine, 8.77 g/l peptone and 0.00 g/l yeast extract.

Production of amylase under submerged fermentation using *Bacillus* sp. was investigated by Zambare (2011) using wheat bran, soybean meal and $CaCO_3$. Response surface methodology (RSM) was used to evaluate the effect of the main variables, i.e., pH (11.35), temperature (35.16°C) and inoculum size (2.95%) on amylase production by applying a full factorial central composite design (CCD). The mutual interaction between these variables resulted into 4.64 fold increase in amylase activity as compared to the non-optimized environmental factors in the basal medium. A work by Gangadharan *et al.* (2008) was aimed to optimize the cultural and production parameters through the statistical approach for the synthesis of α-amylase by *B. amyloliquefaciens* in submerged fermentation (SmF) using a combination of wheat bran and groundnut oil cake (1:1) as the substrate. The process parameters influencing the enzyme production were identified using Plackett–Burman design. Among the various variables screened, the substrate concentration, incubation period and $CaCl_2$ concentration were most significant. The optimum levels of these significant parameters were determined employing the response surface Box–Behnken design, which revealed these as follows: substrate concentration (12.5%), incubation period (42 h) and $CaCl_2$ (0.0275 M).

Screening of important nutrient parameters from selected seven medium components viz. starch, yeast extract, tryptone, K_2HPO_4, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and KCl were carried out in shake flask cultures for enzyme production using Plackett-Burman experimental design of twelve trials (Bansode, 2010). Among these starch, yeast extract and $CaCl_2.2H_2O$ contributed to large extent; tryptone and K_2HPO_4 had moderately significant efffect; while $MgSO_4.7H_2O$ and KCl had minute effect for amylase production by submerged fermentation. Prajapati *et al.* (2014) optimized medium components through the statistical approach for the synthesis of α-amylase by *B. amyloliquefaciens* KCP2 under solid-state fermentation using wheat bran as the substrate. The medium components influencing the enzyme production were identified using a two-level fractional factorial Plackett-Burman design. Among the various variables

screened, starch, ammonium sulphate and calcium chloride were found to be the most significant medium components. The optimum levels of these significant parameters were determined employing the response surface central composite design which significantly increased the enzyme production with the supplementation of starch 0.01 g, ammonium sulphate 0.2 g and 5 mM calcium chloride in the production medium.

Ca-independency with potential activity and stability at low pH are among the most interesting characteristics of α-amylase in starch industry. In this attempt the synergetic effect of low pH on activity of crude Ca-independent α-amylase isolated from a native *Bacillus* sp. KR-8104 in solid-state fermentation (SSF) was studied by Hashemi *et al*. (2010) using wheat bran (WB) as a substrate. The effects of different parameters including moisturizing agents, solid substrate to moisture ratio, particle size, incubation temperature and period, inoculum (v/w) and supplementation with 1% (w/w) different carbon and nitrogen sources on enzyme production were investigated. Response surface methodology (RSM) was applied to find optimum conditions of temperature and pH on crude amylase activity. Using central composite design (CCD) a quadratic mathematical model equation was derived for the prediction of enzyme activity. The results showed that the model was in good agreement with experimental results, with R^2 -0.90 and the low pH has a synergetic effect on enzyme activity at higher temperature.

Rao *et al*. (2013) isolated twenty bacterial strains from the soil collected from Andhra Pradesh and screened them for amylase production, Four isolates showed amylolytic activity. One isolates (N3) displaying the highest activity was selected for further study. The amylolytic bacterium was identified as *Bacillus* sp. VITRKHB. The maximum enzyme activity was observed in case of starch as carbon source and yeast extract as nitrogen source. Response surface methodology was employed to evaluate combined interactive effects of different variables, i.e., starch (0.21%), yeast extract (0.65%), pH (6.13), NaCl (4.63%) and temperature (45°C) on amylase production after 24h of incubation. Determination of co-efficient (R^2 =0.8056) close to 1.0 indicated significant precision and reliability of the response data.

2.5 PURIFICATION OF ENZYME

Industrial enzymes produced in bulk generally require little downstream processing and hence are relatively crude preparations. The commercial use of α-amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases (Gupta *et al.*, 2003). The enzyme in purified form is also a prerequisite in studies of structure-function relationships and biochemical properties. The purification of α-amylase from microbial sources in most cases has involved classical purification methods. These methods involve separation of the culture from fermentation media, selective concentration by precipitation using ammonium sulphate or organic solvents such as chilled acetone (Gupta *et al.*, 2003). The crude enzyme is then subjected to chromatography, usually affinity, ion exchange and/or gel filtration.

The used methods to purify amylases can vary considerably, but most purification protocols involve a series of steps. The design of an appropriate procedure for isolation of a given protein should be tailored in accordance with the objective(s) of the research project, which may require relatively pure product in modest amounts for analytical purposes (e.g. enzyme kinetics) or a highly purified, homogeneous preparation for physicochemical or structural studies. Isolation and purification of a single protein from cells containing a mixture of thousands of unrelated proteins is achievable because of the remarkable variation in the physical and chemical attributes of proteins.

Characteristics unique to each protein—amino acid composition, sequence, subunit structure, size, shape, net charge, isoelectric point, solubility, heat-stability, hydrophobicity, ligand/ metal binding properties and post-translational modifications—can be exploited in formulation of a strategy for purification. Based on these properties a combination of various methods (Table.2) can be used for separation of cellular proteins.

Table 2. Principle separation methods used in purification of enzymes (Deutscher, 1990; Ausubel *et al.*, 1991)

Property	Method
Size or mass	Centrifugation Gel filtration Dialysis, Ultrafiltration
Polarity	
(a) Charge	Ion-exchange chromatography Chromatofocusing Electrophoresis Isolelecteric focusing
(b) Hydrophobic chatracter	Hydriphobic chromatography
Solubility	Change in pH Change in ionic strength Decrease in dielectric constant
Specific binding sites of structural features	Affinity chromatography Immobiolized metal ion chromatography Affinity elution Dye-ligand chromatography Immunoadsorption Covalent chromatography

2.5.1 Precipitation of proteins: Salting Out

Several methods are available for precipitation of proteins utilizing changes in pH and temperature, or addition of salts and organic solvents. Most proteins are less soluble at high concentrations of salt an effect called *salting out*. It is the most frequently used method for precipitation of proteins by using Ammonium sulphate. The salt concentration at which a protein precipitates varies from one protein to another, hence salting out can be used to fractionate proteins. At saturation (3.9 M at 0^oC and 4.04 M at 20^oC) it precipitates most proteins and protects proteins in solution from denaturation and bacterial growth. To avoid surface denaturation, the solution should not be stirred vigorously and $(NH_4)_2SO_4$ should be added gradually, in small amounts, allowing each successive batch to dissolve completely before addition of the next. Salting out is also useful for concentrating dilute solutions of proteins, including active fractions obtained

from other purification steps. Dialysis can be used to remove the salt if necessary (Bollag and Edelstein, 1991a; Wilson, 2010).

2.5.2 Dialysis

Proteins can be separated from small molecules by dialysis through a semipermeable membrane, such as a cellulose membrane with pores. Molecules having dimensions significantly greater than the pore diameter are retained inside the dialysis bag, whereas smaller molecules and ions traverse the pores of such a membrane and emerge in the dialysate outside the bag (Fig. 3). This technique is useful for removing a salt or other small molecule, but it will not distinguish between proteins effectively (Stryer *et al.*, 2002; Wilson, 2010).

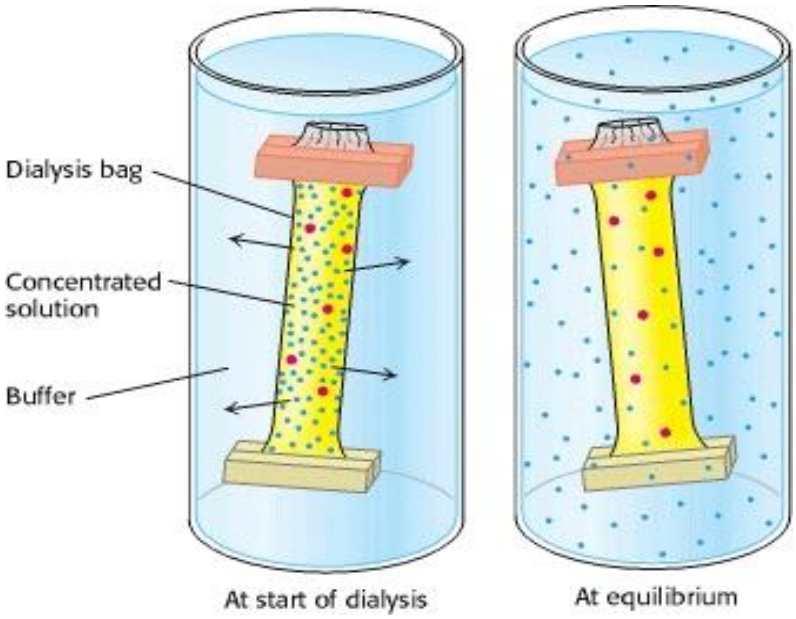


Fig.3 Dialysis. Protein molecules (red) are retained within the dialysis bag, whereas small molecules (blue) diffuse into the surrounding medium (Stryer *et al.*, 2002).

2.5.3 Gel-Filtration Chromatography

More discriminating separations on the basis of size can be achieved by the technique of gel-filtration chromatography (Fig. 4**).** The sample is applied to the top of a column consisting of porous beads made of an insoluble but highly hydrated polymer such as dextran or agarose (which are carbohydrates) or polyacrylamide. Sephadex, Sepharose, and Bio-gel are commonly used

commercial preparations of these beads, which are typically 100 μm (0.1 mm) in diameter. Small molecules can enter these beads resulting in the distribution of small molecules in the aqueous solution both inside the beads and between them. Larger molecules however unable to enter the beads are located only in the solution between the beads. Thus, large molecules flow more rapidly through this column and emerge first because a smaller volume is accessible to them. Molecules that are of a size to enter a bead will flow from the column at an intermediate position, and small molecules, which take a longer path, will exit last (Stryer *et al.*, 2002; Cutler, 2004; Wilson, 2010).

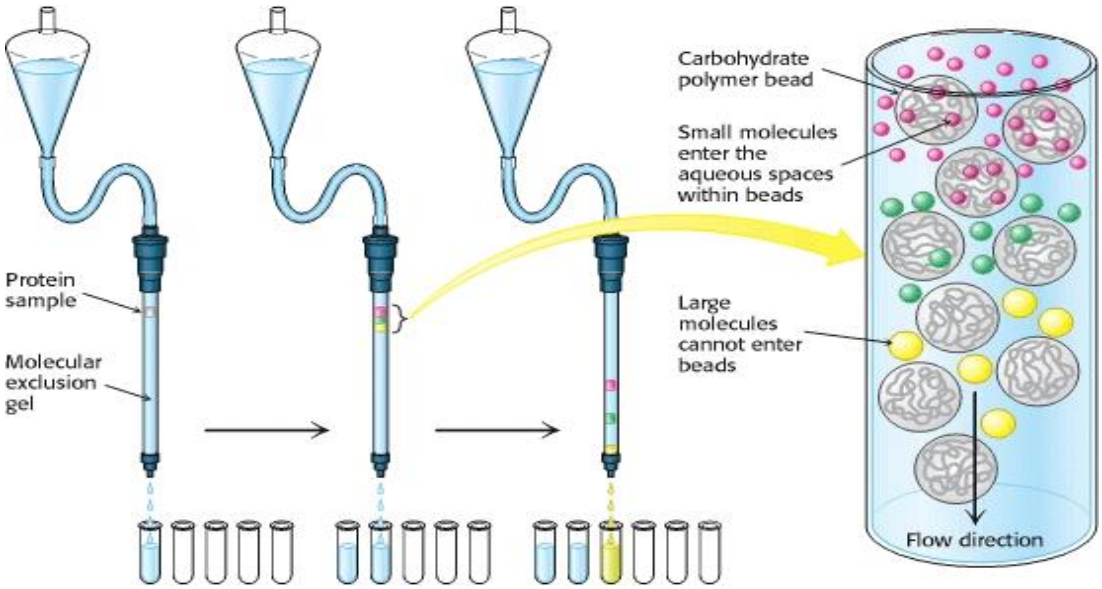


Fig.4 Gel Filtration Chromatography (Stryer *et al.*, 2002)

2.5.4 Ion-Exchange Chromatography

Proteins can be separated on the basis of their net charge by ion-exchange chromatography. If a protein has a net positive charge at pH 7.0, it will usually bind to a column of beads containing carboxylate groups, whereas a negatively charged protein will not. A positively charged protein bound to such a column can then be eluted (released) by increasing the concentration of sodium chloride or another salt in the eluting buffer because sodium ions compete with positively charged groups on the protein for binding to the column. Proteins that have a low density of net positive charge will tend to emerge first, followed by those having

a higher charge density. Positively charged proteins (cationic proteins) can be separated on negatively charged carboxymethyl-cellulose (CM-cellulose) columns. Conversely, negatively charged proteins (anionic proteins) can be separated by chromatography on positively charged diethylaminoethyl-cellulose (DEAE-cellulose) columns (Stryer *et al.,* 2002; Wilson and Walker, 2010).

There are several reports in the literature regarding the purification of α-amylase produced by different bacterial isolates to electrophoretic homogeneity by a series of sequential steps. As stated earlier *Bacillus* sp. is the most sought after for α-amylase production. Various workers have reported successful extraction and then purification and characterization of amylase from *Bacillus* sp. An investigation by Ul-Haq *et al*. (2010) deals with the purification and characterization of α-amylase from a mutant strain of *B. licheniformis* EMS-6. The enzyme was purified up to homogeneity by ammonium sulphate and ion-exchange chromatography using a fast protein liquid chromatography (FPLC) system with a yield of 40.4%. The purification fold by RESOURCE-S was recorded to be 3.58. The molecular weight was found to be 55 KDa and the enzyme was stable over a pH range of 4.5-9.0 with pH optimum of 7.0. Kinetic parameters, V_{max} (2778 U/mg/min) and K_m (8.3mg/ml) of α-amylase were derived from the Lineweaver Burke plot. Hassan *et al.* (2011) isolated *Bacillus* sp. RM16 from a hot spring in Karachi and screened it for the production of α-amylase. Enzyme Amy RM16 was purified by precipitation with ammonium sulfate at 70% saturation, Q-Sepharose, phenyl Sepharose and reversed phase chromatography. Purified enzyme with a 17 fold final purification was obtained with a total yield of 1.35% with specific activity of 6380U/mg. An extracellular amylase from *B. subtilis* was partially purified to 1.28-fold by acid treatment with ice cold 1.0N HCl and 6.4-fold by gel filtration with Sephadex G-150 (Olufunke and Azee, 2013). The purified amylase had a molecular weight of 39.4 kDa .The enzyme had its optimal activity at pH 5.0 and temperature 50^{o}C. The activity of the enzyme was enhanced by Na^{+}, Ca^{2+} and ethylene diaminetetraacetic acid (EDTA), while Hg^{2+} ,Mg^{2+} and Fe^{2+} acted as inhibitors of its activity. The amylase had an apparent Michaelis constant Km of 5.0 mg/ml and maximum velocity (Vmax) of 50 U/mg proteins.

The study by Fincan *et al*. (2014) reported the purification and characterization of thermostable α-amylase (α-1-4 d-glucanglucanohydrolase EC 3.2.1.1) from a newly isolated *Anoxybacillus flavithermus*. Maximum α-amylase activity was obtained at 55^0C at pH 7.0 after 24 h of incubation. Thermostable α-amylase from *A. flavithermus* was purified by 70% $(NH_4)_2SO_4$ and ion-exchange chromatography (5.2-fold; 65.8% yield). The molecular weight of α-amylase was 60 kDa, as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Roy *et al*. (2014) investigated the partial purification and characterization of a new strain of *B. megaterium* strain KAN1. The enzyme was partially purified by ammonium sulphate precipitation having a specific activity of 1.05 Umg^{-1}. The optimum pH of the purified enzyme was 11, but the enzyme can work effectively in the pH range of 5.0–14.0. The temperature optimum for enzyme activity was 60°C, although the effective activity was seen in a wide range of temperature. The K_m and V_{max} values for soluble starch substrate were 0.65 mg/ml and 1.568 mg min^{-1}. Electrophoresis with SDS-polyacrylamide showed that the molecular weight of the enzyme was 67 kDa. Xie *et al*. (2014) purified α –amylase from *B. methylotrophicus* P11-2 to homogeneity by a combination of 80% $(NH_4)_2SO_4$ precipitation, DEAE FF anion exchange, and superdex-75 10/300 GL gel filtration chromatography. The purified α-amylase exhibited specific activity of 330.7 U/mg protein that corresponds to 13.1 fold purification. The relative molecular mass of α -amylase was 44.0 kDa by SDS-PAGE. The optimal pH and temperature for enzyme activity were 7.0 and 70^oC, respectively. The α-amylase activity was stimulated by Mg^{2+}, Ba^{2+}, Al^{3+} and dl-dithiothreitol (DTT), however, Ca^{2+} almost had no activation or inhibition on the α-amylase.

The enzyme obtained from *Pseudomonas* sp. from rhizosphere by Varalakshmi *et al*. (2012) had been used for amylase production at 37 ºC and pH 7.5. It was partially purified using ammonium sulphate precipitation, and was further purified by ion exchange DEAE-cellulose column chromatography. The purity of the enzyme was 45.2 fold greater than the crude enzyme. The molecular weight of the enzyme was found to be ~62 kDa by SDS-PAGE.

Several other reports are also there regarding the isolation and purification of amylase from sources other than microbial origin. Kanwal *et al.* (2004), for an instance have homogenized *Malus pumila* (apple) in buffer for α-amylase extraction. The crude extract showed 3.09 U/ml enzyme activity that was subjected to ammonium sulfate precipitation and sephadex G-150 gel filtration chromatography. A final enzyme activity of 5.02 U/ml and specific activity 38.95 U/ml with 20-fold purification have been observed. SDS-PAGE of enzyme showed that unwanted proteins are removed and clear band of enzyme appeared. Molecular weight was determined by sephadex G-150 column that is 51,180 dalton. Amylase showed a pH optimum of 6.8, temperature 37^{o}C, K_m value of $2.0x10^{-3}$g/ml. Another study by Azad *et al.* (2009) reported isolation and characterization of a novel thermostable α-amylase from Korean pine seed. Enzyme was purified to homogeneity with purification fold of 1286.1 using several techniques such as self-precipitation, $(NH_2)_2SO_4$ fractionation, DEAE anion exchange and starch affinity chromatography. The purified α-amylase showed two bands in SDS-PAGE with molecular weight of 44 and 45 kDa. The apparent molecular weight of native enzyme was calculated to be 46.7 kDa. The optimum pH and temperature for enzyme activity were pH 4.5 and 65^{o}C, respectively. This enzyme was fully stable for 48 h at 50^{o}C and retained 80% activity up to 96 h. The K_m and V_{max} were 0.84 mg/ml and 3.71mmol/min, respectively. α-amylase extracted from (*Solanum tuberosum* L) was purified by successive chromatography on DEAE and CM-cellulose columns with a yield of 24.24% (Sarker*et al.,* 2010). SDS-PAGE showed a molecular weight of 44 kDa for the enzyme that contain 2.8% sugar. The enzyme displayed optimum activity at pH 7.2 and 37^{o}C. The enzyme was strongly inhibited by Cu^{2+}, Fe^{2+} and Zn^{2+}; moderately by Li^{+}, Hg^{+} and Cd^{2+}; and slightly by Ag^{+}, K^{+}, Mn^{2+} and Mg^{2+}. Conversely, Fe^{2+} and Na^{+} appreciably enhanced activity, while adding calcium ion nearly doubled enzyme activity. In a similar study, Usha *et al*. (2011) purified α-amylase to homogeneity from little millet (*Panicum sumatrense* L. Roth ex Roem. et Schult.) by successive chromatography on DEAE-cellulose and Sephadex G-150. Purification achieved was 10.15 fold from the crude extract with a yield of 29% giving a final specific activity of 1001U/mg protein. SDS-PAGE showed a molecular weight of 46 kDa for the enzyme. The enzyme

displayed optimum activity at pH 5.0 and 50°C with an apparent *K*m value of 1.6 mg for soluble starch as substrate and V_{max} 1388 units/min/mg protein. Significant enhancement in the enzyme activity was observed in the presence of metal ions like Ca^{2+} and Ba^{2+} while metal ions such as Fe^{2+}, Hg^{2+} and Al^{3+}completely inactivated the enzyme. Kharkrang and Ambasht (2012) purified α-amylase from pearl millet (*Pennisetum typhoides*) to homogeneity through several steps involving change of pH (pH 7.0 to 6.0), lyophilization (-55 ºC), polyethylene glycol precipitation (3-15%), Sepharose 6B column chromatography and CM-cellulose column chromatography. The 24-fold purified enzyme had specific activity of 580 units/mg protein. Native PAGE revealed a single protein band and activity staining revealed that the band corresponds to alpha amylase. SDS-PAGE also revealed a single protein band. The native molecular mass determined by gel filtration was 33 kDa. The K_m and V_{max} values were 0.5 mg/ml and 603 µmol/min/mg respectively. The k_{cat} was 335/sec. However, divalent cations namely, Co^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+} at 1mM concentration, activated the enzyme activity. Mn^{2+} inhibited the enzyme activity at the same concentration. NH^{4+} showed activation of the enzyme activity, Li^{+} and K^{+} showed inhibition.

2.6 IMMOBILIZATION OF ENZYMES: IMPORTANCE AND ADVANTAGES

Enzymes are protein molecules which serve to accelerate the chemical reactions of living cells (often by several orders of magnitude). Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes display great specificity and are not permanently modified by their participation in reactions. Since they are not changed during the reactions, it is cost-effective to use them more than once. However, if the enzymes are in solution with the reactants and/or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term immobilized means unable to move or stationary.

Over the last few years, there has been considerable interest and progress in the field of immobilized cell technology. In immobilization, the cells are

attached to or entrapped in an inert support material. According to Riaz *et al*. (2009), a biocatalyst is termed as immobilized if its mobility is restricted by chemical means and is a process that often mimics what occurs naturally when cells grow on surfaces or within natural structures. Therefore, the immobilized enzyme may be defined as 'the enzyme physically confined or localized in a certain defined region of space with retention of their catalytic activity'. The basic principle of immobilization is to restrict the gross movement of an enzyme and keep it in relatively defined region through its attachment on a suitable support matrix. This separates the enzymes into a distinct solid phase, which is different from the bulk phase containing substrate and product. Attaching an enzyme to an insoluble matrix facilitates their reuse because large enzyme matrix complex can be recovered easily and non-destructively by coarse filtration (D'Souza, 1989).

Enzymes can be immobilized to a multitude of different carriers by entrapment, adsorption, ionic binding and covalent binding. This is a method for protecting and stabilizing the enzymes, thereby enhancing their properties and their repetitive utilization either in batch, or continuous mode. Immobilization of enzymes prevents their deactivation by various physical and chemical denaturing agents and thereby enhancing their operational stability (D'Souza, 1989; Chaudhary and Chincholkar, 1996; Dhanasekaran *et al.,* 2006; Devi *et al.,* 2012). The main advantages of immobilization include:

- Easy separation from reaction mixture, providing the ability to control reaction times and minimize the enzymes lost in the product.
- Re-use of enzymes for many reaction cycles, lowering the total production cost of enzyme mediated reactions.
- The use of immobilized enzymes facilitates the easy separation of the product from the supernatant broth, thereby making the product extraction much easier and efficient (Fukui and Tanaka 1982; D'Souza 1989; Pandey *et al*. 2000c).
- The ease of conversion of batch processes into a continuous mode and maintenance of high cell density without washout conditions even at very

high dilution rates are the other advantages of this cell system (Pandey *et al*. 2000c).

Immobilizations of enzymes have been used in a wide spectrum of applications such as production of antibiotics, biosensors, degradation of phenols, ethanol fermentation (Manjula *et al.,* 2009) in drugs and pharma industry and other biochemical technologies etc. The immobilized enzyme system has remarkable advantages over the conventional methods especially with respect to production cost, high productivity, reduced duration of fermentation and ease to control fermentation process. Immobilization of enzymes also tends to enhance the stability of the enzymes by retaining their natural catalytic surroundings. The bound cell systems are far more tolerant to the perturbations in the reaction environment like temperature and pH variations and less susceptible to the toxic substances present in the liquid medium in which a soluble enzyme can be rendered inactive (Bihari, 1985). The products formed are generally pure which encourage the utilization of this technique in bioprocessing for food and pharmaceutical industries (Pandey *et al.*, 2000c).

2.6.1 Methods of cell immobilization

The objective of immobilization of enzymes is to allow their extended use as catalysts. The technique for immobilization increases the stability of catalyst inside the porous texture of the support (Kiyohara *et al.,* 2003). Several chemical and physical techniques have been used to immobilize enzymes to accomplish a wide variety of reactions at industrial or analytical levels. These techniques have been used extensively in the process of fermentation, antibiotics and secondary metabolites production, synthesis of organic acids and drugs. These have also been used in food industry, bioremediation and for selective treatment of pollutants (Vullo and Waschman, 2005).

The method of immobilization should be such that enzymes face as little conformational change as possible (Riaz *et al*., 2009). Selection of a technique for immobilization depends upon the nature of enzyme, nature of chemical conversion and the substrate characteristics.

The immobilization methods can be classified into four categories i.e. entrapment, covalent binding, cross linking and adsorption (Vullo and Waschman, 2005). A combination of two or more of these methods can also be employed. For immobilization, enzymes are attached in distinct solid phase or a carrier. The carrier may be defined as the solid phase molecule on which the enzymes attach through various phenomenons like adsorption, cross-linking, entrapment and covalent binding (Ramakrishna and Prakasham, 1999). Alpha-amylase has been immobilized onto a wide variety of organic and inorganic supports, as for example silica gel (Saville *et al.*, 2004), chitosan (El-Ghaffar and Hashem, 2009), and glass.

The most extensively studied and frequently used method in enzyme immobilization is the entrapment of enzyme in the polymer matrices (Pandey *et al*., 2000c). Entrapment of enzyme with natural polymers such as agar, agarose, alginate etc. is done by ionotropic or thermal gelation (Manjula *et al.*, 2009). These entrapment methods are based on the inclusion of enzyme within a rigid network to prevent the enzyme from diffusing into surrounding medium while still allowing the penetration of substrate (Fukui and Tanaka, 1982). Hence, the basic principle behind the entrapment technique is to form a polymeric network around the material to be trapped. The resulting gel must have sufficient porosity to allow the transportation of substrates in and products out while retaining the enzymes. This network is irreversibly formed and is inert. This technique imposes minimal constraints on the enzymes. The enzymes are free within their compartment and can undergo for post immobilization modification with simultaneous entrapment (Ramakrishna and Prakasham, 1999).

Though entrapment of microbial enzymes in polymer gels is the choice for many and has been the most widely accepted technique, it might not be the best as this technique faces many constraints. Excessive enzyme loading in a gel may disrupt the matrix and allow enzyme leakage (Yokotsuka *et al.*, 1997; Fumi *et al*. 1998). The disruption of gel beads due to the formation of CO_2 during ethanol fermentation is another drawback of gel-entrapped enzymes (Godia *et al.*, 1991). In case of aerobic organisms like those employed in antibiotic production,

the limited O_2 supply in gel matrix adversely affects their performance (Dhanasekaran *et al*., 2006). The conversion rate of most of the immobilized enzymes not only depends upon the availability of substrates and microbial kinetics, but also on diffusion rate of metabolites through immobilized enzyme system (Wada *et al.*, 1980; Chibata *et al*., 1983). Hence, in many studies the rate-limiting step is the diffusion of substrate through the solid support (Pilkington *et al*., 1998).

2.6.2 Choice of support material for immobilization

The nature of the solid support or matrix plays an important role in retaining the actual confirmation and activity of enzyme in the processes that utilized immobilized biocatalysts (Riaz *et al.,* 2009). A support material should meet certain criteria for its preference for immobilization. The mechanical stability of the gel plays an important role. The gels must withstand elevated temperature or extremes of pH or high substrate and product concentrations, as reported by D'Souza (1989). The support should present large internal surface to have good geometrical congruence with the enzyme surface (Hanfeld *et al.,* 2009). If the support is formed by very thin fibers, e.g., smaller than the protein, it will hardly be possible to get an intense interaction between the enzyme and the support (Zhang *et al.,* 2013). Another criterion for selecting a carrier is its resistance to chemical degradation. The other common features of a good support material especially in case of enzyme entrapment are the ease of diffusibility of substrates and the products through beads, facilitated activity of entrapped enzyme and mildness of immobilization procedure (Leenan *et al*., 1996). Lack of toxicity in the carriers should be an important consideration in case of food technology (Vullo and Waschman, 2005). The reactive groups in the protein and the support should present minimal steric hindrances in the reaction, because after the initial immobilization, multipoint covalent attachment requires the contact between groups bound to rigid structures (Zhang *et al.,* 2013).

Various natural polymers like alginate, carrageenan, cellulose, chitin, chitosan, agar, agarose, gelatin and synthetic polymers like polyacrylamide have been extensively investigated for the entrapment of cells (Riaz *et al.,* 2009;

Demirkan *et al.*, 2011; Prakash and Jaiswal, 2011; Jaiswal *et al.*, 2012; Talekar and Chavare, 2012; Zhang *et al.*, 2012). For successful immobilization, hydrogels must be conducive to enzyme viability and function (Jen *et al.*, 1996). The enzyme immobilized in these hydrogels is able to survive the immobilization procedures and retain their catalytic activity over a longer period, despite their repeated use (Fukui and Tanaka, 1982).

Table 3. Classification of Supports (Brena and Viera, 2006)

Organic
Natural polymers • Polysaccharides: Cellulose, Dextrans, Agar, Agarose, Chitin, Gelatin • Proteins: Collagen, Albumin • Carbon **Synthetic polymers** • Polystyrene • Other polymers: Polyacrylate Polymethacrylates, Polyacrylamide, Polyamides, Vinyl and Allyl-polymers
Inorganic
• Natural minerals: Bentonite, Silica • Processed materials: Glass (nonporous and controlled pore), Metals, Controlled pore metal oxides.

2.6.2.1 Alginate

Entrapment of enzymes in calcium alginate is one of the most widely used methods for enzyme immobilization. It is a fast and simple technique using mild conditions (Kierstan and Bucke, 1977). Alginate is extracted from algae *Laminaria hyperborean, Macrocystis pyrifera* and *Ascopyllum nodosum*(Gemeiner*et al*., 1994). It is one of the oldest polymers to be used for microbial cell and enzyme entrapment (Bucke, 1987). Alginates constitute a family of unbranched copolymers of 1,4-linked β D-mannuronic (M) acid and 1, 4-linked α L-guluronic acids (G) groups. High guluronic acid (G) alginates have enhanced ability to form gels (Martinsen *et al.*, 1989). The quality and strength of matrix improves with an increasing amount of guluronic acid groups in the alginate (Smidsrod and Skjak-Braek, 1990).

Gels are formed due to binding of divalent cations to guluronic acid groups. Sodium alginate reacts with Ca^{++} ions forming a network (gel) which is thermo-irreversible. The ability of beads to maintain their physical form is directly related to the alginate concentration used in the bead formation. With an increase in alginate concentration, the beads become more stable in retaining the microbial cells. However, with this increase, the diffusion of substrates into and products out of beads is affected (Williams and Munnecke, 1981).

Alpha-amylase from *B. Subtilis* KIBGE-HAR was partially purified by 40% ammonium sulfate upto 7 folds and then immobilized by entrapment in calcium-alginate beads (Riaz *et al.,* 2009). The catalytic properties of the immobilized α-amylase were compared with that of the free enzyme and it was found that the immobilized enzyme was much hardy than the free enzyme. The optimum pH of the free enzyme was 7.0 while that of immobilized enzyme was pH 7.5. The optimum temperature for free and immobilized enzyme was 60^0C and 70^0C respectively. The activity yield of the immobilized enzyme was 65%.

Demirkan *et al*. (2011) immobilized α-amylase from *B. amyloliquefaciens* in an aqueous mixture of sodium alginate in the solution of a Ca^{2+} salt. Optimum alginate and $CaCl_2$ concentrations were found to be 2% and 5% (w/v), respectively. The immobilization yield was 89%. Amylase activity increased with increasing enzyme loading on the beads. The best size and amount of beads for achieving enzyme activity were found to be 3 mm and 0.4 g, respectively. When coated with silicate, amylase-entrapped beads retained the highest catalytic activity. The highest operational stability was six reuses with 51% residual activity.

Immobilization of α-amylase using gelatin matrix has been reported by Jaiswal *et al*. (2012) using glutaraldehyde as an organic hardener. Response surface methodology (RSM) and 3-level-3-factor Box–Behnken design was employed to evaluate the effects of immobilization parameters, such as gelatin concentration, glutaraldehyde concentration and hardening time on the activity of immobilized α -amylase. The results showed that 20% gelatin (w/v), 10%

glutaraldehyde (v/v) and 1 h hardening time yielded an optimum immobilization of 82.5%.

Talekar and Chavare (2012) immobilized α-amylase was by entrapment in calcium alginate beads. Three percent (w/v) sodium alginate, 1M calcium chloride and 2 h curing time were used and 90% immobilization yield of α-amylase was achieved with enhanced thermal and acidic condition stabilities. Significant changes in optimum pH and temperature values of the enzyme were recorded after immobilization. The activity of immobilized enzyme was affected by the size of the bead and bead size of 2.4mm was found to be most effective for starch hydrolysis. From the enzyme kinetic study, decrease in substrate affinity and velocity of enzyme reaction were observed after immobilization of enzyme. Immobilized α-amylase retained 35% activity after 10 reuses with 30 min of each reaction time.

2.6.2.2 Agarose and Agar

Agarose is a neutral linear polysaccharide, extracted from the cell walls of certain *Rhodophyceae* algae, made up of basic repeat units of agarobiose (a diasaccharide consisting of 1,3-linked D-galactopyranose and 1,4-linked 3,6-anhydro-α-L galactopyranose), which gives agarose the ability to form strong bonds even at low concentrations. It has a high capacity of protein entrapment owing to its high porosity. Additional advantages include the absence of charged groups which helps in preventing non-specific adsorption of products and substrates, ease of derivatization, hydrophilic character etc. Moreover, the low melting temperature of agarose (62-65^{o}C) is another major factor which favors its applicability (Prakash and Jaiswal, 2011).

Agar is a polysaccharide having strong gelling ability. It is acid stable and shows no protein reactivity. Moreover, it is comparatively cheaper than other hydrogels used for immobilization.

Use of agar and agraose matrix as a carrier for the immobilization of α –amylase has been reported by Prakash and Jaiswal (2011). The optimum pH of α–amylase immobilized on both matrices was 7.0. 1% of agarose (w/v) and 4% of

agar (w/v) yielded an optimum immobilization of about 75 and 77% respectively. The reusability of agarose and agar immobilized enzyme was found to be upto 5 cycles.

2.6.2.3 Immobilization using nanoparticles

The unique physical properties of nanoparticels are allowing their application in many fields such as biomedicine (Gupta and Gupta, 2005; Atanasijevic *et al.*, 2006; Ya *et al.*, 2006), sensor development, water purification (Savage and Diallo, 2005), environmental remediation (Zhang, 2003; Liu, 2006). The value of coupling biological molecules such as enzymes to solid materials has long been recognized. To date, protein immobilization onto such surfaces often involves covalent coupling, encapsulation, or non-specific adsorption techniques (Johnson *et al.*, 2008).

Researchers are improving current technologies and developing new ones that utilize enzymes immobilized on nanoparticles. Many enzymes currently used in biotechnology, including glucose oxidase and peroxidase, have been covalently immobilized to nanoparticles using several different ligands. Improved techniques for immobilization of enzymes to nanoparticles are needed to allow for greater enzyme loading on the high surface area available. Major challenges of many techniques previously described for enzyme immobilization on nanoparticles inorganic surfaces are nonspecificity, loss of enzyme activity or even complete protein denaturation. Conversely, simple immobilization methods such as non-specific adsorption can result in enzyme leaching making this method inappropriate for applications where stability, both long term and under sub optimal conditions is necessary. Covalent immobilization techniques that use cross linking reagents can result in strong linkages that prevent enzyme leaching; however, the crosslinking reagents can potentially react with amino acids necessary for catalytic activity or substrate recognition and can cause non specific polymerization of protein molecules.

Magnetic nanoparticles (MNP) when are used as support of immobilized enzymes has the following advantages (Halling and Dunill, 1980):

1) higher specific surface area is obtained for the binding of a larger amount of enzymes,

2) lower mass transfer resistance and less fouling, and

3) the immobilized enzymes can be selectively separated from a reaction mixture by application of a magnetic field.

Iron oxide nanoparticles, Fe_3O_4, are one of the widely used types of magnetic nanoparticles and have great potential for applications in biology and medicine due to their strong magnetic properties and low toxicity (Sohn *et al.*, 2008). Different types of biomolecules such as proteins, enzymes, antibodies, and anticancer agents can be immobilized on these nanopaticles (Safarik and Safarikova, 2004). Magnetic supports for immobilization purpose are either prepared by incorporating magnetic particles during the synthesis of the supporting polymer (Robinson *et al.,* 1973; Horisberger, 1976) or magnetic particles itself be coated with common support materials such as dextran or agarose (Rusetski and Ruuge, 1990).

Mukherjee *et al.* (2010) reported the statistical optimization of the immobilization of alkaline α-amylase [E.C. 3.2.1.1] from *B. alcalophilus* onto nano-sized supermagnetic ironoxide nanoparticles (MNPs) for augmenting the cost effective industrial application of MNP-bound α-amylase. A significant improvement (~26-fold) in specific activity, thermal and storage stability, and reusability of α-amylase after binding with iron-oxide MNP reinforced the improved biotechnological potential of the α-amylase iron-oxide MNP bioconjugate compared to free α-amylase. These results open new avenues for applying this MNP immobilized enzyme in different industrial sectors, notably in the paper and brewing industries.

A simple immobilization method was devised by Mukherjee *et al.* (2013) for *Candida rugosa* lipase on super paramagnetic Fe_3O_4 nanoparticles. The Fe_3O_4 nanoparticles were coated with PEI and *Candida rugosa* lipase was adsorbed on these particles via electrostatic interactions. The immobilization resulted in marginal simultaneous purification. However, the immobilized preparation showed 110 × higher transesterification activities in low-water media.

Immobilization of α-amylase covalently on Fe_3O_4 magnetic nanoparticles via carbodiimide activation was studied by Jalal *et al*. (2011). The efficiency of immobilization and residual activity of the immobilized α-amylase was dependent upon the immobilization temperature and mass ratio of nanoparticles: carbodiimide: α-amylase. Confirmation of the binding was demonstrated by Fourier transformation infrared (FTIR) spectroscopy. At room temperature, immobilized amylase displayed more binding efficiency and less residual activity compared with of 4°C, hence 4°C was chosen as optimum immobilization temperature. The optimum pH value for both free and immobilized amylase was at pH 5 but immobilized amylase exhibited more pH sensitivity at the pH values 6, 7 compared with free amylase and there were no differences in pH profiles for amylase that immobilized in room temperature or 4°C.

Several new types of carriers and technologies have been implemented in the recent past to improve traditional enzyme immobilization which aimed to enhance enzyme loading, activity and stability to decrease the enzyme biocatalyst cost in industrial biotechnology (Ansari and Hussain, 2012). These include cross-linked enzyme aggregates, microwave-assisted immobilization, click chemistry technology, mesoporous supports and most recently nanoparticle-based immobilization of enzymes. The union of the specific physical, chemical, optical and electrical properties of nanoparticles with the specific recognition or catalytic properties of biomolecules has led to their appearance in myriad novel biotechnological applications. They have been applied time and again for immobilization of industrially important enzymes with improved characteristics. Enzymes immobilized on nanoparticles showed a broader working pH and temperature range and higher thermal stability than the native enzymes.

Compared with the conventional immobilization methods, nanoparticle based immobilization served three important features (Ansari and Hussain, 2012)

(i) nano-enzyme particles are easy to synthesize in high solid content without using surfactants and toxic reagents,
(ii) homogeneous and well defined core–shell nanoparticles with a thick enzyme shell can be obtained, and

(iii) particle size can be conveniently tailored within utility limits. In addition, with the growing attention paid to cascade enzymatic reaction and *in vitro* synthetic biology, it is possible that co-immobilization of multi-enzymes could be achieved on these nanoparticles.

2.6.3 Effect of enzyme loading on immobilization efficiency

The excessive enzyme loading always causes protein-protein interaction and inhibits the flexible stretching of enzyme conformation, which will result in the steric hindrance and thus the inactivation of an enzyme. That is, the enzyme molecule may be difficult to modulate its most suitable conformation for catching the substrate molecules and releasing product molecules under molecular crowding condition. So enzyme loading is an important factor while immobilizing enzyme by entrapment method (Zhang *et al.,* 2013). Recently, several authors have investigated the effect of enzyme loading on the immobilization of enzymes (Xie and Ma, 2010; Lei and Jiang, 2011; Zhang *et al.*, 2012). For instance, in the study of pectinase immobilization on macroporous polyacrylamide microspheres by Lei and Jiang (2011), they found that the activity of the immobilized pectinase decreased instead when the enzyme amount increased from 10 to12 units/ml. Zhang *et al*. (2012) investigated the effect of steric hindrance on the immobilized lipase. The experiments further demonstrated that an excessive lipase loading had resulted in an intermolecular steric hindrance and greatly affected the lipase activity. In addition, it was also shown by Xie and Ma (2010) that the activity recovery of the immobilized lipase increased at the initial stage with the increase in enzyme amount from 1 to 5mg per 200mg carriers and then remained nearly constant with further increase in lipase amount to 9mg. In general, the amount of enzyme immobilized on carriers has obvious influence on the performance of the immobilized enzyme. Therefore, enough attention must be paid to the amount of enzyme loaded as preparing the immobilized enzyme.

Table 4. Examples of immobilized enzymes in industrial applications

Enzyme	Substrate	Product	Application
Amylase	Glucose	Maltose syrup	Pharmaceutical
Amylase	Glucose	HFCS	Food
Nitrile hydratase	Acrylonitrile	Acrylamide	Chemicals & wastewater treatment
Penicillin amidase	Penicillin G	6-APA	Pharmaceutical
Aspartase	Fumaric acid	L-Aspartic acid	Chemicals
Fumarase	Fumaric acid	L-Malic acid	Chemicals
Aminoacylase	Acyl-D-L-amino acid	L- Amino acid	Food
Lipase	Rac-1-phenylethylamine	S-1-Phenylethylamine	Chemicals & Food
Cephalosporin amidase	Glutaryl-7-ACA	7-ACA	Pharmaceutical
Lactase (Galactosidase)	Lactose	Lactose free milk	Dairy

2.7 INDUSTRIAL APPLICATIONS OF α-AMYLASES

Due of its multifarious applications amylase attracts attention of researchers since centuries of its isolation and identification in the year 1894 from a fungal source that was used as an additive in digestive formulations. Since then, amylases are among the most important hydrolytic enzymes for all starch based industries. In the present day scenario, amylases find applications in all the industrial processes such as in food, detergents, biofuels, textiles and in paper industry, for the hydrolysis of starch. In this light, microbial amylases have completely replaced chemical hydrolysis in the starch processing industry. They can also be of potential use in the pharmaceutical and fine chemical industries. Today, amylases have the major world market share of enzymes. Several different amylase preparations are available with various enzyme manufacturers for specific use in varied industries (Table 5).

Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Couto and Sanroman, 2006). Besides generating fermentable compounds, α-amylases also have an anti-staling effect in bread baking, and they

improve the softness retention of baked goods, increasing the shelf life of these products (van der Maarel, 2002 and Gupta *et al.,* 2003).

Table 5. Uses of amylases in various sectors of industries (Sivaramakrishnan *et al.,* 2006)

Sector	Uses
Food industry	Production of glucose syrups, crystalline glucose
	Production of high fructose syrups
	Production of maltose syrups
	Reduction of viscosity of sugar syrups
	Reduction of haze formation in juices
	Solubilization and saccharification of starch for alcohol fermentation in brewing industries
Biofuel industry	In degradation of starchy substrates for their conversion to bioethanol to be used as power alcohol
Detergent industry	Used as an additive to remove starch based dirts
Paper industry	Reduction of viscosity of starch for appropriate coating of paper
Textile industry	Warp sizing of textile fibres
Pharmaceutical industry	Used as a digestive aid

A thermostable maltogenic amylase of *Bacillus stearothermophilus* is used commercially in the bakery industry (van der Maarel, 2002). Amylases are also used for the clarification of beer or fruit juices and for the pretreatment of animal feed to improve the digestibility of fibre (van der Maarel, 2002; Gavrilescu and Chisti, 2005; Ghorai *et al.,* 2009).

The use of α-amylase for the production of low viscosity, high molecular weight starch for coating of paper is reported. As for textiles, sizing of paper is performed to protect the paper against mechanical damage during processing. It also improves the quality of the finished paper. The size enhances the stiffness and strength in paper. It also improves the erasibilty and is a good coating for the paper. Starch is also a good sizing agent for the finishing of paper. Starch is added to the paper in the size press and paper picks up the starch by passing through two rollers that transfer the starch slurry. The temperature of this process lies in the range of 45-60^{o}C. A constant viscosity of the starch is required for reproducible results at this stage. The mill also has the flexibility of varying the starch viscosity for different paper grades. The viscosity of the natural starch is

too high for paper sizing and is adjusted by partially degrading the polymer with α-amylases in a batch or continuous processes. The conditions depend upon the source of starch and the α-amylase used.

Detergent industries are the primary consumers of enzymes. The use of enzymes in detergent formulations enhances the ability of detergents to remove tough stains making them environmentally safe. 90% of all liquid detergents contain these enzymes rendering them the most important enzymes used in the formulation of detergents (Gupta *et al.,* 2003; Mitidieri *et al.,* 2006; Hmidet *et al.,* 2008). The oxidative stability and activity at alkaline pH exhibited by amylases are among the most important criteria for their use in detergents (Kirk *et al.,* 2002; Chi *et al.,* 2009). One of the limitations of α-amylases in detergents is that the enzyme shows sensitivity to calcium and stability is severely compromised in a low calcium environment.

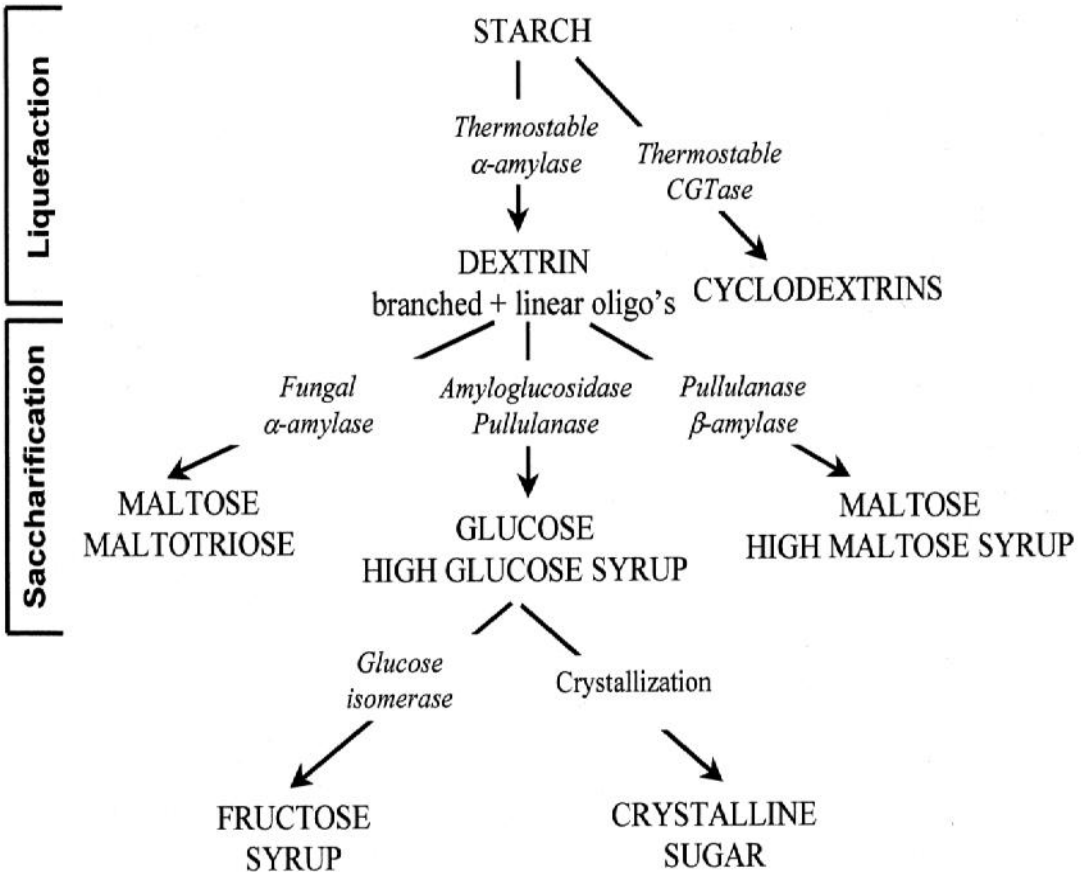


Fig.5 Overview of the industrial processing of starch into cyclodextrins, maltodextrins, glucose or fructose syrups and crystalline sugar

Maltose, a naturally occurring disaccharide widely used as a sweetener and also as intravenous sugar supplement is used in food industries because of low tendency to be crystallized and nonhygroscopic nature. It is prepared from corn, potato, sweet potato, and cassava starches. The concentration of starch slurry is adjusted @ 10-20% for production of medical grade maltose and 20-40% for food grade.

With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistry. There are several processes in the medicinal and clinical areas that involve the application of amylases. The application of a liquid stable reagent, based on α-amylase for the Ciba Corning Express clinical chemistry system has been described (Becks *et al.,* 1995). A process for the detection of higher oligosaccharides, which involved the application of α-amylase, was also developed (Giri *et al.,* 1990). This method was claimed to be more efficient than the silver nitrate test. Biosensors with an electrolyte isolator semiconductor capacitor (EIS-CAP) transducer for process monitoring were also developed (Menzel *et al.,* 1998).

Ethanol is the most utilized liquid biofuel. For ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world. In this production, starch has to be solubilized and then subjected to two enzymatic steps in order to obtain fermentable sugars. The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as α-amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* (Abouzied *et al.,*1996). The production of ethanol by yeast fermentation plays an important role in the economy of Brazil. In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* (Shigechi *et al.,* 2004). Among bacteria, α-amylase obtained from thermoresistant

bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *B. subtilis* is used during the first step of hydrolysis of starch suspensions.

2.8 Fuel alcohol industry

The world is facing a decline of fossil fuel resources, petroleum, natural gas and charcoal while energy requirements are progressively growing up. According to a research report, the world's oil and gas supply will disappear within three decades and also other natural resources such as coal will extinct within a century (Raja *et al.,* 2008). Currently, as an alternative, most of the countries have imposed regulations/ legislations to use alternate/renewable sources such as wind, hydro and solar energy to replace fossil fuel usage. Ethanol has emerged as an attractive alternative to the liquid petroleum resulting in worldwide search for its higher yield to overcome its limited supply (EIA, 2002). It has a number of advantages such as high biodegradability, minimal toxicity, minimal hydrocarbon emission, free from sulphur and aromatic compounds (Demirbas *et al.,* 2011; Atabani *et al.,* 2012). Ethanol is thus the most sought after biofuel which is currently being produced on a large scale, as it can be easily mixed with gasoline and used in internal combustion engine vehicles (Haveren *et al*., 2008).

Moreover, traditional sources of fossil fuels are coupled with accumulation of green house gases derived from their combustion have been turning them into unsustainable pillars of energy supply. The over exploitation of fossil fuels and the increasing greenhouse gases are considered to be the forthcoming crisis for human's population. Especially, carbon dioxide, which is the main component of greenhouse gas emissions, poses great challenge to the world. The inexorable usage of fossil fuels has contributed to approximately 98% of carbon emission from its combustion into the environment. Hence the fossil fuel usage has to be reduced in order to decrease the amount of carbon dioxide and other pollutants being emitted (Demirbas *et al.,* 2011).

Since India is the fourth (energy data) largest global contributor to carbon emissions, GOI transport policy has targeted EURO-III and IV vehicle emission norms for vehicles, which in turn require adoption of clean and green fuel. The

Government of India (GOI) has approved the National Policy on Biofuels on December 24, 2009. The policy encourages use of renewable energy resources as alternate fuel to supplement transport fuels and had proposed an indicative target to replace 20 percent of petroleum fuel consumption with biofuels (bioethanol and biodiesel) by end of 12^{th} Five-Year Plan (2017). Thus the steady demand for ethanol coupled with an expected rise in blending for Ethanol Blending Program (EBP) will push total ethanol consumption heavily. Due to huge gap between the demand and supply of ethanol the research efforts are presently being shifted to the use of forestry and agricultural biomass to generate the second generation biofuels (Bacovsky *et al.*, 2010).

The developed countries like US, Canada and UK have developed the technology to ferment the surplus grains like corn into ethanol leading to sufficient production of alcohol (Bury *et al.*, 2000). But the current data of Renewable Fuel Association showed an increase of demand for fuel ethanol over production level indicating the future growth in cellulosic substrates in this industry (Mosier, 2005).

Traditionally first and second generation biofuels have been produced from substrates such as food and oil crops and the lignocellulosic biomass. However, the liquid fuel consumption and requirement is increasing day by day besides the first and second generation biofuels have limitations to meet the ever increasing demand. The most common concern related to the current first generation biofuels is that as production capacities increase, so does their competition with agriculture for arable land used for food production which leads to severe food shortage (Dragone *et al.,* 2011). Moreover, the use of raw materials belonging to the human and animal food chains for biofuels production resulted in the rise of prices of food all over the world and,consequently, in social disturbance (Xavier *et al*., 2010; Limayemn and Ricke, 2012) . Thus, developing and thickly populated countries like India cannot easily afford to use the items of food chain for ethanol production.

The advent of second generation biofuels is intended to produce fuels from lignocellulosic biomass, the woody part of plants that do not compete with

food production. Sources include agricultural residues, forest harvesting residues or wood processing waste such as leaves, straw or wood chips as well as the non-edible components of corn or sugarcane. However, converting the woody biomass into fermentable sugars requires costly technologies involving pre-treatment with special enzymes, meaning that second generation biofuels have limitations to be produced economically on a large scale (Brennan and Owende, 2010). Therefore, 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 (Chisti, 2007; Nigam and Singh, 2010). Microalgae are able to produce 15–300 times more biofuel 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). Their high photosynthetic rate, i.e. 6.9 × 104 cells/ml/h is approximately 50 times higher as compared to terrestrial plants.

Microalgae, unicellular algae can accumulate a high content of the storage polysaccharide, starch. Presence of certain distinct notable characteristics such as lack of lignin and lack of specific functional parts such as leaves and roots render them a superior alternative over the woody terrestrial biomass. The polysaccharide contained in algal cell wall can be easily hydrolyzed to simple sugars which can further be fermented into bioethanol (Eshaq *et al.,* 2011). Biofuel production using microalgal farming offers the following advantages (Li *et al.,* 2008):

(1) The high growth rate of microalgae makes it possible to satisfy the massive demand on biofuels using limited land resources without causing potential biomass deficit.

(2) Microalgal cultivation consumes less water than land crops.

(3) The tolerance of microalgae to high CO_2 content in gas streams allows high-efficiency CO_2 mitigation.

(4) Nitrous oxide release could be minimized when microalgae are used for biofuel production.

(5) Microalgal farming could be potentially more cost effective than conventional farming.

Since algae mainly are composed of starch along with some of cellulose in their cell wall, therefore amylases are among the most widely used enzymes for their degradation. (Emmanuel *et al.*, 2000). Since thermophilic amylase is in high demand for feasible hydrolysis and bioconversion of algal biomass into ethanol, amylase produced from extremophilic microorganisms, can become the first choice in the process. Microbial communities capable of growing at the extremes of temperature, alkalinity/ acidity are the most sought after extremophiles in this field. (Kiran and Chandra, 2008). Characteristic of thermophilic organisms is their capability to survive in extreme conditions which is strictly related to the enzymes that are mostly thermostable in nature (Khalil, 2011). Thus the isolation of novel species of extremophiles has become crucial in search of thermostable enzymes with high stability to be used for algal biofuel production (Kumar and Swati, 2001).

Himachal Pradesh being a hilly state has a suitable climate as well as many sources of fresh water such as rivers, lakes, ponds and traditional bawris etc. which is a home for varied aquatic life and algae is one of the most prominent forms. Water net (*Hydrodictyon* sp.) being an exotic species to Himachal, is a fresh water weed which is polluting the water bodied thereby causing trouble for aquatic planktonic life. Its removal has become a big problem economically as well as physically. However, its biochemical composition suggests it to be a potential source of easily hydrolysable carbohydrates which can further be converted to bioethanol. In addition to a large number of organic compounds such as vitamins, minerals, lipids and proteins, algal biomass contains up to 64% carbohydrates which are twice the carbohydrate contents of cassava (Sulfahri *et al.*, 2011). So far, the use of algae as an industrial raw material is rare compared with the diversity of algal species in HP. The chemical composition of algal cells renders it a potent industrial raw material and an

alternative solution for the energy crisis. An extensive research is needed to obtain bioethanol from algae by fermentative processes.

There are a few reports of algal biofuel production. Sulfahri *et al.* (2011) have investigated bioethanol production from algae *Spirogyra* by means of fermentation using *Zymomonas mobilis* and *Saccharomyces* cerevisiae. After 96 hrs of fermentation *Z. mobilis* yielded 9.6% ethanol while *S. cerevisiae* yielded 4.42% ethanol. Eshaq *et al.* (2011) reported the production of bioethanol using *Spirogyra* as a raw material as it do not compete with food, fodder and also easily available. *Aspergillus niger* MTCC 2196 and *Saccharomyces cerevisiae* MTCC 170 were employed for fermentation. A maximum of 6.5 g ethanol/ 100g substrate were obtained after 3 days of fermentation.

2.8.1 Pretreatment of algal biomass

The production of bioethanol involves an extensive process and varies depending on the type of biomass. The process mainly encompasses the upstream and downstream operations which involve pre-treatment of the biomass, saccharification, fermentation and product recovery. Drying of the harvested algae, which contains an excess amount of water is the first and foremost step. For the purpose of drying, solar drying, air drying, hot gases and fuel are the most frequently used means. However, a research has mentioned that using gases, fuel for drying procedure consumed nearly 69% of the overall energy input and led to a negative energy balance in producing microalgae biofuels (Sander and Murthy, 2010). Ranjan *et al*. (2010) showed that drying or cell disruption of concentrated algal culture actually enhanced the lipid and/or protein extraction. In a similar report, Nahak *et al*. (2011) observed higher hydrolysable sugars in case of dry algae than the fresh algae. The main advantage of processing dried biomass is the better percolation of solvents or fluids through the cell to improve extraction efficiency. However, drying is not considered as an economically viable option for biomass production because of the high-energy requirements (Lardon *et al.*, 2009). In other words, high dependency on fossil fuels for drying process will jeopardize commercial viability of microalgae biofuels and thus, new

technologies or approaches (e.g. development of efficient dryers) are urgently needed to ensure the sustainability of microalgae biofuel production.

The biomass pre-treatment is a crucial process because it is essential for the fermentable sugars to be released and made available for the fermentation process. However, since the pre-treatment process contributes significantly to the cost of production of bioethanol, the most economical pretreatment protocol must be selected in order to make bioethanol more economically attractive. An efficient biomass pre-treatment procedure must be energy efficient, cost effective, simple to apply and must not degrade the fermentable sugars (Rabelo *et al.*, 2009). Biomass can be pre-treated in three different ways; physical, biological and chemical. The physical pre-treatment process normally uses mechanical comminution (such as chipping, milling and griding) and pyrolysis, where the biomass is exposed to high temperatures. Although this procedure is quite simple, the high energy consumption associated with it makes it not preferable to be implemented in a commercial scale production. The chemical pre-treatment process has been successfully proven for various biomasses: corn, switchgrass, sugar cane and straw. The chemicals commonly used are either acidic (acetic acid, nitric acid, hydrochloric or sulphuric acid) or alkaline (Sodium hydroxide and lime), which are everyday industrial chemicals (Harun and Danquah, 2011). The biological pre-treatment involves the utilisation of microbes and enzymes to degrade the biomass in order to release the fermentable sugars. This mechanism has been reported to have the potential to be applied in the pre-treatment of biomass (Akin *et al.*, 1995). However, biological pre-treatment is associated with low hydrolysis rate which prolongs the completion time of the process step.

In the case of microalgal biomass, it is well known that a high content of complex carbohydrates entrapped in the cell wall of algae makes it essential to incorporate a pretreatment stage to release and convert these complex carbohydrates into simpler sugars prior to the fermentation process (Harun and Danquah, 2011). Several workers have investigated various pretreatment methods.

Among the physical pretreatment methods, Asada *et al*. (2012) reported ultrasonication for extraction of starch from dried algae. A 30 min of sonication resulted in extraction of 93.8% starch at 68 ^{o}C. However, crushing algae in a mortar for 30 min resulted in lowered starch extraction only upto 14%. Nahak *et al*. (2011), performed steam soaking of the biomass and reported more sugars in case of dry algae than the fresh algal biomass. Kim *et al*. (2014) studied several physical pretreatment methods such as bead beating, autoclaving and sonication etc. Bead-beating pretreatment increased hydrolysis by 25% compared with the processes lacking pretreatment.

Similarly for chemical pretreatment, various acids such as dilute acetic acid, H_2SO_4, HCl and HNO_3 have been used by several workers. Harun and Danquah (2011) explored the influence of dilute H_2SO_4 exposure as a microalgal pre-treatment strategy for bioethanol production. Different parameters were investigated: acid concentration, temperature, microalgae loading and pre-treatment time. A central composite design technique was employed to optimize the acid pre-treatment conditions. Results showed that the highest bioethanol concentration obtained was 7.20 g/L and this was achieved when the pre-treatment step was performed with 15 g/L of microalgae at 140^{o}C using 1% (v/v) of sulphuric acid for 30 min. Gupta *et al.* (2012) reported 0.70% H_2SO_4, pretreatment for marine algae. Eshaq *et al*. (2011) reported alkali pretreatment with 1% NaOH for 2h at room temperature. Ho *et al.* (2013) studied final sulphuric acid concentrations ranging from 0.1-5% and found 0.1% to be the best for *Chlorella vulgaris* biomass. Markou *et al.* (2013) explored the potential of bioethanol production using carbohydrate-enriched biomass of the cyanobacterium *Arthrospira platensis*. For the saccharification of the carbohydrate-enriched biomass, four acids (H_2SO_4, HNO_3, HCl and H_3PO_4) were investigated. Each acid was used at four concentrations, 2.5N, 1N, 0.5N and 0.25N, and for each acid concentration the saccharification was conducted under four temperatures (40°C, 60°C, 80°C and 100°C). Hydrolysates produced with 0.5 N HNO_3 and 0.5 N H_2SO_4respectively were found to yield highest ethanol. Wang *et al.* (2011) studied sulphuric acid pretreatment with an acid concentration

of 0.5, 1.0, 2.5 and 5% and found 2.5% to be the best concentration yielding maximum glucose.

Physical or chemical pretreatment of the biomass is followed by enzymatic hydrolysis to get fermentable sugars out of the complex carbohydrates present in the biomass. Enzymatic hydrolysis was reported to increase saccharification yields. Liquid-phase enzymatic hydrolysis (alpha-amylase and glucoamylase) has been used for starch hydrolysis. Wang *et al*. (2011) utilized cellulase for further hydrolysis of the pretreated biomass. Trivedi *et al.* (2013) also reported the use of different commercial cellulases for the hydrolysis of algal biomass. However, a cocktail of enzymes has also been reported to be used by several workers. α-amylase, amyloglucosidase and xylanase for hydrolysis of *Chlorella* sp. A mixture of amylase and cellulase has been used by Eshaq *et al*. (2011). Kim *et al*. (2011) used a mixture of cellulase, β-glucanase, amyloglucosidae, endoglucanase to obtain an efficient hydrolysis of an acid pretreated algal biomass into simplest sugars. Mollers *et al*. (2014) evaluated a mixture of lysozyme, α-amylase and amyloglucosidase for enzymatic hydrolysis of cyanobacterial biomass.

A study by Ho *et al*. (2013) demonstrated the efficiency of acid hydrolysis over enzymatic hydrolysis. The self-made enzyme mixture from *Pseudomonas* sp. CL3 containing suitable amylase/cellulase composition could effectively hydrolyze *C. vulgaris* FSP-E biomass for subsequent ethanol production via the SHF and SSF processes. Ethanol production by the SHF process with dilute acid hydrolysis (1% H_2SO_4) of the microalgal biomass was more effective, producing 11.66 g/L of ethanol within 12h with a yield of 87.59%.

In the next step, the fermentable sugars are converted to ethanol by a suitable yeast strain e.g. *Saccharomyces cerevisiae*. In order to achieve the highest possible fermentation yield (theoretically 0.51 g of ethanol/1 g of glucose) an appropriate nutrition of yeast has to be ensured (source of N, P, S etc.). Eshaq *et al.* (2011), Asada *et al.* (2012), Gupta *et al*. (2012), Kim *et al*. (2013), Trivedi *et al*. (2013) and Mollers *et al*. (2014) utilized *Saccharomyces cerevisiae* for fermentation of algal hydrolysate. Ho *et al.* (2013) utilized

Zymomonas mobilis for fermentation of algal hydrolysate.The processes of enzymatic hydrolysis and fermentation can be carried out either separately as reported by Eshaq *et al.* (2011), Trivedi *et al*. (2013) and Ho *et al*. (2013) or simultaneously as reported by Doran-Peterson *et al.* (2009), Kim *et al.* (2011), Asada *et al*. (2012) and Mollers *et al*. (2014).

An attempt on to the scale up of bioethanol production from algal biomass in a bioreactor has also been done. Kim *et al*. (2014) reported scale-up of ethanol production from microalga *Chlorella vulgaris* by enzymatic hydrolysis in a 10 l capacity fluidized bed bioreactor at 30^{o}C for 48 h. However, a very little amount of research work has been reported on the pretreatments of microalgae, further conversion of the fermentable sugars into bioethanol and the scale up of bioethanol. Studies on the effects and optimization of process variables that influence the performance of the microalga will be an essential outlook of bioethanol development from microalgae.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled **"Purification and characterization of amylase produced from potential extremophiles for industrial application"** was conducted in the Microbiology section, Department of Basic Sciences, Nauni, Solan (H.P.) – India, during the year 2011-2015. The chapter provides insight into methods employed and materials used during the course of research period which has been discussed under following heads:

3.1 Isolation and screening of hyperamylolytic microorganisms from mushroom compost

3.2 Identification of selected isolates

3.3 Production and optimization of extracellular α-amylase using one variable at a time (OVAT) approach and Response Surface Methodology (RSM)

3.4 Purification of extracellular α-amylase

3.5 Biochemical and kinetic characterization of purified α-amylase

3.6 Internal amino acid sequence of amylase determined by MALDI-TOF Mass Spectrometry

3.7 Immobilization of purified α-amylase on different substrates and its characterization

3.8 Application of purified α-amylase in bioethanol production using algal biomass

3.9 Physicochemical pretreatment of algal biomass

3.10 Enzymatic hydrolysis of algal biomass

3.11 Bioethanol production under SHF (Separate hydrolysis and fermentation) and SSF (Simultaneous saccharification and fermentation)

3.12 Scale-up of bioethanol production in a stirred tank bioreactor

3.1 ISOLATION AND SCREENING OF AMYLOLYTIC MICROORGANISMS FROM MUSHROOM COMPOST

3.1.1 Collection of samples

Mushroom compost was collected from Directorate of Mushroom Research, Chambaghat, Solan, Himachal Pradesh in clean polythene bags and was brought to the laboratory.

3.1.2 Enrichment of soil samples

To 100g of mushroom compost, 2% of soluble starch was added and water was sprinkled in the petriplate and mixed thoroughly. The petriplates containing the above mixture were incubated at 50 ±2 °C for three days.

3.1.3 Isolation of amylolytic microorganisms

One gram of enriched sample was serially diluted from 10^{-2} to 10^{-8} times using sterilized 9 ml dilution blanks. Each of diluents (0.1 ml) was placed on the surface of starch agar medium in a petriplate and evenly spread with the help of a spreader and the plates were incubated at 50±2°C for 3 days. The pure line cultures were obtained by streak plate method. The bacterial pure line cultures were maintained on nutrient agar slants and preserved in refrigerator at 4°C and were also glycerol preserved for further use. The periodic subculturing was done once in a month.

The following basal medium was used for screening of amylolytic activity (Shaw *et al*., 1995): Peptone- 5.0 g, Yeast extract- 1.5 g, Beef extract-1.5 g, Starch soluble -2.0 g, Sodium chloride -5.0 g, Agar-15.0 g, Distilled water-1000 mL, pH- 9.0 ± 0.1.

3.1.4 Screening of hyper amylase producing strains

Potential amylase producing microorganisms were screened by employing following qualitative and quantitative assays:

3.1.4.1 Qualitative assay: Starch hydrolysis test (Shaw *et al*., 1995)

Starch hydrolysis test was performed on isolated strains to screen hyper amylolytic strains. Starch agar plates were spot inoculated with the isolated

strains and incubated at 50 ^{0}C for 72 h. The growth was then flooded with 10 ml of iodine solution for about 20 min. Area of hydrolysis appeared as a clear zone among the blue stained unhydrolysed starch. The diameter of the clear zone obtained was the measure of extent of amylolytic activity of the strain.

3.1.4.2 Quantitative assay: Starch-Iodine method (Xiao *et al.*, 2006)

3.1.4.2.1 Production of extracellular enzyme by microbial isolates

Each bacterial isolate was grown in 100 ml of starch broth at 50±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 autoclaved distilled water. 5 ml of inoculum was added to 45 ml of standard enzyme production medium (Bajpai *et al*., 1990) which comprised (gl^-): starch, 10.0; yeast extract, 5.0; peptone, 2.0; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 0.1; $MgSO_4.7H_2O$, 0.12; NaCl, 1.5; pH, 9.0 in 250 ml of Erlenmeyer flasks. An uninoculated culture medium was kept as a control. The inoculated and uninoculated enzyme production media were then incubated for 3 days at 50±2°C at 120 rpm. After incubation, the culture contents were centrifuged at 12,000 rpm for 20 min (4°C). The supernatant thus obtained was then used as the crude enzyme for quantifying extracellular amylolytic activity.

3.1.4.2.2 α-Amylase assay (Xiao *et al.*, 2006)

Reagents

i. 1N acetic acid solution

ii. Iodine reagent (1g iodine and 0.2 g potassium iodide dissolved in 100 mL distilled water)

iii. Soluble starch solution (as standard) 0.2%

Procedure

1.0 ml of the enzyme solution with 1.0 ml of 0.2% starch was incubated at 37 °C for 30 min. To this, 5.0 ml of 1N acetic acid was added to stop the reaction, followed by its dilution to 200 ml with distilled water. Finally 5.0 ml of iodine reagent was added and the preparation were read at 580 nm using spectronic® GENESYS™5 Spectrophotometer. Standard curve was prepared by using 0.2% soluble starch as a standard and by preparing dilutions of the standard solution of

0.5, 1.0, 1.5, 2.0, 2.5 ml and final volume was made 2.5 ml with distilled water in each tube.

One International Unit (IU) of amylase activity is defined as the disappearance of an average of 1µmol of iodine binding starch material per minute in the assay reaction.

Specific activity represents µmoles of starch released/min/mg of protein.

3.1.4.2.3 β-Amylase assay (Miller, 1959)

Reagents

i. Dinitrosalicylic acid (DNSA) Reagent

ii. Standard solution of glucose (0.4 mg/ml)

To 0.5 ml of enzyme solution was incubated with 0.2% starch at 37 ^{o}C for 15 min. 3 ml of DNSA reagent was added to it and the mixture was heated on boiling water bath for 15 min. After cooling down to room temperature, absorbance of reaction mixture was read at 540 nm. 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 acivity (SA).

One International Unit (IU) of enzyme activity represents µ moles of glucose released/min/ml of enzyme.

Specific Activity (SA) represents µ moles of glucose released/min/mg of protein.

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

Reagents

i. 1N Folin Ciocalteau'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 Ciocalteau'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 670 nm 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.

3.2 MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF AMYLASE PRODUCING ISOLATES

3.2.1 Phenotypic characterization

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

3.2.1.1 Morphological characterization

Morphological characteristics of isolates including colony morphology, cell shape, Gram's reaction, and cultural conditions of different isolates were studied.

3.2.1.2 Metabolic fingerprinting

Major biochemical tests such as fermentation pattern of different sugars viz. glucose, galactose, sucrose, mannose, arabinose, raffinose, inulin, trehalose, cellobiose, starch, etc. were performed. Peptone broth was used as a medium for fermentation of sugar. Phenol red was used as an indicator to depict the colour change from red to yellow due to decline in pH. Other than this, catalase, urease, indole test, MR/VP test and H_2S production test etc. were carried out following standard procedures for the preliminary identification of amylolytic bacterial isolates on suitable media.

3.2.2 Molecular taxonomic characterization

Screened bacterial isolates were identified at genomic level by using 16S rRNA gene technique as given below:

3.2.2.1 Extraction of genomic DNA

Bacterial isolates GC6 and GV2 were grown overnight at 50 ^{0}C in nutrient broth at 200 rpm. The cells were harvested and processed for DNA isolation by conventional method of Sambrook and Rusell (1989) as given below:

Requirements:

96-100% Ethanol
Sterile, DNase- free pipette tips and microcentrifuge tubes
RNase A (50 mg/ml)
10% SDS
Phenol: Chloroform (1:1)
Extraction buffer (20mg/ml lysozyme, 100mM Tris- HCl, 50mM EDTA, 500 mM NaCl)
TE buffer (10mM Tris HCl, 1mM EDTA)

Procedure

1. 5ml of overnight grown culture was transferred to a micro-centrifuge tube and centrifuged at 13,000 rpm for 1 min and supernatant was discarded.
2. Bacterial pellet thus obtained was suspended in 500 µl of extraction buffer and 50 µl of 10% SDS. Cell pellet was resuspended by vortexing or pipetting.
3. The samples were then incubated at 65^{0}C in a water bath for 30 min until the sample lysate became clear. During incubation, tubes were inverted gently every 5 min.
4. After incubation, 2 µl of RNase A (50 mg/ml) was added to the sample lysate and was mixed by vortexing. The preparation was then incubated at room temperature for 5 min.
5. To the lysate equal volume of phenol: chloroform (1:1) was added and mixed well.
6. The above mixture was centrifuged at 10,000 rpm for 5 min at room temperature. Two layers were formed. Upper aqueous layer was collected in new eppendorf tube with the help of pipette.

7. The phenol: chloroform step was repeated.
8. The above mixture was again centrifuged at 10,000 rpm for 5 min at room temperature. Two layers were formed. Upper aqueous layer was collected in new eppendorf tube with the help of pipette.
9. 1/10 volume of 5 M NaCl and 2.5 volume of absolute ethanol was added to the aqueous phase collected in an eppendorf tube.
10. The tubes were incubated at -20 ^{0}C overnight.
11. Next day, the above mixture was centrifuged at 12,000 rpm for 20 min at room temperature and supernatant was discarded.
12. The DNA pellet was washed with 1ml of 70% ethanol.
13. Again the mixture was centrifuged at 12,000 rpm for 5 min room temperature and supernatant was discarded.
14. DNA pellet thus obtained was air dried for about 15 min until all the residual ethanol got evaporated.
15. Finally the DNA pellet was suspended in appropriate amount (90µl) of TE and quantified.

3.2.2.2 Agarose gel electrophoresis (Bollag and Edelstein, 1991b)

3.2.2.2.1 Material required

1. Horizontal gel electrophoresis plates
2. Power pack
3. Water bath
4. Microfuge
5. Deep Freezer (-20^{o}C)
6. Ice making machine
7. Gel documentation system

3.2.2.2.2 Solutions/ Regents

1. Agarose powder 1 %

2. TAE buffer stock 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 $4^{o}C$

4. Ethidium bromide 10mg/ml

3.2.2.2.3 Protocol

1. 250 ml of TAE buffer (1X) was taken in a 500 ml Erlenmeyer flask.
2. 2.5 g of agarose was weighed and added in to the TAE buffer (1X).
3. The contents were heated on hot plate till agarose dissolves completely. Contents were cooled to $60^{o}C$. 5µl of ethidium bromide was mixed with precooled agarose.
4. Gel casting tray was fixed properly and kept in an electrophoretic tank.
5. Comb was inserted in the gel casting tray.
6. Agarose solution was poured into the casting tray and was allowed 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, Bangalore)
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 (Genei Make)

3.2.2.2.3 Quantification of DNA (Sambrook and Russell, 1989)

1. 2 µl of DNA samples of each strain were taken and diluted with 1 ml of TE buffer.

2. The absorbance of DNA samples were measured at a wavelength of 260 nm against TE buffer blank in quartz cuvette.

3. DNA concentration was calculated as given below:

Calculations

$$\text{Total Quantity of DNA (µg/µl)} = \frac{\text{Measured } OD_{260} \times 50 \times \text{dilution factor}}{1000}$$

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

3.2.2.2.4 PCR Amplification of 16S rRNA Region

PCR reaction mixture

Taq buffer (10X)	:	5.0µl
dNTP 2mM	:	2.5 µl
Primer (F)	:	1.0 µl
Primer (R)	:	1.0 µl
Taq Polymerase	:	0.2 µl
Glycerol	:	0.5 µl
Water	:	12.8 µl
DNA	:	1 µl
$MgCl_2$	:	1 µl

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'. The PCR analysis was carried out with a volume of 25 µl mixture in a thermocycler. It consisted of 35 cycles, 92^{o}C for 1 min, 55^{o}C for 1 min, 72 ^{o}C for 1 min.

3.2.2.2.5 Agarose gel electrophoresis

Same as section 3.2.2.2

3.2.2.2.6 Purification of PCR product

The PCR product (1500bp) was purified from contaminating products by electroelution of the gel slice containing the excised desired fragment with Qiaquick gel extraction kit (Qiagen, USR). The elution was carried out in 30µl of nuclease free water.

3.2.2.2.7 Sequencing

The PCR products of GC6 and GV2 were sequenced by commercially available services of (BioServe Biotechnologies (India) Pvt. Ltd., Hyderabad, India). The sequences so obtained after sequencing were used to identify GC6 and GV2 by BLASTN bioinformatics tool.

3.2.2.2.8 Identification

On the basis of 16S rRNA gene technique, GC6 and GV2 were identified as *Bacillus aerius* and *Bacillus sonorensis* respectively. The sequences so obtained were submitted in National Centre for Biotechnology Information (NCBI) to get an accession number. *Bacillus aerius* GC6 and *Bacillus sonorensis* GV2 were registered under the accession numbers |KJ775810.1| and |KJ775811.1| respectively.

3.2.2.2.9 Clustal Omega analysis

Translated nucleotide sequences were then analyzed by using tool www.ebi.ac.uk/services.

3.3 OPTIMIZATION OF PROCESS PARAMETERS AND MEDIUM COMPONENTS FOR ENHANCED AMYLASE PRODUCTION

Strains GC6 and GV2 owing to their maximum amylase production efficiencies, were selected for further optimization studies. An increase in the titers of amylase by varying different fermentation variables and by fortifying medium components was aimed.

3.3.1 One variable at a time (OVAT) approach

Before using statistical optimization tool, Response Surface Methodology (RSM) for the optimization of various parameters, preliminary studies with selected variables such as fermentation medium, inoculum size, incubation period, pH, temperature, divalent ions concentration, detergents and media additives were carried out by using conventional approach i.e. one-variable-at-a-time (OVAT).

3.3.1.1 Effect of Media

3.3.1.1a Inoculum preparation

B. aerius GC6 and *B. sonorensis* GV2 were grown in 100 ml of starch broth separately and were incubated at 50±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 starch broth as blank.

3.3.1.1b Production of amylase

5 ml of inoculum thus prepared was added to each 45 ml of different medium (given below) in 250 ml of Erlenmeyer flasks separately (1.0 OD) and the flasks were incubated at 50±2°C under shaking conditions for 3 days. After 3 days, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C. The supernatant thus collected was used for the quantitative tests.

3.3.1.1c Composition of media used

1. Babu and Satyanarayana (1993)

Soluble starch- 20.0 g, Beef extract- 10.0 g, Yeast extract- 2.0 g, $CaCl_2$- 0.2 g, $MgSo_4$. $7H_2O$- 0.1 g, dH_2O- 1000mL, pH- 9.

2. Kwan *et al*. (1993)

Soluble starch- 4.0 g, $(NH_4)_2HPO_4$- 0.5 g, Yeast extract- 0.5 g, Sodium citrate- 0.2 g, $MgSO_4.7H_2O$- 0.05 g, $CaCl_2.2H_2O$- 0.008 g, Distilled water- 100 mL, pH- 9.0± 0.2.

3. Starch Agar medium (Saxena *et al*., 2007)

Soluble starch- 1.0 g, Yeast extract- 0.4 g, KH_2PO_4– 0.1 g, $MgSO_4.7H_2O$- 0.15 g, dH_2O- 1000 ml, pH- 9.0

4. Modified starch medium (Jyoti *et al*., 2011)

Starch, 10.0; yeast extract, 5.0; peptone, 5.0; KH_2PO_4, 0.12; $CaCl_2.2H_2O$, 0.12; $MgSO_4.7H_2O$, 0.12; $MnSO_4.4H_2O$, 0.02, pH, 9.0.

5. Modified yeast medium (Fooladi and Sajjadian, 2010)

Soluble starch, 10.0 g, Yeast extract, 2.0 g, Peptone, 5.0 g, $MgSO_4$, 0.5 g, NaCl, 0.15 g, $CaCl_2$, 0.15 g, dH_2O- 1000mL, pH- 9.0

6. Modified medium (Jyoti *et al*., 2006)

Soluble starch- 5.0 g, Na_2HPO_4- 1.5 g, $MgSO_4.7H_2O$- 0.8 g, KH_2SO_4- 1.0 g, $FeSO_4.7\ H_2O$- 0.03 g, $CaCl_2.2H_2O$- 0.06 g, Yeast extract- 0.1 g, Peptone- 0.5 g, dH_2O- 1000mL, pH- 9.0

3.3.1.1d Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.1e Protein assay

Protein estimation was as given in section 3.1.4.3.

3.3.1.1f Statistical analysis

All the experiments were conducted in triplicates and results are presented as mean ± SD. Further the significant differences among the mean values were evaluated by one way ANOVA and Tukey's analysis for comparison of significant means using GraphPad Prism 5.0. Standard error of mean has been given in the end of the table.

Starch agar medium and Modified medium were found best for amylase production by *B. aerius* GC6 and *B. sonorensis* GV2 respectively. These media were selected for the next step of optimization.

3.3.1.2 Effect of Incubation Time

3.3.1.2a Inoculum preparation: Same as given in 3.3.1.1a

3.3.1.2b Production of amylase

5 ml of inoculum (1.0 OD) was added to 45 ml of above mentioned respective selected media in each of 250 ml of Erlenmeyer flasks. The flasks were incubated at different incubation time ranging from 24, 48…,144 h at 120 rpm. After mentioned incubation time, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C. Supernatants were collected and quantitative tests were performed.

3.3.1.2c Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.2d Protein estimation

Protein estimation was done as given in section 3.1.4.3.

3.3.1.2e Statistical analysis: Same as given in section 3.3.1.1f

Incubation period of 72 h showed highest amylase production and was thus selected for further studies.

3.3.1.3 Effect of Incubation Temperature

3.3.1.3a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.3b Production of amylase

5 ml of inoculum (1.0 OD) of both strains was added to 45 ml of respective selected media in 250 ml Erlenmeyer flasks separately. The flasks were incubated at different temperatures ranging from 30, 40,....., 80 °C at 120 rpm. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative amylase assay was performed with the supernatants.

3.3.1.3c Enzyme assays

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.3d Protein estimation

Protein estimation was done as given in section 3.1.4.3.

3.3.1.3e Statistical analysis: Same as given in section 3.3.1.1f

The temperature showing optimum amylase production i.e. 50°C was selected for further studies.

3.3.1.4 Effect of pH

3.3.1.4a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.4b Production of amylase

5 ml inoculum (1.0 OD) of both strains was added separately to 45 ml of respective optimized media in 250 ml of Erlenmeyer flasks. The flasks were incubated at 50°C with different pH ranging from 7 to 12 at 120 rpm while keeping other studied variables at their optimum levels to get maximum amylase yield. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative amylase assay was performed with the supernatant.

3.3.1.4c Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.4d Protein estimation

Protein estimation was done as given in section 3.1.4.3.

3.3.1.4e Statistical analysis: Same as given in section 3.3.1.1f

pH 9.0 showing optimal amylase production by both the strains was thus chosen for further studies.

3.3.1.5 Effect of Inoculum Size

3.3.1.5a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.5b Production of amylase

45 ml of above mentioned selected media were seeded separately with variable inoculum sizes of screened bacteria ranging from 2.5, 5.0……, 15.0 % in each of 250 ml of Erlenmeyer flasks. The flasks were incubated at 50°C at 120 rpm. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative amylase assay was performed with the supernatant.

3.3.1.5c Enzyme assays

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.5d Protein estimation

Protein estimation was done as given in section 3.1.4.3.

3.3.1.5e Statistical analysis: Same as given in section 3.3.1.1f

The best fermentable inoculum size of 7.5% was thus chosen for further studies.

3.3.1.6 Effect of Different Carbon Sources

3.3.1.6a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.6b Production of amylase

5 ml of inoculum (1.0 OD) of both strains was added to 45 ml of respective optimized media in 250 ml of Erlenmeyer flasks. The flasks were incubated at 50°C at 120 rpm with different substrates viz. mannose, arabinose, starch, maltose, sucrose and glucose. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative amylase assay was performed with the supernatant.

3.3.1.6c Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.6d Protein estimation

Protein estimation was done as given in section 3.1.4.3.

3.3.1.6e Statistical analysis: Same as given in section 3.3.1.1f

Starch was adjudged as a best carbon source for *B. sonorensis* GV2 while maltose was the best carbon source for *B. aerius* GC6.

3.3.1.7 Effect of Substrate Concentration

3.3.1.7a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.7b Production of amylase

5 ml of inoculum (1.0 OD) of both strains was added to 45 ml of respective optimized media with a substrate concentration ranging from 0.5, 1.0….., 3.0% in 250 ml Erlenmeyer flasks. The flasks were incubated at 50°C at 120 rpm. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative amylase assay was performed with the supernatant.

3.3.1.7c Enzyme assays

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.7d Protein estimation

Protein estimation was done as given in section 3.1.4.3.

3.3.1.7e Statistical analysis: Same as given in section 3.3.1.1f

The best fermentable substrate concentration of 0.5% for *B. aerius* GC6 and 1.0% for *B. sonorensis* GV2 thus chosen was used for further studies.

3.3.1.8 Effect of Nitrogen Sources

3.3.1.8a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.8b Production of amylase

5 ml of inoculum (1.0 OD) of both strains was added separately to 45 ml of respective optimized media in 250 ml Erlenmeyer flasks. The medium were supplemented with various organic and inorganic nitrogen sources such as gelatin, tryptone, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3 and $NaNO_3$. The flasks were incubated at 50°C at 120 rpm. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative assay was performed with the supernatant.

3.3.1.8c Enzyme assays

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.8d Protein estimation

Protein content was determined as given in section 3.1.4.3

3.3.1.8e Statistical analysis: Same as given in section 3.3.1.1f

Ammonium sulphate and sodium nitrate respectively were adjudged as the best nitrogen source aiding in amylase production from *B. aerius* GC6 and *B. sonorensis* GV2.

3.3.1.9 Effect of Divalent Ions

3.3.1.9a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.9b Production of amylase

5 ml of inoculum (1.0 OD) of both strains was added separately to 45 ml of respective optimized media in 250 ml Erlenmeyer flasks. The media were supplemented with various divalent ions (5mM) such as Ca^{2+}, Mg^{2+}, Cu^{2+}, Ba^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+}. The flasks were incubated at 50°C at 120 rpm. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative amylase assay was performed with the supernatant.

3.3.1.9c Enzyme assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.3.1.9d Protein estimation

Protein content was determined as given in section 3.1.4.3

3.3.1.9e Statistical analysis: Same as given in section 3.3.1.1f

Ca^{2+} and Mg^{2+} were included in the production medium as they had been found to enhance enzyme activity in both the strains.

3.3.1.10 Effect of Media Additives

3.3.1.10a Inoculum preparation: Same as given in section 3.3.1.1a

3.3.1.10b Production of amylase

5 ml of inoculum (1.0 OD) of both strains was added separately to 45 ml of respective optimized media in 250ml Erlenmeyer flasks. The media were supplemented with various media additives (1%) such as SDS, EDTA, TritonX 100, Tween 20, Tween 80 and Glycerol. The flasks were incubated at 50°C at 120 rpm. After 72 h, the culture contents were centrifuged at 8,000 rpm for 20 min at 4°C and quantitative amylase assay was performed with the supernatant.

3.3.1.10c Enzyme assay

Amylase estimation was done by Starch-Iodine method (Xiao *et al.*, 2006) as mentioned in section 3.1.4.2.

3.3.1.10d Protein estimation

Protein estimation was done by the method of Lowry *et al*. (1951) as given in section 3.1.4.3.

3.3.1.10e Statistical analysis: Same as given in 3.3.1.1f

None of the medium additives have resulted in an enhanced amylase production.

3.3.1.11 Percent (%) Increase in Enzyme Activity after Optimization of Different Parameters

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

3.3.2 OPTIMIZATION OF AMYLASE PRODUCTION USING RESPONSE SURFACE METHODOLOGY (RSM)

Using 'One Variable At a Time (OVAT) approach', the effect of media types, incubation time, temperature, pH, inoculum size, carbon sources, substrate concentration, nitrogen sources, media additives and divalent ions etc. were studied. Based on these optimization results, six independent variables were chosen for further optimization studies by employing Central Composite Design (CCD) of Response Surface Methodology (RSM). The dependent variable was amylase activity and the independent variables chosen were substrate concentration (X_1), pH (X_2), temperature (X_3), inoculum size (X_4), Ca^{2+} concentration (X_5) and Mg^{2+} concentration (X_6). The central composite design

(CCD) with six factors at five levels was employed to investigate the first and higher order main effects of each factor and the interactions among them. The experiment divided into 5 blocks contained 90 runs. The design involved 14 centre points with an alpha value being 2.83. The minimum and maximum ranges of the independent variables are given in Table 1. The experimental design included 90 flasks with three replicates at their central coded values. The mathematical relationship of response (enzyme production) and variables i.e. X_1, X_2, X_3, X_4, X_5 and X_6 was approximated by a quadratic model equation. The response value in each case is the average of triplicate experiments.

Table 1. Range of values for independent variables used in Central Composite Design (CCD) of RSM

Independent variables	Units	Low	High
Substrate concentration (X_1)	%	0.5	3.0
pH (X_2)	-	7	12
Temperature (X_3)	°C	30	65
Inoculum size (X_4)	%	2.5	15
Ca^{2+} concentration (X_5)	%	0.2	1.0
Mg^{2+} concentration (X_6)	%	0.2	1.0

3.3.2.1 Central composite design (CCD)

Central Composite Design (CCD) of Response Surface Methodology (RSM) was used to optimize the fermentation parameters for an enhanced production of amylase. RSM has four steps: procedures to move into the optimum region, behavior of the response in the optimum region, estimation of the optimal conditions and verification (Tanyildizi *et al*., 2005). A Central Composite Design (CCD) (Box and Wilson, 1951; Coman and Bahrim, 2011) was employed in this study. According to the CCD, the total number of experimental combinations is $2^k + 2k + n_{0,}$ where k is the number of independent variables and n_0 is the number of repetitions of the experiments at the centre point.

3.3.2.2 Enzyme assays

Amylase estimation was done by Starch-Iodine method (Xiao *et al.*, 2006) as mentioned in section 3.1.4.2.

3.3.2.3 Protein Estimation

Protein content was determined by the method of Lowry *et al*. (1951) as given in section 3.1.4.3

3.3.2.4 Statistical analysis

The statistical software package Design Expert® version 7.0 (Stat Ease, Inc, Minneapolis, USA) was used for regression analysis of the experimental data to obtain working parameters and to generate polynomials and the contour plots. A second-order polynomial equation was established based on analysis of variance and the optimum ratio of the medium components was found using the Design-Expert 7.1 software optimization toolbox. Standard deviation, PRESS, R^2 values were also analyzed.

3.3.2.5 Model validation

The mathematical model generated during RSM implementation was validated by conducting check point studies. The experimentally obtained data were compared with the predicted one and the prediction error was calculated.

3.3.3 SCALE UP OF α-AMYLASE IN A BIOREACTOR FROM *B. aerius* GC6

Among the isolates, *B. aerius* GC6 yielding highest titers of α-amylase was chosen for further scale-up in a stirred tank bioreactor, utilizing the conditions optimized in a shake flask experiment.

3.3.3.1 Inoculum preparation

B. aerius GC6 was grown in 300 ml of starch broth and incubated at 50±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 starch broth as blank.

3.3.3.2 Batch cultivation of α-amylase

Batch cultivation for the production of α-amylase 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 50±2^{o}C and pH 9.0. The conditions optimized for enzyme

production in the shake flask experiment (Section 3.3) were utilized for pilot scale production of amylase. The medium was sterilized in situ for 20 min at 121^{o}C.

3.3.3.3 Amylase assay: Same as given in section 3.1.4.2

3.3.3.4 Protein estimation: Same as given in section 3.1.4.3

3.3.3.5 Determination of growth kinetics in a bioreactor

Cell biomass in the fermentation broth was quantified by dry-cell weight analysis and by measurement of the optical density of the broth. For dry weight determinations, the cells were recovered by centrifugation at 4°C, 8000 rpm and washed twice with distilled water. The recovered biomass was dried to constant weight in an oven at 80°C for 24 h. Absorbance was read at 620 nm using a UV/Vis spectrophotometer for optical density.

3.3.3.6 Mathematical and Kinetic Modelling

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

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

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

Where μ = specific growth rate, / μ_{max} = maximum specific growth rate, S = substrate concentration, Ks = substrate, saturation constant (i.e. substrate concentration at half μmax).

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters μ_{max} and Ks.

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

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

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

In addition to this, Monod also related the yield coefficient (Yx/s) (equation 2) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (q) (equation 3).

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

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

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

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

3.4 PURIFICATION OF AMYLASE OBTAINED FROM *B. aerius* GC6 and *B. sonorensis* GV2

3.4.1 Production of amylase

Each of *B. aerius* GC6 and *B. sonorensis* GV2 culture (1.0 OD) was separately inoculated in their respective best selected medium @ 7.5% respectively. The flasks were incubated at 50±2^{o}C and 120 rpm for 72 h. The culture contents of both of the organisms were centrifuged separately at 12,000 rpm for 20 min at 4°C. Cell free supernatant as crude enzyme extract thus obtained was collected and stored at 4°C in refrigerator for further use.

3.4.2 Ammonium sulfate precipitation

The cell free crude extracts of the enzyme were subjected to sequential ammonium sulfate saturations. Different concentrations of ammonium sulfate in the range of 0-80 % were evaluated to attain saturation point for each of *B. aerius* GC6 and *B. sonorensis* GV2 amylase. The preparations were kept overnight at 4°C and then centrifuged that resulted in separation of precipitates and supernatants. Precipitates from each preparation so obtained were dissolved in minimum volume of Tris HCl buffer (20 mM, pH 8.0) separately and were refrigerated until further use.

3.4.3 Dialysis

Ammonium sulpahte salted out protein precipitates each of *B. aerius* GC6 and *B. sonorensis* GV2 amylase were dialyzed against 20mM Tris HCl (pH-8.0) buffer with five changes overnight at 4 ^{0}C using 14 kDa cut-off dialysis membrane.

3.4.4 Gel Exclusion Column Chromatography (Bollag and Edelstein, 1991a)

Sephadex G-75 and G-100 were weighed (5g) separately and suspended in 500 ml of 20mM Tris HCl (pH-8.0) buffer overnight for swelling. The packing was done with a precaution to avoid entrapment of any air bubbles in the gel bed. The packed column was first washed with distilled water and then pre-equilibrated with 20mM Tris HCl buffer (pH-8.0). The proteins obtained from ammonium sulfate saturation, after dialyzing were loaded on to the Sephadex G-75 column for *B. aerius* GC6 and G-100 column for *B. sonorensis* GV2 packed column and 60 fractions of 2 ml volume each were collected with a flow rate of 2ml/3.5min. The fractions were analyzed for protein contents by measuring OD at 280 nm. Based on highest OD, the most active fractions were pooled and purified enzyme was kept under refrigeration for further use.

3.4.5 Ion Exchange Chromatography (Bollag and Edelstein, 1991a)

The gel exclusion chromatography purified enzyme was further purified by anion exchange chromatography. DEAE-cellulose (2g) was suspended in 200 ml of distilled water for 48 h and packed in a glass column (10x1.0cm) avoiding layering of the gel or entrapment of any air bubbles in the gel bed. The column was first eluted with 100 ml distilled water to wash out any impurities. The column was then extensively washed with 500 ml of 20mM Tris HCl buffer (pH 8.0) containing 200mM NaCl, 1mM DTT and 1mM EDTA to remove contaminating proteins and then eluted with 20mM Tris HCl buffer (pH 8.0). Concentrated supernatant containing enzyme was loaded on the ion exchange column. Bound proteins were eluted with linear salt gradients (2 bed volumes) of 0.1 M, 0.2 M…., 1.0M NaCl in the same buffer at a flow rate of 0.2 ml min^{-1}. Fractions of 3 ml each were collected and monitored for amylase activity and the

protein content was measured at 280 nm. The active fractions were pooled and used for electrophoresis. The enzyme activity and soluble protein were estimated after each step of purification and purification fold was calculated.

3.4.6 SDS- PolyAcrylamide Slab Gel Electrophoresis (Laemmli, 1970)

SDS- PAGE was done on 12% polyacrylamide slab gel with 0.1% sodium dodecyl sulphate according to the protocol given below:

3.4.6.1 Equipment

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

3.4.6.2 Reagents

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

3.4.6.3 Stock solutions

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

3.4.6.4 Working solutions

Solution A (Acrylamide stock solution), 100 ml

30 % w/v acrylamide, 0.8 % (w/v) bis-acrylamide
29.2 g acrylamide
0.8 g bis-acrylamide
Add distilled water to make 100ml and stir until completely dissolved

Solution B (4 x separating gel buffer), 100 ml

75 ml 2 M Tris HCl (pH 8.8) -1.5 M
4 ml 10 % SDS -0.4 %
21 ml H_2O
Stable for months in refrigerator.

Solution C (4 x stacking gel buffer), 100 ml

50 ml 1 M Tris HCl (pH 6.8) - 0.5 M
4 ml 10 % SDS -0.4 %
46 ml H_2O
Stable for months in refrigerator.

10 % ammonium persulfate, 5 ml

0.5 g ammonium persulfate
5 ml H_2O
Stable for months in a capped tube in refrigerator.

Electrophoresis buffer, 1 l (pH 8.3)

3 g Tris	-	25 mM
14.4 g Glycine	-	92 mM
1g SDS	-	0.1 %

H_2O to make 1 l.

Stable indefinitely at room temperature

5 x sample buffer, 10 ml

0.6 ml 1 M Tris –HCl (pH 6.8)	-	60 mM
5 ml glycerol (50%, v/v)	-	25 %
2 ml SDS (10%, w/v)	-	2 %
0.5 ml 2- mercaptoethanol	-	14.4 mM
1 ml bromophenol blue (1%, w/v)	-	0.1 %

0.9 ml H_2O

Stable for weeks in the refrigerator for months at -20^{o}C.

3.4.6.5 Composition of stacking and resolving gel for SDS-PAGE

Table 2. Composition of stacking and resolving gel for SDS-PAGE

Working solution	**Stacking gel (5%)**	**Resolving gel (12%)**
Solution A	0.67 ml	4.0 ml
Solution B	-	2.5 ml
Solution C	1.0 ml	-
H_2O	2.3 ml	3.5 ml
Ammonium persulfate (10%)	30 µl	50 µl
TEMED	10 µl	10 µl
Total Volume	**4.0 ml**	**10 ml**

3.4.6.6 Protein molecular weight marker

The protein molecular weight marker PAGEmark^{TM} Protein Marker (medium range 14.3-97.4 kDa) from G-Biosciences already mixed with protein dye was used for SDS-PAGE.

3.4.6.7 Assembling of gel sandwich

Glass plates were washed well with distilled water and were oven dried. The bottom of both gel plates and spacer were perfectly flushed against a flat surface before tightening clamp assembly avoiding any leakage or air bubble entrapment. Various components of the resolving gel were mixed as specified in Table 3.2 and poured into the gel sandwich using a pipette after addition of TEMED. When appropriate amount of separating gel solution had been added, 1

cm of water was gently layered on top of separating gel solution to keep the gel surface flat. Gel was allowed to polymerize (30–60 min). After polymerization, water covering the separating gel was poured off. Components of stacking gel were mixed by gently swirling or inverting the container. Stacking gel solution was then poured onto separating gel until solution reached top of front plate. The comb was inserted into the gel sandwich until bottom of teeth reached top of front plate. Care was taken in order to avoid formation of any bubbles. Stacking gel was set to polymerize (about 30 min). After stacking gel polymerized, the comb was removed carefully. After the polymerization of gel, the whole assembly was placed into reservoir buffer chamber. Tank was filled with prechilled electrophoresis making sure that both top and bottom of gel were immersed in buffer. Air bubbles clinging to bottom of gel were removed to ensure even current flow.

3.4.6.8 Preparing and loading samples

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

Electrophoresis was carried out at a constant current of 3mA/ well. The protein bands were visualized by staining with Coomassie Brilliant Blue R 250 stain.

3.4.6.9 Staining and destaining a gel with Coomassie Blue

Staining with Coomassie Blue R 250 can detect as little as 0.1 µg of protein in a single band.

Table 3. Composition of staining and destaining solution

Coomassie Gel Stain (1l)		**Coomassie Gel Destain (1l)**	
Coomassie Blue R-250	1.0 g	Methanol	100 ml
Methanol	450 ml	Glacial acetic acid	100 ml
Distilled water	450 ml	Distilled water	800 ml
Glacial acetic acid	100 ml		

3.4.6.10 Staining procedure

After electrophoresis, the gel was transferred to a small gel staining box containing coomassie blue stain (approx. 50 ml) (Table 3) and agitated for 10-20 min on a slow rotary shaker at room temperature. The stain was then poured out and the gel was washed twice with distilled water. Coomassie destain (about 50 ml) was added and agitated gently on a slow rotary shaker for an hour. To destain completely, destain solution was changed 2-3 times and the gel was allowed to destain overnight with mild agitation.

3.4.6.11 Zymogram analysis (Kumar *et al*., 2004)

Zymogram analysis was performed by using the same conditions that of Native PAGE. Amylase activity was detected by incubating the native agarose gel into 1% starch solution at 50 ^{o}C for 1h. Gel was then removed from starch solution and was stained with iodine reagent and amylase bands were visualized as transparent bands on the dark blue background.

Alternatively, agarose gel bands were cut from the gel and were placed onto starch agar plates and the plates were incubated at 50 $^{\circ}$C for 24h. The plates were then flooded with iodine reagent. Amylase containing agarose gel gave a clear halo around the gel in the blue background.

3.5 CHARACTERIZATION OF α-AMYLASE FROM *B. aerius* GC6 AND *B. sonorensis* GV2

3.5.1 Effect of pH on the activity and stability

The effect of pH on enzyme activity was determined by incubating the reaction mixture at various pH ranging from 7.0 to 12.0 at 50±2 ^{0}C for 30 min. The buffers used were Citrate phosphate buffer (pH 7.0), Tris HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0 to 12.0).

3.5.1a Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.5.1b Protein estimation

Protein content was determined as given in section 3.1.4.3

3.5.2 Temperature optimum and thermal stability

To evaluate the optimal temperature for the enzyme activity, the assay was conducted at varying temperatures ranging from 35-121 ^{0}C.

To determine the thermostability of enzyme 0.5 ml aliquot of each purified amylase was added to 2.5 ml of freshly prepared Tris HCl (20 mM, 8.0 pH) buffer. The preparations were pre incubated at different temperatures ranging from 30, 40 ……121^{o}C for 0-180 min. Half life of the enzyme was determined as the time taken to reduce to half of the original activity.

3.5.2a Amylase assay

Amylase estimation was as mentioned in section 3.1.4.2.

3.5.2b Protein estimation

Protein content was determined as given in section 3.1.4.3

3.5.3 Effect of metal ions

0.5 ml of purified amylase in 2.5ml 20mM Tris HCl buffer (pH 8.0) was incubated for 30 min with various metal ions (5 mM) K^{+}, Hg^{2+}, Na^{+}, Ca^{2+}, Cu^{2+}, Mn^{2+},Zn^{2+}, Mg^{2+}.

3.5.3a Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.5.3b Protein estimation

Protein content was determined as given in section 3.1.4.3

3.5.4 Substrate specificity

0.5 ml of aliquot of purified amylase in Tris HCl buffer (pH- 8.0) was analyzed by incubating the samples at 50±2 $^{\circ}$C for 30 min with soluble starch, amylopectin, amylose, glycogen, xylose and maltodextrin etc. to evaluate the substrate specificity of the enzyme.

3.5.4a Amylase assay

Amylase estimation was done by Starch-Iodine method (Xiao *et al.*, 2006) as mentioned in section 3.1.4.2.

3.5.4b Protein estimation

Protein content was determined by the method of Lowry *et al*. (1951) as given in section 3.1.4.3

3.5.5 Effect of different concentrations of substrate

To evaluate the effect of different concentrations of substrate on enzyme activity, varying substrate concentrations ranging from 0.2%, 0.4%..... 2.0% in 20 mM Tris HCl buffer (pH 8.0) were incubated at 50 ±2^{o}C with enzyme for 30 min.

3.5.5a Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.5.5b Protein estimation

Protein content was determined as given in section 3.1.4.3

3.5.6 Raw starch adsorption and hydrolysis (Li *et al*., 2007)

1 ml of the purified amylase in 20 mM (pH 8.0) Tris HCl buffer was mixed with 0.2 g of raw potato starch and raw corn starch. The mixture was incubated at 50±2 ^{0}C for 15 min at 120 rpm. After the expiry of the mentioned time, the preparation was centrifuged at 8,000 g for 20 min and amylase activity was estimated as mentioned in section 3.1.4.2. The adsorption rate (AR) was calculated according to the equation:

$$AR\ (\%) = \frac{O - R}{O} \times 100$$

Where, R and O stand for the residual and original amylase activity, respectively.

3.5.7 Analysis of hydrolyzed products (Li *et al*., 2007)

Hydrolysis products of soluble starch by purified α-amylase from both the

strains were analyzed by ascending thin layer chromatography using silica gel plates. 1% starch was incubated with purified enzyme separately at 50 ºC for 12h. TLC was run with the solvent system of n-butanol-pyridine water (6:4:3) and a detection reagent comprising 2.0% (w/v) diphenylamine in acetone- 2.0% (w/v) aniline in acetone- 85% (w/v) phosphoric acid (5:5:1, v/v/v).

3.5.8 Determination of enzyme kinetics

The effect of soluble starch concentration ranging from 2.0, 4.0…… 20.0 mg/ml on partially purified amylase activity in 20mM Tris HCl buffer (pH 8.0) were incubated at 50^{o}C for 10 min under optimal assay conditions. The kinetic parameters of Michaelis- Menton constant, K_m and maximal reaction velocity, Vmax were determined by linear regression according to Lineweaver and Burk double-reciprocal plot. Catalytic turnover number (K_{cat}) was determined.

3.5.9 Shelf stability of amylase

Shelf stability of amylase was determined by pre incubating the enzyme at 4 ^{o}C and at room temperature in 20mM Tris HCl buffer (pH 8.0). Enzyme activity was determined every 7 days till 70 days.

3.5.9a Amylase assay

Amylase estimation was as mentioned in section 3.1.4.2.

3.5.9b Protein estimation

Protein content was as given in section 3.1.4.3

3.5.10 Effect of media additives

To determine the influence of different additives viz. SDS, EDTA, CTAB, Tween 20, Tween 80, Triton X 100 and Glycerol etc., purified α-amylase in 20mM Tris HCl buffer (pH 8.0) was pre-incubated for 30 min at 50±2 ^{0}C. Activity in the absence of additives was taken as 100%.

3.5.10a Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.5.10b Protein estimation

Protein content was determined as given in section 3.1.4.3

3.6. DETERMINATION OF INTERNAL AMINO ACID SEQUENCE OF AMYLASE BY MALDI-TOF MASS SPECTROMETRY

The internal amino acid sequence of purified α-amylase was determined by subjecting the digested mixture of peptides by trypsin to Matrix Assisted Laser Desorption Ionization- Time of Flight (MALDI-TOF) analysis for peptide mass fingerprinting and protein sequencing of the digestion products by mass spectrometry using Bruker Ultraflex MALDI-TOF MS. The mass-to-charge ratio of the resulting peptides gave information to identify the proteins in a sample. The resulting mass to charge ratio was matched against theoretical obtained mass data from already identified protein sequences and a score depending upon the correlation was given. The data was analyzed using MASCOT search engine. Search parameters for MALDI-TOF MS has been given in Table 4.

Table 4. Search parameters for MALDI-TOF MS

Type of search	Peptide mass fingerprint
Enzyme	Trypsin
Fixed modifications	Carbamidomethyl (C)
Variable modifications	Oxidation (M), Propionamide (C)
Mass value	Monoisotopic
Protein mass	Unrestricted
Peptide mass tolerance	0.5 Da
Peptide charge state	1+
Maximum missed cleavages	1

3.7 IMMOBILIZATION OF PURIFIED AMYLASE FROM *B. aerius* GC6 and *B. sonorensis* GV2

3.7.1 Immobilization by using natural hydrogels

3.7.1.1 Calcium alginate (Rajagopalan and Krishnan, 2008)

A 0.5, 1.0…., 3.0% percent solution of sodium alginate was prepared in 20mM warm Tris HCl buffer (pH 8.0). After cooling down to room temperature,

1ml of enzyme stock solution was mixed with 9ml of sodium alginate solution. The mixture was then suspended drop wise into prechilled 0.1, 0.2…..0.5M $CaCl_2$ solution with gentle stirring at 4 ^{0}C for 2h. The formed beads were recovered by filtration and thoroughly washed with distilled water. These beads were then stored in 20mM Tris HCl buffer (pH 8.0) at 4 ^{0}C.The filtered $CaCl_2$ solution was collected for enzyme activity determination.

3.7.1.2 Agar (Prakash and Jaiswal, 2011)

A 0.5, 1.0…., 3.0% solution of agar was prepared in 20mM warm Tris HCl buffer (pH 8.0). After cooling down to room temperature, 1ml of enzyme stock solution was mixed with 9ml of agar solution. The gel preparation was then casted on preassembled glass plates. After solidification, 10 ml of 10% solution of glutaraldehyde was added and left for complete hardening. The gel was then cut into small chips of equal size and washed thoroughly with distilled water. These chips were then stored in 20mM Tris HCl buffer (pH 8.0) at 4 ^{0}C.

3.7.1.3 Agarose (Prakash and Jaiswal, 2011)

0.5, 1.0…., 3.0% solution of agarose was prepared in 20mM warm Tris HCl buffer (pH 8.0). After cooling down to room temperature, 1ml of enzyme stock solution was mixed with 9ml of agarose solution. The gel preparation was then casted on preassembled glass plates. After solidification, 10 ml of 10% solution of glutaraldehyde was added and left for complete hardening. The gel was then cut into small chips of equal size and washed thoroughly with distilled water. These chips were then stored in 20mM Tris HCl buffer (pH 8.0) at 4 ^{0}C.

3.7.1.4 Gelatin (Jaiswal *et al*., 2012)

10, 20…, 50% solution of gelatin was prepared in 20mM Tris HCl buffer (pH 8.0) by heating at 50 ^{0}C. After cooling down to room temperature, 1ml of enzyme stock solution was mixed with 9ml of gelatin solution. The gel preparation was then casted on preassembled glass plates. After solidification, 10 ml of 10% solution of glutaraldehyde was added and left for complete hardening. The gel was then cut into small chips of equal size and washed thoroughly with distilled water. These chips were then stored in 20mM Tris HCl buffer (pH 8.0) at 4 ^{0}C.

3.7.1.5 Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.7.1.6 Enzyme Immobilization Yield

Efficiency of different carriers used for immobilization was determined by calculating % Immobilization yield as:

Immobilization Yield (%) = TA_I / TA_S x 100

where, TA_I and TA_S stand for total activity of enzyme in immobilized gel and total activity of the soluble enzyme loaded.

3.7.2 Immobilization by using Nanoparticles (Wang *et al*., 2012)

3.7.2.1 Requirements

Dried ascorbic acid modified nano-particles

Tris HCl Buffer (20mM, pH 8.0)

Amylase-Tris HCl Buffer stock solution: 5 ml of purified amylase was added to 15 ml of Tris HCl buffer, pH 8.4

3.7.2.2 Procedure

For immobilization of amylase onto nano-particles, 30 mg of nano particles were suspended in Tris HCl buffer, pH 8.4 and were ultrasonicated for 5 min at room temperature. 5 ml of amylase-Tris HCl Buffer stock solution was added to it and was incubated with N_2 for 5 min. the mixture was then kept for shaking at 100 rpm for 24 h at 37 ^{0}C for immobilization of amylase onto magnetic nano-particles (Wang *et al*., 2012). Amylase immobilized nano-particles were then separated by a magnet and supernatant was collected. Collected nano-particles were given several washings with the buffer and were dispersed into Tris HCl buffer for further use.

3.7.2.3 Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.7.2.4 Enzyme immobilization yield

Efficiency of different carriers used for immobilization was determined by calculating % Immobilization yield as:

Immobilization Yield (%) = TA_I / TA_S x 100

where, TA_I and TA_S stand for total activity of enzyme in immobilized gel and total activity of the soluble enzyme loaded.

3.7.3 Reusability of the immobilized enzyme

The reusability of different carrier immobilized amylase from *B. aerius* GC6 and *B. sonorensis* GV2 was studied by assaying the activity in the beads/ nano particles several times for starch hydrolysis. Taking the activity of the first batch as 100%, the immobilized enzyme in the form of beads or nano particles was reassayed for enzyme activity. After each activity assay, the beads were removed and washed thoroughly with distilled water prior to the subsequent assay procedure.

3.7.3.1 Amylase assay

Amylase estimation was done as mentioned in section 3.1.4.2.

3.7.3.2 Enzyme immobilization yield

Efficiency of different carriers used for immobilization was determined by calculating % Immobilization yield as:

Immobilization Yield (%) = TA_I / TA_S x 100

where, TA_I and TA_S stand for total activity of enzyme in immobilized gel and total activity of the soluble enzyme loaded.

3.8 APPLICATION OF PURIFIED α-AMYLASE IN BIOETHANOL PRODUCTION USING ALGAL BIOMASS

3.8.1 Collection of algal biomass

The fresh water green algae were collected from fresh water ponds of Solan, Himachal Pradesh. The algal samples were washed thoroughly with tap

water to remove salts, epiphytes and debris and dried at 50 ^{0}C. After drying the samples were powdered to attain 1 mm mesh size using grinder for further use.

3.8.2 Identification of algal species

Identification of collected green algae was done by applying a two-step approach:

- In field identification by utilizing Algae Identification Field Guide (Huynh and Serediak, 2006)
- Microscopic identification was done in laboratory using imaging microscope (Zeiss, Germany) at 20X and 45X. Wet mount of the sample was prepared on a clean, dry glass slide by putting cover slip over the sample by avoiding air bubbles.

3.8.3 Chemical composition determination of algal biomass

The chemical composition analysis of biomass was done by using the following methods:

3.8.3.1 Estimation of Cellulose (Sadasivam and Manickam, 1991)

3.8.3.1.2 Reagents

Acetic & Nitric acid reagent: 150 ml of 80% acetic acid was mixed with 15 ml of concentrated nitric acid.

Anthrone Reagent:200 mg anthrone was dissolved in 100 ml conc. H_2SO_4. It was prepared fresh and was prechilled for 2h.

Sulphuric Acid: Sulphuric acid 67%

Standard cellulose stock: 100mg in 100 ml water

Working standard: 10 ml stock diluted to 100 ml with water

3.8.3.1.2 Procedure

0.5 g sample was taken in a test tube and 3 ml acetic & nitric acid reagent was added to it. The test tube was placed in a water bath at 100 $^{\circ}$C for 30 min.

The contents were then cooled and were centrifuged at 8,000 rpm for 15-20 min. Supernatant was discarded and the residue was washed with distilled water. 10 ml of 67% H_2SO_4 was added to the residue and allowed to stand for 1 h. 1ml of the above preparation thus formed was diluted to 100 ml. For the preparation of standard curve 0.2, 0.4….1.0 ml of the working standard was pipetted out and volume was made up to 1ml with distilled water. To 1 ml of this solution, 10 ml of anthrone reagent was added and mixed well. The tubes were kept in a boiling water bath for 10 min and cooled and the contents were read spectrophotometrically at 630 nm.

3.8.3.2 Estimation of Hemicellulose (Ververis *et al*., 2007)

3.8.3.2.1 Reagents

- 72% H_2SO_4

3.8.3.2.2 Procedure

0.7 g of algal biomass was boiled in 72% H_2SO_4 for 4.5 h in order to hydrolyse cellulose and hemicelluloses. The residue thus formed was filtered and dried at 105 ^{0}C for 24h. The residue after drying was transferred to a preweighed crucible and heated at 600 ^{0}C for 5h. Leftover ash was then weighed. % (w/w) hemicellulose was calculated by taking the weight of the ash and by diving it by initial weight.

3.8.3.3 Estimation of Pectic Substances (Sadasivam and Manickam, 1991)

3.8.3.3.1 Reagents

- 1N Acetic Acid
- 1N Calcium chloride solution
- 1% Silver nitrate
- 0.01N HCl
- 0.05N HCl
- 0.3N HCl

3.8.3.3.2 Procedure

50 g of sample was weighed in 1000 ml beaker and 300 ml of 0.01N HCl was added to it. It was boiled for 30 min and filtered. Residue was washed with warm water and filtrate was collected. To this residue, 100 ml 0.05 N HCl was added and boiled for 20 min. Residue was washed with warm water and filtrate was collected. To the residue was then added 100 ml 0.3 N HCl and was boiled for 10 min. Residue was washed with warm water and filtrate was collected. The filtrates were pooled, cooled and the volume was made up to 500 ml & 100 to 200ml of aliquots were pipetted into 1000 ml beaker. 250 ml of water was added to it and the acid was neutralized using 1N NaOH using phenolphthalein indicator and allowed to stand overnight. Afterwards 50 ml of 1N acetic acid was added to it and then after 5 min, 25 ml 1N calcium chloride solution was added with continuous stirring. It was allowed to stand for 1 h and boiled for 1-2 min. It was then filtered through a pre-weighed Whatman No. 1 filter paper. Filtrate was then washed with almost boiling water until the filtrate is free from chloride. Filter paper with calcium pectate was dried at 100 ^{0}C in a weighing dish. The pectin content is expressed as % calcium pectate

$$\% \text{ Calcium pectate} = \frac{\text{Weight of calcium pectate x 500 x 100}}{\text{ml of filtrate taken} \times \text{Weight of sample for estimation}}$$

3.8.3.4 Estimation of Starch (Sadasivam and Manickam, 1991)

3.8.3.4.1Reagents

Anthrone Reagent: 200 mg of anthrone was dissolved in 100ml of ice-cold 95% sulfuric acid.

Ethanol 80%

Perchloric acid 52%

Standard Glucose Stock: 100 mg in 100 ml water.

Working standard: 10 ml stock diluted to 100 ml with water.

3.8.3.4.2 Procedure

0.5 g of sample was homogenized in hot 80% ethanol to remove sugars. It was then centrifuged to retain the sugars. The residue was washed repeatedly

with hot 80% ethanol until the washings did not give color with ethanol. The residue was dried over a water bath. To this residue, 5.0 ml of water and 6.5 ml of 52% perchloric acid was added. It was then extracted at 0 ^{0}C, centrifuged and supernatant was saved. The extractions were repeated using fresh perchloric acid and supernatants were pooled to make volume 100 ml. 0.1 or 0.2 ml of the supernatant was pipetted out and volume was made up to 1ml. For the preparation of standard curve 0.2, 0.4…., 1.0 ml of the working standard was pipetted out and volume was made to 1ml with distilled water. 4 ml of Anthrone reagent was added to each tube and the tubes were heated for 8 min in a boiling water bath. The contents were then cooled and color intensity was read at 630 nm.

3.9 PHYSIOLOGICAL PRETREATMENTS OF ALGAL BIOMASS

3.9.1 The following pretreatments were used for pretreating the algal biomass.

3.9.1.1 NaOH + H_2O_2 pretretament (Sharma and Sharma, 2013)

5 g of algal biomass was immersed in 1% NaOH + H_2O_2 in the ratio of 9:1 for 4 h. After the pretreatment the slurry (supernatant and residue) was neutralized and were investigated for reducing sugars.

3.9.1.2 H_2SO_4 + steam pretreatment (Kaushal, 2014)

5 g of algal biomass with 1.0 % H_2SO_4 was taken in a flask and was steamed in an autoclave at a pressure of 1.1 bar, at 121°C for 60 min. After pretreatment, the pH of the slurry (supernatant and residue) was set to 7.0 with NaOH, which was then tested for reducing sugars.

3.9.1.3 NaOH + Urea pretreatment (Tandon, 2014)

5 g of algal biomass was immersed in 50 ml 1% NaOH + Urea (1:1) in a ratio of 3:1 and incubated at room temperature for 24 h. slurry was then neutralized to pH 7.0 and was then tested for reducing sugars..

3.9.1.4 H_2SO_4 pretreatment (Harun and Danquah, 2011)

5 g sample was immersed in 50 ml of 1% H_2SO_4 and incubated at 100 ^{0}C in a water bath for 30 min. The contents were then cooled and pH was neutralized. The slurry thus obtained was then tested for reducing sugars.

3.9.1.5 Nitric acid/ Acetic acid treatment (Nahak *et al*., 2011)

5 g sample was immersed in 50 ml of nitric acid/ acetic acid (1:1) and incubated at 100 ^{0}C in a water bath for 30 min. The contents were then cooled neutralized and tested for reducing sugars.

3.9.1.6 NaOH pretreatment (Eshaq *et al*., 2011)

5 g sample was immersed in 50 ml of 1% NaOH and incubated at room temperature for a period of 2 h. pH was neutralized and the slurry was then tested for reducing sugars.

3.9.1.7 Saturated Steam pretreatment (Trivedi *et al*., 2013)

5 g sample was steam pretreated at 121 ^{o}C for 30 min. contents were cooled and was then tested for reducing sugars.

3.9.1.8 Estimation of Reducing sugars (Miller, 1959)

Reagents

i. Dinitrosalicylic acid (DNSA) Reagent

ii. Standard solution of glucose (0.4 mg/ml)

To 1.0 ml of supernatant, 3 ml of DNSA reagent was added and the mixture was heated on boiling water bath for 15 min. After cooling down to room temperature, absorbance of reaction mixture was read at 540 nm.

Among the pretreatments tested on the basis of reducing sugars produced, 1% H_2SO_4 pretreatment was adjudged as the best so was selected for further studies.

3.9.2 Optimization of conditions for dil. H_2SO_4 pretreatment

Varying concentrations of H_2SO_4 i.e. 0.5, 1.0 and 1.5% were tested for variable time ranging from 30 min to 180 min for selecting best concentration and best time combination. 0.5% H_2SO_4 for 180 min was adjudged as the best pretreatment yielding maximum sugars which was taken up for further studies.

3.10 ENZYMATIC HYDROLYSIS OF ALGAL BIOMASS

3.10.1 Source of enzymes

The following purified and sterilized (using 0.22 µm-pore size filters) enzymes from potential in house microorganisms were used for enzymatic hydrolysis of pretreated algal biomass by mixing them in varied ratios.

Table 5. Enzymes used for preparing mixture of enzymes for enzymatic hydrolysis of *Hydrodictyon* biomass

Enzyme	Isolate	Enzyme activity (IU)	Reference
α-amylase	*B. aerius* GC6 |KJ775810.1|	106.46 IU	Present study
β-amylase	*B. amyloloqiefaciens* SH8 |JX129360.1|	18.40 IU	Kumar, 2012
Cellulase	*B. stratosphericus* N12 |KC995118|	2.00 IU	Sharma, 2013
Pectinase	*Bacillus* sp. C6	6.28 IU	Ongoing study

3.10.2 Optimization of conditions for enzymatic hydrolysis by using CCD of Response Surface Methodology (RSM)

Enzymatic hydrolysis conditions were optimized by employing Central Composite Design (CCD) of Response Surface Methodology (RSM). The independent variables selected were biomass (X_1), enzyme loading (X_2) and time (X_3) and the dependent variable was reducing sugars. The central composite design (CCD) with three factors at five levels was employed to investigate the first and higher order main effects of each factor and the interactions among them. The experiment contained 20 runs. The design involved 6 centre points with an alpha value being 1.68. The minimum and maximum ranges of the independent variables are given in Table 6. The mathematical relationship of response (reducing sugars) and variables i.e. X_1, X_2 and X_3 was approximated by a quadratic model equation. The response value in each case is the average of triplicate experiments.

Table 6. Range of values for independent variables used in Central Composite Design (CCD) of the RSM

Independent variables	Units	Low	High
Biomass concentration (X_1)	g/l	5	30
Enzyme loading (X_2)	ml/g biomass	5	20
Time (X_3)	h	0	48

3.10.3 Estimation of reducing sugars (Miller, 1959)

Reducing sugars in the slurry was estimated by the method described in section 3.9.1.8.

3.10.4 Statistical analysis

The statistical software package Design Expert® version 7.0 (Stat Ease, Inc, Minneapolis, USA) was used for regression analysis of the experimental data to obtain working parameters and to generate polynomials and the contour plots. A second-order polynomial equation was established based on analysis of variance and the optimum ratio of the medium components was found using the Design-Expert 7.1 software optimization toolbox. Standard deviation, PRESS, R^2 values were also analyzed.

3.10.5 Model validation

The mathematical model generated during RSM implementation was validated by conducting check point studies. The experimentally obtained data were compared with the predicted one and the prediction error was calculated.

3.11 BIOCONVERSION OF HYDROLYZED ALGAL BIOMASS INTO BIOETHANOL UNDER SHF (SEPARATE HYDROLYSIS AND FERMENTATION) AND SSF (SIMULTANEOUS SACCHARIFICATION AND FERMENTATION)

Bioethanol production from hydrolyzed algal biomass was carried out by opting for different processes of fermentation i.e. Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) as given below:

3.11.1 Substrate: Algal hydrolysate, grinded and pretreated with 0.5% H_2SO_4 as mentioned in section 3.9.2

3.11.2 Source of hydrolytic enzymes: As mentioned in section 3.10.1

3.11.3 Separate Hydrolysis and Fermentation (SHF)

3.11.3.1 Enzymatic hydrolysis

Same as given in section 3.10.2

3.11.3.2 Ethanol Fermentation

3.11.3.2.1 Optimization of fermentation conditions for Separate Hydrolysis and Fermentation (SHF)

3.11.3.2.1.1Optimization of fermentation period

Fermentation period spanning from 24 to 72h has been studied at an interval of 12h each.

Fermentation period of 36 h was adjudged as the best and was selected for further optimizations.

3.11.3.2.1.2 Optimization of inoculum size of fermenting organism

Inoculum size for fermenting organism was optimized in the range spanning 10, 15…30%.

20% inoculum size of fermenting microorganism was found to be the best, was thus selected for further studies.

3.11.3.2.1.3 Ethanologens used

Fermenting microbes i.e. *S. cerevisiae* I (MTCC-3089) procured from MTCC Chandigarh –India, was used for fermentation in the experiments. The inoculum was prepared by growing cells aerobically in 250 ml flask containing 100 ml of the preculture medium (Struch *et al*., 1995) comprised of (gl^-): glucose, 20g; yeast extract, 10g; $(NH_4)_2SO_4$, 1g; K_2HPO_4, 1g; $MgSO_4.7H_2O$, 0.5g in a rotary shaker incubator for 24 h at 25±2^oC to make 1.0 O.D culture.

3.11.4 Simultaneous Saccharification and Fermentation (SSF)

3.11.4.1 Optimization of fermentation conditions for SSF

3.11.4.1.1 Optimization of fermentation pH

Fermentation pH ranging from 5.2, 5.4…6.0 was optimized for efficient fermentation of algal biomass into ethanol.

Best pH of 5.6 yielding highest ethanol was selected for further studies.

3.11.4.1.2 Optimization of inoculum size and fermentation time

Inoculum size in the range spanning 10, 15….30% was optimized starting from fermentation time of 12h at a regular interval of 4h till 40^{th} h.

15% inoculum, at 28 h of fermentation period yielded maximum ethanol thus was selected for further strudies.

3.11.4.1.3 Optimization of fermentation temperature

Fermentation temperature in the range spanning 25, 30….45 ^{o}C was tested for efficient ethanol production.

35 °C was adjudged as best temperature yielding maximum ethanol thus was selected for further studies.

3.11.4.1.4 Process

25 g/l of 0.5% H_2SO_4 pretreated algal biomass (pH 5.6) was taken in a 250 ml flask. To this preculture medium (Struch *et al*., 1995) comprised of (gl^{-}): glucose, 20g; yeast extract, 10g; $(NH_4)_2SO_4$, 1g; K_2HPO_4, 1g; $MgSO_4.7H_2O$, 0.5g was added and the contents were autoclaved at 121 ^{o}C for 20 min. The hydrolytic enzymes were added after sterilization using a 0.22 µm pore size filter. *S. cerevisiae*-I (MTCC-3089) was added @ 15%. SSF was performed in a 300 ml flask with 100 ml of medium in an orbital shaker at 100 rpm for 28 h at 35 ^{o}C.

3.11.5 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 1000 ml by adding distilled water. To 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 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.

3.12 SCALE UP OF ETHANOL PRODUCTION IN A STIRRED TANK BIOREACTOR

3.12.1 Separate Hydrolysis and Fermentation (SHF)

3.12.1.1 Inoculum Preparation

S. cerevisiae I (MTCC-3089) was grown in 300 ml of preculture medium and incubated at 25±2°C for 24 h. As soon as the substantial growth of the strain was observed in the broth, optical density was set to 1.0 using broth as blank.

3.12.1.2 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 of the enzymatic hydrolysate at 30°C and pH 5.6, utilizing the conditions optimized in the shake flask experiment. The enzymatic hydrolysate was seeded with 20% inoculum of *S. cerevisiae*-I (MTCC-3089) under aseptic conditions. The fermentation was carried out under anaerobic conditions. The agitation speed 100 rpm was used for batch cultivation in bioreactor. The samples were withdrawn regularly at different intervals ranging from 3, 6……..42 h and ethanol yield, reducing sugars were estimated.

3.12.1.3 Determination of Growth Kinetics in a Bioreactor

Cell biomass in the fermentation broth was quantified by measurement of the optical density of the broth and absorbance was read at 600 nm.

3.12.1.4 Ethanol estimation: Ethanol analysis in the fermentation broth was carried out using a gas chromatograph through direct sample injection using an external standard for calibration. The sample was distilled before injection to the GC column.

3.12.1.4.1 Conditions for GC MS

Column temperature	100^{o}C
Injector temperature:	220^{o}C
Detector:	Flame Ionization Detector (FID)
Injection volume:	1.0 µl
Run time	13.74 min

3.12.1.5 Reducing sugars: Reducing sugars were estimated by high performance liquid chromatography (HPLC)

3.12.1.5.1Conditions for HPLC

Column:	Ultra C18 (Restek Corp.), 250mm × 4.6 mm, 5µm
Mobile Phase A:	90: 10 water: methanol, 10mM ammonium formate
Flow:	0.5 ml /min
Temperature:	30^{o}C
Detector:	UV@ 280 nm
Injection volume:	10µl
Standard dilution:	1mg/ml

3.12.1.5.2 Formula to calculate the concentration of reducing sugars

$$\text{Concentration of reducing suagrs} = \frac{\text{Area of Sample}}{\text{Area of Standard}} \text{ X Standard dilution}$$

3.12.2 Simultaneous Saccharification and Fermentation (SSF)

3.12.2.1 Inoculum Preparation

S. cerevisiae I (MTCC-3089) was grown in 300 ml of preculture medium and incubated at 25±2°C for 24 h. As soon as the substantial growth of the strain was observed in the broth, optical density was set to 1.0 using broth as blank.

3.12.2.2 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. Conditions optimized for shake flask experiment were utilized in bioreactor. The fermentation was carried out under anaerobic conditions. The agitation speed 100 rpm was used for batch cultivation in bioreactor. The samples were withdrawn regularly at different intervals ranging from 3, 6……..42 h and ethanol yield, reducing sugars were estimated.

3.12.2.3 Determination of Growth Kinetics in a Bioreactor

Cell biomass in the fermentation broth was quantified by dry measurement of the optical density of the broth and absorbance was read at 600 nm.

3.12.2.4 Ethanol Estimation: same as given in section 3.12.1.4

3.12.2.5 Reducing sugars: same as given in section 3.12.1.5

3.12.3 Mathematical and Kinetic Modeling for Ethanol Production

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

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

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

Where μ = specific growth rate, / μ_{max} = maximum specific growth rate, S = substrate concentration, Ks = substrate, saturation constant (i.e. substrate concentration at half μ_{max}).

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters μ_{max} and Ks.

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

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

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

In addition to this, Monod also related the yield coefficient (Yx/s) (equation 2) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (q) (equation 3).

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

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

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

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

Chapter-4

RESULTS AND DISCUSSION

The present investigation entitled **"Purification and characterization of amylase produced from potential extremophiles for industrial application"** was undertaken in Microbiology Research Laboratory, Department of Basic Sciences, Dr. YS Parmar University of Horticulture and Forestry, Nauni -Solan, Himachal Pradesh, during the year 2011-15. Here we present the experimental results discussed with 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 screening of amylolytic microorganisms from mushroom compost

4.2 Identification of screened hyperamylolytic isolates

4.3 Production and optimization of α-amylase using one variable at a time (OVAT) approach and Response surface methodology (RSM)

4.4 Sequential purification of α-amylase

4.5 Biochemical and kinetic characterization of purified α-amylase

4.6 Internal amino acid sequence of amylase determined by MALDI-TOF Mass Spectrometry

4.7 Immobilization of purified α-amylase on different natural substrates and its characterization

4.8 Immobilization of purified α-amylase on synthetic iron oxide nano particles and its characterization

4.9 Application of purified α-amylase in bioethanol production using algal biomass

4.10 Physicochemical pretreatment of algal biomass

4.11 Enzymatic hydrolysis of algal biomass

4.12 Fermentation of ethanol in a shake flask experiment under SHF (Separate hydrolysis and fermentation) and SSF (Simultaneous saccharification and fermentation)

4.13 Scale up of bioethanol production in a stirred tank bioreactor

4.1 ISOLATION AND SCREENING OF HYPER AMYLOLYTIC MICROORGANISMS FROM MUSHROOM COMPOST

4.1.1 Isolation and qualitative screening of amylolytic microorganisms

The natural niches like soil, compost degrading litter and hot water springs etc. are rich reservoirs of industrially important potential enzyme producing microorganisms. Among them, the least explored and highly probable source of hydrolytic enzyme producing microorganisms, mushroom compost has been selected in the present study for isolation of thermoalkalophilic amylogenic microorganisms. Mushroom compost mainly comprised of agro-residues such as wheat straw, paddy straw, sugarcane bagasse, represents a thermogenic, solid state fermentation process that results from succession of microbial community. It is an eco-system which harbours a multitude of microbial diversity ranging from mesophiles to thermophiles, which could be attributed to the decomposition of organic matter (Agrawal, 2014).

Mushroom compost samples collected from the Directorate of Mushroom Research (DMR), Chambaghat, Solan, HP were processed for isolation of amylase producing microorganisms by directly plating the serially diluted samples onto the starch agar plates. The plates were flooded with iodine reagent for the appearance of clear zone post incubation, which indicated the presence of thermoalkalophilic amylogenic strains. In total, 13 thermoalkalophilic amylogenic bacterial isolates showing clear zones of hydrolysis with varying diameters were obtained from the pooled samples of mushroom compost on Starch Agar medium at 50±2°C having pH 9.0.

Nowadays, amylase is one of the most important enzymes in industry and its application even increase with the discovery of thermostable enzymes (Pandey *et al.,* 2000a; Raharjo *et al.,* 2010). Microbial sources are solely used for the commercial production of this enzyme in economical bulk and due to simple preparation processes. It has been established that the use of thermophilic enzymes in starch processing industry could deliver major benefits related to the reduced risk of contamination, reduced viscosity and reaction time and increased solubility of starch (Bhabla and Chtanta, 2000). Thermophilic strains *B. licheniformis, B. stearothermophilus* and *B. amyloliquefaciens* have been reported to be used for thermophilic amylase production (Wind *et al.,* 1994; Mithu *et al.,* 2005). However, new trials need to be focused on finding new sources of this enzyme with distinct properties for diverse industrial application. It has been well documented that the enzymes of an organism are adapted to function optimally near or at its growth conditions (Khalil, 2011) as the microorganisms surviving in extreme conditions are having certain special features, which mainly consist of novel enzymes or biochemical pathways. In this light, the thermoalkalophilic amylogenic strains isolated in this study have an immense industrial importance.

All the thermoalkalophilic amylogenic isolates were preliminarily characterized based on their morphological and cultural characteristics. Among the bacterial isolates, all were Gram positive. Morphological fingerprinting of the colonies revealed that 38% (Fig. 1) were white in color, 23% were cream and 31% were brown while 8% were yellow in color. As far as shape of the colonies was concerned, 69% of the colonies were circular in form, 23% were wrinkled while 8% were punctiform. On the basis of the colony elevation and margin, 85% of the isolates were flat, while 8% were each umbonate and raised whereas 54% were entire, 38% were undulated and 8% were lobulate (Fig. 1). Based on the cell shape, 61% were rod shaped, 23% were coccobacillus while 15% were small rods in shape. The pattern of growth in the broth culture majorly revealed the formation of smooth sediments and pellicle by 46% and 38% of the isolates respectively while 8% of the isolates represented growth each as loose sediments and film formation.

Table 1. Biochemical characteristics of screened alkalothermophilic amylolytic bacteria isolated from mushroom compost

Characteristics	Screened Amylolytic Isolates												
	GC1	GC2	GC3	GC4	GC5	GC6	GC7	GC8	GV1	GV2	GV3	GV4	GV5
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	-	-	+	-	-	-	+	-	+	-	-	-
H_2S	+	-	+	+	-	+	-	-	+	-	+	+	-
Methyl Red	-	-	+	+	+	+	-	+	+	-	-	+	+
Voges Proskeur	-	-	-	-	-	-	-	-	-	-	-	-	-
DNase activity	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	-	-	-	-	+	+	-	+	-	+	+	+
Cellulolytic	-	-	-	-	-	-	-	-	-	-	-	-	-
Sugar fermentation													
• Glycerol	A	A	A	AG	A	A	AG	A	A	AG	A	AG	A
• Sorbitol	-	-	AG	-	A	AG	-	-	-	A	-	-	-
• Dextrose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
• Galactose	AG	A	A	A	AG	A	A	AG	A	A	AG	A	A
• Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
• Mannose	A	A	A	A	AG	A	A	AG	A	A	AG	A	A
• Sucrose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
• Arabinose	-	-	A	-	A	-	AG	-	-	AG	A	-	-
• Lactose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
• Raffinose	A	A	A	A	A	AG	A	A	A	A	A	A	A
• Inulin	A	A	A	A	-	-	-	-	-	-	A	-	A
• Maltose	AG	AG	AG	AG	A	A	AG	AG	AG	A	AG	AG	AG
• Sucrose	A	A	A	A	AG	-	A	-	A	-	-	-	-
Tentative identification	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.

A- acid production G- gas production

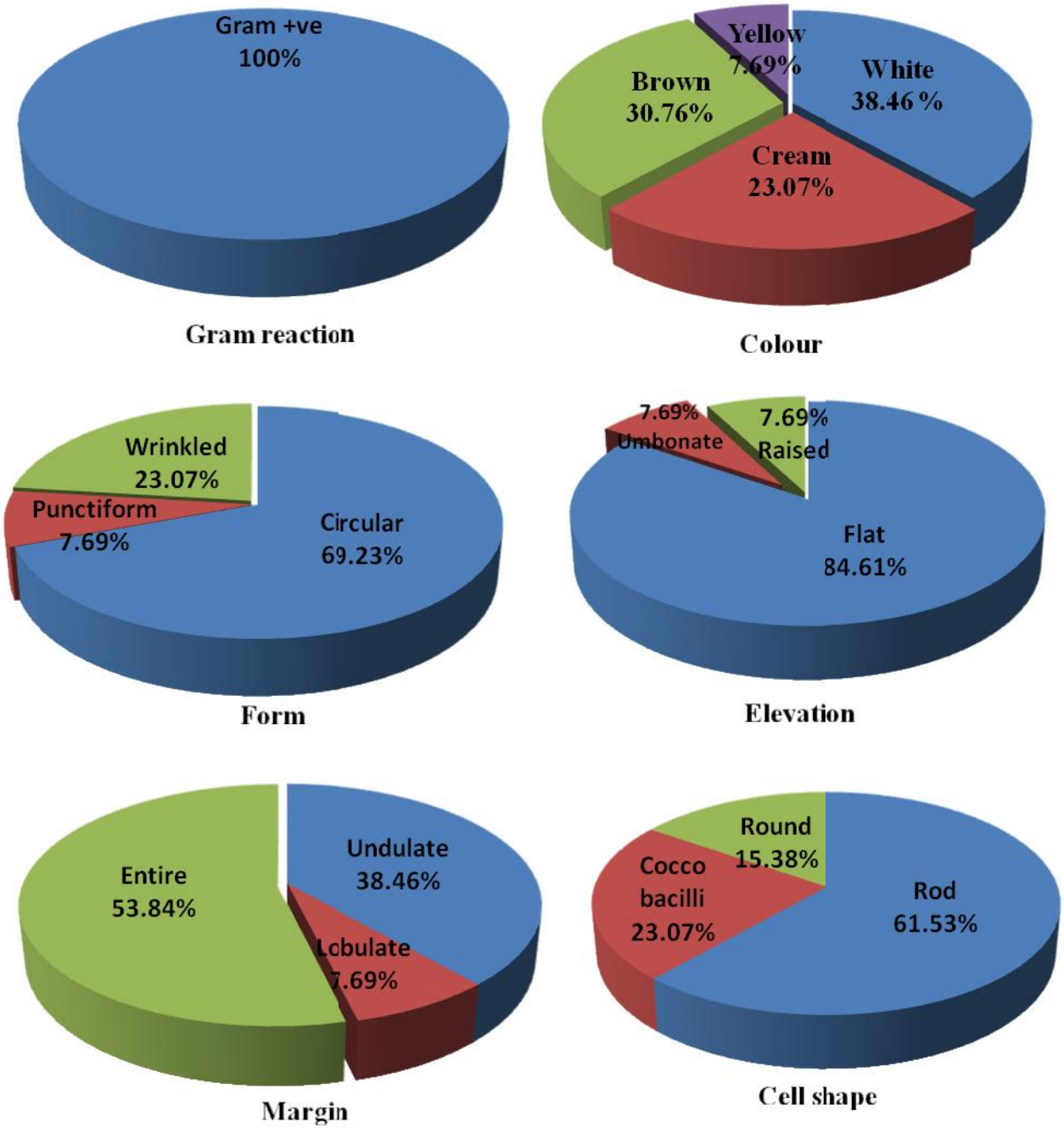


Fig 1. Representation of consolidated morphological and cultural characteristics (in percentage) of screened population of alkalothermophilic amylolytic bacteria isolated from mushroom compost

Table 1 represents the biochemical and metabolic characteristics of all the bacterial isolates. Different biochemical tests performed were citrate utilization test, catalase test, H_2S production test, urease test, MR/VP test and indole test etc. Fermentation pattern of different sugars has also been tested for characterization of the screened isolates. Among them, all the isolates were found negative for indole production, VP test, gelatin and cellulose hydrolysis and for DNAase activity. All the isolates were found to be positive for citrate utilization and catalase production. Among all, 62% isolates were found to be positive for methyl red test while 54% were positive for nitrate reduction and 56% were positive for H_2S production.

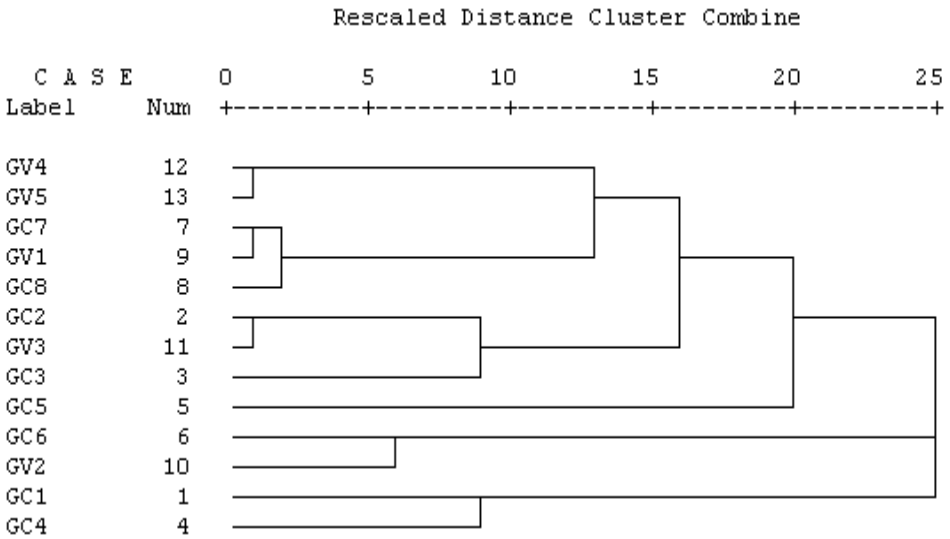


Fig. 2 Dendrogram depicting the metabolic and biochemical profiling based similarity and differences among the screened thermoalkalophilic amylolytic bacterial isolates based on rescaled distance

The fermentation pattern of sugars was also very different for all the isolates with some of them producing both acid and gas while most of them producing either acid or gas (Table 1). Glycerol, glucose, galactose, maltose, raffinose, mannose and sucrose were fermented by all the isolates. Rest of the sugars such as sorbitol, arabinose, inulin exhibited a varied pattern where some of the isolates showed fermentation whereas others did not. Xylose was the sugar which was not fermented by any of the isolates. Fig. 2 represents the cluster formed by employing SPSS software of the screened amylogenic isolates based

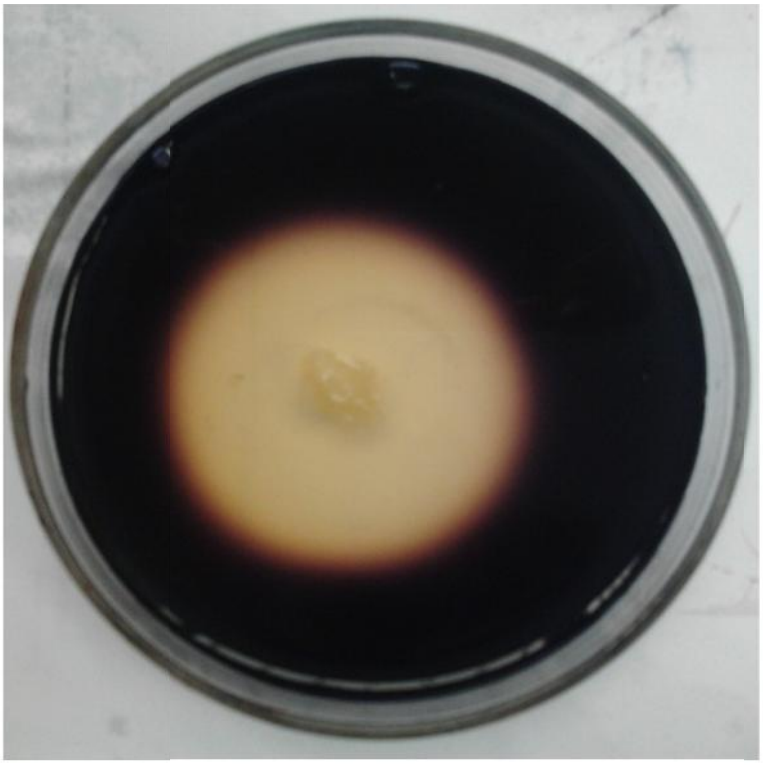
on their biochemical and metabolic fingerprinting. The figure depicts similarity among all the isolates as evident from the clusters formed. The unrooted dendrogram inferred that all the isolates were similar physiologically as well as metabolically.

Based on the above mentioned morphological, cultural, biochemical and metabolic fingerprinting, the amylolytic isolates were tentatively identified using ABIS-online software (http://www.tgw1916.net/bacteria_abis.html). On the basis of similarity the isolates showed 92% homology to genus *Bacillus*. Among the isolates, GC6 (Plate1a) and GV2 (Plate1b) exhibited biggest zone of hydrolysis around the colony among the blue stained starch plate.

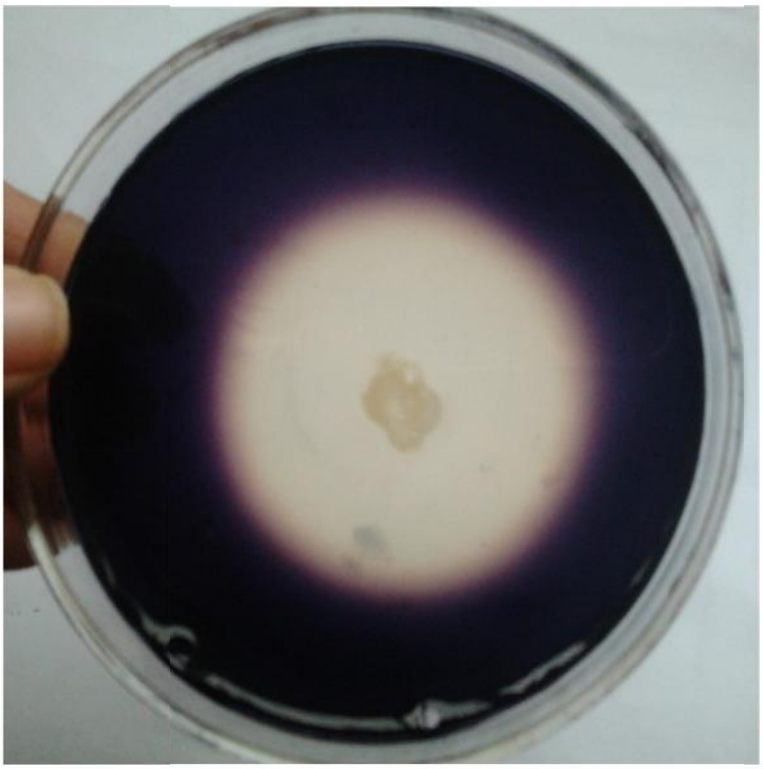
4.1.2 Quantitative screening of thermoalkalophilic α-amylolytic microorganisms

Qualitatively screened thermoalkalophilic amylogenic isolates based on formation of zone of hydrolysis on starch agar plates, were subjected to quantitative screening by assaying their enzyme activity. Maximum activity of 74.82 IU was possessed by GC6 closely followed by 68.24 IU from GV2 while minimum activity of 7.36 IU was observed by GC4 (Table 2). Thus, GC6 and GV2 were finally selected for further studies. Cluster analysis by using SPSS software of the amylogenic potential of the screened isolates was conducted. Fig. 3 depicts the dendrogram giving a comparative account of all the isolates with respect to their amylogenic potential. The cluster analysis divided all the isolates into three clusters depending upon their enzyme production potential. Among the clusters, GC6 and GV2 had formed a cluster that was entirely different from rest of the dendrogram indicating their comparatively very high potential as amylogenic isolates.

One of the most striking features of isolates GC6 and GV2 was their ability to grow at high temperature till 65^{o}C and produce maximum enzyme titres of 74.82 IU and 68.24 IU respectively at 50^{o}C, justifying their selection for further studies. Literature survey revealed similar reports for thermophilic amylolytic microorganisms. Al-Qodah *et al.* (2007) isolated amylolytic bacterial isolate from a hot spring in Jordan, identified as *Bacillus sphaericus* producing α-amylase at 50^{o}C. Adiguzel *et al.* (2009) reported *Bacillus* sp. from the hot springs



(a) GC6



(b) GV2

Plate 1 (a, b) Zone of starch hydrolysis formed by the amylolytic strains

of Turkey growing at 50°C. Amylolytic *Bacillus* sp. isolated from Iranian hot springs by Fooladi and Sajjadian (2010) was able to survive up to 80 °C with optima for enzyme production at 65°C. Asad *et al.* (2010) isolated amylolytic *Bacillus* sp. WA21 bacteria from hot springs of Karachi surviving up to 65°C, with 4 U/ml amylase at an optimum temperature of 45°C. Fincan *et al.* (2014) isolated a thermophilic amylolytic bacterial isolate *Anoxybacillus flavithermus* yielding maximum amylase at 55°C. Neelima and Reddy (2015) reported a *B. subtilis* ss7 isolated from flour mill waste yielding 31U/ml at 40°C. However, thermophiles producing such high titres of α-amylase as that of GC6 and GV2 have rarely been reported in literature. Such enzymes with high temperature tolerance and good enzyme titres are much sought after in the starch processing industry.

Table 2. Production of α-amylase from alkalothermophilic bacteria isolated from mushroom compost

Isolates	***Protein**	****Amylolytic activity**	*****Specific activity**
GC1	8.99±0.11[a]	13.50±0.11[a]	1.50±0.04[a]
GC2	5.34±0.09[b]	8.63±0.09[b]	1.61±0.02[a]
GC3	11.09±0.13[c]	19.47±0.17[c]	1.76±0.06[b]
GC4	4.99±0.21[d]	7.36±0.11[d]	1.47±0.10[a]
GC5	7.04±0.17[e]	11.98±0.13[e]	1.70±0.11[a]
GC6	**26.08±0.21[f]**	**74.82±0.34[f]**	**2.86±0.07[c]**
GC7	6.23±0.04[g]	9.32±0.10[g]	1.49±0.10[a]
GC8	17.67±0.11[h]	43.24±0.14[h]	2.44±0.11[d]
GV1	12.87±0.13[i]	34.01±0.11[i]	2.64±0.11[e]
GV2	**24.36±0.12[j]**	**68.24±0.23[j]**	**2.80±0.06[f]**
GV3	12.34±0.09[k]	20.40±0.11[k]	1.65±0.10[a]
GV4	3.67±0.04[l]	7.36±0.084[l]	2.07±0.09[g]
GV5	8.01±0.10[m]	13.23±0.11[m]	1.65±0.09[a]
SE(m)	0.11	0.14	0.08

*Protein concentration: mg/ml
** Amylolytic activity (IU): µmoles of starch hydrolysed/min/ml of enzyme.
***Specific activity (IU/mg): enzyme activity/mg of protein

Another important property of GC6 and GV2 is being alkalophile producing maximum enzyme at pH 9.0. This unique combination of two most

cherished attributes of amylase producing isolates is majorly sought after for wide variety of applications in industries such as paper, textile, detergent and biofuel. Acidic or neutral α-amylase producers finding application in food and fermentation industries have been reported by many workers (Sohail *et al.*, 2005; Thippeswamy *et al.*, 2006; Asgher *et al.*, 2007; Vidyalakshmi *et al.*, 2009; Asad *et al.*, 2010). However, reports on alkalophiles producing α-amylase despite their high utility are very scarce in the literature. Khalil (2011) reported α-amylase production upto pH 8.5 whereas Alkando and Ibrahim (2011) reported extracellular α-amylase activity of 0.795 U/mg/ml from *B. licheniformis* isolated from soil. The organism was found active at 70^{o}C and pH 9.0. Thus owing to their interesting and industrially important characteristic properties, alkalothermophiles GC6 and GV2 isolated from mushroom compost yielding reasonably high titres are potential candidates for use in industry.

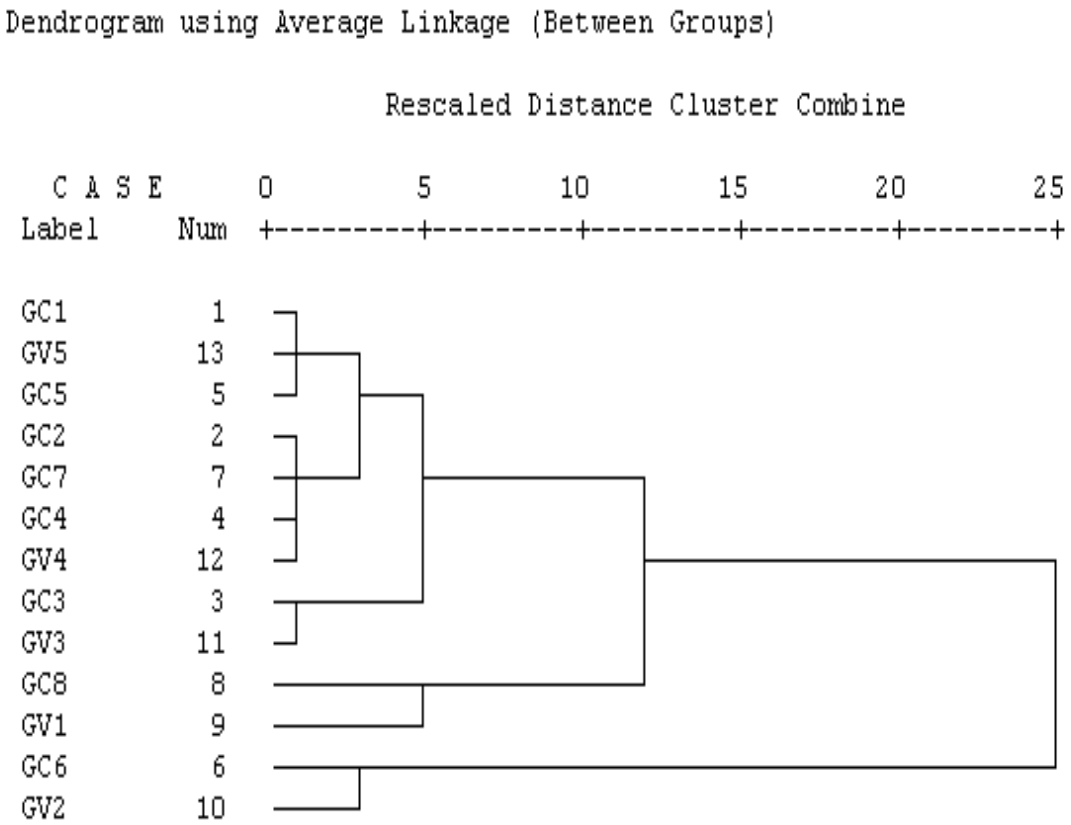


Fig. 3 Dendrogram depicting the comparative account of amylogenic potential of the screened thermoalkalophilic amylolytic bacterial isolates based on rescaled distance

4.1.3 Quantitative screening of thermoalkalophilic β-amylolytic microorganisms

Quantitative screening for β-amylase production has also been done for the isolated strains. It has been found that maximum β- amylase activity has been shown by GC1 with 3.50 IU followed by GC8 with 3.02 IU. Least β- amylase activity has been shown by GV5 with 0.27 IU (Table 3). Literature survey revealed that Olufunke *et al.* (2013) reported 479 U/ml β-amylase from *B. subtilis.* Awoyinka *et al.* (2014) extracted beta amylase having 721.09 U/ml activity from Nigerian maize cultivar.

Table 3. Production of β-amylase from alkalothermophilic bacteria isolated from mushroom compost

Isolates	*Protein	**Amylolytic activity	**Specific activity
GC1	**1.23±0.11^{a}**	**3.50±0.11^{a}**	**2.84±0.09^{a}**
GC2	1.02±0.21^{a}	0.86±0.09^{b}	0.84±0.11^{b}
GC3	1.03±0.09^{a}	1.21±0.12^{c}	1.17±0.12^{c}
GC4	0.84±0.05^{b}	1.30±0.11^{d}	1.54±0.13^{d}
GC5	0.84±0.01^{c}	1.29±0.13^{e}	1.53±0.14^{e}
GC6	1.8±0.1^{d}	2.41±0.08^{f}	1.33±0.16^{f}
GC7	1.64±0.21^{e}	2.12±0.11^{g}	1.29±0.21^{g}
GC8	**1.23±0.07^{a}**	**3.02±0.21^{h}**	**2.45±0.15^{a}**
GV1	0.48±0.09^{f}	1.03±0.2^{i}	2.14±0.18^{h}
GV2	1.03±0.08^{a}	0.28±0.07^{j}	0.27±0.11^{i}
GV3	1.64±0.11^{g}	2.45±0.17^{k}	1.49±0.09^{j}
GV4	1.09±0.12^{a}	1.31±0.08^{l}	1.20±0.11^{k}
GV5	1.03±0.13^{a}	0.27±0.09^{m}	0.26±0.07^{l}
SE(m)	**0.26**	**0.18**	**0.36**

*Protein concentration
** Amylolytic activity
***Specific activity
} same as given in Table 2

Keeping in view the meagre amylase units being produced by the screened isolates when compared to that available in literature, α-amylase was decided as the enzyme of interest for further studies. Moreover the ever

increasing industrial demand of α-amylase due to is multifaceted applications justified its selection as an enzyme for further detailed studies.

4.2. IDENTIFICATION OF SCREENED THERMOALKALOPHILIC HYPER AMYLOLYTIC MICROORGANISMS

4.2.1 Phenotypic Identification of Bacterial Isolates

Preliminary identification of the screened thermoalkalophilic hyper amylolytic bacteria has been done on the basis of their morphological, physiological, biochemical and metabolic characteristics by using ABIS-online software. All the bacterial isolates were tentatively identified (Table 1) and were placed under the group of Genus *Bacillus* as per Bergey's Manual of Determinative Bacteriology, 7th Edn.

4.2.2 Genotypic Characterization

Isolation and identification of bacterial isolates from different sources have been studied by several workers and their identification has been done by various methods such as numerical taxonomy (Sahin *et al.*, 2002), fatty acid composition analysis (McNabb *et al.*, 1997) and ribosomal protein analysis (Ochi, 1992). Recent advances in molecular biology techniques such as sequencing a stable part of genetic code have provided an excellent opportunity for the precise identification and characterization of bacteria at species and subspecies levels (Adiguzel *et al.,* 2009). Among them, 16S rRNA is considered the best evolutionary chronometer because it is the most conserved and universally present in all prokaryotes and is large enough for bioinformatic analyses (Patel, 2001). 16S rRNA gene sequencing has thus been considered at present a gold standard for identification of prokaryotes.

Thus, the selected alkalothermophilic hyperamylogenic strains GC6 and GV2 were identified at genomic level by using 16S rRNA gene technique. In literature also, 16S rRNA method has been followed by most of the workers for accurate identification of isolates (Al-Zazaee *et al.*, 2011; Ashwini *et al.*, 2011; Demirkan *et al.*, 2011; Kaur *et al.*, 2012). Genomic DNA of bacteria was isolated by using conventional DNA isolation method. DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The small subunit 16S rRNA of

isolated genomic DNA was amplified using PCR using universal primers having product size of approximately 1500 bp. The PCR product so obtained after amplification was visualized using ethidium bromide on 2% agarose gel (Plate 3). Amplified PCR products were purified and sequenced by the services provided by BioServe Biotechnologies (India) Pvt. Ltd., Hyderabad, India to confirm the results.

4.2.2.1 Nucleotide Sequencing

Following sequences of isolates GC6 and GV2 were obtained after sequence analysis

Nucleotide sequence of GC6

>0913_378_01_F1_27F-A01.ab1

TGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCAT
GGTTCAATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACAAAAGTCTGACGGA
GCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGT
TCGAATAGGGCGGTACCTTGACGGTACCTAACCAAAAACCCACGGCTAACTACGTGCCACCAGCCGCGGT
AAGACGTAGGTGGCAAGCGTTGTCCGGAATTATGGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAACTCT
GATGTGAAAGCCCGCGGCTCAAC

>0913_378_01_F1_1492R-A08.ab1

GGCTCCAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAG
GCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGT
TGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTG
TTCTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTC
CTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCG
CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGC
CCCCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGC
TTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGAC
CGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAG
CACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGC
GTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACA

CGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGGTTGAGCCGG
GGGCTTTCACATCAGACTTAAGAAAACCGCCTGGCGCGCGCTTTACGCCCAATAAT

Nucleotide sequence of GV2

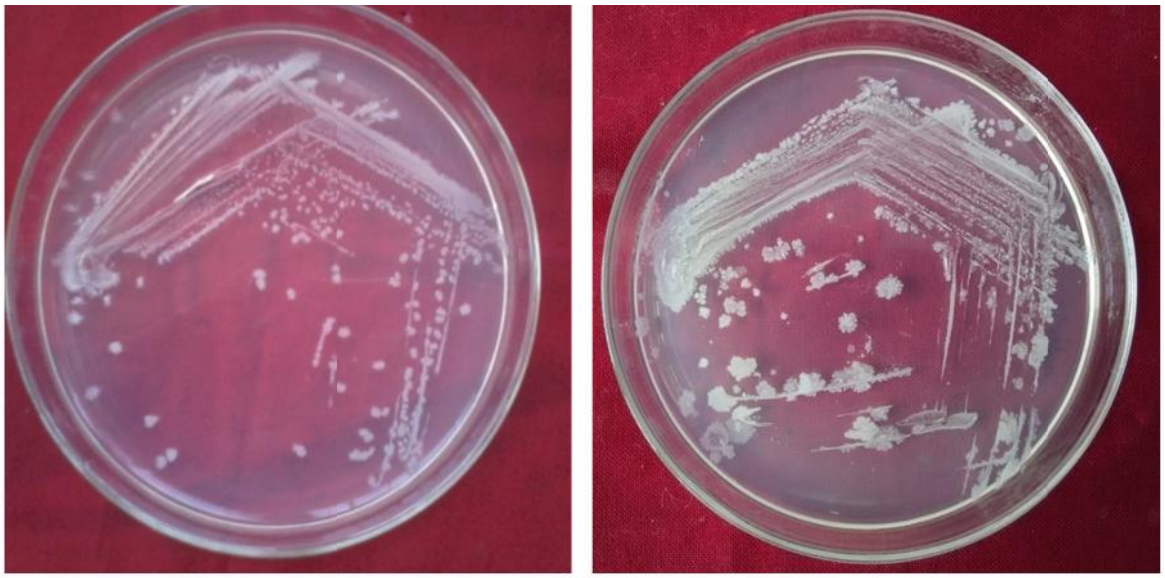
>0114_305_001_1_27F-C07.ab1

TAAGACTGGGATAACTCCGGGAAACCGGGACTAATACCGGATGCTTGATTGAACCGCATGGTTCAATTCT
AAAAGGTGGCTTTTAACTTCCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCGACGATGCGTAGCCGACCTGAGGAGGGTGATCGGCCACACTGGGACTGAGACACGGCAA
CAAACTCATACGGGAGGCAGCTGTAGGGAAACTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTTCCGTTCGAATAGG
GCGGTACCTTGACGGTTCCTAACCAGAAAGCCACGGCTAACCACGTGCCAGCAGCCGCGGTCATACGTAT
GTGGCAAGCGTTGTCCGGTATTATTGGGCGTAAAGCGCGCGCAGGCTGTTTCTTTAGTCTGATGTGAAAG
CCCCCGGCTCAAGCGGGGAGGGTCATTGG

>0114_305_002_1_1492R-D07.ab1

TGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCGACCGCAT
GTGCGGGCCCCCGTTTATTCCTTTGAGTTTCAAACTTGCCACCGTACTCGGGGAGGCGGAGTGCTTAATG
CGTTTGCTGCAGCGCTAAAGAGGGGGAAACGCTCTAACACTTAGCACTCATCGTTTACAGCGTGGACTAC
GAAGGTATCTAATCCTGTTCACTCCCCACGCTTTCGCGCCTCAGGGGAGTTAAAGACCAGAGAGTCGCCT
TGGCTACTGGCGTTGGTCCACATCTCTA

Sequences of these isolates so obtained were submitted to NCBI database and matched with already existing sequences. Partial 16S rRNA gene sequence was quire to NCBI BLAST (http:// www.ncbi.nlm.nih.gov/Blast) and the nearest neighbour of the isolate was determined. Sequence similarity search (mBLAST, NCBI) for GC6 showed 98% homology with nucleotide sequence of *Bacillus aerius* 24K |NR042338.1| 16S ribosomal RNA gene, partial sequence while sequence similarity search (mBLAST, NCBI) for GV2 showed 97% homology with nucleotide sequence of *Bacillus sonorensis* NRRLB 23154 |NR116189.1| 16S ribosomal RNA gene, partial sequence. The 16S rRNA gene sequences of the selected isolates have been deposited to National Centre for Biotechnology Information (NCBI) gene bank using Bankit program and has been registered in the databases vide accession numbers *B. aerius* GC6 |KJ775810.1| and *B. sonorensis* GV2 |KJ775811.1|.



(a) GC6 (b) GV2

Plate 2 (a,b) Morphology of screened hyperamylogenic isolates

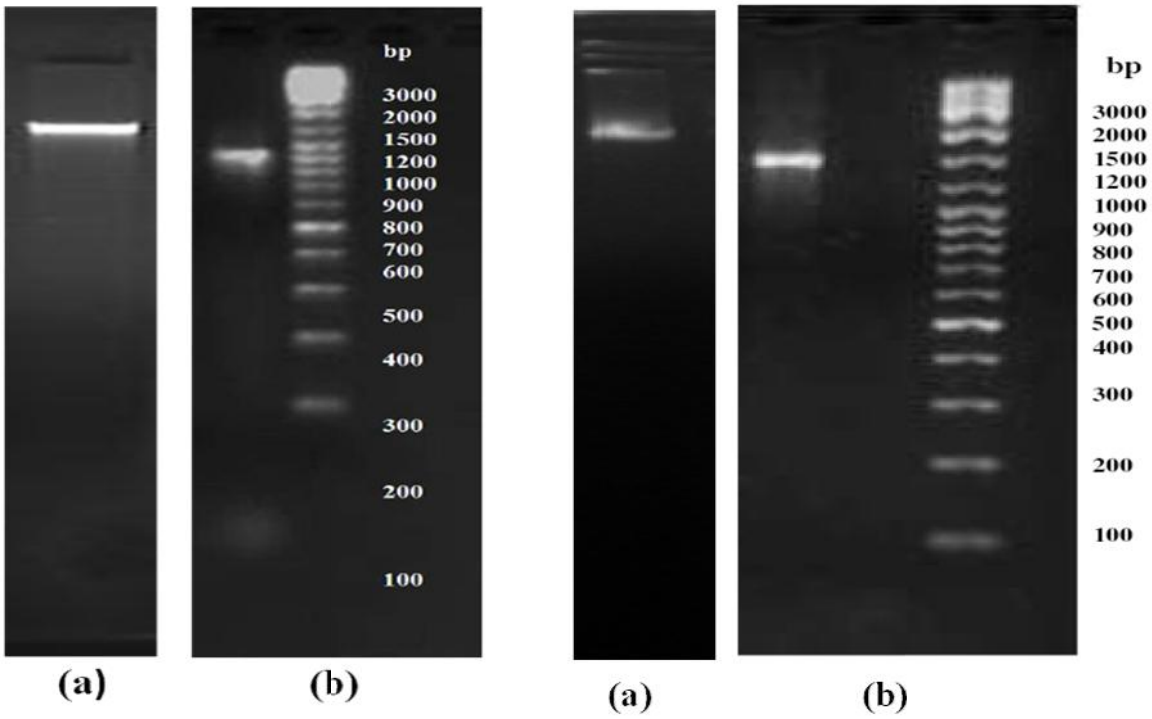


Plate 3 (a) Genomic DNA and (b) PCR product of screened hyperamylolytic isolates (i) GC6 and (ii) GV2

These two strains have been reported for the first time in literature for α-amylase production and produces exceptionally high titres of amylase. The newly isolated strains offer interesting hydrolytic properties, being active up to pH 9.0 and temperature 50^{o}C therefore being alkalophilic, and thermophilic as well. These unique set of properties make them potential candidates for industrial applications. In literature also, several reports are available on the thermoalkalophilic amylolytic microorganisms.

4.2.2.2 Phylogenetic analysis

Unrooted phylogenetic tree based on comparison of 16S rRNA sequence data of *B. aerius* GC6 |KJ775810.1| (Fig. 4) and *B. sonorensis* GV2 |KJ775811.1| (Fig.5) with its closest phylogenetic relatives in the NCBI, GenBank, USA, was constructed by Neighbour joining (NJ) tree algorithm using boot strap value of the MEGA 6.0 (http:// www.megasoftware.net/) (Tamura *et al.*, 2007). Numbers on the tree indicates percentage of bootstrap sampling derived from 1000 random samples.

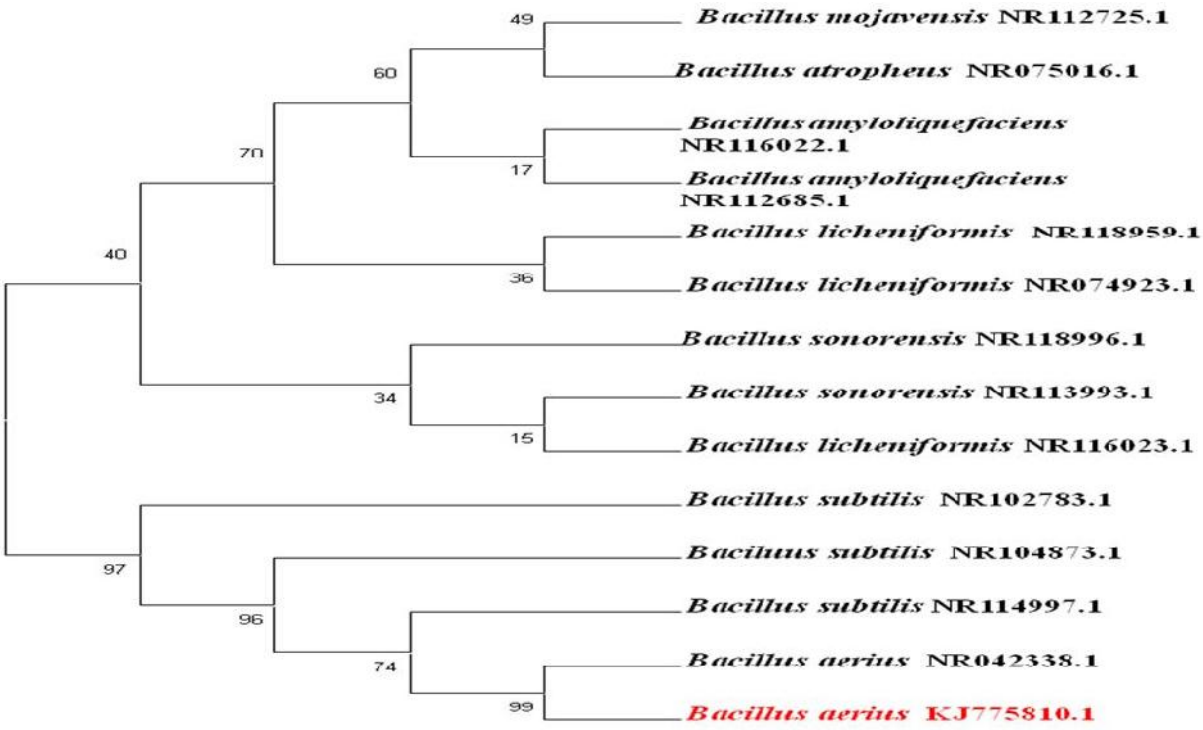


Fig. 4 Neighbor-joining tree with 1000 bootstrap values in MEGA 6.0 showing phylogenetic relationship of *Bacillus aerius* GC6 based on a distance matrix analysis of 16S rRNA sequences.

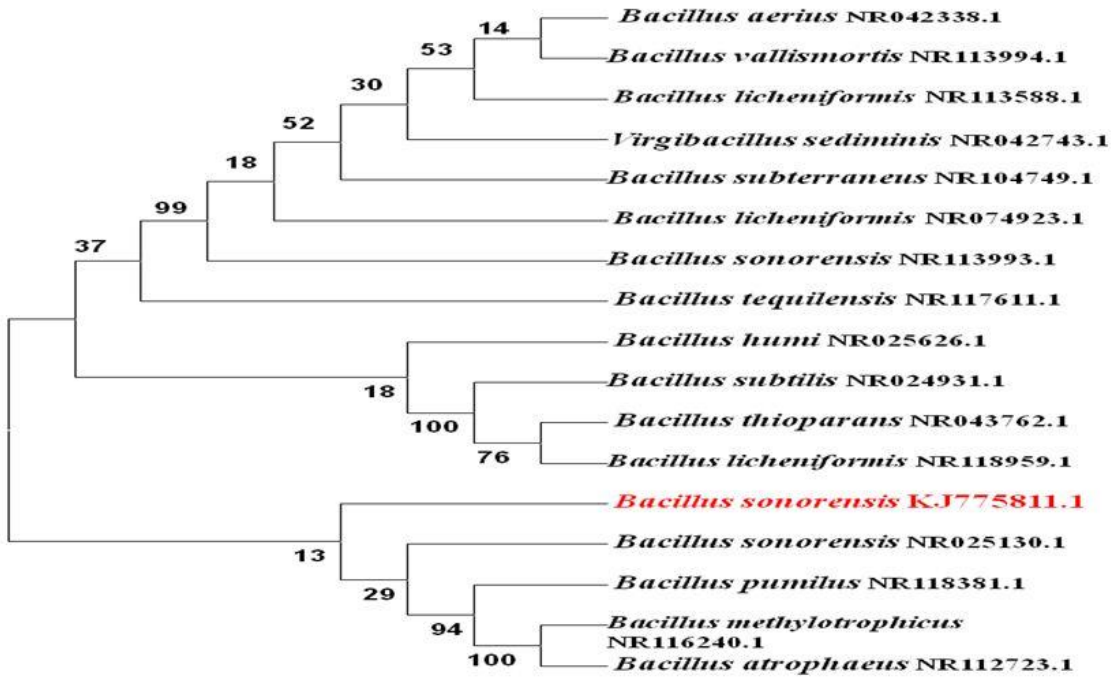


Fig. 5 Neighbor-joining tree with 1000 bootstrap values in MEGA 6.0 showing phylogenetic relationship of *Bacillus sonorensis* GV2 based on a distance matrix analysis of 16S rRNA sequences.

4.3 PRODUCTION AND OPTIMIZATION OF α-AMYLASE FROM *B. aerius* GC6 AND *B. sonorensis* GV2

To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production (Gangadharan *et al.*, 2008). Since, the growth and enzyme production of the organisms are strongly influenced by different process parameters viz. temperature, pH, incubation period, inoculum size, divalent ions, surfactants, media types etc. Therefore, in the present study, enzyme production has been optimized by following two approaches:

4.3.1 One Variable at a Time (OVAT) approach

4.3.2 Response Surface Methodology (RSM)

4.3.1 Optimization using one variable at a time (OVAT) approach

4.3.1.1 Effect of media

Data pertaining to the effect of media types on α-amylase production from *B. aerius* GC6 and *B. sonorensis* GV2 has been given in Tables 4 and 5.

Table 4. Effect of different media on extracellular α-amylase production from *B. aerius* GC6

Medium	***Protein**	****Amylase activity**	*****Specific activity**
Babu and Satyanarayana	12.04±0.23[a]	54.65±0.26[a]	4.53±0.26[a]
Kwan *et al.*	18.00±0.56[b]	63.05±0.23[b]	3.50±0.14[a]
Starch agar medium	**26.10±0.37[c]**	**74.35 ±0.06[c]**	**2.84±0.18[a]**
Modified starch medium	19.67±0.35[d]	67.19±0.17[d]	3.41±0.31[a]
Starch citrate medium	8.88±0.19[a]	14.42±0.36[e]	1.62±0.26[a]
Modified medium	12.75±0.21[a]	37.76±0.16[f]	2.96±0.18[a]
SE(m)	**0.34**	**0.50**	**0.42**

*Protein concentration
**Enzyme activity
**Specific activity
} same as given in Table 2

Table 5. Effect of different media on extracellular α-amylase production from *B. sonorensis* GV2

Medium	***Protein**	****Amylase activity**	*****Specific activity**
Babu and Satyanarayana	9.10±0.11[a]	18.42±0.36[a]	2.02±0.06[a]
Kwan *et al.*	18.10±0.09[b]	49.60±0.32[b]	2.7±0.11[b]
Starch agar medium	23.04±0.13[c]	66.78±0.09[c]	2.89±0.13[c]
Modified starch medium	12.75±0.36[d]	38.05±0.11[d]	2.98±0.14[d]
Starch citrate medium	20.81±0.18[e]	61.74±0.13[e]	2.96±0.19[e]
Modified medium	**20.87±0.51[f]**	**67.19±0.15[f]**	**3.21±0.16[f]**
SE(m)	**0.42**	**0.34**	**0.33**

* Protein concentration
* Enzyme activity
**Specific activity
} same as given in Table 2

B. aerius GC6 showed maximum growth in Starch Agar medium containing Soluble starch- 5.0 g, Na_2HPO_4- 1.5 g, $MgSO_4.7H_2O$- 0.8 g, KH_2SO_4- 1.0 g, $FeSO_4.7\ H_2O$- 0.03 g, $CaCl_2.2H_2O$- 0.06 g, Yeast extract- 0.1 g, Peptone- 0.5 g, dH_2O- 1000mL, pH- 9.0. Cell free broth obtained from this medium showed highest enzyme yield of 74.35 IU with a specific activity of 2.84 IU/mg. Other media however, showed lower yields of enzyme which could be attributed to solution of complex ions which might have induced inhibitory effect on enzyme production. *B. sonorensis* GV2 showed maximum growth in Modified Medium containing Soluble starch- 1.0 g, Yeast extract- 0.4 g, KH_2PO_4– 0.1 g, $MgSO_4.7H_2O$- 0.15 g, $CaCl_2.2H_2O$- 0.08 g, dH_2O- 1000 ml, pH- 9.0 with an enzyme activity of 67.19 IU and specific activity of 3.21 IU/mg.

Enhanced enzyme production using defined medium may be due to the presence of nitrogen, carbohydrate and other compounds in appropriate concentration as per the physiological requirements of the organism that would have resulted in enhanced ability of the cells to synthesize most of the extracellular amylase (Barsar *et al.,* 2010). Starch being the sole carbon source in both the media might act as an inducer of enzyme production by the isolates. Presence of yeast extract and peptone in the medium probably provided microorganisms with easily available sources of nitrogen which would have acted as stimulators for amylase production. Moreover, the presence of metal ions i.e Na^+, Mg^{2+}, Ca^{2+}, K^+ and Fe^{2+} etc. in the production medium revealed these α-amylases as metalloenzymes. The metal ions act as co-factors for the catalytic activity and stability of enzyme.

Similar reports of using a complex media for enzyme production are available in the literature. Tanyildizi *et al.* (2005), Lily *et al.* (2012) and Deb *et al.* (2013) observed the requirement of Ca^{2+}, Mg^{2+}, Na^+ and K^+ for efficient α-amylase production from *Bacillus* sp., *B. subtilis* BMT4i and *B. amyloliquefaciens* P-001 respectively. Gangadharan *et al.* (2008), Khalil (2011) and Tamilarasan *et al.* (2012) observed starch as the best carbon source for induction of α-amylase by the microorganisms. Nguyen *et al.* (2000) and Vidyalakshmi *et al.* (2009) have reported the production of amylase from *Thermomyces lanuginosus* and *Bacillus* sp. in the presence of yeast extract and

peptone. These reports are in consonance with the results of the present experiment.

4.3.1.2 Effect of incubation period

To determine the effect of incubation period on amylase production from *B. aerius* GC6 and *B. sonorensis* GV2, enzyme activity was measured at regular intervals from 24 h to a period of 144 h.

For *B. aerius* GC6, maximum amylase activity of 74.95 IU was noticed at 72nd h with a specific activity of 3.38 IU/mg. A similar trend has been shown for α-amylase production by *B. sonorensis* GV2. Incubation period of 72 h showed maximum amylase activity of 68.36 IU with a specific activity of 3.27 IU/mg. Table 6 and Table 7 shows the effect of incubation period on the enzyme production potential of *B. aerius* GC6 and *B. sonorensis* GV2 respectively. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given at the end of the table. For *B. aerius* GC6, initial incubation period of 24 and 48 h have resulted in a lesser enzymatic activity of 60.88 IU and 64.47 IU respectively. Similarly, for *B. sonorensis* GV2, an activity of 56.93 IU and 62.98 IU respectively had been exhibited at these periods.

Table 6. Effect of incubation period on extracellular α-amylase production from *B. aerius* GC6

Incubation period (h)	***Protein**	****Amylase activity**	*****Specific activity**
24	19.00±0.16^{a}	60.88±0.21 ^{a}	3.20±0.12^{a}
48	20.37±0.08 ^{a}	64.47±0.17^{b}	3.16±0.09^{a}
72	**22.10±0.11** ^{a}	**74.95±0.11^{c}**	**3.38±0.10** ^{a}
96	23.87±0.14 ^{a}	73.79±0.13^{d}	3.09±0.15^{a}
120	21.81±0.12 ^{a}	72.65±0.12^{e}	3.33±0.17^{a}
144	19.78±0.13 ^{a}	72.08±0.14^{f}	3.64±0.11^{a}
SE(m)	**0.28**	**0.45**	**0.34**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

Table 7. Effect of incubation period on extracellular α-amylase production from *B. sonorensis* GV2

Incubation period (h)	*Protein	**Amylase activity	***Specific activity
24	19.00±0.21 [a]	56.93±0.23[a]	2.99±0.12 [a]
48	21.17±0.16[b]	62.98±0.21[b]	2.97±0.00[a]
72	**20.87±0.14[b]**	**68.36±0.18[c]**	**3.27±0.14[a]**
96	23.06±0.20[c]	65.46±0.13[d]	2.97±0.09[a]
120	16.81±0.17[d]	52.89±0.12[e]	3.14±0.11[a]
144	12.75±0.10[e]	41.78±0.14[f]	3.27±0.20 [a]
SE(m)	**0.31**	**0.47**	**0.34**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

When growth of the selected isolates was studied, it was revealed that both the isolates exhibited an exponential phase from 12 to 48 h followed by stationary phase till 72 h (Fig 6, 7). The lesser enzyme activity for 48 h could be attributed to the lag and the log phase of the organismal growth where the time was taken by the microorganism to get stabilized in a new medium and then start multiplying. Amylase being an inducible enzyme is generally produced during the stationary phase. Thus the peak in enzyme yield has been found to be at 72nd h of growth by both the isolates. Afterwards incubation beyond 72 h resulted in a decreased enzyme activity that could be due to depletion of nutrients available causing a stressed microbial physiology eventually resulting in an inactivation of enzyme (Flores *et al.,* 1997). Another reason could be the catabolite repression i.e. the increase in the production of reducing sugars, which after a certain period of growth could exhibit inhibitory effect on enzyme production as α-amylase is an inducible enzyme (Kiran and Chandra, 2008; Premila, 2013). Till 144th h, enzyme activity decreased to 72.08 IU for *B. aerius* GC6 and 41.78 IU for *B. sonorensis* GV2.

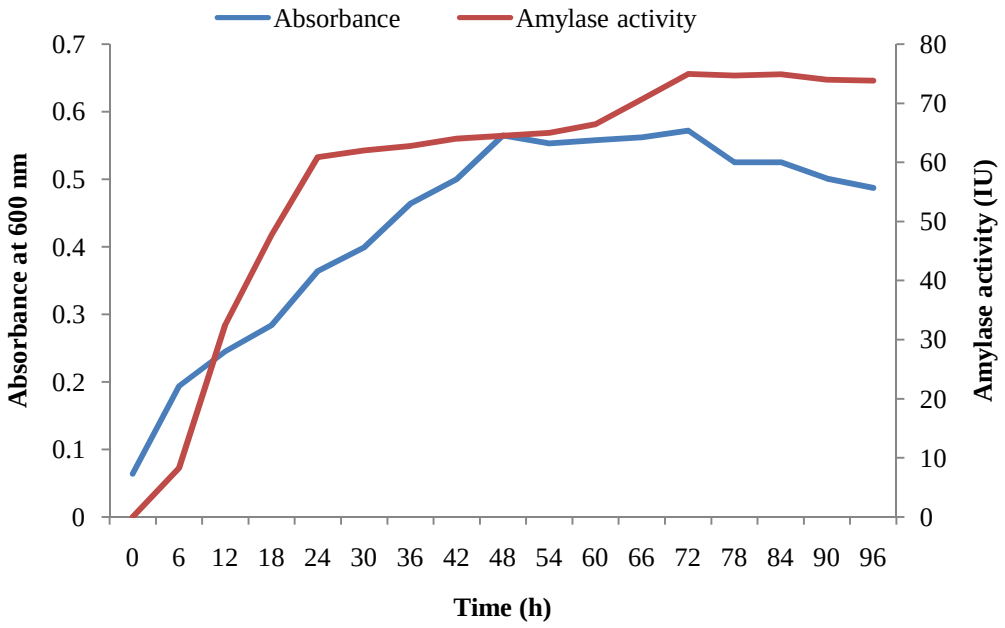


Fig. 6 Growth curve with amylase activity profile of *B. aerius* GC6

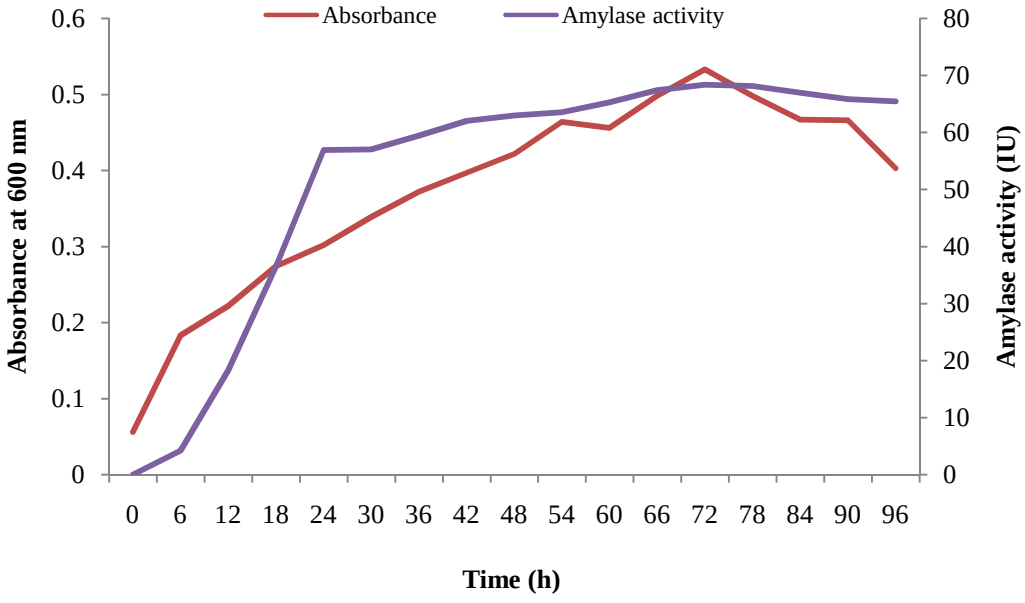


Fig. 7 Growth curve with amylase activity profile of *B. sonorensis* GV2

Hamza (2004) isolated *B. subtilis* from local soil of Iraq yielding 3.96 U/ml amylase at 72 h of incubation period. Najafi *et al.* (2005) observed a maximum amylase activity of 38 U/ml at stationary phase exhibited by *B. subtilis* AX20 isolated from soil. *B. coagulans* isolated from soil by Allah *et al.* (2011)

exhibited an amylase activity of 80.33 U/ml at 72 h. Amutha and Priya (2011) reported *B. subtilis* KCX006 yielding 38 U/ml amylase in its stationary phase. Khalil (2011) isolated *Deinococcus geothermals* sp. M4 from hot springs of Iraq showing highest amylase activity after 72 h of incubation.

4.3.1.3 Effect of different carbon sources and substrate concentration

Carbon source in the form of either monosaccharides or polysaccharides influence the production of amylase. α-amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose. Tables 8 and 9 respectively depict the effect of various carbon sources on α-amylase production from *B. aerius* GC6 and *B. sonorensis* GV2. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given at the end of the table. The responses are all significant.

Table 8. Effect of different carbon sources on extracellular α-amylase production from *B. aerius* GC6

Carbon source	***Protein**	****Amylase activity**	*****Specific activity**
Starch	20.20±0.11 [a]	74.95±0.08 [a]	3.68±0.12[a]
Mannose	17.37±0.11[a]	62.01±0.12[b]	3.56±0.09[a]
Arabinose	14.19±0.21[a]	56.77±0.13[c]	4.00±0.21[a]
Glucose	13.07±0.09[a]	47.87±0.17[d]	3.66±0.16[a]
Maltose	**24.98±0.13**[a]	**75.66±0.16**[d]	**3.02±0.11**[a]
Sucrose	19.35±0.12[a]	60.87±0.20[e]	3.14±0.12[a]
SE(m)	**0.31**	**0.34**	**0.26**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

Perusal of the Table 8 revealed that *B. aerius* GC6 exhibited highest α-amylase activity of 75.66 IU with maltose as a carbon source with a specific activity of 3.02 IU/mg. Other carbon sources such as mannose, arabinose and sucrose failed to significantly induce α-amylase production. Starch, in spite of being the most probable substrate has also shown a comparatively lowered

enzyme activity of 74.95 IU in *B. aerius* GC6, thus inferring a very different nature of α-amylase than the usual ones. Table 9 represents the effect of carbon sources on the enzyme production potential of *B. sonorensis* GV2. α-amylase production was induced by the presence of starch as a carbon source yielding 69.13 IU enzyme. Starch has also been found to increase enzyme production in *B. subtilis* IMG22, *Bacillus* sp. PS-7 and *Bacillus* sp. I-3 as reported by Tanyildizi *et al.* (2005); Sodhi *et al.* (2005) and Goyal *et al.* (2005) respectively. Other carbon sources viz. arabinose, maltose, mannose and sucrose did not result in a considerable α-amylase induction. α-amylase production is subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes. Similar repression in enzyme production has been observed in both the isolates with glucose yielding a lowered 47.87 IU and 50.57 IU α-amylase respectively, from *B. aerius* GC6 and *B. sonorensis* GV2. Synthesis of amylases in most species of the genus *Bacillus* is repressed by readily metabolizable substrates such as glucose and fructose as reported by Haseltine *et al.* (1996); Lin *et al.* (2007); Kiran and Chandra (2008); Premila (2013). A reduced enzyme activity in our strains is in consonance with these reports.

Table 9. Effect of different carbon sources on extracellular α-amylase production from *B. sonorensis* GV2

Carbon source	*Protein	**Amylase activity	***Specific activity
Starch	**21.08±0.13** [a]	**69.13±0.08**[a]	**3.27±0.08** [a]
Mannose	22.90±0.42[b]	58.37±0.13[b]	2.98±0.10 [a]
Arabinose	24.04±0.15[c]	64.83±0.31[c]	2.69±0.13[a]
Glucose	19.87±0.12[d]	50.57±0.24[d]	2.54±0.20[a]
Maltose	16.81±0.23[e]	46.87±0.53[e]	2.78±0.12[a]
Sucrose	12.75±0.28f	34.23±0.25[f]	2.68±0.10[a]
SE(m)	**0.64**	**0.37**	**0.28**

*Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

Varying concentrations of the best substrate i.e. maltose for *B. aerius* GC6 and starch for *B. sonorensis* GV2 ranging from 0.5 to 3.0% were explored for its effect on the enzyme yield. The results have been given in Tables 10 and

11 for *B. aerius* GC6 and *B. sonorensis* GV2 respectively. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given at the end of the tables. *B. aerius* GC6 at a concentration of 1.0 % starch yielded a maximum enzyme activity of 76.20 IU with 3.29 IU/mg specific activity. For *B. sonorensis* GV2, an induction in α-amylase production at 0.5% concentration was observed with yield of 70.12 IU with as specific activity of 3.31 IU/mg.

Table 10. Effect of substrate concentration on extracellular α-amylase production from *B. aerius* GC6

Substrate concentration (%)	***Protein**	****Amylase activity**	*****Specific activity**
0.5	21.10±0.09 [a]	69.63±0.11[a]	3.3±0.12[a]
1	**23.10±0.07** [a]	**76.20±0.16[b]**	**3.29±0.07** [a]
1.5	18.04±0.11 [a]	60.93±0.13[c]	3.37±0.11[a]
2	12.87±0.12[b]	42.88±0.09[d]	3.33±0.13[a]
2.5	9.34±0.16[c]	28.47±0.11[e]	3.04±0.14[a]
3	5.67±0.21[d]	12.61±0.13[f]	2.22±0.08[a]
SE(m)	**0.30**	**0.33**	**0.26**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

There were statistically significant variations among the different substrate concentrations used for amylase production ranging from 0.5-3.0%. 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 causes substrate inhibition, which substantially lowers enzyme production (Liu and Yang, 2007; Singhania *et al.*, 2007). An increase in substrate concentration from optimum upto 3.0% has resulted in decreased α-amylase activity. A decreased activity of 12.61 IU has

been shown by *B. aerius* GC6 at 3.0% substrate concentration where as *B. sonorensis* GV2 exhibited 32.09 IU of enzyme activity at the same substrate concentration.

Several workers have optimized substrate concentrations for efficient α-amylase production. Thippeswamy *et al.* (2006), Asgher *et al.* (2007), Ashwini *et al.* (2011) and Deb *et al.* (2013) adjudged 1% substrate concentration as optimum yielding 72 U/ml, 0.46µmole/min/ml and 8 U/ml amylase from *B. subtilis* JS-2004, *Bacillus* sp., *B. marini* and *B. amyloliquefaciens* P-001 respectively. Bai *et al.* (2012) however reported 0.5% starch as optimum for amylase production from *Alicyclobacillus* sp. A4. Perusal of these results revealed that potential amylogenic strains *B. aerius* GC6 and *B. sonorensis* GV2 yielding 76.20 IU and 70.12 IU respectively of amylase at 1.0% and 0.5% substrate concentration are potential candidates for cost effective amylase production.

Table 11. Effect of substrate concentration on extracellular α-amylase production from *B. sonorensis* GV2

Substrate concentration (%)	***Protein**	****Amylase activity**	*****Specific activity**
0.5	**21.13±0.06^{a}**	**70.12±0.12^{a}**	**3.31±0.11** ^{a}
1	23.10±0.13^{b}	62.64±0.11^{b}	2.71±0.13^{a}
1.5	25.43±0.15^{c}	64.98±0.36^{c}	2.55±0.08^{a}
2	18.66±0.13^{d}	47.28±0.19^{d}	2.53±0.14^{a}
2.5	16.81±0.09^{e}	46.78±0.00^{e}	2.78±0.12^{a}
3	9.35±0.11^{f}	32.09±0.13^{f}	3.43±0.20^{a}
SE(m)	**0.24**	**0.34**	**0.33**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

4.3.1.4 Effect of inoculum size

The size of inoculum plays an important role in the fermentation of enzymes.Different inoculum sizes studied for enhanced enzyme production were 2.5, 5.0…, 15.0% (v/v). Table 12 and 13 reveal the effect of inoculum size on enzyme production by *B. aerius* GC6 and *B. sonorensis* GV2 respectively. All the

values of response variables are the mean of triplicate values with standard deviation as calculated by GraphPad Prism software. Standard error of mean has been given in the end of the table.

Table 12. Effect of inoculum size on extracellular α-amylase production from *B. aerius* GC6

Inoculum size (%)	*Protein	**Amylase activity	***Specific activity
2.5	16.66±0.08 [a]	69.36±0.11 [a]	4.16±0.12[a]
5	19.80±0.11[a]	71.13±0.11[b]	3.59±0.09[a]
7.5	**18.03±0.11**[a]	**76.99±0.21** [a]	**4.22±0.21**[a]
10	21.53±0.16[a]	71.47±0.09 [a]	3.31±0.16[a]
12.5	16.81±0.13[a]	67.57±0.13[c]	4.01±0.11[a]
15	9.67±0.09[a]	58.36±0.12[d]	6.03±0.7[a]
SE(m)	**0.28**	**0.45**	**0.83**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

Highest amylase titres of 76.99 IU from *B. aerius* GC6 and 71.82 IU from *B. sonorensis* GV2 were observed at 7.5% inoculum size having statistically significant differences over the range of variable inoculum sizes. There was a steady increase in the enzyme production from 2.5 to 7.5% in both the cases however there was a steep decrease in the enzyme activity beyond this inoculum size because of an overload of the cells in the production medium resulting in nutrient starvation (Deb *et al.*, 2013). Maximal enzyme activity at optimal level was observed because at this point there is equilibrium between the number of cells and availability of nutrients for growth and substrate to act upon 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.

In contrary to our findings, variable inoculum sizes have been reported by workers. 10% of inoculum has been reported optimum for amylase production from *Thermomyces lanuginosus* by Kunamneni *et al.* (2005). Ashwini *et al.* (2011) reported an enzyme activity of 8 U/ml from *B. marini* at an inoculum size

of 10%. A lower inoculum size of 1% has been reported by Deb *et al.* (2013) for amylase production by *B. amyloliquefaciens* P-001 yielding 39.25 U/ml of enzyme.

Table 13. Effect of inoculum size on extracellular α-amylase production from *B. sonorensis* GV2

Inoculum size (%)	***Protein**	****Amylase activity**	*****Specific activity**
2.5	21.08±0.13 [a]	49.33±0.08[a]	2.34±0.13[a]
5	18.69±0.24 [b]	58.07±0.10[b]	3.10±0.11[a]
7.5	**21.08±0.15** [a]	**71.82±0.53[c]**	**3.40±0.20[b]**
10	22.03±0.20[c]	66.07±0.24[d]	2.99±0.12[a]
12.5	9.08±0.21[d]	21.99±0.35[e]	2.42±0.14[a]
15	7.85±0.12[e]	18.13±0.14[f]	2.30±0.09 [a]
SE(m)	**0.26**	**0.38**	**0.42**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

4.3.1.5 Effect of pH

Enzyme activity is markedly affected by pH. The effect of pH is crucial in terms of growth of the producing organism and the biosynthesis of α-amylase. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and, in particular enzyme molecules (Shah and Madamwar, 2005). The pH of the growth medium plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannan, 2006). Keeping in view its importance, the effect of pH in the range of 7.0-12.0 was evaluated on α-amylase production. Table 14 and 15 represent effect of pH on the enzyme production potential from *B. aerius* GC6 and *B. sonorensis* GV2 respectively. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given at the end of the table.

The highest α-amylase activity of 77.66 IU was observed for *B. aerius* GC6 at alkaline pH 9.0 with specific activity 4.71 IU/mg. At lower pH of 7.0 and 8.0, a respective amylase activity of 43.46 IU and 61.72 IU has been observed. Similarly, at increased pH of 10.0 and 11.0, enzyme activity of 47.36 IU and 23.29 IU respectively had been recorded.

Table 14. Effect of different pH on extracellular α-amylase production from *B. aerius* GC6

pH	*Protein	**Amylase activity	***Specific activity
7	19.24±0.11	43.46±0.18	2.25±0.21
8	22.08±0.13	61.72±0.8	2.79±0.1
9	**16.46±0.16**	**77.66±0.12**	**4.71±0.04**
10	19.24±0.17	47.36±0.09	2.46±0.09
11	7.43±0.12	23.29±0.12	3.13±0.11
12	2.45±0.08	1.28±0.16	0.52±0.17
SE(m)	**0.33**	**0.31**	**0.45**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

Table 15. Effect of different pH on extracellular α-amylase production from *B. sonorensis* GV2

pH	*Protein	**Amylase activity	***Specific activity
7	21.08±0.11[a]	43.00±0.12[a]	2.04±0.04 [a]
8	19.33±0.12[b]	57.99±0.09[b]	3.00±0.14 [a]
9	**22.08±0.06[a]**	**71.96±0.11[c]**	**3.41±0.08 [a]**
10	21.03±0.14[a]	67.89±0.16[d]	3.20±0.11 [a]
11	8.34±0.16[c]	31.34±0.16[e]	3.75±0.13 [b]
12	4.26±0.17[d]	6.03±0.21[f]	1.41±0.15 [a]
SE(m)	**0.33**	**0.21**	**0.21**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

On the other hand, for *B. sonorensis* GV2, maximum enzyme activity of 71.96 IU was observed at pH 9.0 with a specific activity of 3.41 IU/mg. At pH 7.0 and 8.0, an amylase activity of 43.00 and 57.99 IU respectively was observed. Above optimum, pH 11.0 showed a dipped amylase activity of 31.34 IU. This implies that the 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).

There are several reports available in the literature regarding the production of amylase at neutral and acidic pH (Sohail *et al.*, 2005; Asgher*et al.*, 2007; Asad *et al.*, 2010; Fooladi and Sajjadian, 2010; Lily *et al.*, 2012). However, at alkaline pH, a few reports are available. Maximum amylase production from *B. amyloliquefaciens* P-001 of 35.84 U/ml at pH 9.0 has been reported by Deb *et al.* (2013). Pathak and Narula (2013) reported amylolytic fungus *Aspergillus niger* producing amylase at pH 9.0. Andualem (2014) reported 99% starch hydrolysis at pH 8.0 by an amylase isolated from thermophilic spore former *Baillus* sp. Alkalophilic amylogenic isolates *B. aerius* GC6 and *B. sonorensis* GV2 reported in the present study yielding high titres of amylase at pH 9.0 are thus organisms of special interest for use in textile, detergent and biofuel industries.

4.3.1.6 Effect of temperature

Table 16 and 17 represents the effect of temperature on α-amylase production by *B. aerius* GC6 and *B. sonorensis* GV2. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given in the end of the table.

B. aerius GC6 and *B. sonorensis* GV2 exhibited highest amylase activity of 77.96 and 72.76 IU at an optimum temperature of 50°C which was significantly higher than others. Maximum enzyme activity at optimum

temperature may be due to an increased metabolic activity of the cells resulting in an increased extracellular enzyme production in culture supernatant. Lowered incubation temperature of 40 and 45°C resulted in a decreased amylase activity of 36.38 IU and 72.56 IU respectively for *B. aerius* GC6. Similarly for *B. sonorensis* GV2, a decreased activity of 27.98 and 57.22 IU was observed at a temperature of 40 and 45°C respectively. At temperatures lower than optimum, membranes solidify by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity (Willey *et al.*, 2008). At higher temperatures of 55 and 60°C, a respective amylase activity of 66.76 IU and 32.45 IU was observed for *B. aerius* GC6. A similar decrease in enzyme activity i.e. 18.13 IU with increase in incubation temperature to 65°C of *B. sonorensis* GV2 is evident from the findings of Bakri *et al.* (2009), who observed a decrease in amylolytic potential of *Bacillus* sp. strain isolated from soil samples beyond 37^{o}C temperature, which yielded maximum amylase. Vidyalakshmi *et al.* (2009) reported a maximum amylase yield of 7 U/ml at 35^{o}C from *Bacillus* sp., which reduced considerably to 4.58 U/ml at 40^{o}C. Andualem (2014) observed a decrease in starch hydrolysis efficiency of alpha amylase beyond 60^{o}Cextracted from *Bacillus* sp. strain isolated from starch rich soil.

Table 16. Effect of different temperature on extracellular α-amylase production from *B. aerius* GC6

Temperature (^{o}C)	***Protein**	****Amylase activity**	*****Specific activity**
40	17.78±0.07^{a}	36.38±0.11^{a}	2.04±0.09^{a}
45	19.24±0.09^{b}	72.56±0.^{13b}	3.77±0.11^{b}
50	**18.09±0.11^{a}**	**77.96±0.16^{c}**	**4.02±0.13^{c}**
55	19.63±0.16^{c}	66.76±0.21^{d}	3.40±0.17^{d}
60	13.06±0.12^{d}	32.45±0.12^{e}	2.48±0.14^{e}
65	6.08±0.08^{e}	21.01±0.17^{f}	3.45±0.21^{f}
SE(m)	**0.41**	**0.31**	**0.26**

* Protein concentration
**Amylase activity
*** Specific activity
} same as given in Table 2

Table 17. Effect of different temperature on extracellular α-amylase production from *B. sonorensis* GV2

Temperature (^{o}C)	*Protein	**Amylase activity	***Specific activity
40	16.93±0.21 [a]	27.98±0.07[a]	1.65±0.09 [a]
45	25.43±0.11 [b]	57.22±0.15[b]	2.25±0.13 [a]
50	**18.09±0.07 [c]**	**72.76±0.11[c]**	**4.02±0.17[b]**
55	16.80±0.14 [a]	64.37±0.08[d]	3.83±0.21[c]
60	11.58±0.13 [d]	26.34±0.13[e]	2.28±0.22 [a]
65	7.88±0.11 [e]	18.13±0.08[f]	2.30±0.24 [a]
SE(m)	**0.17**	**0.26**	**0.31**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

The reduction in enzyme activity at higher temperature could be due to damaged and deformed enzyme structures. Incubation temperature is the most important physical factor which affects structure and function of macromolecules. Increase in temperature not only leads to an increase in activity reaction kinetics but also accelerates the denaturation induced by higher physiological temperatures. Any temperature beyond the optimum range is found to have some adverse effects on the metabolic activities of the microorganisms and it is also reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperatures (Okolo *et al.*, 1995).

Asgher *et al.* (2007) isolated a moderately thermophilic strain *B. subtilis* for starch processing industry yielding 72 U/ml at 50°C. In accordance to our findings, Khalil (2011) reported amylase production at 50°C by a thermophilic bacterial strain isolated from hot springs of Saudi Arabia. *Bacillus* sp. isolated from soil by Senthilkumar *et al.* (2012) yielded 3.5 U/ml enzyme at 50°C. *B. subtilis* yielding 0.038 U/ml of amylase at 50°C have been isolated from soil by Sani *et al.* (2014).

4.3.1.7 Effect of various nitrogen sources

Nitrogen sources are the most important secondary energy compounds for the growth and metabolism of microorganisms. The nature of these compounds and the concentration used may stimulate or down regulate the production of enzymes (Sharma and Singh, 2012). Table 18 and 19 represents the effect of nitrogen sources on α-amylase production by *B. aerius* GC6 and *B. sonorensis* GV2. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given in the end of the table.

B. aerius GC6 enzyme production medium supplemented with sodium nitrate exhibited an increased amylase activity of 78.78 IU (specific activity 4.62 IU/mg). For *B. sonorensis* GV2 enzyme production medium supplemented with sodium nitrate showed an increased enzyme activity of 73.08 IU (specific activity of 3.86 IU/mg). Of the rest other nitrogen sources used viz. gelatin, tryptone, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$ however showed a decline in enzyme activity. It has been observed that inorganic sources expressed comparatively higher enzyme yields than organic nitrogen sources (Sankaralingam *et al.*, 2012).

Table 18. Effect of different nitrogen sources on extracellular α-amylase production from *B. aerius* GC6

Nitrogen source	*Protein	**Amylase activity	***Specific activity
Gelatin	18.23±0.13^{a}	60.65±0.21^{a}	3.32±0.8^{a}
Tryptone	22.65±0.18^{a}	72.22±0.08^{b}	3.18±0.76^{a}
$(NH_4)_2SO_4$	**28.11±0.11^{b}**	**78.78±0.11^{c}**	**4.62±0.73^{a}**
NH_4Cl	22.22±0.08^{a}	61.37±0.12^{a}	2.76±0.11^{a}
NH_4NO_3	22.65±0.12^{a}	72.96±0.09^{d}	3.22±0.21^{a}
$NaNO_3$	29.15±0.16^{c}	73±0.17^{e}	2.50±0.17^{a}
SE(m)	**0.36**	**0.91**	**0.45**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

The nature and relative concentration of different complex nitrogenous sources in the growth medium are important for the synthesis of amylase. Lower levels as well as excess of nitrogen are equally detrimental causing enzyme inhibition (Sharma and Singh, 2012). The presence of more than one nitrogen source in the medium has been reported to give better results which are evident from the results of the experiment. Yeast extract has been shown to induce amylase production by several workers (Ashwini *et al.*, 2011; Sharma *et al.*, 2012; Deb *et al.*, 2013) for *B. marini, B. amyloliquefaciens* and *B. amyloliquefaciens* P-001.

Table 19. Effect of different nitrogen sources on extracellular α-amylase production from *B. sonorensis* GV2

Nitrogen source	*Protein	**Amylase activity	***Specific activity
Gelatin	24.56±0.12[a]	57.98±0.23[a]	2.36±0.00 [a]
Tryptone	16.182±0.16[b]	27.22±0.22[b]	1.68±0.12 [a]
$(NH_4)_2SO_4$	18.58±0.14[c]	71.72±0.13[c]	3.86±0.14 [b]
NH_4Cl	16.80±0.20[d]	64.37±0/05[d]	3.83±0.09 [c]
$NH_4 NO_3$	20.58±0.17[e]	56.34±0.16[e]	2.28±0.13 [a]
$NaNO_3$	**19.35±0.11[f]**	**73.08±0.14[f]**	**3.86±0.11 [a]**
SE(m)	**0.37**	**0.18**	**0.34**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

Coleman and Elliott (1962) have reported ammonium salts to enhance amylase production. In consonance to our findings, ammonium salts along with yeast extract has been reported to yield enhanced amylase titres by Mahmood and Rahman (2008) from *B. subtilis* DM-03 and Sivakumar *et al.* (2012) from *B. cereus*. Similarly, sodium nitrate along with yeast extract has been reported to yield elevated titres of amylase from *B. amyloliquefaciens* isolated from industrial waste as reported by Sharma *et al.* (2012). Yeast extract amended media showed the maximum production of α-amylase from *B. subtilis, B. marini* and *B. cereus* probably due to its high content in minerals, vitamins, coenzymes

and nitrogen components (Asgher *et al.*, 2007; Ashwini *et al.*, 2011; Vijayabaskar *et al.*, 2012). In consonance with these findings, starch agar medium used for enzyme production in the present study having yeast extract, when supplemented with an inorganic nitrogen source ammonium sulphate and sodium nitrate resulted in an elevated enzyme yield.

4.3.1.8 Effect of divalent ions

Table 20 and 21 represent the effect of various metal ions on α-amylase production by *B. aerius* GC6 and *B. sonorensis* GV2. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given in the end of the table.

Table 20. Effect of different divalent ions on extracellular α-amylase production from *B. aerius* GC6

Divalent ions	***Protein**	****Amylase activity**	*****Specific activity**
Ca^{2+}	**22.34±0.8^{a}**	**78.99±0.11^{a}**	**3.50±1.09^{a}**
Mg^{2+}	**20.93±0.05^{a}**	**77.58±0.13^{b}**	**3.65±0.87 ^{a}**
Cu^{2+}	14.35±0.18^{b}	68.47±0.14^{c}	4.77±0.66 ^{a}
Ba^{2+}	11.24±0.11^{c}	19.27±0.17^{d}	1.71±0.23 ^{a}
Fe^{2+}	11.24±0.09^{d}	36.43±0.21^{e}	3.24±0.12 ^{a}
Mn^{2+}	17.32±0.14^{e}	46.48±0.09^{f}	2.68±0.09 ^{a}
Co^{2+}	19.99±0.4^{a}	70.98±0.07^{g}	3.55±0.11 ^{a}
Zn^{2+}	8.28±0.6^{f}	12.34±0.14^{h}	1.49±0.25 ^{a}
Ni^{2+}	6.57±0.6^{g}	11.76±0.22^{i}	1.78±0.17 ^{a}
SE(m)	**0.43**	**0.52**	**0.32**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

Enzyme action is specific to the presence or absence of a particular metal ion. Likewise, *B. aerius* GC6 and *B. sonorensis* GV2 exhibited quite a variable behaviour towards extracellular α-amylase secretion in the presence of various

metal ions in the production medium. As metal ions accept or donate electrons and act as electrophiles, mask nucleophiles to prevent unwanted side reactions, bind enzyme and substrate by coordinate bonds, hold the reacting groups in the required 3D orientation, and simply stabilize a catalytically active conformation of the enzyme (Palmer, 2001). Most of the enzymes are metalloenzymes by nature and exhibit an increased activity in the presence of metal ions.

Table 21. Effect of different divalent ions on extracellular α-amylase production from *B. sonorensis* GV2

Divalent ions	*Protein	**Amylase activity	***Specific activity
Ca^{2+}	**21.98±0.16[a]**	**73.44±0.16[a]**	**3.34±0.09 [a]**
Mg^{2+}	**14.23±0.18[c]**	**73.43±0.26[c]**	**4.94±0.12 [b]**
Cu^{2+}	19.88±0.09[b]	68.49±0.34[b]	3.44±0.07 [a]
Ba^{2+}	11.23±0.22[d]	28.98±0.11[d]	2.56±0.20 [a]
Fe^{2+}	11.99±0.22[e]	36.43±0.09[e]	3.03±0.10 [a]
Mn^{2+}	14.90±0.11[f]	51.34±0.13[f]	3.44±0.14 [a]
Co^{2+}	15.48±0.14[g]	34.28±0.21[g]	2.21±0.15[c]
Zn^{2+}	11.23±0.09[h]	2.23±0.12[h]	0.19±0.03[d]
Ni^{2+}	11.23±0.15[i]	8.97±0.09[i]	0.79±0.17[e]
SE(m)	**0.28**	**0.44**	**0.30**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

Ca^{2+} and Mg^{2+} showed a positive effect on enzyme production by both the isolates. For *B. aerius* GC6 an amylase activity of 78.99 and 77.58 IU respectively has been observed whereas for *B. sonorensis* GV2 an amylase activity of 73.44 and 73.43 IU respectively has been noticed. Ca^{2+} and Mg^{2+} have been reported to enhance enzyme activity by Goyal *et al.* (2005); Asgher *et al.* (2007), Kiran and Chandra (2008), Tamilarasan *et al.* (2012) and Deb *et al.* (2013) from *Bacillus* sp. I-3, *B. subtilis* JS-2004, *Bacillus* sp. TSCVKK, *Aspergillus oryzae* MTCC 1847 and *B. amyloliquefaciens* P-001 respectively. Catalytic activity and stability of amylase have been reported to be calcium

dependent. Qader *et al.* (2006) reported maximum amylase production in the presence of 0.02% $CaCl_2$ from *Bacillus* sp. AS-1.

In accordance to the findings of Burhan *et al.* (2003) and Singh *et al.* (2014), Ni^{2+} and Zn^{2+} almost inhibited the enzyme production while in the medium. Ba^{2+}, Fe^{2+}, and Co^{2+} showed a negative effect on enzyme production lowering the titres considerably. Mn^{2+} and Cu^{2+} however did not affect the enzyme production upto a greater extent as reported by Rao and Satyanarayana (2007).

4.3.1.9 Effect of surfactants

Surfactants in the fermentation medium are known to increase the secretion of proteins by increasing cell membrane permeability (Hashemi *et al.*, 2010). Therefore, addition of these surfactants is used for the production of extracellular enzymes (Sivaramakrishnan *et al.*, 2006). Most organisms are stimulated to increased enzyme production; however, enzyme production by some organisms is only slightly affected in the presence of surfactants. In general, the percentage increases is greatest for organisms which produce small amounts of enzyme in the control lacking surfactant. The increase was less pronounced in the organisms which are normally good producers of the enzyme (Reese and Maguire, 1969).

Table 22 and 23 represent the effect of surfactants on α-amylase production by *B. aerius* GC6 and *B. sonorensis* GV2. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given in the end of the table. For *B. aerius* GC6 a decrease in enzyme production was observed while supplementing the media with surfactants. All the surfactants resulted in considerable inhibition of enzyme activity to varying extents. α-amylase production by *B. sonorensis* GV2 also showed the same trend with the addition of surfactants such as Tween 80. With Tween 80 in the medium, enzyme activity was observed as 69.46 IU with a specific activity of 3.26 IU/mg protein.

Table 22. Effect of surfactants on extracellular α-amylase production from *B. aerius* GC6

Surfactants	*Protein	**Amylase activity	***Specific activity
SDS	11.34±0.12[a]	59.58±0.09[a]	5.25±0.6[a]
EDTA	3.54±0.04[b]	2.43±0.08[b]	0.68±0.16[b]
CTAB	6.54±0.08 [a]	8.55±0.13[c]	1.30±0.11[c]
Tween 20	11.34±0.11 [a]	36.78±0.21[d]	3.24±0.34 [a]
Tween 80	10.46±0.13 [a]	38.34±0.17[e]	3.66±1.09 [a]
Triton X 100	12.78±0.07 [a]	41.02±0.09[f]	3.20±1.32 [a]
Glycerol	18.83±0.17[c]	51.22±0.08[g]	2.72±0.34 [a]
SE(m)	**0.28**	**0.31**	**0.42**

* Protein concentration
** Amylase activity
***Specific activity
} same as given in Table 2

Table 23. Effect of surfactants on extracellular α-amylase production from *B. sonorensis* GV2

Surfactants	*Protein	**Amylase activity	***Specific activity
SDS	20.02±0.16[a]	72.90±0.11[a]	3.64±0.23[a]
EDTA	3.23±0.08[b]	1.23±0.09[b]	0.38±0.15[b]
CTAB	8.82±0.06[c]	5.43±0.17[c]	0.61±0.09[c]
Tween 20	16.43±0.17[d]	41.98±0.21[d]	2.55±0.13[d]
Tween 80	21.30±0.11[a]	69.46±0.13[e]	3.26±0.14[a]
Triton X 100	11.89±0.09[e]	38.82±0.7[f]	3.26±0.07[a]
Glycerol	21.03±0.13[a]	56.89±0.14[g]	2.70±0.06[e]
SE(m)	**0.40**	**0.27**	**0.31**

* Protein concentration
**Amylase activity
***Specific activity
} same as given in Table 2

An increase in enzyme activity on addition of surfactants in the medium has been reported by Rao and Satyanarayana (2007) and Sodhi *et al.* (2005). However the addition of EDTA and CTAB in the medium resulted in inhibition

of activity proving it to be α-amylase. Tween 20, Triton X100 and glycerol also failed to increase the enzyme activity considerably thus were not added in the medium. Addition of surfactants would lead to an increased additional cost of production, thus an inhibition of enzyme activity in the presence of these additives would lead to a cost effective enzyme production process.

Classical approach i.e. one variable at a time (OVAT) used for optimization of α-amylase production from *B. aerius* GC6 and *B. sonorensis* GV2 has resulted in statistically significant increase in the production of amylase. Six significant variables chosen from one variable at a time optimization were further taken up for precise standardization by using a statistical tool of Response Surface Methodology (RSM).

4.3.2 Optimization of α-amylase production using Central Composite Design (CCD) of Response Surface Methodology (RSM) for *B. aerius* GC6 and *B. sonorensis* GV2

Optimization of medium by classical OVAT approach involves changing one independent variable at a time keeping all other variables at their constant levels. These optimization studies do not consider the interaction effects among the variables. In fermentation process, the operational variables interact and influence each other affecting the response, it is thus important that the optimization method accounts for interactions among the variables as well so that best optimal set of conditions can be determined (Silva and Roberto, 2001). This limitation of single variable optimization process can be eliminated by employing the statistical optimization technique of Response Surface Methodology (RSM), which is used to explain the individual as well as combined effects of all the factors in a fermentation process (Elibol, 2004). Single factor optimization methods are not only tedious, but also can lead to misinterpretation of results especially due to the interaction effects between different factors are overlooked (Wenster-Botz, 2013). Response Surface Methodology may be summarized as a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables (Liu and Tzeng, 1998).

Table 24. Complete experimental design with coded independent and response variables for optimization of α-amylase production from *B. aerius* GC6 by applying CCD of RSM

Std	Run	Block	Factors						Response 1 Amylase Activity (IU)
			Factor 1 A:Substrate concentration %	Factor 2 B: pH	Factor 3 C:Temperature ºC	Factor 4 D:Inoculum size %	Factor 5 E:Calcium ions %	Factor 6 F:Magnesium ions %	
2	1	1 0 0 0 0	1.000	-1.000	-1.000	-1.000	-1.000	-1.000	38.65
7	2	1 0 0 0 0	1.000	1.000	1.000	1.000	1.000	-1.000	5.44
16	3	1 0 0 0 0	1.000	1.000	1.000	1.000	-1.000	1.000	0.99
1	4	1 0 0 0 0	-1.000	-1.000	-1.000	-1.000	1.000	-1.000	57.17
17	5	1 0 0 0 0	0.000	0.000	0.000	0.000	0.000	0.000	84.45
15	6	1 0 0 0 0	-1.000	1.000	1.000	1.000	1.000	1.000	0.55
12	7	1 0 0 0 0	1.000	1.000	-1.000	-1.000	1.000	1.000	4.95
10	8	1 0 0 0 0	-1.000	-1.000	-1.000	-1.000	-1.000	1.000	46.50
13	9	1 0 0 0 0	1.000	-1.000	1.000	1.000	-1.000	1.000	0.89
4	10	1 0 0 0 0	-1.000	1.000	-1.000	-1.000	1.000	-1.000	2.17
14	11	1 0 0 0 0	-1.000	-1.000	1.000	1.000	1.000	1.000	1.48
8	12	1 0 0 0 0	-1.000	1.000	1.000	1.000	-1.000	-1.000	0.48
3	13	1 0 0 0 0	1.000	1.000	-1.000	-1.000	-1.000	-1.000	0.51
11	14	1 0 0 0 0	-1.000	1.000	-1.000	-1.000	-1.000	1.000	5.59
5	15	1 0 0 0 0	-1.000	-1.000	-1.000	1.000	-1.000	-1.000	1.77
18	**16**	**1 0 0 0 0**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**84.65**
6	17	1 0 0 0 0	1.000	-1.000	-1.000	1.000	1.000	-1.000	8.49
9	18	1 0 0 0 0	1.000	-1.000	-1.000	-1.000	1.000	1.000	42.92
24	19	0 1 0 0 0	-1.000	-1.000	-1.000	1.000	-1.000	-1.000	16.61
33	20	0 1 0 0 0	1.000	1.000	1.000	1.000	-1.000	1.000	10.51
32	21	0 1 0 0 0	1.000	-1.000	-1.000	1.000	-1.000	1.000	1.48
29	22	0 1 0 0 0	1.000	1.000	1.000	-1.000	1.000	1.000	4.96
20	23	0 1 0 0 0	-1.000	-1.000	-1.000	-1.000	1.000	-1.000	56.67
19	24	0 1 0 0 0	1.000	-1.000	-1.000	-1.000	-1.000	-1.000	51.05
27	25	0 1 0 0 0	-1.000	-1.000	1.000	-1.000	-1.000	1.000	52.73
30	26	0 1 0 0 0	-1.000	1.000	-1.000	-1.000	-1.000	1.000	14.58
34	27	0 1 0 0 0	-1.000	1.000	1.000	1.000	1.000	1.000	10.22

25	28	0 1 0 0 0	-1.000	1.000	0.000	1.000	-1.000	-1.000	14.06
36	29	0 1 0 0 0	0.000	0.000	0.000	0.000	0.000	0.000	83.39
35	30	0 1 0 0 0	0.000	0.000	0.000	0.000	0.000	0.000	84.21
21	31	0 1 0 0 0	-1.000	1.000	1.000	-1.000	1.000	-1.000	11.22
22	32	0 1 0 0 0	1.000	1.000	1.000	-1.000	-1.000	-1.000	7.69
26	33	0 1 0 0 0	1.000	1.000	1.000	1.000	1.000	-1.000	17.49
23	34	0 1 0 0 0	1.000	-1.000	-1.000	1.000	1.000	-1.000	11.53
28	35	0 1 0 0 0	1.000	-1.000	-1.000	-1.000	1.000	1.000	59.44
31	36	0 1 0 0 0	-1.000	-1.000	-1.000	1.000	1.000	1.000	1.08
39	37	0 0 1 0 0	-1.000	1.000	1.000	-1.000	-1.000	-1.000	3.99
49	38	0 0 1 0 0	-1.000	-1.000	-1.000	1.000	-1.000	1.000	0.00
47	39	0 0 1 0 0	1.000	1.000	1.000	-1.000	-1.000	1.000	1.72
54	40	0 0 1 0 0	0.000	0.000	0.000	0.000	0.000	0.000	83.49
40	41	0 0 1 0 0	1.000	1.000	1.000	-1.000	1.000	-1.000	2.25
51	42	0 0 1 0 0	1.000	1.000	1.000	1.000	1.000	1.000	1.49
50	43	0 0 1 0 0	1.000	-1.000	-1.000	1.000	1.000	1.000	1.11
52	44	0 0 1 0 0	-1.000	1.000	1.000	1.000	-1.000	1.000	6.89
45	45	0 0 1 0 0	-1.000	-1.000	-1.000	-1.000	1.000	1.000	41.94
44	46	0 0 1 0 0	1.000	1.000	1.000	1.000	-1.000	-1.000	7.55
41	47	0 0 1 0 0	1.000	-1.000	-1.000	1.000	-1.000	-1.000	1.39
38	48	0 0 1 0 0	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	49.40
46	49	0 0 1 0 0	1.000	-1.000	-1.000	-1.000	-1.000	1.000	45.28
42	50	0 0 1 0 0	-1.000	-1.000	-1.000	1.000	1.000	-1.000	7.58
37	51	0 0 1 0 0	1.000	-1.000	-1.000	-1.000	1.000	-1.000	51.71
53	52	0 0 1 0 0	0.000	0.000	0.000	0.000	0.000	0.000	83.39
48	53	0 0 1 0 0	-1.000	1.000	1.000	-1.000	1.000	1.000	1.47
43	54	0 0 1 0 0	-1.000	1.000	1.000	1.000	1.000	-1.000	0.49
70	55	0 0 0 1 0	1.000	1.000	1.000	1.000	1.000	1.000	0.05
62	56	0 0 0 1 0	-1.000	1.000	1.000	1.000	1.000	-1.000	0.13
58	57	0 0 0 1 0	-1.000	1.000	1.000	-1.000	-1.000	-1.000	4.94
57	58	0 0 0 1 0	1.000	1.000	1.000	-1.000	1.000	-1.000	0.00
56	59	0 0 0 1 0	1.000	-1.000	-1.000	-1.000	1.000	-1.000	42.46
72	60	0 0 0 1 0	0.000	0.000	0.000	0.000	0.000	0.000	82.02

68	61	0 0 0 1 0	-1.000	-1.000	-1.000	1.000	-1.000	1.000	0.49
63	62	0 0 0 1 0	1.000	-1.000	-1.000	-1.000	-1.000	1.000	39.71
55	63	0 0 0 1 0	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	46.25
61	64	0 0 0 1 0	1.000	1.000	1.000	1.000	-1.000	-1.000	1.10
60	65	0 0 0 1 0	1.000	-1.000	-1.000	1.000	-1.000	-1.000	0.49
65	66	0 0 0 1 0	-1.000	1.000	1.000	-1.000	1.000	1.000	1.61
71	67	0 0 0 1 0	0.000	0.000	0.000	0.000	0.000	0.000	65.66
67	68	0 0 0 1 0	1.000	-1.000	-1.000	1.000	1.000	1.000	1.58
66	69	0 0 0 1 0	1.000	1.000	1.000	-1.000	-1.000	1.000	1.97
69	70	0 0 0 1 0	-1.000	1.000	1.000	1.000	-1.000	1.000	0.28
59	71	0 0 0 1 0	-1.000	-1.000	-1.000	1.000	1.000	-1.000	1.44
64	72	0 0 0 1 0	-1.000	-1.000	-1.000	-1.000	1.000	1.000	45.72
81	73	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	47.47
90	74	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	83.49
78	75	0 0 0 0 1	0.000	0.000	0.000	2.828	0.000	0.000	0.00
85	76	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	83.29
76	77	0 0 0 0 1	0.000	0.000	0.000	0.000	2.828	0.000	0.00
84	78	0 0 0 0 1	2.828	0.000	2.828	0.000	0.000	2.828	34.57
89	79	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	84.59
73	80	0 0 0 0 1	0.000	2.828	0.000	0.000	0.000	0.000	25.42
75	81	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	0.00
86	82	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	82.59
88	83	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	84.56
77	84	0 0 0 0 1	0.000	0.000	0.000	-2.828	0.000	0.000	12.23
80	85	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	0.00
79	86	0 0 0 0 1	0.000	-2.828	0.000	0.000	0.000	0.000	0.00
87	87	0 0 0 0 1	0.000	0.000	0.000	0.000	-2.828	0.000	84.46
83	88	0 0 0 0 1	-2.828	0.000	-2.828	0.000	0.000	0.000	44.97
74	89	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	-2.282	64.46
82	90	0 0 0 0 1	0.000	0.000	0.000	0.000	0.000	0.000	32.19

Table 25. Complete experimental design with coded independent and response variables for optimization of α-amylase production from *B. sonorensis* GV2 by applying CCD of RSM

Std	Run	Block	Factors						Response 1 Amylase Activity (IU)
			Factor 1 A:Substrate concentration %	Factor 2 B: pH	Factor 3 C:Temperature (°C)	Factor 4 D:Inoculum size %	Factor 5 E:Calcium ions %	Factor 6 F:Magnesium ions %	
12	1	1 0 0 0 0	1.000	1.000	-1.000	1.000	1.000	1.000	10.98
5	2	1 0 0 0 0	0.000	-1.000	1.000	-1.000	-1.000	-1.000	3.76
9	3	1 0 0 0 0	0.000	-1.000	-1.000	1.000	1.000	1.000	24.78
11	4	1 0 0 0 0	0.000	1.000	-1.000	-1.000	1.000	-1.000	12.56
16	5	1 0 0 0 0	1.000	1.000	1.000	-1.000	1.000	1.000	1.14
3	6	1 0 0 0 0	-1.000	1.000	-1.000	-1.000	-1.000	1.000	1.77
6	7	1 0 0 0 0	1.000	-1.000	1.000	1.000	-1.000	1.000	42.16
15	8	1 0 0 0 0	-1.000	1.000	1.000	1.000	1.000	-1.000	0.78
7	9	1 0 0 0 0	-1.000	1.000	1.000	1.000	-1.000	1.000	0.04
10	10	1 0 0 0 0	1.000	-1.000	-1.000	-1.000	1.000	-1.000	49.73
14	11	1 0 0 0 0	1.000	-1.000	1.000	1.000	1.000	-1.000	47.90
8	12	1 0 0 0 0	1.000	1.000	1.000	-1.000	-1.000	-1.000	1.21
2	13	1 0 0 0 0	1.000	-1.000	-1.000	-1.000	-1.000	1.000	45.61
4	14	1 0 0 0 0	1.000	1.000	-1.000	1.000	-1.000	-1.000	1.44
18	15	1 0 0 0 0	0.000	0.000	0.143	0.000	0.000	0.000	63.47
13	16	1 0 0 0 0	-1.000	-1.000	1.000	-1.000	1.000	1.000	3.26
1	17	1 0 0 0 0	-1.000	-1.000	-1.000	1.000	-1.000	-1.000	44.65
17	18	1 0 0 0 0	0.000	0.000	0.143	0.000	0.000	0.000	54.55
28	19	0 1 0 0 0	1.000	-1.000	-1.000	1.000	1.000	1.000	57.74
26	20	0 1 0 0 0	1.000	1.000	1.000	1.000	-1.000	1.000	1.48
34	21	0 1 0 0 0	1.000	1.000	1.000	1.000	1.000	-1.000	11.09
20	22	0 1 0 0 0	1.000	-1.000	-1.000	1.000	-1.000	-1.000	55.56
22	23	0 1 0 0 0	1.000	1.000	-1.000	-1.000	-1.000	1.000	12.21
32	24	0 1 0 0 0	1.000	-1.000	1.000	-1.000	1.000	1.000	32.92
33	25	0 1 0 0 0	-1.000	1.000	1.000	-1.000	1.000	1.000	0.87
19	26	0 1 0 0 0	-1.000	-1.000	-1.000	-1.000	-1.000	1.000	57.34
36	27	0 1 0 0 0	0.000	0.000	0.143	0.000	0.000	0.000	80.05

25	28	0 1 0 0 0	-1.000	1.000	1.000	-1.000	-1.000	-1.000	0.17
30	29	0 1 0 0 0	1.000	1.000	-1.000	-1.000	1.000	-1.000	9.21
29	30	0 1 0 0 0	-1.000	1.000	-1.000	1.000	1.000	1.000	2.12
24	31	0 1 0 0 0	1.000	-1.000	1.000	-1.000	-1.000	-1.000	44.48
27	32	0 1 0 0 0	0.000	-1.000	-1.000	-1.000	1.000	-1.000	37.08
23	33	0 1 0 0 0	-1.000	-1.000	1.000	1.000	-1.000	1.000	16.79
21	34	0 1 0 0 0	-1.000	1.000	-1.000	1.000	-1.000	-1.000	10.14
35	35	0 1 0 0 0	0.000	0.000	0.143	0.000	0.000	0.000	79.75
31	36	0 1 0 0 0	-1.000	-1.000	1.000	1.000	1.000	-1.000	6.44
45	37	0 0 1 0 0	-1.000	-1.000	-1.000	1.000	1.000	-1.000	17.79
46	38	0 0 1 0 0	1.000	-1.000	-1.000	-1.000	1.000	1.000	32.94
41	39	0 0 1 0 0	0.000	-1.000	1.000	-1.000	-1.000	1.000	1.74
47	40	0 0 1 0 0	0.000	1.000	-1.000	-1.000	1.000	1.000	11.09
48	41	0 0 1 0 0	1.000	1.000	-1.000	1.000	1.000	-1.000	2.47
43	42	0 0 1 0 0	-1.000	1.000	1.000	1.000	-1.000	-1.000	0.49
44	43	0 0 1 0 0	1.000	1.000	1.000	-1.000	-1.000	1.000	0.04
53	44	0 0 1 0 0	0.000	0.000	0.143	0.000	0.000	0.000	68.74
54	45	0 0 1 0 0	0.000	0.000	0.143	0.000	0.000	0.000	69.55
37	46	0 0 1 0 0	-1.000	-1.000	-1.000	1.000	-1.000	1.000	32.49
39	47	0 0 1 0 0	-1.000	1.000	-1.000	-1.000	-1.000	-1.000	49.39
51	48	0 0 1 0 0	-1.000	1.000	1.000	1.000	1.000	1.000	0.39
52	49	0 0 1 0 0	1.000	1.000	1.000	-1.000	1.000	-1.000	1.74
38	50	0 0 1 0 0	1.000	-1.000	-1.000	-1.000	-1.000	-1.000	61.67
50	51	0 0 1 0 0	1.000	-1.000	1.000	1.000	1.000	1.000	47.24
49	52	0 0 1 0 0	-1.000	-1.000	1.000	-1.000	1.000	-1.000	1.47
40	53	0 0 1 0 0	1.000	1.000	-1.000	1.000	-1.000	1.000	1.76
42	54	0 0 1 0 0	1.000	-1.000	1.000	1.000	-1.000	-1.000	47.07
65	55	0 0 0 1 0	-1.000	1.000	-1.000	1.000	1.000	-1.000	1.02
60	56	0 0 0 1 0	1.000	-1.000	1.000	-1.000	-1.000	1.000	1.49
57	57	0 0 0 1 0	-1.000	1.000	-1.000	1.000	-1.000	1.000	1.37
62	58	0 0 0 1 0	1.000	1.000	1.000	1.000	-1.000	-1.000	0.49
71	59	0 0 0 1 0	0.000	0.000	0.143	0.000	0.000	0.000	81.33
58	60	0 0 0 1 0	1.000	1.000	-1.000	-1.000	-1.000	-1.000	0.66

66	61	0 0 0 1 0	1.000	1.000	-1.000	-1.000	1.000	1.000	0.50
72	62	0 0 0 1 0	0.000	0.000	0.000	0.000	0.000	0.000	81.31
63	63	0 0 0 1 0	-1.000	-1.000	-1.000	-1.000	1.000	1.000	39.71
55	64	0 0 0 1 0	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	59.97
56	65	0 0 0 1 0	1.000	-1.000	-1.000	1.000	-1.000	1.000	59.71
70	66	0 0 0 1 0	1.000	1.000	1.000	1.000	1.000	1.000	0.04
59	67	0 0 0 1 0	0.000	-1.000	1.000	1.000	-1.000	-1.000	7.47
61	68	0 0 0 1 0	0.000	1.000	1.000	-1.000	-1.000	1.000	0.39
69	69	0 0 0 1 0	0.000	1.000	1.000	-1.000	1.000	-1.000	0.04
68	70	0 0 0 1 0	1.000	-1.000	1.000	-1.000	1.000	-1.000	0.14
67	71	0 0 0 1 0	-1.000	-1.000	1.000	1.000	1.000	1.000	5.79
64	72	0 0 0 1 0	1.000	-1.000	-1.000	1.000	1.000	-1.000	52.72
78	73	0 0 0 0 1	0.000	0.000	2.828	0.000	0.000	0.000	0
86	74	0 0 0 0 1	0.000	0.000	0.143	0.000	0.000	0.000	82.47
73	75	0 0 0 0 1	-2.828	0.000	0.143	0.000	0.000	0.000	11.46
82	76	0 0 0 0 1	0.000	0.000	0.143	0.000	2.828	0.000	49.47
80	77	0 0 0 0 1	0.000	0.000	0.143	2.828	0.000	0.000	0.77
75	78	0 0 0 0 1	0.000	-2.828	0.143	0.000	0.000	0.000	0
88	**79**	**0 0 0 0 1**	**-1.000**	**0.000**	**0.143**	**0.000**	**0.000**	**0.000**	**82.77**
74	80	0 0 0 0 1	2.828	0.000	0.143	0.000	0.000	0.000	32.24
76	81	0 0 0 0 1	0.000	2.828	0.143	0.000	0.000	0.000	0
77	82	0 0 0 0 1	0.000	0.000	-2.828	0.000	0.000	0.000	12.87
83	83	0 0 0 0 1	-1.000	0.000	0.143	0.000	0.000	-2.828	79.44
89	84	0 0 0 0 1	-1.000	0.000	0.143	0.000	0.000	0.000	81.36
90	85	0 0 0 0 1	-1.000	0.000	0.143	0.000	0.000	0.000	81.34
84	86	0 0 0 0 1	-1.000	0.000	0.143	0.000	0.000	2.828	81.37
79	87	0 0 0 0 1	0.000	0.000	0.143	-2.828	0.000	0.000	0
85	88	0 0 0 0 1	-1.000	0.000	0.143	0.000	0.000	0.000	82.77
81	89	0 0 0 0 1	-1.000	0.000	0.143	0.000	-2.828	0.000	55.47
87	90	0 0 0 0 1	-1.000	0.000	0.143	0.000	0.000	0.000	82.47

Statistical methods have been applied for optimization of α-amylase by several workers (Dey *et al.*, 2001; Francis *et al.*, 2002; Ahuja *et al.*, 2004; Kunamneni *et al.*, 2005; Tamilarasan *et al.*, 2012).

4.3.2.1 Regression model of response

In this method, prior knowledge of significant culture conditions and media components obtained from previous One Variable at a Time (OVAT) approach experiment was necessary for achieving a more realistic model. Table 1 (Section 3.3.2) shows the maximum and minimum levels of variables chosen for trials (Run) in the CCD for both *B. aerius* GC6 and *B. sonorensis* GV2. For RSM based on Central Composite Design, used for the optimization of independent variables for amylase production, 90 experimental runs with different combinations of six factors were carried out. The independent variables used for the factorial analysis were substrate concentration (X_1), pH (X_2), temperature (X_3), inoculum size (X_4), Ca^{2+} concentration (X_5) and Mg^{2+} concentration (X_6) while the dependent variable selected was enzyme activity (Y). The complete experimental design with coded factors and response values has been given in Table 24 for *B. aerius* GC6 and in Table 25 for *B. sonorensis* GV2, which shows considerable variation in the amount of amylase production depending upon the interaction of various levels of six independent variables in the medium.

By applying multiple regression analysis on the experimental data obtained for *B. aerius* GC6 α-amylase, a quadratic model was generated for the response of amylase activity. The significant model terms were evaluated by analysis of variance (ANOVA) (Table 26) in the optimization study ($p<0.05$) and were identified as X_1, X_2, X_3, X_5, X_1^2, X_2^2, X_3^2, X_4^2, X_5^2, X_6^2, X_1X_2, X_1X_4,X_2X_3, X_2X_4, X_2X_6, X_3X_4, X_3X_6, X_4X_6, X_5X_6.

Enzyme Activity (Y) = $77.08+1.96 * X_1-9.64 * X_2-9.72 * X_3+0.14 * X_4-1.14 *X_5-0.97 * X_6-7.95 *X_1^2-11.98 * X_2^2-11.98 * X_3^2-11.98 * X_4^2-7.00 * X_5^2-7.01 * X_6^2+0.34 * X_1 * X_2+0.11* X_1 * X_3+0.090 * X_1 * X_4+0.21 * X_1 * X_5 +0.014 * X_1 * X_5+10.78* X_2 * X_3 -0.25* X_2 *X_4 +0.32 * X_2 * X_5+0.50 * X_2 * X_6-0.17 *X_3 *X_4-0.44 *X_3 * X_4+1.10 *X_3 *X_5-0.73*X_4*X_5+1.11*X_4*X_5+0.41*X_5*X_6$ **Eq.1**

Table 26. ANOVA for response surface quadratic model for α-amylase production from *B. aerius* GC6

Source	Sum of Squares	df	Mean Sum of Square	F-Value	Prob > F
Block	66.57	4	16.64		
Model	799.14	27	29.60	29.04	< 0.0001 significant
X_1	3.86	1	3.86	3.79	0.0005*
X_2	74.30	1	74.30	72.90	< 0.0001*
X_3	116.97	1	116.97	114.77	< 0.0001*
X_4	4.998E-003	1	4.998E-003	4.904E-003	0.9444
X_5	3.26	1	3.26	3.20	0.0008*
X_6	0.45	1	0.45	0.44	0.5094
X_1^2	26.22	1	26.22	25.73	< 0.0001*
X_2^2	196.03	1	196.03	192.35	< 0.0001*
X_3^2	135.14	1	135.14	132.60	< 0.0001*
X_4^2	196.03	1	196.03	192.35	< 0.0001*
X_5^2	29.87	1	29.87	29.31	< 0.0001*
X_6^2	29.71	1	29.71	29.15	< 0.0001*
X_1X_2	4.941E-003	1	4.941E-003	4.848E-003	0.0007*
X_1X_3	0.087	1	0.087	0.085	0.7714
X_1X_4	5.234E-003	1	5.234E-003	5.136E-003	0.0001*
X_1X_5	0.023	1	0.023	0.022	0.8814
X_1X_6	0.15	1	0.15	0.14	0.7062
X_2X_3	111.04	1	111.04	108.95	< 0.0001*
X_2X_4	2.51	1	2.51	2.46	0.0001*
X_2X_5	1.64	1	1.64	1.61	0.2101
X_2X_6	6.703E-003	1	6.703E-003	6.577E-003	0.0006*
X_3X4	0.18	1	0.18	0.17	0.0009*
X_3X_5	2.90	1	2.90	2.85	0.0970
X_3X_6	1.94	1	1.94	1.90	0.0009*
X_4X_5	0.30	1	0.30	0.29	0.5899
X_4X_6	1.90	1	1.90	1.86	0.0006*
X_5X_6	0.011	1	0.011	0.011	0.0003*
Residual	59.11	58	1.02		
Lack of Fit	58.64	49	1.20	22.96	< 0.0001 significant
Pure Error	0.47	9	0.052		
Cor Total	924.82	89			

where Y is the enzyme activity (IU), X_1 is substrate concentration, X_2 is pH, X_3 is temperature, X_4 is inoculum size, X_5 is Ca^{2+} ion concentration and X_6 is Mg^{2+} ion concentration. By optimizing the above parameters by using RSM, maximum enzyme activity obtained was 84.65 IU/ml at 50°C temperature and 9.0 pH @ 8.75% inoculum using 1.75% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration.

Similarly, when multiple regression analysis was applied on the experimental data for *B. sonorensis* GV2 α-amylase, a similar quadratic model was generated for the response of amylase activity. The significant model terms were evaluated by analysis of variance (ANOVA) (Table 27) in the optimization study (p<0.05) and X_1, X_3, X_4, X_5, X_1^2, X_2^2, X_3^2, X_4^2, X_5^2, X_6^2, X_1X_3, X_1X_4, X_1X_6,X_2X_3, X_2X_4, X_2X_5, X_3X_4, X_3X_5, X_4X_5, X_5X_6 were found to contribute to the response.

Enzyme Activity (Y) = 74.25+4.17 * X_1-10.32 * X_2-7.38 * X_3+0.53 * X_4-2.20 * X_5-0.90 * X_6-8.77 *X_1^2-12.33 * X_2^2-11.68 * X_3^2-12.28 * X_4^2-5.23* X_5^2-0.61* X_6^2-5.94* X_1*X_2+2.87*X_1* X_3+4.21*X_1*X_4+1.68 * X_1 * X_5-0.055 *X_1 * X_6+5.40* X_2 * X_3-2.10 * X_2 *X_4+1.25* X_2 * X_5-0.52 * X_2 * X_6 +4.17 *X_3*X_4+3.35 * X_3 * X_5+1.46 * X_3 * X_6+1.01 * X_4 * X_5+1.14 * X_4 * X_6+1.72 * X_5 * X_6 **Eq. 2**

where Y is enzyme activity (IU), substrate concentration (X_1), pH (X_2), temperature (X_3), inoculum size (X_4), Ca^{2+} concentration (X_5) and Mg^{2+} concentration (X_6).By optimizing the above parameters by using RSM, maximum enzyme activity obtained was 82.78 IU/ml at 50°C temperature and 9.0 pH @ 8.75% inoculum using 1.0% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration.

The statistical significance of Eq.1 and 2 was checked by *F*-test, and the analysis of variance (ANOVA) of the response surface quadratic model for *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase has been shown in Table 26 and 27 respectively. It is evident that both the models are highly significant, as suggested by the model *F*- values and a low probability values (P_{model}>F= 0.0001). The coefficient of determination (R^2) was calculated as 0.9311 for *B aerius* GC6 and 0.8829 for *B. sonorensis* GV2, indicating that the statistical

Table 27. ANOVA for response surface quadratic model for optimum production of α-amylase from *B. sonorensis* GV2

Source	Sum of Squares	df	Mean Sum of Square	F-Value	Prob > F
Block	4703.63	4	1175.91		
Model	34450.24	27	1275.93	7.75	< 0.0001 significant
X_1	735.84	1	735.84	4.47	0.0009*
X_2	1518.86	1	1518.86	9.22	0.0036
X_3	3988.95	1	3988.95	24.21	< 0.0001*
X_4	530.13	1	530.13	3.22	0.0001*
X_5	108.68	1	108.68	0.66	0.4200
X_6	4.59	1	4.59	0.028	0.8681
X_1^2	123.15	1	123.15	0.75	0.3908
X_2^2	9718.23	1	9718.23	58.99	< 0.0001*
X_3^2	9553.88	1	9553.88	58.00	< 0.0001*
X_4^2	7983.73	1	7983.73	48.46	< 0.0001*
X_5^2	1234.33	1	1234.33	7.49	0.0082
X_6^2	114.61	1	114.61	0.70	0.4076
X_1X_2	1.40	1	1.40	8.488E-003	0.9269
X_1X_3	185.50	1	185.50	1.13	0.0003*
X_1X_4	122.97	1	122.97	0.75	0.0002*
X_1X_5	1.02	1	1.02	6.192E-003	0.9375
X_1X_6	1.15	1	1.15	6.983E-003	0.0007*
X_2X_3	9.025E-003	1	9.025E-003	5.479E-005	0.9941
X_2X_4	429.47	1	429.47	2.61	0.0008*
X_2X_5	1.23	1	1.23	7.479E-003	0.0004*
X_2X_6	4.07	1	4.07	0.025	0.8756
X_3X_4	90.27	1	90.27	0.55	0.0001*
X_3X_5	107.17	1	107.17	0.65	0.0002*
X_3X_6	48.16	1	48.16	0.29	0.5908
X_4X_5	203.90	1	203.90	1.24	0.0005*
X_4X_6	50.30	1	50.30	0.31	0.5827
X_5X_6	456.68	1	456.68	2.77	0.0003*
Residual	9554.49	58	164.73		
Lack of Fit	9237.02	51	181.12	3.99	0.0302 significant
Pure Error	317.47	7	45.35		
Cor Total	48708.36	89			

*Significant values

model can explain a respective variability of 93.11% and 88.29% in the response. Normally, a regression model having an R^2 value > 0.9 is considered to have a very high correlation. The closer the R^2 (correlation coefficient) is to 1.0, the stronger the model and the better it predicts the response (Haaland, 1989). The value of R= 0.9971 for Eq. 1 and R= 0.9936 for Eq. 2 indicates a close agreement between the experimental results and the theoretical values predicted by the model equations. An adequate precision value for amylase production by *B. aerius* GC6 was 21.65 whereas for *B. sonorensis* GV2 an adequate precision value 12.42 was recorded. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually the higher value of CV, the lower the reliability of the experiment is (Gangadharan *et al.*, 2008). A lower value of CV i.e. 4.09 for *B. aerius* GC6 and 4.95 for *B. sonorensis* GV2 indicated a better precision and reliability of the experiment.

P-value, along with the parameter estimate have been given in Table 26 for *B. aerius* GC6 and in Table 27 for *B. sonorensis* GV2. The *P*-values are used as a tool to check the significance of each of the coefficients which are necessary to understand the pattern of the mutual interactions between the variables. The smaller the *P*-values, the bigger the significance of the corresponding coefficient. For α-amylase production by *B. aerius* GC6, the parameter estimates and the corresponding *P*-values suggested that among the independent variables X_1 (substrate concentration), X_2 (pH), X_3 (temperature) and X_5 (Ca^{2+} concentration) have significant effect on enhancement of amylase production. A positive *P*-values for X_4 (inoculum size), X_6 (Mg^{2+} concentration) indicated a linear effect of these variables on amylase production. Quadratic terms of all the variables were significant. Among the interactions, X_1X_2, X_1X_4, X_2X_3, X_2X_4, X_2X_6, X_3X_4, X_3X_6, X_4X_6, X_5X_6 were found to contribute to the response at a significant level. The parameter estimates and the corresponding *P*-values for *B. sonorensis* GV2 on the other hand suggested that among the independent variables X_1 (substrate concentration), X_3 (temperature), X_4 (inoculum size) and X_5 (Ca^{2+} concentration) have significant effect on enhancement of amylase production. A positive *P*-values for X_2 (pH), X_6 (Mg^{2+} concentration) indicated a linear effect of these variables on amylase production. Quadratic terms of all the

variables were significant. Among the interactions, X_1X_3, X_1X_4, X_1X_6, X_2X_3, X_2X_4, X_2X_5, X_3X_4, X_3X_5, X_4X_5, X_5X_6 were found to contribute to the response at a significant level.

4.3.2.2 Localization of optimum conditions

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables on the response variable. The shape of the corresponding contour indicates whether the mutual interactions between the independent variables are significant or not. An elliptical contour plot indicates that the interactions are significant. Fig. 8 depicts the expected responses for amylase production and correlation between the independent variables for *B. aerius* GC6 in three dimensional plots, with substrate concentration, temperature and pH respectively, at the X-axis. Fig. 8a and 8b shows the curve between substrate concentration at X-axis and pH and temperature at Y- axis respectively while keeping all other variables at their 'O' central levels. The plots depicted a moderate interaction between the considered variables towards an enhanced amylase activity. Fig. 8c shows a curve between temperature at X-axis and inoculum size at Y-axis while keeping all other variables at their 'O' central levels. With an increased inoculum size, an increased amylase activity has been observed irrespective of the lower value of temperature. The pair of factors showed a non- additive effect due to the significant interaction between them. Fig. 8d shows a curve between substrate concentration at X-axis and inoculum size at Y-axis while keeping all other variables at their 'O' central levels. This curve shows a negative interaction between the two variables suggesting the additive effect of the pair of factors towards the response variable. Fig. 8e and 8f shows a curve between pH at X-axis and inoculum size and temperature at Y-axis respectively, while keeping all other variables at their 'O' central levels. A lower level of pH and temperature has resulted in an increased amylase activity thus suggesting the factors to be non-additive as they show a positive and significant interaction among them. However, increasing pH and inoculum size simultaneously had a negative effect on amylase activity thus suggesting their non significant effect as a pair.

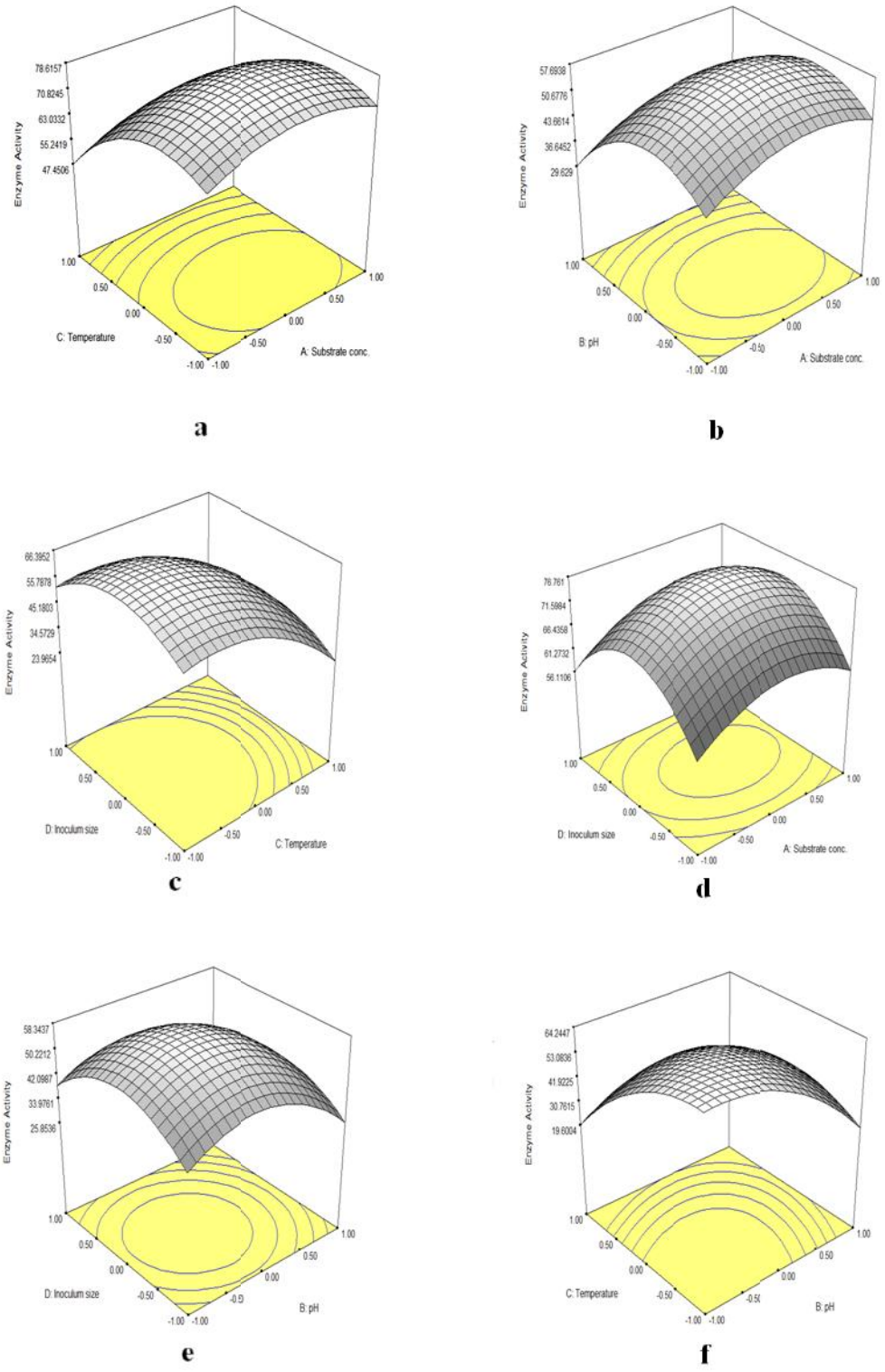


Fig. 8 Response surface curves for *B. aerius* GC6 α-amylase showing interactions between a) substrate concentration and temperature, b) substrate concentration and pH, c) temperature and inoculum size, d) substrate concentration and inoculum size, e) pH and inoculum size and f) pH and temperature

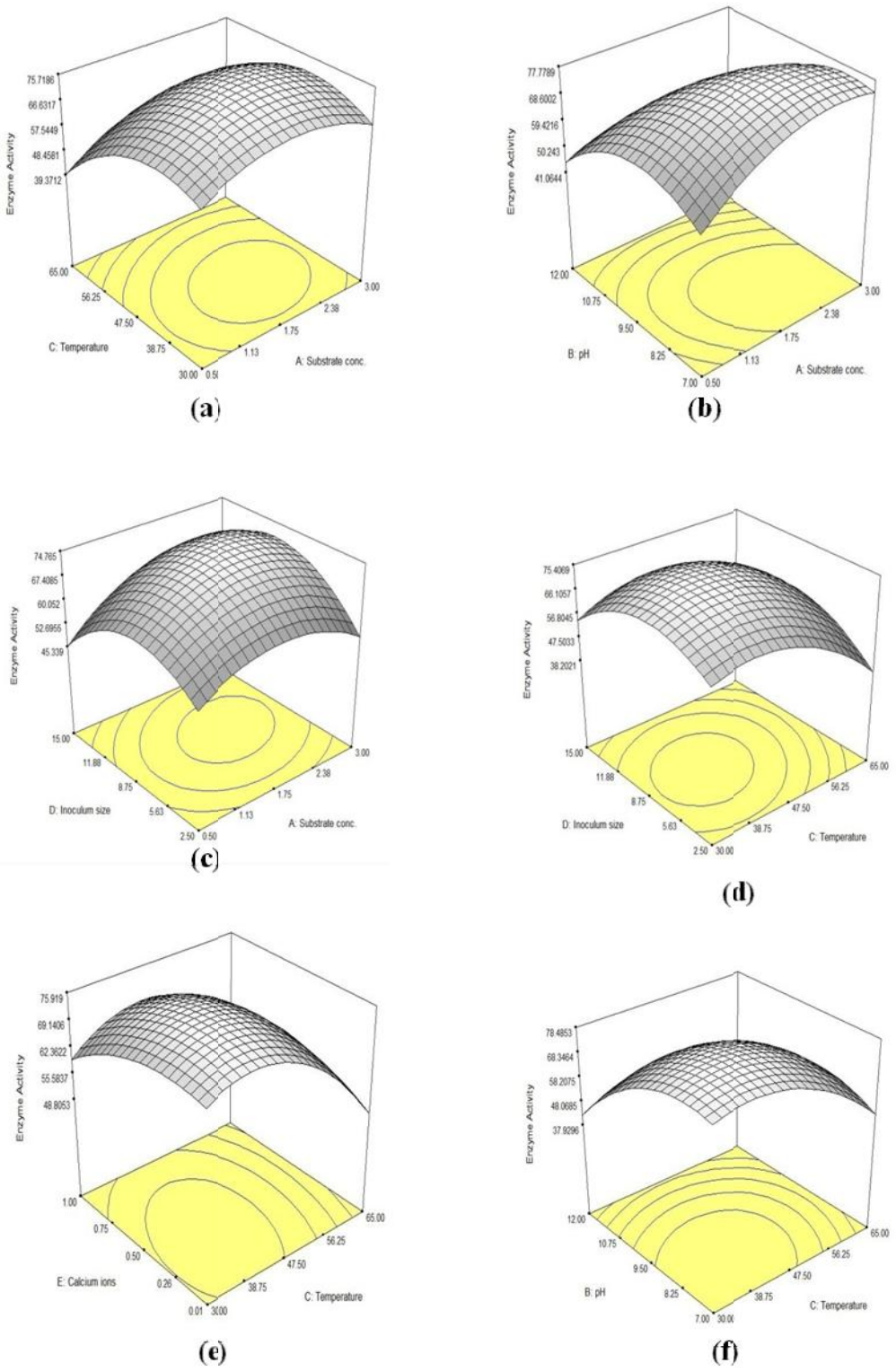


Fig. 9 Response surface curves showing interactions between a) substrate concentration and temperature, b) substrate concentration and pH, c) substrate concentration and inoculum size, d) temperature and inoculum size, e) temperature and calcium ions and f) pH and temperature

Fig. 9 represents the expected responses for amylase production by *B. sonorensis* GV2 and correlation between the independent variables in three dimensional plots, with substrate concentration and temperature respectively, at the X-axis. Fig. 9a shows the curve between substrate concentration at X-axis and temperature at Y-axis respectively while keeping all other variables at their 'O' central levels. Higher levels of substrate concentration and lower levels of temperature have resulted in an enhanced amylase production. The products showed a moderate level of interaction. Fig. 9b and 9c represents the interaction between substrate concentration and inoculum size and pH respectively. Higher levels of substrate concentration and inoculum size and lower levels of pH has resulted in decreased amylase activity thus showing negative interaction between the pair of factors proving the factors to be additive in nature towards the activity of amylase. Fig. 9d and 9e shows a curve between temperature at X-axis and calcium ions and pH respectively, at Y-axis while keeping all other variables at their 'O' central levels. With an increased temperature on the X-axis and calcium ions and pH respectively, at Y-axis an increase in amylase activity has been observed. The pair of factors has shown a positive interaction and thus is non-additive due to the significant interaction between them.

4.3.2.3 Comparison of observed and predicted amylase activity

A regression model can also be used to predict future observations on the response Y (amylase activity) corresponding to the particular values of the regression variables. Fig. 10 shows observed amylase activity versus those from the empirical model Eq. 1 i.e. for *B. aerius* GC6. The predicted R^2 of 0.7508 is in reasonable agreement with the adjusted R^2 of 0.8991. This indicated good agreement between the experimental and predicted values for amylase production. For Eq. 2 i.e. for *B. sonorensis* GV2, Fig. 11 shows the observed and predicted amylase activity. The predicted R^2 of 0.7874 is in reasonable agreement with the adjusted R^2 of 0.6818. The adjusted R^2 corrects the R^2 value for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the predicted R^2. The model F-value of 29.04 for *B. aerius* GC6 and 7.75 for *B. sonorensis* GV2 and values of prob > F (<0.05)

indicated that the model terms are significant. The figures prove the predicted data for both *B. aerius* GC6 and *B. sonorensis* GV2 of the response from the empirical model Eq. 1 and Eq. 2 is in agreement with the observed ones in the range of the operating variables.

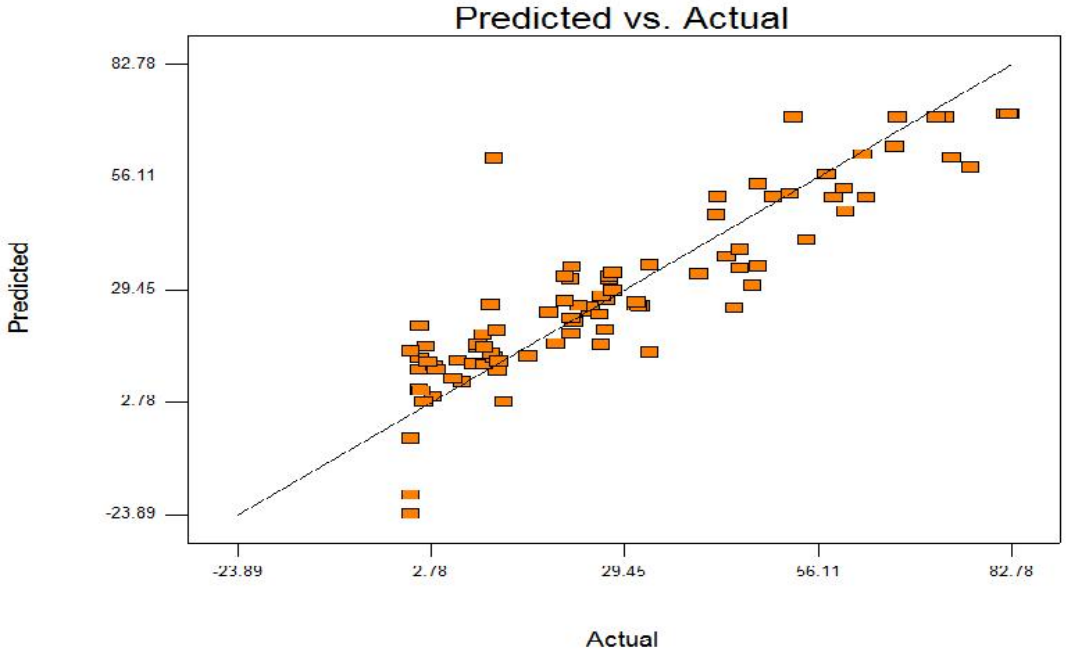


Fig. 10 Parity plot showing distribution of experimental and predicted values of amylase production by *B. aerius* GC6

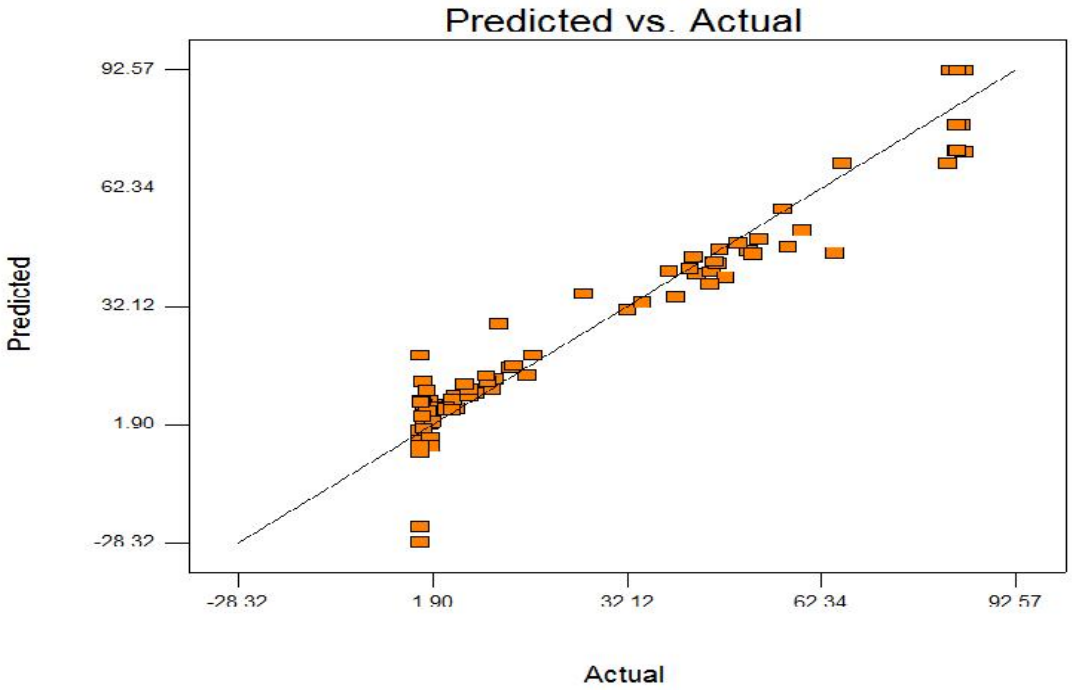


Fig. 11 Parity plot showing distribution of experimental and predicted values of amylase production by *B. sonorensis* GV2

4.3.2.4 Model adequacy checking

Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface likely give poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy. By constructing a normal probability plot for the residuals, a check was made for the normality assumption, as given in Fig. 12 and Fig. 13 for *B. aerius* GC6 and *B. sonorensis* GV2 respectively, the normality assumption was satisfied as the residual plot approximated along a straight line. The general impression is that the residuals scatter randomly on the display, suggesting that the variance of the original observation is constant for all values of predicated response (Y). Fig. 12 and Fig. 13 are satisfactory, so we conclude that the empirical model is adequate to describe the amylase activity by response surface.

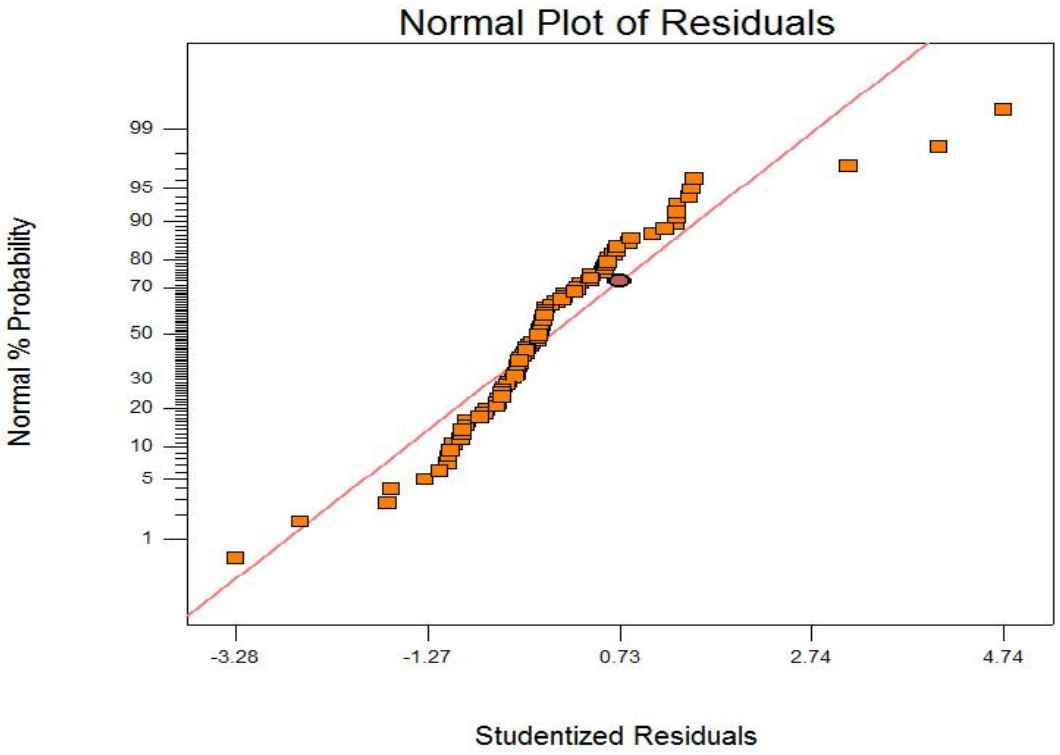


Fig. 12 Normal probability of internally studentized residuals for *B. aerius* GC6

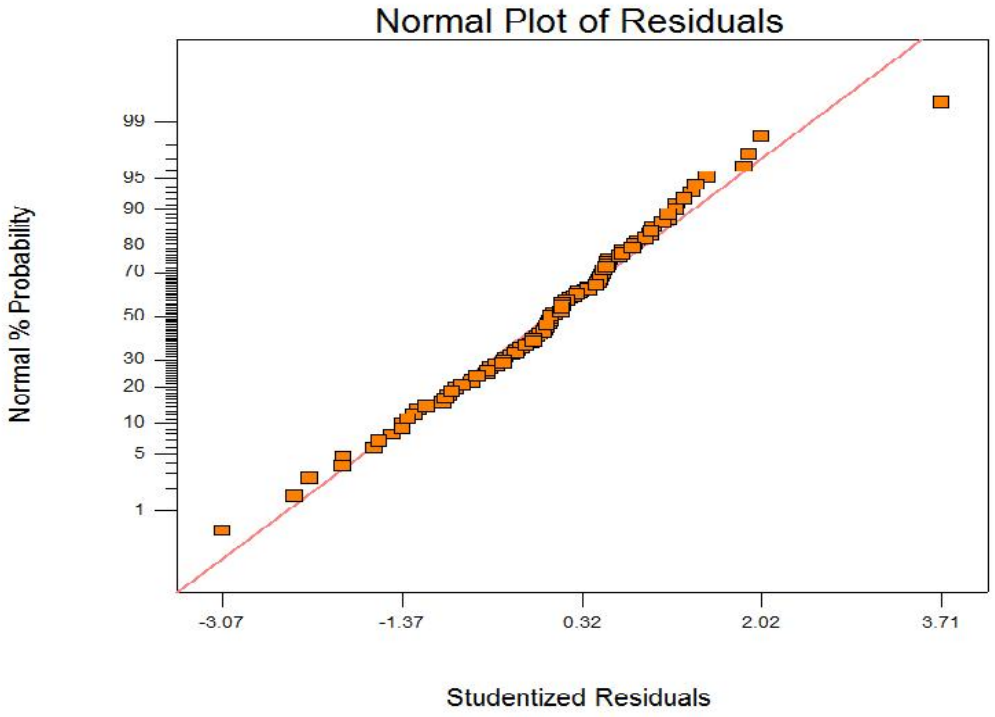


Fig. 13 Normal probability of internally studentized residuals for *B. sonorensis* GV2

4.3.2.5 Validation of the model

The statistically optimal values of variables were obtained when moving along the major and minor axis of the contour, and the response at the centre point yielded maximum amylase production. These observations were also verified from canonical analysis of the response surface. The canonical analysis revealed a minimum region for the model. The stationary point presenting a maximum amylase activity for *B.* aerius GC6 had the critical values as 84.65 IU/ml at 50°C temperature and 9.0 pH @ 8.75% inoculum using 1.75% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration. For *B. sonorensis* GV2, critical Values observed were 82.78 IU/ml at 50°C temperature and 9.0 pH @ 8.75% inoculum using 1.0% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration.

Numerical optimization was carried out to obtain the best combination for maximum amylase production. The desired goals for each variable and response were chosen and different weights were assigned to each goal to adjust the shape of its particular desirability function. Table 28 and Table 29 shows software generated three optimum conditions of independent variables with the predicted values of responses for *B. aerius* GC6 and *B. sonorensis* GV2 respectively.

Solution number 1 having the maximum desirability value of 0.93 for *B. aerius* GC6 and a value of 0.97 for *B. sonorensis* GV2 was selected for production of α-amylase.

Table 28. Software generated three optimum conditions of independent variables with the desirability values for optimum α-amylase production from *B. aerius* GC6

Sr. No.	Substrate concentration	pH	Temperature	Inoculum size	Calcium ions	Magnesium ions	Desirability
1	**1.00**	**9.0**	**50.0**	**8.75**	**0.48**	**0.33**	**0.93**
2	1.00	9.0	50.0	8.78	0.49	0.61	0.91
3	1.00	9.0	50.0	5.31	1.00	0.48	0.89

Response surface methodology (RSM) is a statistical tool used for the modelling and optimization of multiple variables, which determines the optimum process conditions by combining experimental designs with interpolation by first or second order polynomial equations in a sequential testing procedures (Ferreira *et al.*, 2009). Central composite experimental design maximizes the amount of information that can be obtained while limiting the number of individual experiments (Kunamneni *et al.*, 2005). Thus smaller and less time consuming experimental designs could generally suffice for the optimization of many processes in very less time. The major objective of RSM is to determine the optimum region of the factor space in which operating specifications are satisfied.

Table 29. Software generated three optimum conditions of independent variables with the desirability values for optimum production of α-amylase from *B. sonorensis* GV2

Sr. No.	Substrate concentration	pH	Temperature	Inoculum size	Calcium ion	Magnesium ions	Desirability
1	**1.00**	**9.0**	**50.0**	**8.75**	**0.48**	**0.33**	**0.97**
2	1.00	9.0	50.0	8.78	0.49	0.61	0.96
3	1.00	9.0	50.0	5.31	1.00	0.48	0.94

An overall increase of 11.61% has been observed in amylase production by *B. aerius* GC6 where as an increase of 17.55% (Fig.14) has been observed in

case of *B. sonorensis* GV2 after the factors were optimized by using RSM. Similarly, process parameters which influence the enzyme activity significantly have been observed with an increase in the enzyme production by several workers. Tanyildizi *et al.* (2005) studied the combined effect of macronutrients of media viz. starch, glycerol, yeast extract and peptone on α-amylase production from *B. subtilis.* They found that among the variables screened, glycerol had the principal effect while yeast extract was completely non-significant. The optimal combinations of all the response variables were then determined. In agreement to our findings, among all the variables screened, Gangadharan *et al.* (2008) observed substrate concentration, incubation period and $CaCl_2$ concentration as the variables influencing amylase production by *B. amyloliquefaciens*. The optimum levels of the said variables were then determined by employing RSM. Present findings are also in accordance with the findings of Zambare (2011) in terms of the independent variables obtained. They optimized production of amylase from *Bacillus* sp. under submerged fermentation and used Response Surface Methodology (RSM) to evaluate the effect of the main variables i.e. pH, temperature and inoculum size on amylase production. The mutual interaction between these variables resulted into 4.64 fold increase in amylase activity as compared to the non-optimized environmental factors in the basal medium.

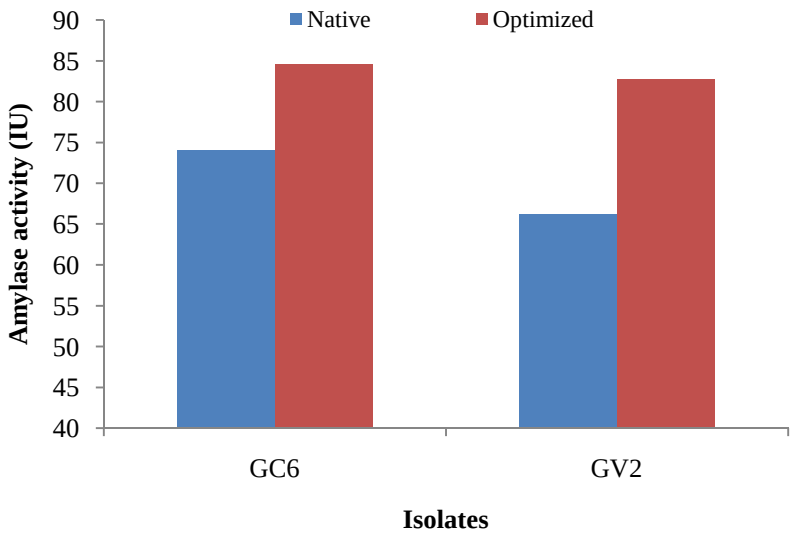


Fig. 14 Increase in α-amylase activity of *B. aerius* GC6 and *B. sonorensis* GV2 before and after optimization

4.3.3 SCALE UP OF α-AMYLASE OF *B. aerius* GC6 IN A STIRRED TANK BIOREACTOR

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully the selected microorganisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution. Due to the development of large-scale fermentation technologies, the production of microbial enzymes accounts for a significant proportion of the biotechnology industry total output. Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic metres.

α-amylase is among the most sought after commercially important hydrolytic enzymes. These are used in biofuel production, paper and pulp industry, textile and detergent industry and food and fermentation industry, etc. Among the selected isolates and after their enzyme optimization studies, *B. aerius* GC6 producing high titres of α-amylase was chosen for scale up in a 7.5 l stirred tank bioreactor having 3.0l working volume capacity. A maximum amylase activity of 90.26 IU has been observed at 8^{th} h of fermentation at 50^{o}C and pH 9.0.

4.3.3.1 Mathematical Modelling of *B. aerius* GC6 for Cell Growth

Most of the growth processes are explained in terms of Monod or logistic equations (unstructured models). Logistic equation is simple for calculation of fermentation parameters related to biological and geometrical significance using sigmoid profiles independent of substrate concentration (Garcia-Ochoa *et al.*, 1995). The sigmoid growth pattern of *Bacillus aerius* GC6 was analyzed using logistic equation for its variation against time during log phase. The idea of microbial growth kinetics has been dominated by an empirical model originally proposed by Monod (1942). The Monod model introduced the concept of a growth limiting substrate.

$\mu = \mu_{max}\, S/Ks+S$ (Batch growth)

$1/\mu = Ks/\mu_{max}.1/s + 1/\mu_{max}$

where μ = specific growth rate, μ_{max} = maximum specific growth rate, S = substrate concentration, Ks = substrate saturation constant (i.e. substrate concentration at half μ_{max}).

B. aerius GC6 showed a conventional growth pattern as shown in Fig. 15 during batch fermentation in which exponential phase lasted for 8h followed by stationary phase upto 10h. The production of amylase started from 2nd h reaching maxima at 8th h. Enzyme production increased rapidly during late exponential phase and early stationary phase, while it was slowed down during late stationary phase as shown in Fig. 15.

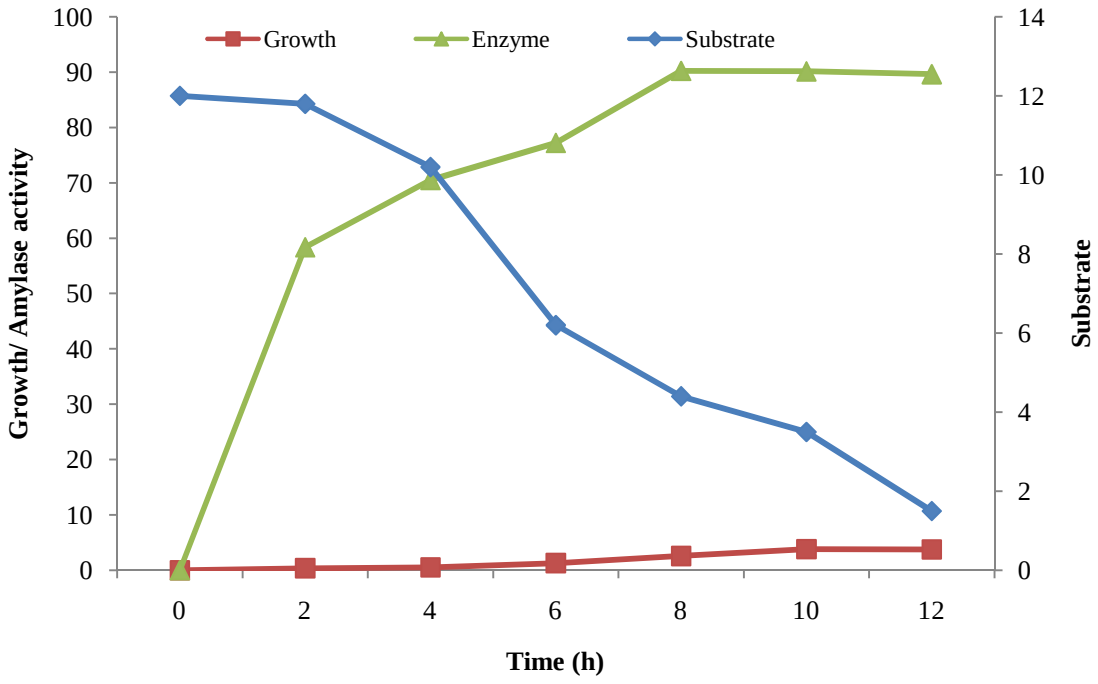


Fig. 15 Growth kinetics for enzyme scale-up for *B. aerius* GC6 in a stirred tank bioreactor

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters μmax and Ks. In addition

to this, Monod also related the yield coefficient ($Y_{x/s}$) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (Q)

$$Y_{x/s} = dx/ds$$

$$Y_{x/s} = X_m - X_0/S_0\text{-}S_m$$

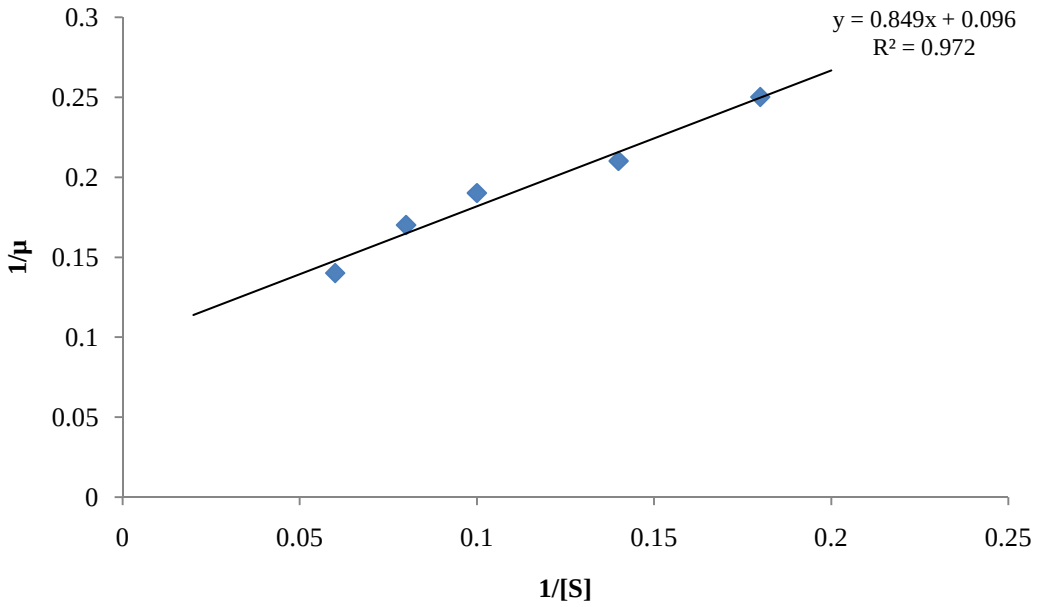


Fig. 16 Comparison between experimental results of specific growth rate (μ) and Substrate utilization [S] and model prediction

Model prediction for cell growth was followed with the experimental results with coefficient of determination, $R^2 = 0.972$ (Fig. 16), which revealed that this kinetic model is appropriate for predicting the experimental cell growth.

$Y_{x/s} = dx/ds$

$Y_{x/s} = 0.96$

$1/\mu_{max} = 0.06$

$\mu_{max} = 14.92\ min^{-1}$

Ks/ μmax = 0.0006 from slope

Ks = 0.00892 mg/ml

4.3.3.2 Kinetic Analysis for Amylase Production

The Luedeking-Piret equation (Luedeking and Piret, 1959) was applied for the kinetic modeling of α-amylase production. The Luedeking-Piret model

indicates that the rate of α-amylase production (Q_p) is linearly proportional to the *B. aerius* GC6 cell growth rate and cell biomass as shown in Fig. 17.

$$Q_p = \alpha\mu + \beta$$

where, α = growth associated constant and β non growth associated constant, both may vary under different fermentation conditions.

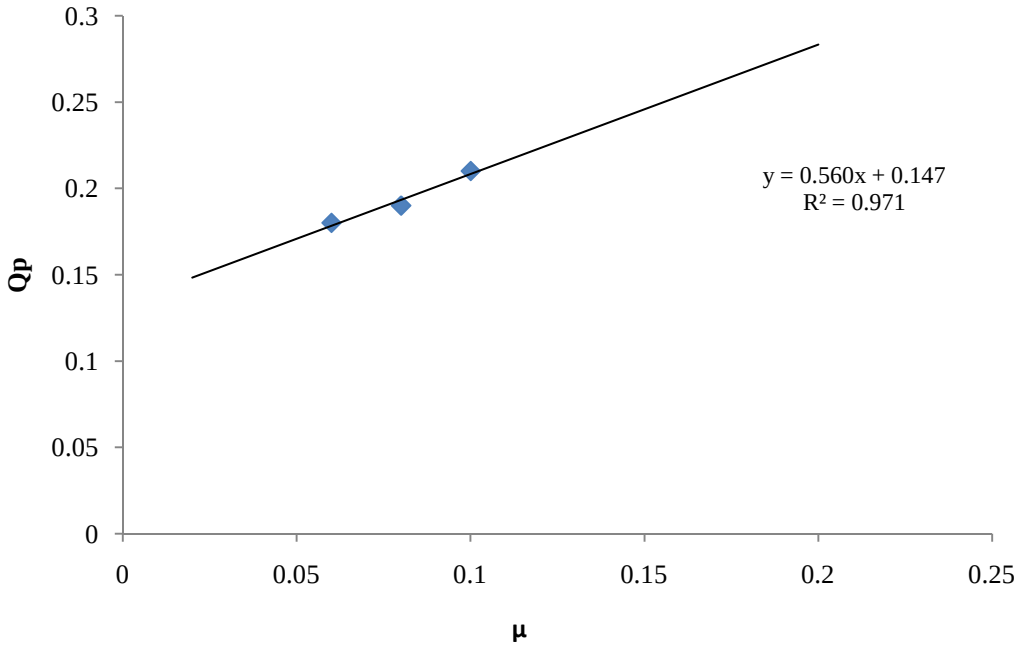


Fig. 17. Comparison between experimental results and model prediction for product formation

The comparison between model predictions and the experimental data of the product formation during batch fermentation is shown in Fig 17. Results indicate that this model is evidently very appropriate for describing the product formation rate (coefficient of determination, R^2 was 0.971).

α-amylase scale-up from *B. aerius* GC6 revealed that in a stirred tank bioreactor where all the process parameters can be maintained at their optimum levels an enhanced amylase activity of 96. IU has been observed after 8 h of fermentation. In comparison to shake flask conditions, an enhanced enzyme production at a considerable reduced fermentation time of 8 h makes the enzyme production very economical and efficient.

4.4 PURIFICATION OF α-AMYLASE FROM *B. aerius* GC6 and *B. sonorensis* GV2

The cell free supernatant of both the cultures was prepared in one litre respective culture media after 72 h of incubation at 50 ± 2°C applying the set of optimized conditions for enhanced amylase production. All the purification experiments were conducted at 4°C.

4.4.1 Ammonium sulphate fractionation

The cell free extracts of *B. aerius* GC6 and *B. sonorensis* GV2 were subjected to sequential ammonium sulphate saturations from 0-80%. *B. aerius* GC6 α-amylase got precipitated from 30-80% saturation of ammonium sulphate, with 33.56 mg protein and a specific activity of 2.75 IU/mg (Table 30). 1.38 fold purification of enzyme was achieved with 54.74% amylase yield after 80% ammonium sulphate fractionation.

Table 30. Purification profile of α-amylase from *B. aerius* GC6

Purification step	Total activity	Total protein	Specific activity	Purification fold	%Recovery/ Yield
Crude enzyme	844.97	42.56	1.98	1	100
Ammonium sulphate fractionation (30-80%)	462.55	33.564	2.75	1.38	54.74
Dialysis	413.84	31.24	3.31	1.67	48.97
Gel exclusion chromatography (Sephadex G-100)	382.44	12.47	10.22	15.16	45.26
Anion exchange chromatography (DEAE-cellulose)	225.78	3.19	35.38	17.86	26.72

*Total activity : Enzyme activity in given volume (IU)
**Total protein: mg/ml
***Specific activity : Enzyme activity per unit protein concentration (IU/mg)
****Purification fold: is increase in specific activity.
*****Percent recovery is remaining protein concentration as % of the initial protein concentration

In literature also, advanced purification steps followed by ammonium sulphate precipitation has been reported by several workers. In agreement to our results, majority of them reported 80% saturation as optimum for efficient precipitation of α-amylase. 80% saturation of ammonium sulphate has been adjudged as best for precipitation of *B. subtilis* α-amylase by Hamza (2004).

Gangadharan *et al.* (2008) reported 30-90% saturation as optimum for salting out maximum amylase isolated form *B. amyloliquefaciens.* Shafiei *et al.* (2010), observed 80% ammonium sulphate saturation as best for precipitation of α-amylase from a moderate halophile. Lily *et al.* (2012) also reported 80% saturation as best for α-amylase form *B. subtilis* MTCC 9447. *B. methylotrophicus* P11-2 α-amylase was efficiently precipitated at 80% concentration of ammonium sulphate with purification fold of 2.3 by Xie *et al.* (2014).

B. sonorensis GV2 α-amylase on the other hand got precipitated from 20-70% saturation of ammonium sulphate, with 27.63 mg protein and a specific activity of 3.21 IU/mg (Table 31) and 1.47-fold purification of enzyme was achieved with 53.73% amylase yield after 70% ammonium sulphate fractionation. Najafi *et al.* (2005) and Fincan *et al.* (2014) have reported 70% saturation as optimum for precipitation of extracellular α-amylase form *B. subtilis* AX20 and *B. subtilis* respectively. In contrary to our findings, 40-60% saturation has been adjudged as best for precipitating α-amylase from *B. licheniformis* by Hmidet *et al.* (2008).

Table 31. Purification profile of α-amylase from *B. sonorensis* GV2

Purification step	Total Activity	Total protein	Specific activity	Purification fold	%Recovery/ Yield
Crude enzyme	827.70	37.64	2.18	1	100
Ammonium sulphate fractionation (20-70%)	444.75	27.63	3.21	1.47	53.73
Dialysis	371.96	21.23	4.37	2.00	44.93
Gel exclusion chromatography (Sephadex G-75)	298.38	4.24	25.05	11.46	36.04
Anion exchange chromatography (DEAE-cellulose)	213.52	3.37	31.67	14.52	25.79

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same as given in Table 30

For *B. aerius* GC6, below 30% and above 80% salt saturation, amylase activity was found considerably reduced. Similarly, for *B. sonorensis* GV2, below 20% and above 70% saturation of ammonium sulphate, amylase activity was minimally low. This showed that the protein forming amylase enzymes were rich in hydrophobic amino acids. Based on these results, a respective ammonium sulphate saturation of 30-80% and 20-70% was applied to precipitate α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 for further experimentation. Protein samples between the respective cuts for *B. aerius* GC6 and *B. sonorensis* GV2 were collected, pooled and dialyzed for further concentration.

4.4.2 Dialysis

Ammonium sulphate salted out protein precipitates of *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase were dialyzed against 20 mM Tris HCl (pH-8.0) buffer overnight at 4°C using dialysis membrane of cut-off 14 kDa. Dialysis of a protein sample involves the removal of excess salts thereby concentrating the protein. Literature survey also reveals the significance of dialysis in purification of proteins. Several workers have dialysed their samples before loading onto the column for size exclusion chromatography (Li *et al.*, 2007; Gangadharan *et al.*, 2008; Michelin *et al.*, 2010; Kolawole *et al.*, 2011; Lily *et al.*, 2012; Chavan and Wadatkar *et al.*, 2014).

Dialyzed *B. aerius* GC6 α-amylase had a protein content of 31.24 mg and a specific activity of 3.31 IU/mg protein with 1.67 fold purification of enzyme was achieved with 48.97% amylase yield (Table 30). Similarly, dialyzed *B. sonorensis* GV2 α-amylase has a protein content of 21.23 mg protein and a specific activity of 4.37 IU/mg and 2 fold purification of α-amylase was achieved with 44.93% amylase yield (Table 31).

4.4.3 Gel filtration chromatography

Chromatographic techniques are the most reliable and easy to handle techniques which are widely applied for protein separation and purification. Gel chromatographic technique also known as size exclusion chromatography is a technique for the separation of molecules on the basis of their size and shape

exploiting the molecular sieving properties of a variety of porous materials. This is the most frequently used technique for purification of biological macromolecules by facilitating their separation from larger and smaller molecules in the supernatant (Bollag and Edelstein, 1991a; Cutler, 2004; Wilson, 2010).

The enzyme rich samples obtained after dialysis were applied onto Sephadex G-75 (1.5 x 40 cm) column for *B. aerius* GC6 and Sephadex G-100 (1.5 x 40 cm) column for *B. sonorensis* GV2 for gel exclusion chromatography pre-equilibrated with 20mM Tris HCl buffer (pH-8.0). 60 fractions of 2 ml volume each were collected with a flow rate of 2 ml/3.5min. Most of the low expressed background proteins were removed but the major contributing proteins responsible for starch hydrolysis, incurred in amylase rich fractions as revealed by the optical density of the collected fractions. Fraction number 16-22 for *B. aerius* GC6 (Fig. 18) and fraction number 22-28 for *B. sonorensis* GV2 exhibited amylase activity (Fig. 19). Maximum specific amylase activity for *B. aerius* GC6 was observed in 20^{th} fraction where as for *B. sonorensis* GV2 it was observed in 26^{th} fraction as depicted by amylase activity values. Gel chromatographic separation resulted in 45.26 and 36.04% yield and 15.16 and 11.46 fold purification for *B. aerius* GC6 (Table 30) and *B. sonorensis* GV2 respectively (Table 31). Most active fractions for both the organisms were pooled and utilized for anion exchange chromatography.

A survey of literature reveals the extensive use of gel exclusion chromatography for purifying enzymes. Rao and Satyanarayana (2007) reported purification of amylase obtained from extreme thermophile *Geobacillus thermoleovorans* with 54.8 fold purification by Sephadex G-100. Hmidet *et al.* (2008) performed gel filtration chromatography on Sephadex G-100 column yielding 2.21 fold purified enzyme isolated from *B. licheniformis* NH1 with 36.62% recovery. α-amylase from *B. subtilis* AX20 was purified to homogeneity with 8.8 fold purification by Najafi *et al.* (2007). Michelin *et al.* (2010) reported 8.4 fold purification and 9.2% recovery of the enzyme after Sephadex G-100 gel filtration chromatography isolated from a fungus *Paecilomyces variotii*. Xie *et al.* (2014) purified α-amylase from *B. methyloitrophicus* P11-2 strain by Sephadex gel filtration column to 9.0 fold homogeneity.

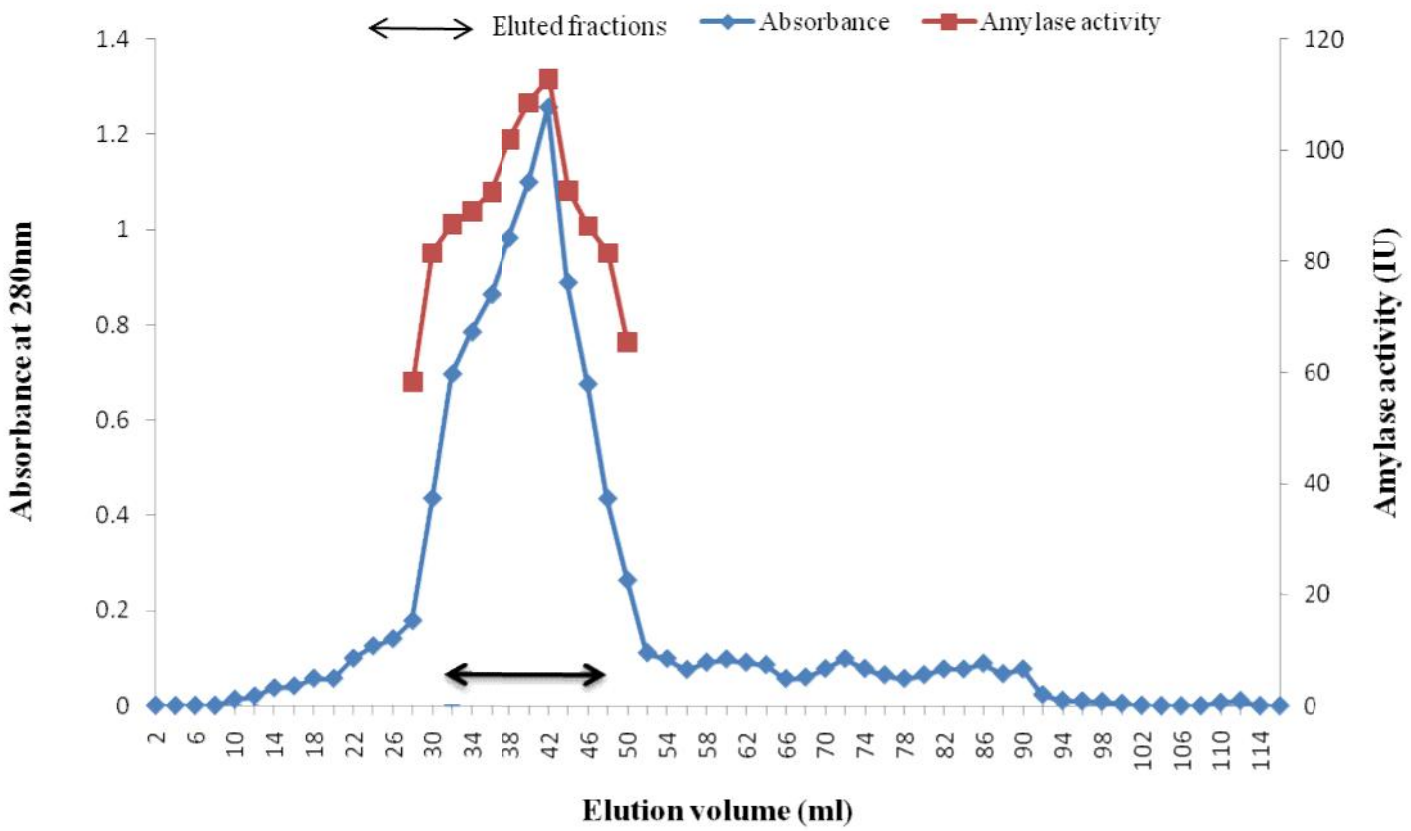


Fig. 18 Protein and enzyme activity profile of fractions of Sephadex G-100 column chromatography of the dialyzed α-amylase of *B. aerius* GC6

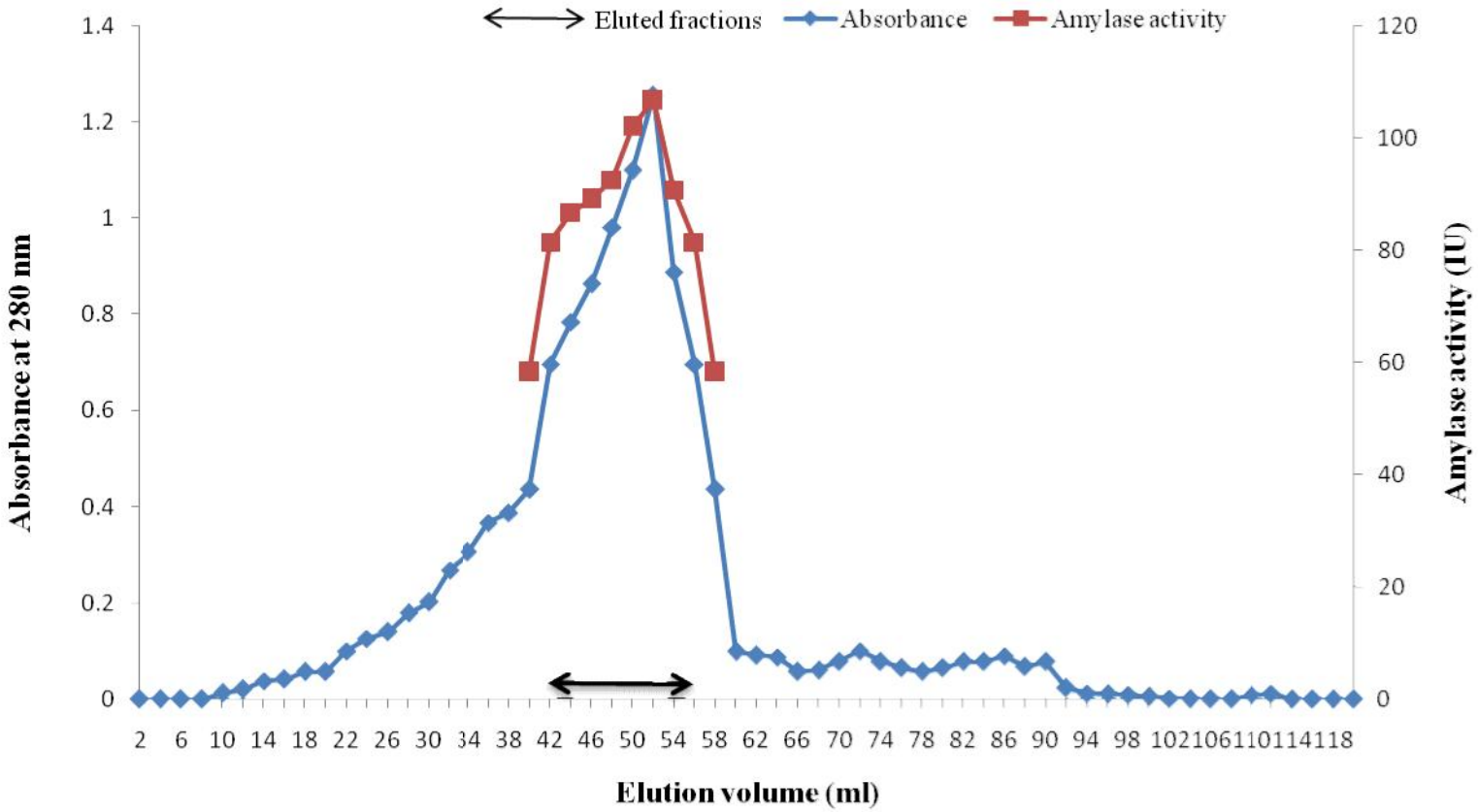


Fig. 19 Protein and enzyme activity profile of fractions of Sephadex G-75 column chromatography of the dialyzed α-amylase of *B. sonorensis* GV2

4.4.4 Anion exchange chromatography

Anion exchange chromatography relies upon the attraction between the oppositely charged stationary phase and analyte. It is widely utilized for protein purification due to its high resolving power and high efficiency (Bollag and Edelstein, 1991b; Cutler, 2004; Wilson, 2010). The pooled sample from Sephadex G-100 and G-75 column were applied onto DEAE-cellulose anion exchange column. The protein profile in terms of optical density and amylase activity has been shown in Fig. 20 and 21 respectively, for *B. aerius* GC6 and *B. sonorensis* GV2. Gradient elution has been reported to be more efficient as compared to isocratic elution. Hence, the amylase active fractions in both the organisms were eluted with 0.3 M to 0.5M potassium chloride gradient.

Fraction number 7-11 for *B. aerius* GC6 and fraction number 4-8 for *B. sonorensis* GV2 exhibiting highest α-amylase activity were pooled for further characterization studies. Anion exchange chromatography of *B. aerius* GC6 α-amylase resulted in 26.72% yield (Table 30) and 17.86 fold purification with a raised specific activity of 35.38 IU/mg whereas for *B. sonorensis* GV2 it resulted in 25.79% yield (Table 31) and 14.52 fold purification with a specific activity of 31.67 IU/mg.

A multistep approach has been successfully exploited in the present study for the purification of α-amylase from both the strains. *B. aerius* GC6 α-amylase have been purified to homogeneity with a final purification fold of 17.86 and 26.72% yield. An increase in α-amylase activity from 84.49 IU to 112.89 IU has been attained with a final specific activity of 35.38 IU/mg protein. Similarly, *B. sonorensis* GV2 α-amylase has also been purified to homogeneity with a final purification fold of 14.52 and 25.79% yield. An increase in α-amylase activity from 82.77 IU to 106.76 IU has been observed with a final specific activity of 31.67 IU/mg protein.

Al-ZaZaee *et al.* (2011) purified α-amylase from *B. cereus* to 27 fold purification by anion exchange chromatography. α-amylase from *B. licheniformis* JAR-26 isolated from spoiled tomato was purified by Jyoti *et al.* (2011) by applying DEAE cellulose anion exchange chromatography with a purification

fold of 76.41. Bai *et al.* (2012) performed ion exchange chromatography to purify α-amylase isolated from *Alicyclobacillus* sp. A4 with yield of 21.8%. Fincan *et al.* (2014) purified α-amylase from a thermophile *Anoxybacillus flavithermus* to 5.2 fold homogeneity by applying DEAE- cellulose anion exchange chromatography.

In literature, several reports are available regarding the multi-step purification of amylase to homogeneity. Rao and Satyanarayana (2007) purified α-amylase from *Geobacillus thermoleovorans* by following a five step approach to a purification fold of 54.8. Gangadharan *et al.* (2008) utilized a three step approach for purification of *B. amyloliquefaciens* α-amylase to homogeneity with a purification fold of 38.46. *Nesterenkonia* sp. strain F produced a novel SDS stable α-amylase, which was purified to homogeneity by Shafiei *et al.* (2010) by a three step purification technique with a 10.8-fold increase in specific activity. Xie *et al.* (2014) utilized a four step regime to a purify α-amylase from *B. methylotrophicus* P11-2 to a purification fold of 13.

4.4.5 Molecular weight determination of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

The purity and molecular weight of the purified α-amylase of *B. aerius* GC6 and *B. sonorensis* GV2 was determined on SDS-PAGE. Purified α-amylase of both the organisms exhibited single band with only one sub unit. The molecular weight of purified enzyme was calculated according to the RF values of the standards used in the protein molecular weight marker. Most of the microbial α-amylases have a molecular weight in the range of 50-60 kDa (Gupta *et al.*, 2003). The molecular mass of denatured α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 estimated from the relative mobility of proteins on SDS-PAGE was~43 kDa (Fig. 22) and~45 kDa (Fig. 23) respectively.

4.4.6 Zymogram analysis

Zymogram of α-amylase showed a prominent activity band against blue background corresponding to 43 kDa for *B. aerius* GC6 (Fig. 22) α-amylase and 45 kDa for *B. sonorensis* GV2 (Fig. 23) α-amylase. The alternative method utilized for zymography i.e. the portion of gel cut and plated onto the starch agar

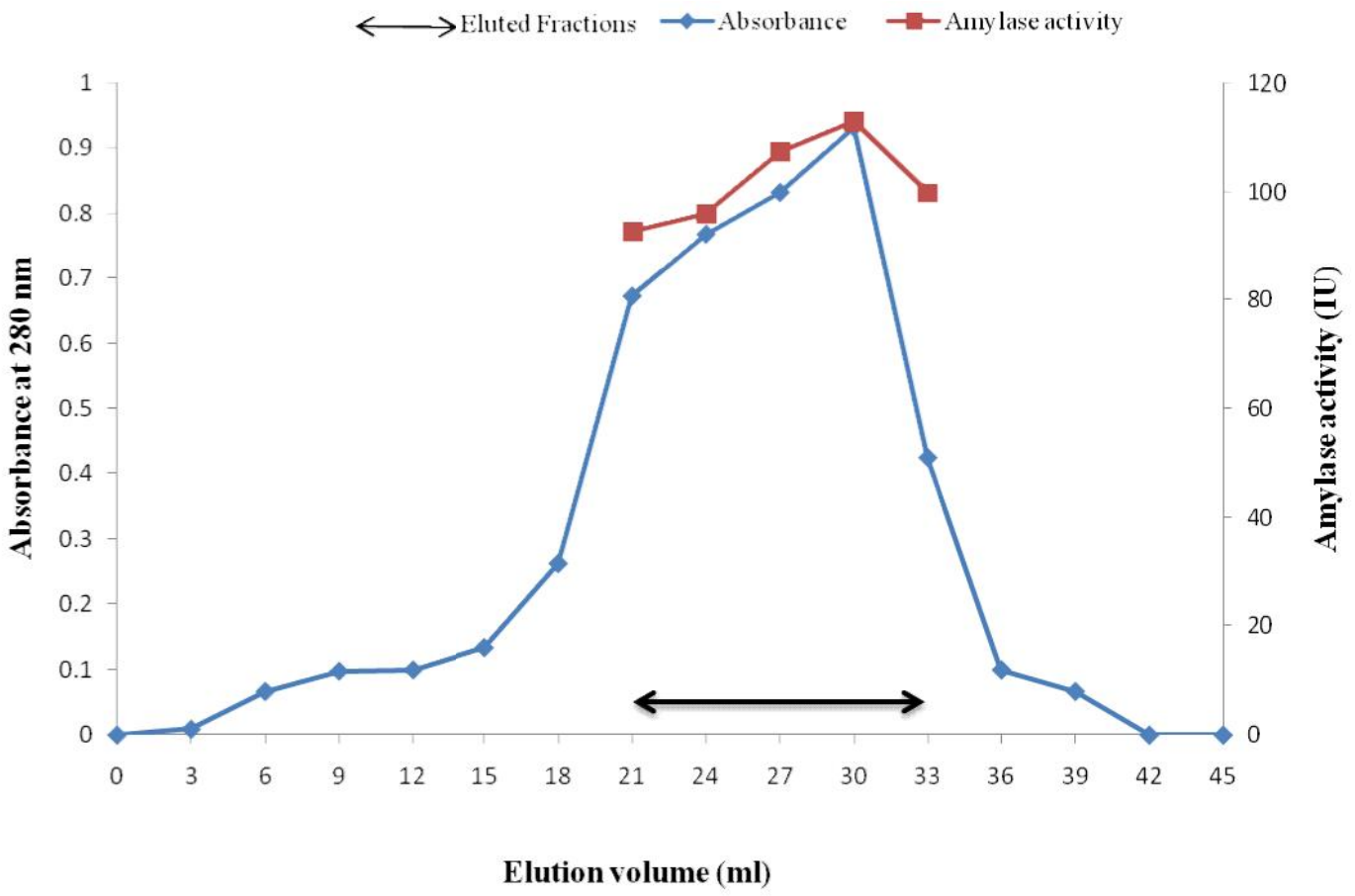


Fig. 20 Protein and enzyme activity profile of fractions of DEAE-cellulose anion exchange chromatography of the gel chromatographed α-amylase of *B.aerius* GC6

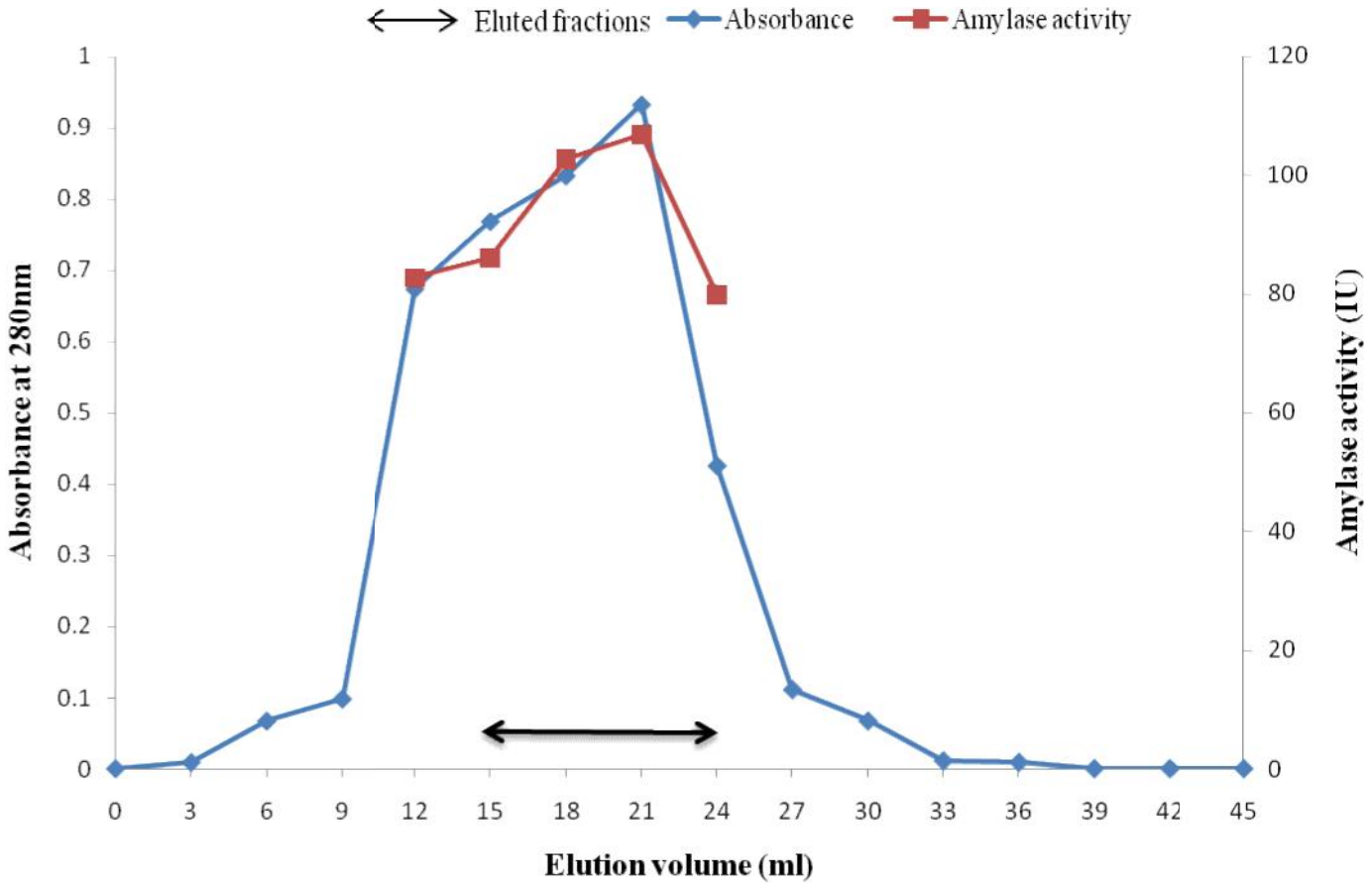


Fig. 21 Protein and enzyme activity profile of fractions of DEAE-cellulose anion exchange chromatography of the gel chromatographed α-amylase of *B. sonorensis* GV2

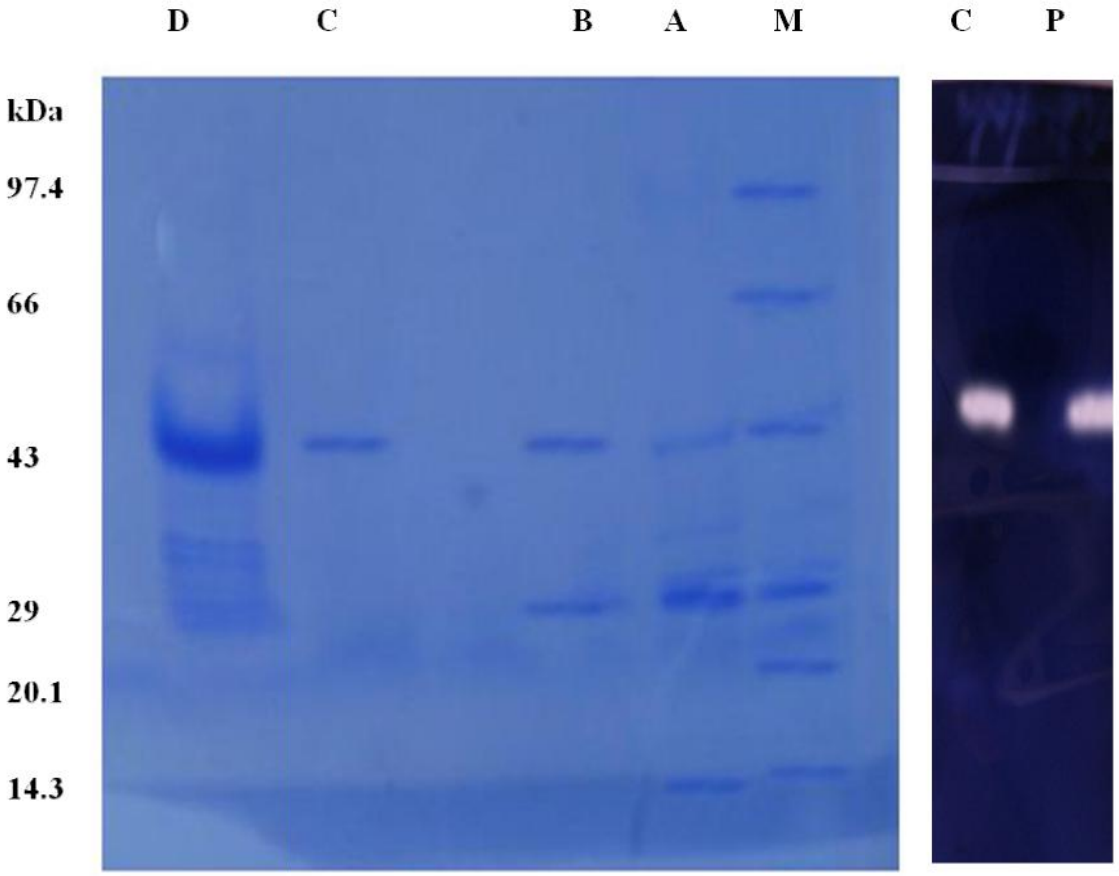


Fig. 22 (a) SDS-PAGE of *B. aerius* GC6 α- amylase at various stages of purification and (b) zymogram of crude and purified amylase

M- medium range molecular weight marker

Phosphorylase B	(97.4 kDa)
Bovine serum albumin	(66 kDa)
Ovalbumin	(43 kDa)
Carbonic anhydrase	(29 kDa)
Soyabean trypsin inhibitor	(20 kDa)
Lysozyme	(14.3 kDa)

Lane A: Ammonium sulphate fractionated enzyme
Lane B: Sephadex G-100 column chromatographey pooled fractions
Lane C: DEAE Cellulase anion exchange chromatography pooled fractions
Lane D: Crude enzyme

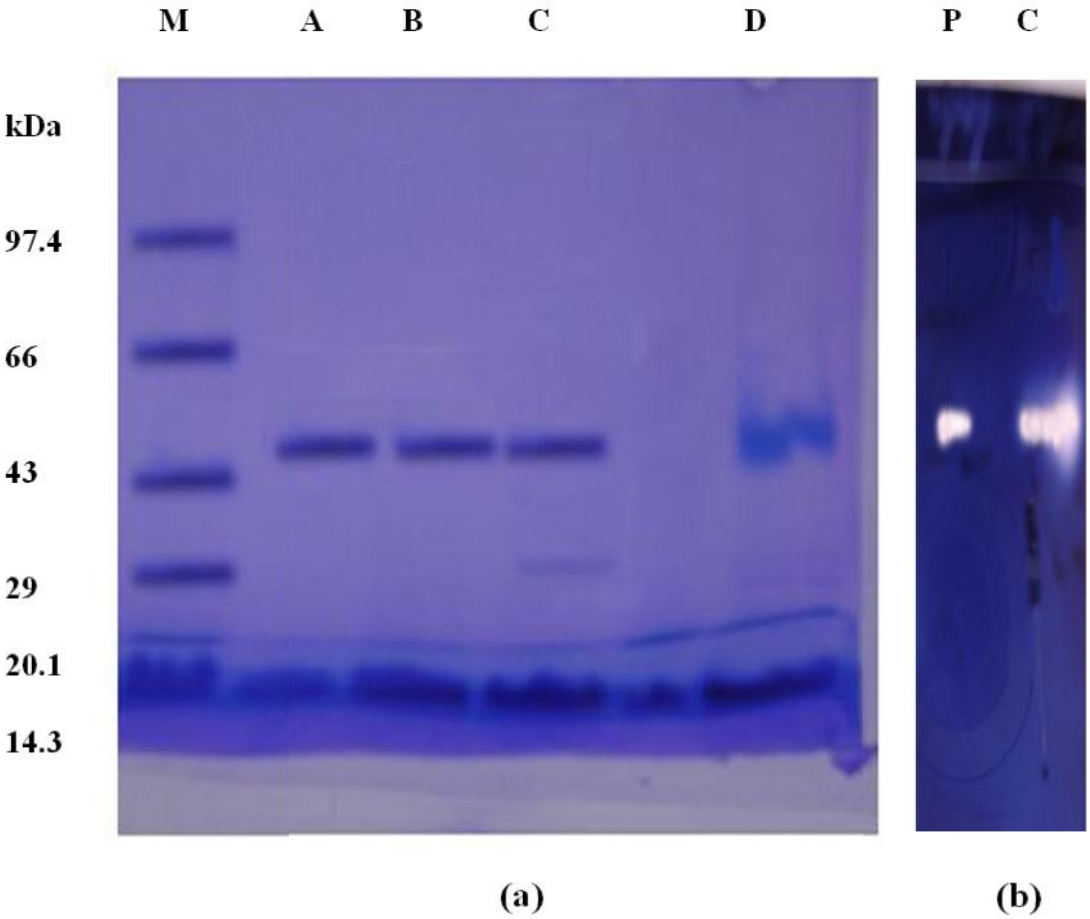


Fig. 23 (a) SDS-PAGE of *B. sonorensis* GV2 α- amylase at various stages of purification. (b) zymogram of purified and crude amylase

M- medium range molecular weight marker

Phosphorylase B	(97.4 kDa)
Bovine serum albumin	(66 kDa)
Ovalbumin	(43 kDa)
Carbonic anhydrase	(29 kDa)
Soyabean trypsin inhibitor	(20 kDa)
Lysozyme	(14.3 kDa)

Lane A: DEAE Cellulase anion exchange chromatographed enzyme
Lane B: Sephadex column chromatographed enzyme
Lane C: Ammonium sulphate fractionated enzyme
Lane D: Crude enzyme

plate also produced a clear zone of hydrolysis around the band confirming the presence of starch hydrolysing α-amylase. Iodine interacts strongly with the polysaccharide containing α (1-4) and α (1-6) linked reducing sugar units. The resulting dye-glucan complexes are intensely coloured making them very sensitive for detection of such polysaccharides. Zymogram activity of α-amylase represented by a clear zone among the blue background for *B. aerius* GC6 (Plate 4a) and *B. sonorensis* GV2 (Plate 4b). Several workers (Burgess-Cassler and Imam, 1991; Arikan, 2008) have reported similar pattern of zymogram analysis on the non-reducing gels.

4.5 CHARACTERIZATION OF PURIFIED *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Although the stability of enzyme is its intrinsic property dictated by its primary structure, many external factors such as physical factors and chemical reagents influence the overall stability and thus the activity of enzyme. Keeping in view these facts, effect of various physical and chemical factors viz. pH, temperature, divalent ions, surfactants, organic solvents, substrate, substrate concentration etc. were evaluated on the behaviour of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase.

4.5.1 Effect of pH on activity and stability

Enzymes being proteins are sensitive to changes in the environment in which they work. Any change in hydrogen ion concentration (pH) can profoundly affect the activity of an enzyme. This is very important in the industrial use of enzymes to be active at varied pH levels. The pH must be controlled and industrial processes usually try to get the maximum rates of activity by choosing the appropriate pH at which the enzyme is active but is not denatured.

pH optimum for α-amylase activity was explored at a constant temperature of 50^{o}C with a substrate concentration of 1.75% for *B. aerius* GC6 and 1.0% for *B. sonorensis* GV2. Purified *B. aerius* GC6 α-amylase was revealed to have a broad optimal pH range (7.0-10.0) with maximum activity at pH 9.0 (Fig. 24). More than 85% of the maximum observed activity was achieved at pH 9.0 and 10.0, proving alkaline nature of α-amylase. Below pH 7.0 and above

10.0, amylase activity decreased considerably. For *B. sonorensis* GV2, the optimal pH ranged from pH 7.0-11.0, with maximum activity at pH 10.0 (Fig. 25). More than 75% of the maximum observed activity was achieved at pH 8.0, 9.0 and 10.0. At higher pH of 11.0, 42.36% of the original activity was retained.

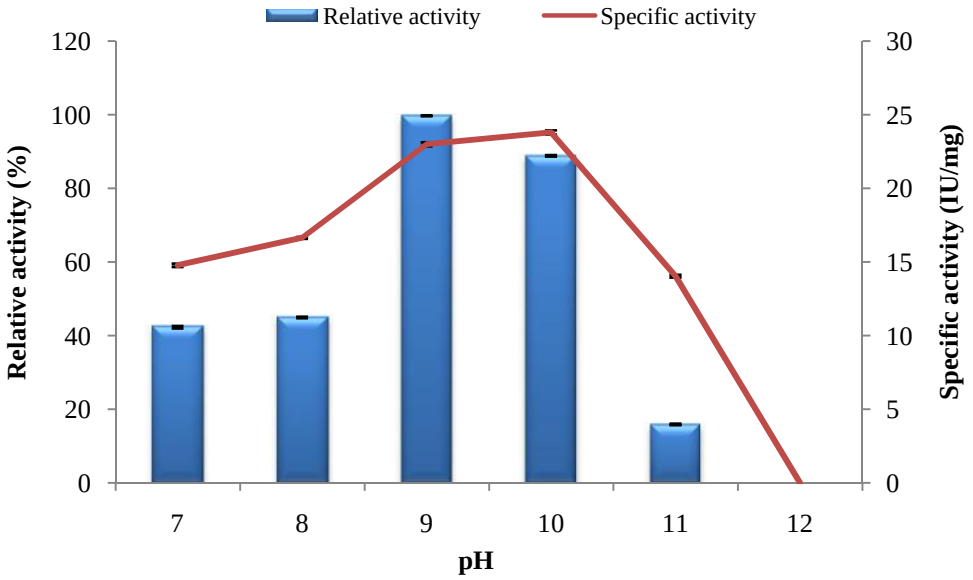
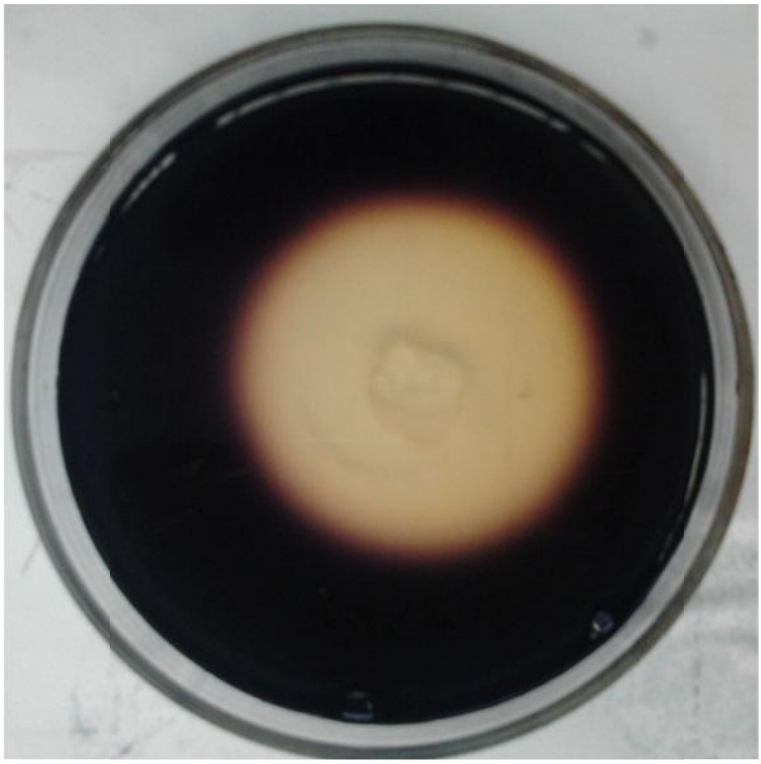
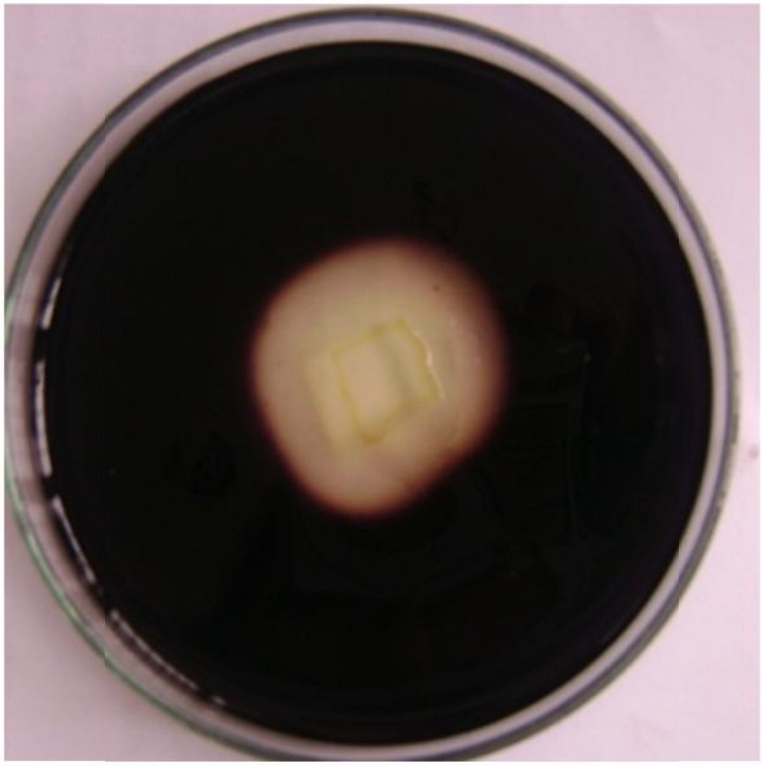


Fig. 24 Effect of pH on purified α-amylase of *B. aerius* GC6

Most bacterial amylases are active at slightly acidic to near neutral pH (Collins *et al.*, 1993). In accordance to this statement, acidic to neutral α-amylase finding great applications in food and baking industries from fungi and bacteria has been reported in vast majority in the literature (Pandey *et al.*, 2000a; Gupta *et al.*, 2003; Goyal *et al.*, 2005; Gangadharan *et al.*, 2008; Michelin *et al.*, 2010; Singh *et al.*, 2014). However, there are a very few reports on alkaline amylase giving activity at pH 9.0 and 10.0. Hmidet *et al.* (2008) reported α-amylase from *B. licheniformis* NH1 which was highly active in the wide pH range of 5.0-10.0, with maximum activity at pH 9.0. α-amylase isolated from *Bacillus* sp. TSCVKK showed stability in the pH range spanning 6.0-9.5 with maximum amylase activity at pH 7.5 (Kiran and Chandra, 2008). Similarly, Roy *et al.* (2014) reported a *B. megaterium* Strain KAN1 showing elevated amylase activity at pH



B. aerius **GC6**



B. sonorensis **GV2**

Plate 4 Zymogram analysis of purified α-amylase of *B. aerius* GC6 and *B. sonorensis* GV2 on starch agar plates with gel cut from Native PAGE showing zone of hydrolysis

11.0. In this regard, alkaline α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 is a potent candidate in industry.

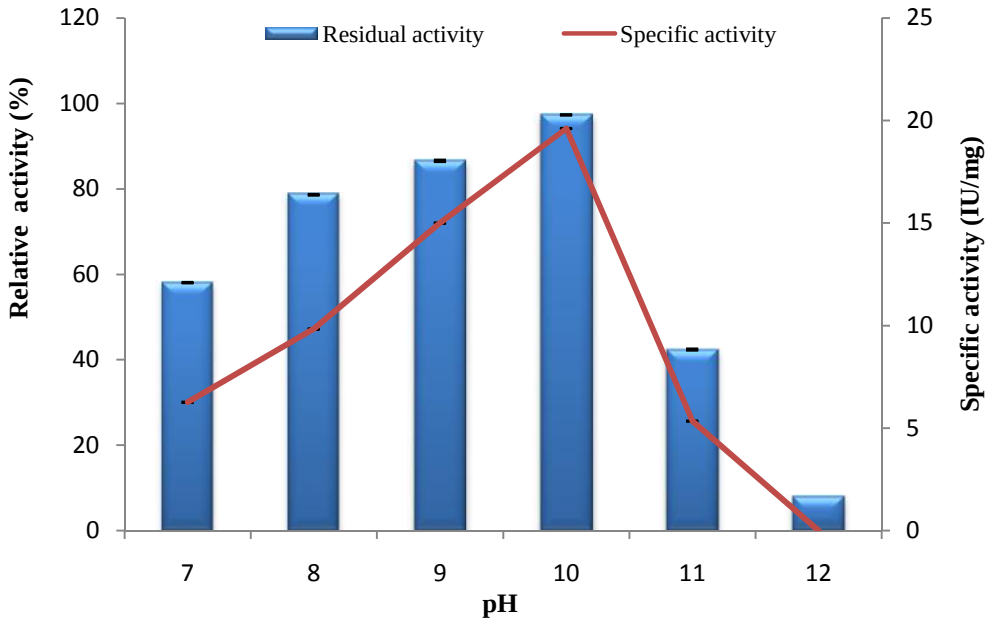


Fig. 25 Effect of pH on purified α-amylase of *B. sonorensis* GV2

Many industrial processes involving enzymatic processes do not have an adequate pH control and thus the pH usually fluctuates around the required value. Thus it is important to determine the pH activity curve in quantitative detail for a particular substrate enzyme system under consideration. In such conditions, an enzyme with a wider pH stability range is sought after. The wide pH stability range for *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase spanning 7.0-11.0 makes them a promising candidate in paper, detergent, textile and biofuel industries requiring near neutral to alkaline pH. It has been documented that 90% of all liquid detergents contain α-amylases (Dehkordi and Javan, 2013).

4.5.2. Effect of temperature on activity of purified α-amylase of *B. aerius* GC6 and *B. sonorensis* GV2

The effects of temperature on the activity of an enzyme are complex and can be considered as two forces acting simultaneously but in opposite directions. As the temperature is raised, the rate increases, but at the same time there is a

progressive inactivation (denaturation) of the enzyme protein. This becomes more pronounced as the temperature increases, so that an apparent temperature optimum is observed.

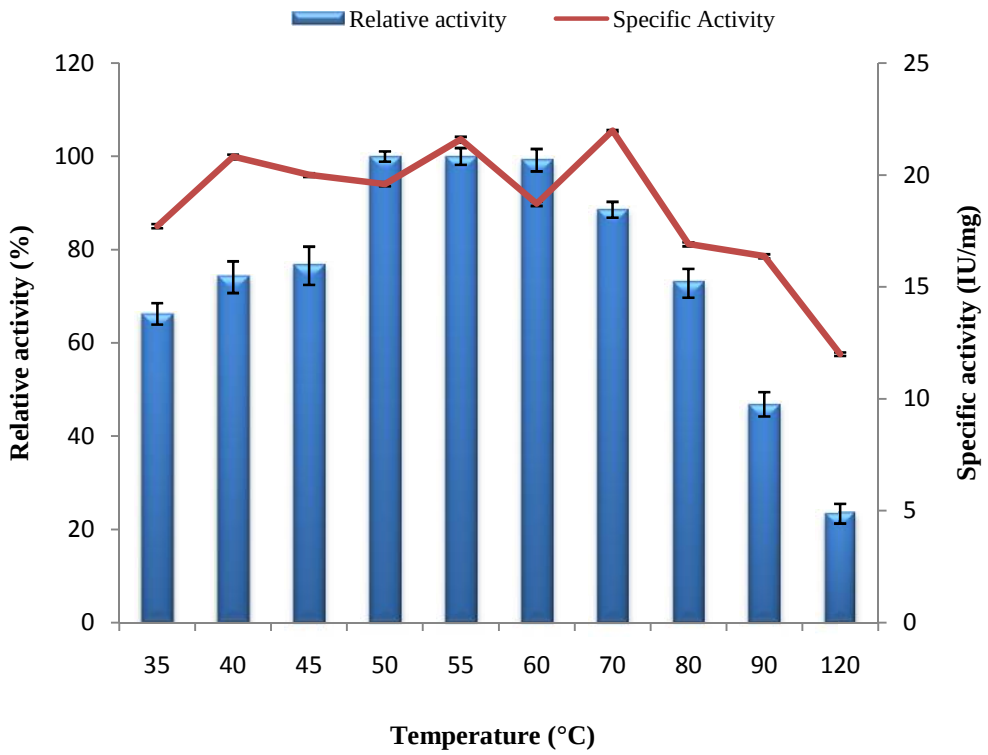


Fig. 26 Effect of temperature on purified α-amylase of *B. aerius* GC6

Behaviour of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 at varying temperatures ranging from 35-121°C was explored. Enzyme activity showed a clear increase from 35°C to 60°C for *B. aerius* GC6 (Fig. 26) and 35 to 50°C for *B. sonorensis* GV2 (Fig. 27). Highest enzyme activity was observed at 50°C for both the isolates. Optimum α-amylase production at 50°C is a characteristic of moderately thermostable microorganisms (Tonkova, 2006) and has been reported in literature by several workers. Demirkan *et al.* (2011) reported an amylase from *B. amyloliquefaciens* with 70% relative activity at 50°C. α-amylase from *Aspergillus oryzae* S2 has been shown to exhibit maximum activity at 50°C with a retention of 70% activity between 40-55°C (Sahnoun *et al.*, 2012). *B. subtilis* α-amylase purified by Sani *et al.* (2014) exhibited maximum activity at 55°C.

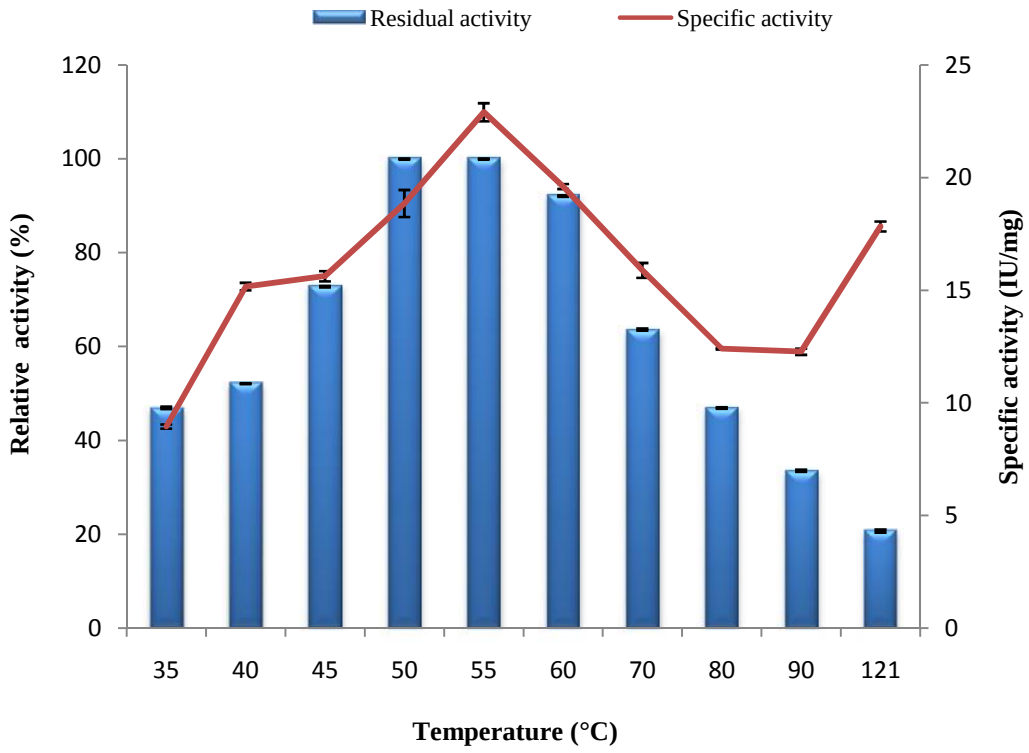


Fig. 27 Effect of temperature on purified α-amylase of *B. sonorensis* GV2

Beyond 50°C a sudden decrease has been documented in the activity of enzyme. However, α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 showed a wide range of temperature tolerance with 96% and 92% activity at 60°C respectively and 74% and 63% respective activity at a temperature of 70°C. In consonance with these results, Bai *et al.* (2012) observed retention of >95% of initial activity at 75^{o}C but a complete loss in activity was observed at 80^{o}C. However, α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 have shown considerable retention of activity up to 90^{o}C. Thermostability of purified α-amylase from both the isolates is thus a striking feature which makes this enzyme a potential candidate to be used in industry. The enzyme was even active at higher temperature of 121°C with a relative activity approximately 20%.

Thermostable *a*-amylases are generally preferred in industry as their application minimizes contamination risk and reduces reaction time, thus providing considerable energy saving (Dehkordi and Javan, 2013). The major utility of α-amylases is in the starch industry for the liquefaction process that

converts starch into fructose and glucose syrups. This process requires the use of a highly thermostable α-amylase which can act at temperatures around 70-100^{o}C.

4.5.3 Thermostability of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2

Enzymes that are stable at high temperatures may often be more resistant to organic solvents or extremes of pH and there is considerable interest in thermostable enzymes. By allowing processes to occur at higher temperature than normal, reaction rates are elevated and the risk of microbial contamination is reduced.

Thermostability is one of the crucial properties of enzymes for industrial applications. Thermostable α-amylases which are stable and active at high temperature and can withstand organic solvent, detergent, low and high pH and other denaturing agents (Gupta and Khare, 2006) are highly valued in the industries for their commercial exploitation. According to the data which have been published in the last 20 years, amylases can act in the temperature range of 40-100°C (Koch *et al.*, 1991; Chung *et al.*, 1995; Lee *et al.*, 1996; Kwak *et al.*, 1998; Haki and Rakshit, 2003; Ramachandran *et al.*, 2004; Thippeswamy *et al.*, 2006; Tiwari *et al.*, 2014).

4.5.3.1 Thermal inactivation parameters

Thermostability studies were carried out by pre incubating purified α-amylase for 180 min at various temperatures ranging from 35°C to 121°C. Thermal stability of the enzyme was determined by studying the time dependent inactivation of enzyme at temperatures it was subjected to. As shown in Fig. 28, purified *B. aerius* GC6 α-amylase was stable at a range spanning 50-70^{o}C with retention of approximately 50% of the maximum activity for 60 min; however at 80°C, 46% relative activity for 30 min was observed. Activity retention of upto 50% was observed at higher temperatures of 90 and 121°C for 15 min. A similar trend was observed for *B. sonorensis* GV2 α-amylase, which showed 50% retention in amylase activity from 50–70^{o}C (Fig. 29) for 60 min. At 80°C, 50% relative activity for 30 min was observed. Activity retention of upto 50% was observed at higher temperatures of 90 and 121°C for 15 min.

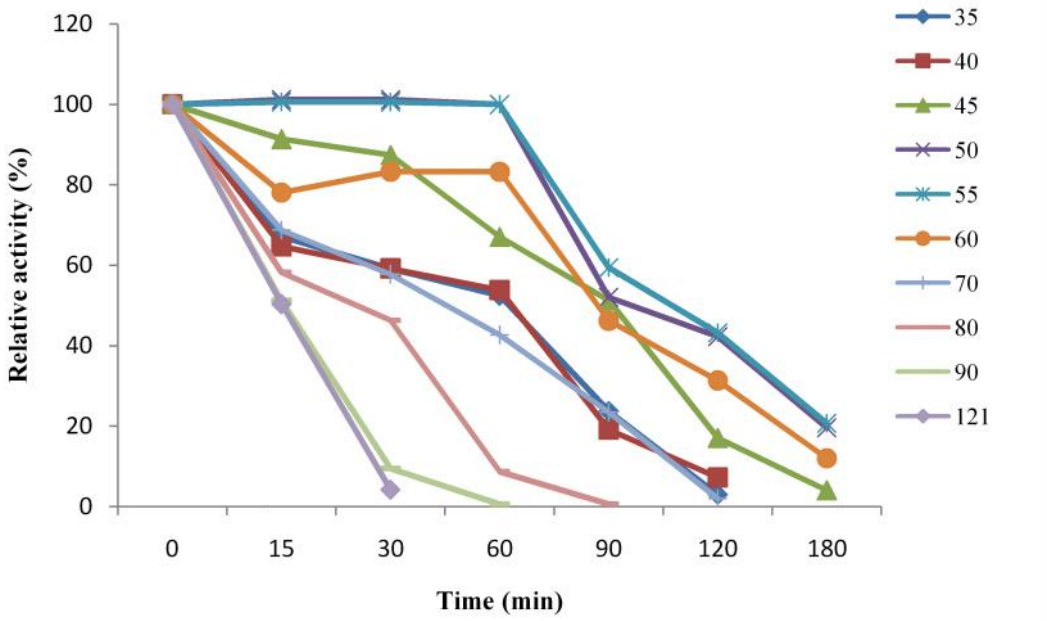


Fig. 28 Thermostability profile of purified α-amylase of *B. aerius* GC6

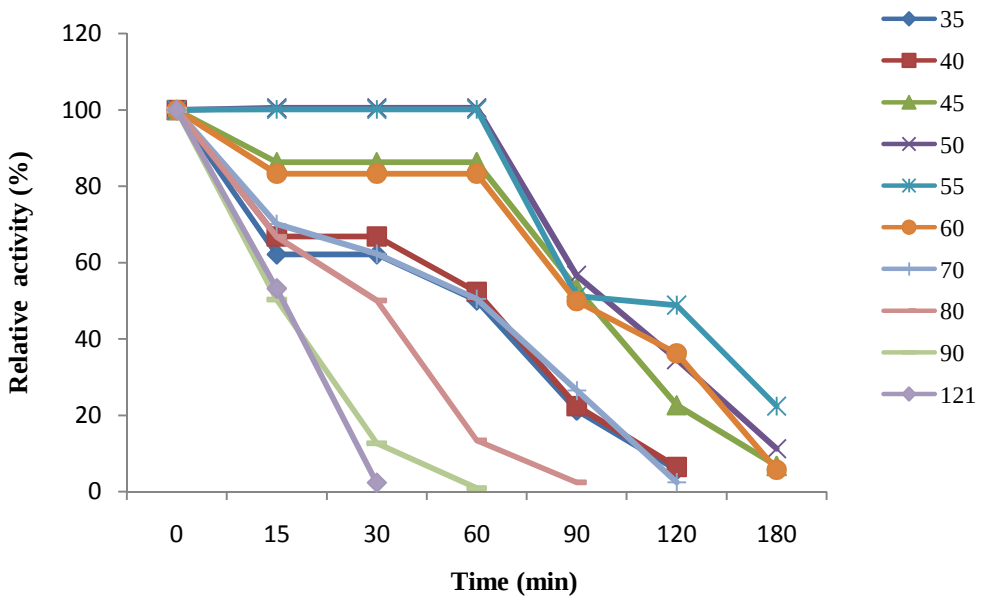


Fig. 29 Thermostability profile of purified α-amylase of *B. sonorensis* GV2

Jyoti *et al.* (2011) reported retention of 55% activity at 100°C for 30 min from α-amylase isolated from *B. licheniformis* JAR-26. Abdel-Fattah *et al.* (2013) purified *B. licheniformis* AI20 α-amylase. Thermal stability studies showed that purified enzyme retained 75, 50 and 20% of its activity at 70, 75, and

80^{o}C. Fincan *et al.* (2014) studied thermal stability of α-amylase purified from *Anoxybacillus flavithermus* and found that the enzyme was stable from 40-45^{o}C for 120 min however, at 50^{o}C; up to 45 min a reduction in activity up to 50% was observed. In conformity to these findings, *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase were found to be quite stable at comparatively wider range of temperature, thereby proving its applicability in industrial processes being carried out at higher temperatures.

4.5.3.2 Half-life ($t_{½}$)

Half-life ($t_{½}$) of the enzyme is defined as the time it takes for the activity to reduce to a half of the original activity. Half-life of purified α-amylase at a temperature of 45-60°C was 90 min while it was 60 min at 70°C. Enzyme activity reduced to its half in just 15 min at higher temperatures of 90 and 121°C for both *B. aerius* GC6 (Fig. 28) and *B. sonorensis* GV2 (Fig. 29). Survey of literature of the half-life studied revealed that Gangadharan *et al.* (2008) in their report, have shown a 50% reduction in enzyme activity purified from *B. amyloliquefaciens* at a temperature of 50^{o}C after 30 min. α-amylase purified from *Alicyclobacillus* sp. by Bai *et al.* (2012) showed a retention of 50% of activity for 10 min at 70^{o}C. Considering these studies, purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 showed a good retention of activities having considerably higher half-lives.

4.5.3.3 Shelf stability

Shelf stability of the enzyme was studied at 4°C and at room temperature for 70 days. *B. aerius* GC6 α-amylase was found to be quite stable with a relative activity of 73% after 70 days (Fig. 30) at 4°C. Purified α-amylase from *B. sonorensis* GV2 showed 64% relative activity at 4°C for 70 days (Fig. 30). However, at room temperature, a rapid loss in activity was observed. A respective relative activity of 40% and 25% of the original was retained after 49 days of incubation at room temperature by *B. aerius* GC6 and *B. sonorensis* GV2 (Fig. 31). Kiran and Chandra (2008) studied shelf life of amylase from *Bacillus* sp. TSCVKK for 2 months and found it stable at 4^{o}C, however a loss of 15% activity was observed after 48 h when it was incubated at 30^{o}C.

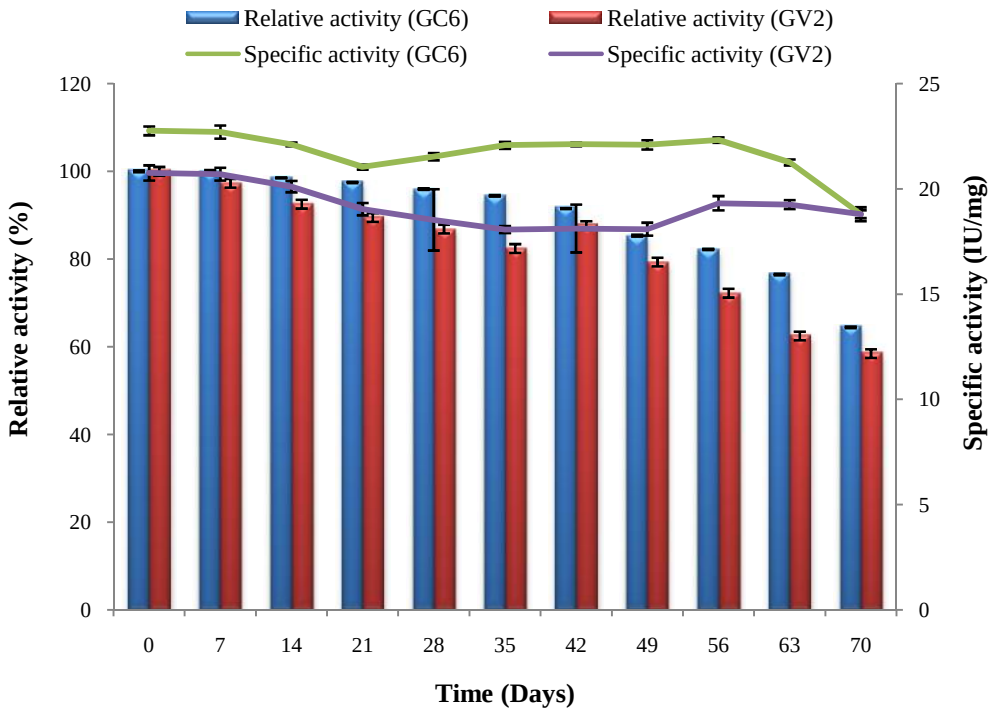


Fig. 30 Shelf stability of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase at 4°C

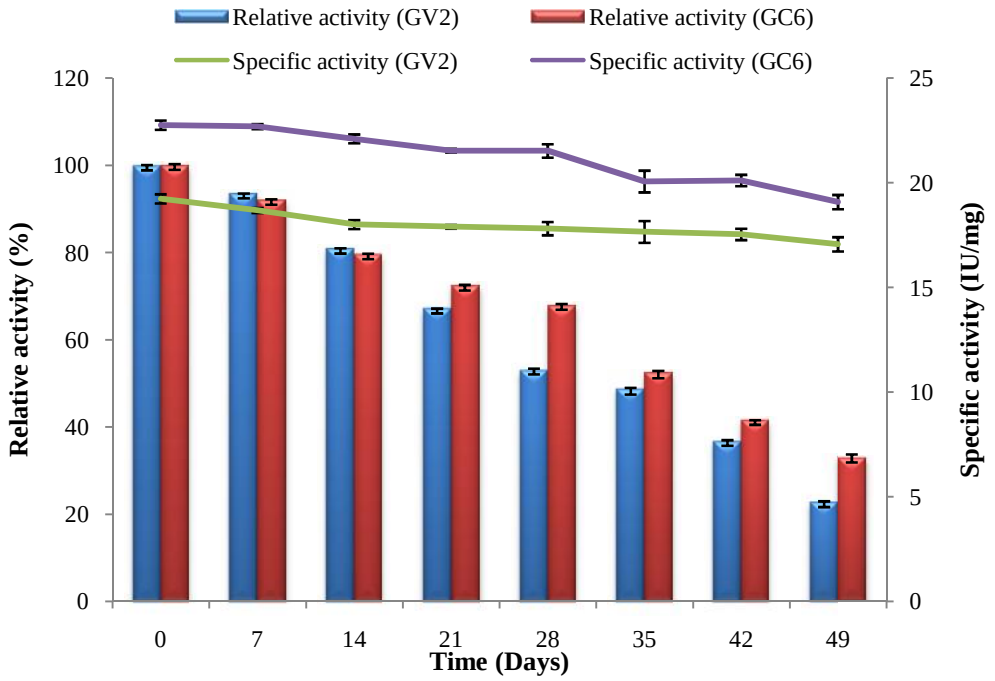


Fig. 31 Shelf stability of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase at room temperature

4.5.4 Effect of metal ions

The activity of various enzymes is influenced by the presence of metal ions either by directly involving in their catalysis or by structural modifications (Bano *et al.*, 2009). Some enzymes require certain metal ions as cofactors and thus called metalloproteins while others are inhibited by their presence in the reaction mixture. Thus, to investigate their effect on enzyme activity, α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 was assayed in the presence of various divalent ions at a concentration of 5 mM. α-amylase activity of *B. aerius* GC6 was strongly influenced on incubation in the presence of divalent ions (Table 32). An enhanced activity of 152.89% and 136.66% respectively was observed with Ca^{2+} and Mg^{2+} suggesting the enzyme to be a metalloprotein needing a cofactor for its maximum activity. In case of *B. sonorensis* GV2, an increased activity of 146.48% has been shown in the presence of Ca^{2+} in the medium, while 138.67% activity has been shown in the presence of Mg^{2+}(Table 32).

Similar increase in enzyme activity has been shown by Bano *et al.* (2009). A relative activity of 111 and 117% has been reported in the presence of Ca^{2+} and Mg^{2+} respectively. A 21% increase in original activity was observed in *Bacillus* sp. TSCVKK α-amylase by Kiran and Chandra (2008) in the presence of $CaCl_2$ in the medium.

Table 32. Effect of divalent ions on the activity of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Divalent ions	**Relative activity (%)**	
	***B. aerius* GC6**	***B. sonorensis* GV2**
Ca^{2+}	**152.89±0.15^{a}**	**146.48±0.08^{a}**
Mg^{2+}	**136.66±0.11^{b}**	**138.67±0.19^{b}**
Cu^{2+}	91.02±0.23^{c}	93.27±0.20^{c}
Ba^{2+}	11.32±0.39^{d}	20.11±0.11^{d}
Fe^{2+}	58.89±0.17^{e}	62.36±0.32^{e}
Mn^{2+}	81.93±0.14^{f}	86.90±0.12^{f}
Co^{2+}	0±0.00^{g}	0±0.00^{g}
Zn^{2+}	2.78±0.16^{g}	11.87±0.32^{h}
Ni^{2+}	91.02±0.13^{c}	96.24±0.29^{i}
Control	100.00±0.09^{h}	100.00±0.10^{j}
SE(m)	**0.16**	**0.23**

The stabilizing effect of Ca^{2+} on thermo stability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus, causing the adoption of a compact structure (Goyal *et al.*, 2005). Metal ions may also stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme. It is suggested that Ca^{2+} increases the thermal stability of the enzyme due to the presence of more binding sites (Saboury *et al.*, 2005). These results suggested that Ca^{2+} and Mg^{2+} both stimulants were required for the stability of enzyme.

A few divalent ions viz. Co^{2+}, Zn^{2+} and Ba^{2+} were inhibitory for *B. aerius* GC6 α-amylase while Co^{2+}, Zn^{2+}, Cu^{2+} and Ba^{2+} were inhibitory for *B. sonorensis* GV2 α-amylase. The inhibition in enzyme activity may be due to the binding of these metal ions to the catalytic residues in the active site of the enzyme rendering the enzyme non-functional or it might be due to the competition between the exogenous cations and the protein associated cations resulting in the decreased enzyme activity (Leveque *et al.*, 2000).

α-amylase retained an activity of 91%, 59%, 81.93% and 91.02% with Cu^{2+}, Fe^{2+}, Mn^{2+} and Ni^{2+} respectively for *B. aerius* GC6 α-amylase while an activity retention of 62.36%, 86.90% and 96.24% has been observed in the presence of Fe^{2+}, Mn^{2+} and Ni^{2+} respectively. The behaviour of α-amylase towards different metal ions as reported in the literature is very different from one another, which could be attributed to the source of enzyme, origin of enzyme and the nature of enzyme.

4.5.5 Effect of surfactants and inhibitors

Substances that reduce the activity of an enzyme catalyzed reaction are known as inhibitors. They act by either directly or indirectly influencing the catalytic properties of the active site. Inhibitors can be foreign to the cell or natural components of it. In the latter instance, they can represent an important element of the regulation of cell metabolism.

For application in detergent industries, α-amylase must be stable in various detergent ingredients such as surfactants and chelators. Various

surfactants were tested for their effect on purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase. Perusal of Table 33 revealed that presence of anionic surfactant SDS was able to retain 94.14% of the original activity of *B. aerius* GC6 α-amylase and 99.52% of the original activity of *B. sonorensis* GV2 α-amylase. An increase in activity by 2.9 fold has been observed for *B. subtilis* KIBGE-HAS in the presence of SDS (Bano *et al.*, 2005). Kiran and Chandra (2008) reported retention of 80% of the original activity in the presence of SDS for *Bacillus* sp. strain TSCVKK. However, reduction or complete inhibition of enzyme activity in the presence of SDS has also been reported by several workers (Li *et al.*, 2007; Rao and Satyanarayana, 2007; Gangadharan *et al.*, 2008;Bai *et al.*, 2012).

Non-ionic surfactants like Tween 20, Tween 80 and Triton X 100 also stabilized enzyme with a relative activity of 101.31, 101.31 and 99.56% respectively for *B. aerius* GC6 α-amylase (Table 33). For *B. sonorensis* GV2 α-amylase, a relative activity of 102.36, 102.93 and 101.59% was observed in the presence of Tween 20, Tween 80 and Triton X 100 (Table 34). These results are in consonance with the reports of Rao and Satyanarayana (2007) who have reported an increased enzyme activity i.e. 126%, 120% and 80% in the presence of Tween 20, Tween 80 and Triton X 100. Kiran and Chandra (2008) also reported retention of 90% activity in the presence of Tween 20, Tween 80 and Triton X 100.

Table 33. Effect of surfactants on the activity of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Surfactant	**Relative activity (%)**	
	***B. aerius* GC6**	***B. sonorensis* GV2**
SDS	94.14 ± 0.13^{a}	99.52 ± 0.21^{a}
EDTA	9.84 ± 0.24^{b}	7.619 ± 0.09^{b}
CTAB	17.9 ± 0.17^{b}	13.24 ± 0.12^{b}
Tween 20	101.31 ± 0.22^{c}	102.36 ± 0.31^{a}
Tween 80	101.31 ± 0.27^{c}	102.93 ± 0.23^{a}
Triton X 100	99.56 ± 0.19^{c}	101.59 ± 0.33
Glycerol	87.09 ± 0.14^{d}	89.38 ± 0.15
Control	100.00 ± 0.11^{c}	100 ± 0.09
SE(m)	**0.19**	**0.24**

Some researchers reported Tween 20 and Tween 80 as inhibitors of enzyme activity (Bano *et al.*, 2005) however; a few others have reported stability in enzyme activity in the presence of these surfactants (Hmidet *et al.*, 2008; Shafiei *et al.*, 2010). Ion chelator EDTA inhibited the enzyme strongly, as reported by Li *et al.* (2007) and Shafiei *et al.* (2010) thus proving it to be a metalloenzyme.

Surfactant stability of α-amylase is comparatively a rare feature and is very important at the same time considering the applicability of amylase in industry. The surface active agents in the medium increase the turn over number of the α-amylase thereby increasing its efficiency by increasing the contact frequency between the active site of the enzyme and the substrate by lowering the surface tension of the reaction mixture (Rao and Satyanarayana, 2007). Variable behaviour of the enzyme towards these chemical reagents arose from the chemical nature of the enzyme, its origin and the constituent amino acids. The inhibitory and stimulatory effect of these additives however may be an important factor in the commercial exploitation of the enzyme where stability and activity of the enzyme are paramount (Kitabatake *et al.*, 2003).

4.5.6 Effect of organic solvents

Purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 was evaluated for the effect of organic solvents on the enzymatic activity. *B. aerius* GC6 α-amylase exhibited high tolerance to ethanol and acetone i.e. 99.55 and 96.35% respectively (Table 34). Thermostable enzymes are known to be resistant to organic solvents (Rao and Satyanarayana, 2007). However, the presence of benzene and chloroform resulted in decreased activity to 56.21% and 76.78% respectively. Purified α-amylase of *B. sonorensis* GV2 exhibited high activity in the presence of ethanol, methanol and acetone i.e. 95.48%, 90.84% and 95.68% respectively. The enzyme however showed lesser tolerance towards benzene and chloroform (Table 35). This trend shows the stability of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 towards organic solvents, which make them an eligible candidate in the industry using organic solvents in medium.

Table 34. Effect of organic solvents on the activity of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Organic solvetnts	Relative activity (%)	
	***B. aerius* GC6**	***B. sonorensis* GV2**
Acetone	96.35±0.28[a]	95.68±0.11[a]
Methanol	90.41±0.37[b]	90.84±0.23[b]
Ethanol	99.55±0.18[a]	95.48±0.17[a]
Benzene	56.21±0.16[c]	57.32±0.21[c]
Chloroform	76.78±0.47[d]	82.77±0.13[d]
Xylene	11.09±0.11[e]	0±0.00[e]
Control	100±0.07[a]	100±0.09[f]
SE (m)	**0.22**	**0.17**

4.5.7 Substrate specificity

Substrate specificity studies for *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase has been conducted using various substrates viz. corn starch, amylase, amylopectin glycogen, dextran and cellulose. For *B. aerius* GC6 α-amylase, results revealed that corn starch and amylopectin were better substrates for purified amylase than glycogen, dextran and cellulose yielding 56.26% and 48.30% activities respectively. This indicated that soluble starch, corn starch and amylopectin were the physiological substrates for the enzyme (Table 35). Soluble starch was kept as control. This enzyme was highly specific to soluble starch compared to other starches used giving comparatively lower enzyme yields. Similar results have been reported by several workers showing the substrate specificity of α-amylase for starch soluble. Azad *et al.* (2009) isolated and purified amylase which showed maximum activity with soluble starch as substrate. Ashwini *et al.* (2011) reported starch soluble as a substrate of choice for amylase isolated and purified from *B. marini.*

B. sonorensis GV2 α-amylase however, showed a bit of different behaviour with 116.53% relative activity for corn starch considering its activity 100% with soluble starch (Table 35). This is a rare feature as most of the amylases reported till date showed maximum activity towards soluble starch which is easily hydrolysable. Rao and Satyanarayana (2007) have shown

retention of 80% whereas Gangadharan *et al.* (2008) showed retention of 62% of the original activity with corn starch as substrate. 43.96% of the original activity was observed for amylose, thus indicating that corn starch, soluble starch and amylose are the physiological substrates for *B. sonorensis* GV2 α-amylase.

Table 35. Substrate specificity of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Substrate	Relative activity (%)	
	***B. aerius* GC6**	***B. sonorensis* GV2**
Soluble starch (control)	100.00	100.00
Corn starch	56.26	116.53
Amylose	-	43.96
Amylopectin	48.30	-
Dextran	-	-
Cellulose	-	-
Glycogen	-	-

4.5.8 Determination of Enzyme Kinetics

K_m is a useful and fundamental characteristic for an enzyme and a particular substrate. It can be viewed as an index of how easily the enzyme can be saturated by the substrate (i.e. the affinity) under defined conditions of temperature and pH. The smaller the value of K_m the more readily the enzyme may be saturated with substrate.

The kinetic parameters for hydrolysis of soluble starch were determined by plotting the initial velocities towards different concentrations of soluble starch as substrate, incubated with the fixed amount of enzyme. *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase followed the Michaelis Menton kinetics of catalysis. The V_{max} and K_m values of *B. aerius* GC6 enzyme were found to be 1404 µmol/ mg/min and 4.40 mMol/min (Table 36). Lineweaver Burk plot for enzyme action has been shown in Fig. 32. The V_{max} and K_m values of *B. sonorensis* GV2 α-amylase were found to be 1347 µmol/mg/min and 3.46 mMol/ml. Lineweaver Burk plot for enzyme action has been shown in Fig. 33. Low values of K_m indicate high affinity of the enzyme for substrate. The V_{max} and K_m values are enzyme specific for different enzymes and are difficult to compare due to the

difference in origin, conditions and the substrate used. Gangadharan *et al.* (2008) reported V_{max} and K_m values of 3.04 mg/min and 2.34 mg respectively for purified α-amylase of *B. amyloliquefaciens.* In another study, Shafiei *et al.* (2010) reported V_{max} and K_m values of 1.18mg/ml/min and 4.5 mg/ml respectively. Rasiah and Rehm (2009) studied the kinetics of purified α-amylase from *B. licheniformis* and V_{max} and K_m values were found to be 506 U/mg and 5µmol respectively. Perusal of these reports revealed that purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase have shown very good kinetic characteristics thereby making them efficient enzymes in terms of quality and quantity.

Table 36. Kinetic parameters of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Isolate	K_m	V_{max}	K_{cat}	K_{cat}/K_m	$t_{1/2}$ (45-60°C)
***B. aerius* GC6**	4.40 mMol/min	1404µmol/mg/min	154.1/min	35.02	90 min
***B. sonorensis* GV2**	3.46 mMol/min	1374µmol/mg/min	136.56/min	38.88	90 min

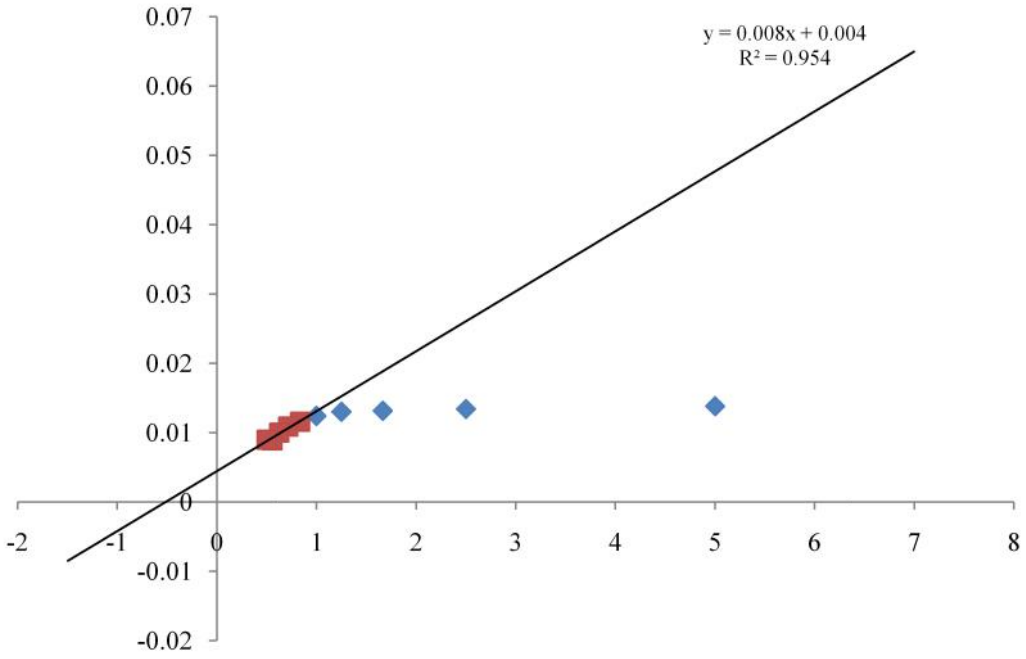


Fig. 32 Lineweaver Burk Plot for enzyme kinetics of *B. aerius* GC6

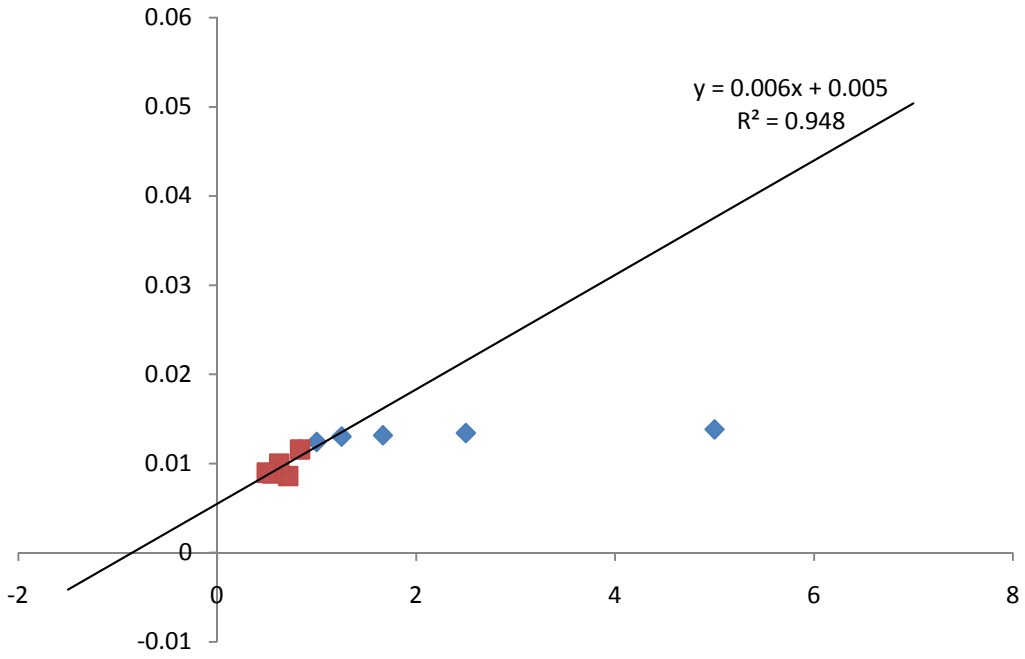


Fig. 33 Lineweaver Burk Plot for enzyme kinetics of *B. sonorensis* GV2

Turnover number K_{cat} corresponds to the maximum number of substrate molecules converted to product per active site per unit of time. The ratio of K_{cat} /K_m often referred to as the 'specificity constant' is a useful index for comparing the relative rates of enzyme action on different substrates (Eisenthal *et al.*, 2007). In the present study *B. aerius* GC6 α-amylase exhibited a turnover number (K_{cat}) 154.1/min and specificity constant of 35.02 whereas *B. sonorensis* GV2 α-amylase exhibited a turnover number (K_{cat}) 136.56/min and specificity constant of 38.88 (Table 36).

4.5.9 Raw starch adsorption and hydrolysis

There has been a considerable interest in the potential use of raw starch degrading enzymes for industrial applications in food industry, especially those capable of hydrolysing starch molecules in large raw starch granules. Conventionally, conversion of starch to glucose and other oligosaccharides required a two-step process namely liquefaction and saccharification before addition of α-amylase and glucoamylase (Goyal *et al.*, 2005). As a replacement of these steps, efforts are being made to exploit microorganisms to produce raw starch degrading enzymes. Enzymes that are capable of digesting raw potato

starch are economically attractive for they can increase the range of starch sources for direct saccharification (Hayashida *et al.*, 1990). It has been well documented that raw starch adsorption is a must for raw starch digestion (Goyal *et al.*, 2005; Li *et al.*, 2007). Thus, raw starch adsorption of the α-amylase produced by *B. aerius* GC6 and *B. sonorensis* GV2 was carried out. Studies on the affinities of the enzyme from *B. aerius* GC6 showed 94.29% adsorbability to raw potato starch and 68.46% adsorbability towards raw corn starch granules (Table37) thus indicating an additional property of the enzyme which makes it very useful in efficient hydrolysis of raw starch granules. This is a highly cherished property in industry as the enzymes capable of digesting raw starches are economically attractive for they can increase the range of starch sources for direct saccharification (Hayashida *et al.*, 1990).

Table 37. Raw starch hydrolysis efficiency of purified *B. aerius* GC6 and *B.sonorensis* GV2 α-amylase

Raw starch	**% Hydrolysis**	
	***B. aeiurs* GC6**	***B. sonorensis* GV2**
Corn starch	68.46±0.23[a]	46.23±0.21[a]
Potato starch	94.29±0.31[b]	-
SE(m)	**0.21**	**0.21**

However, *B. sonorensis* GV2 α-amylase showed 46.23% adsorbability towards corn starch but no adsorption has been shown for potato starch (Table 37). Binding of the raw starch digesting amylases to the starch granules is usually affected by C-terminal domain within the enzyme. Kelly *et al.* (1995) showed that raw starch digesting amylase of *Bacillus* sp. IMD 370 showed no adsorbability to the raw starch thus showing that adsorbability was apparently not necessary for raw starch digesting α-amylase. Raw starch adsorbing yeast has been reported in literature (Iefui *et al.*, 1996; Nagasaka *et al.*, 1998; Li *et al.*, 2007) however, there are apparently very few reports on the adsorbability of raw starch by bacterial amylases. Gangadharan *et al.* (2008) have reported anα-amylase from *B. amyloliquefaciens* which exhibited a respective adsorbability of 65% and 50% towards raw potato and corn starch. α-amylase from *Alicyclobacillus* sp. showed a respective hydrolysis efficiency of 58% and 52%

for raw potato and corn starches, as reported by Bai *et al.* (2012). In this view, α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 has a novel feature of raw starch digestion which can be of great interest to the food industry.

4.5.10 Analysis of hydrolysed products

The hydrolysis products of soluble starch, hydrolysed separately by purified *B. aerius* GC6 and *B. sonorensis* GV2 amylase were identified by thin layer chromatography. Glucose, maltose and maltooligosaccharides were used as standards. The RF values of the samples and standards were determined and it was observed that the main hydrolysis products for both the enzymes were maltooligosaccharides (Fig. 34). The hydrolysis patterns presented by both *B. aeiurs* GC6 and *B. sonorensis* GV2 purified amylase showed that the enzymes are typical endo-acting α-amylases. In conformity to our results, Hmidet *et al.* (2008) have purified an endo-acting α-amylase from *B. licheniformis* NH1 yielding similar hydrolysis pattern. Kiran and Chandra (2008) also reported the production of maltooligosaccharides as the hydrolysis products by purified *Bacillus* sp. TSCVKK α-amylase.

4.6 INTERNAL AMINO ACID SEQUENCE OF α-AMYLASE DETERMINED BY MALDI-TOF MS

4.6.1 Internal amino acid sequence of *B. aerius* GC6 α-amylase determined by MALDI-TOF MS

An amino acid sequence from the digested protein was analyzed by MALDI-TOF mass spectrum to identify the purified enzyme. The MALDI-TOF mass spectrum (peptide mass fingerprinting) analysis of the reduced and carboxymethylated amylase digested with chymotrypsin has been shown in Table 38. MALDI-TOF mass spectrum of a tryptic protonated peptide molecular ions map has been shown in Fig. 35.

The results of Mascot Search Engine from Matrix-Science search indicated that this partial amino acid sequence was little homologous by a score of 5 with UDP N-acetylglucosamine transferase. Amino acid sequence of amylase UDP N-acetylglucosamine transferase from *Geobacillus thermodenitrificans* NG80-2 has been shown in Fig. 36 and the matched peptides are shown in bold. The protein sequence coverage had been found only up to 5%.

The peptide sequence obtained is: Ser-Gly-Ser-Ser-Asp-Pro-Leu-Gln-Thr-Leu-Leu-Ala-Leu-Ile-Gln-Asn-Thr-Ala-Arg.

Table 38. The MALDI TOF mass spectrum (peptide mass fingerprinting) analysis of the reduced and carboxymethylated *B. aerius* GC6 α-amylase digested with chymotrypsin

m/z	Resolution	Intensity	Area
770.092	7233	4362	792
995	6380	735	153
1013.017	6486	749	155
1157.021	7205	836	202
1175.018	7356	836	211
1319.022	7710	893	261
1337.025	8560	983	279
1481.032	8153	1034	368
1499.040	8711	1108	384
1607.040	7749	746	266
1643.045	8691	1126	463
1661.045	9508	1332	546
1805.057	8921	1114	529
1823.057	9522	1430	703
1967.062	8859	1058	557
1985.067	9580	1447	787
2129.069	9100	1005	557
2147.077	9349	1333	789
2291.074	8803	861	510
2309.083	9397	1208	769
2453.087	8630	765	472
2471.088	9150	1048	718
2615.093	8631	678	425
2633.098	9207	906	634
2777.103	8349	583	364
2795.101	8826	776	576
2957.114	8890	651	465
3119.121	8240	543	397

* range from 700-3200m/z

4.6.2 Internal amino acid sequence of *B. sonorensis* GV2 α-amylase determined by MALDI-TOF MS

An amino acid sequence from the digested protein was analyzed by MALDI-TOF mass spectrum to identify the purified enzyme. The MALDI-TOF masss spectrum (peptide mass fingerprinting) analysis of the reduced and carboxymethylated amylase digested with chymotrypsin has been shown in Table 39. MALDI-TOF mass spectrum of a tryptic protonated peptide molecular ions map has been shown in Fig. 37. The results of Mascot Search Engine from

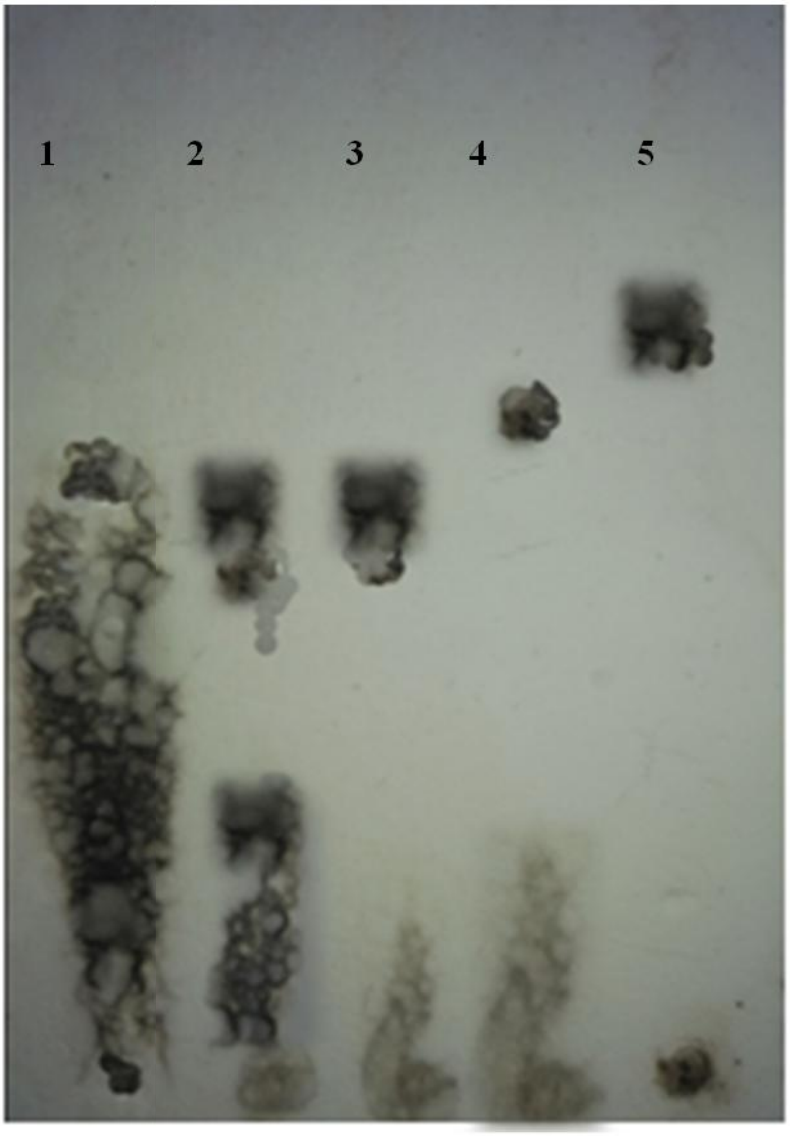


Fig. 34 Thin layer chromatogram for the hydrolysis products of purifed *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Lane 1	Hydrolysed starch by purified *B. aerius* GC6
Lane 2	Hydrolysed starch by purified *B. sonorensis* GV2
Lane3	Mixture of maltooligosaccharides
Lane 4	Glucose
Lane 5	Maltose

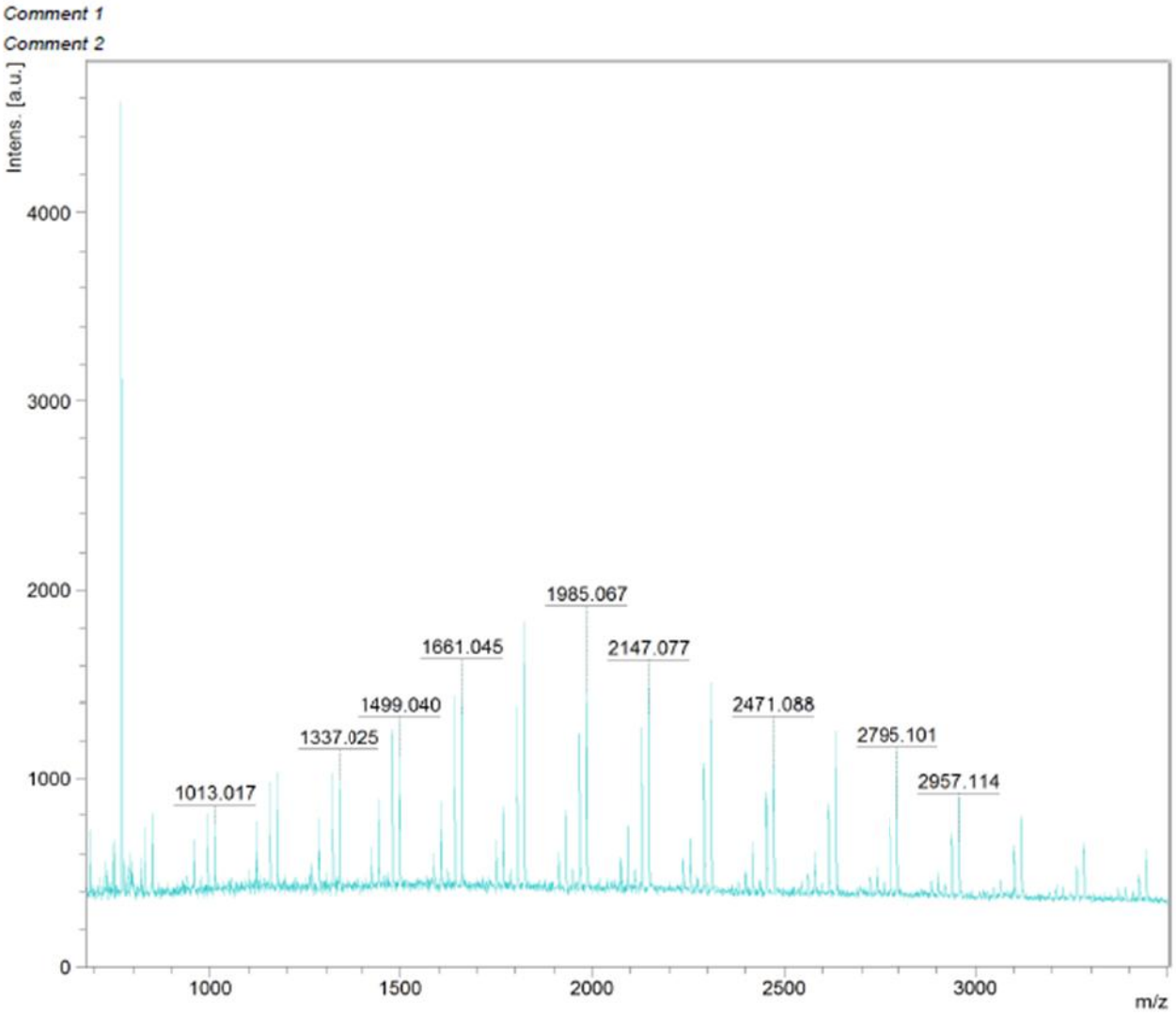


Fig. 35 MALDI-TOF mass spectrum of purified α-amylase from *B. aerius* GC6. The mass spectrum shows a series of protonated peptide molecular ions

1	MRKKIVLTGG	GTAGHVMVNV	ALIPKLKEQG	WDIVYIGSHE	GIEREIIGRI
51	DGVPYYSVST	GKLRRYFDWK	NFKDPFNVLK	GVWQAYRLIQ	KEKPDIVFSK
101	GGFVSVPVIL	GAWLNGVPSV	IHESDLTPGL	ANKIAMPFAT	KICLTFPETK
151	QHVNTDKAVY	VGAVVREELK	HGSAEQGRKL	CQFDGQKPVL	LAMGGSLGSK
201	KINDALRASL	PVLLSEFDIV	HICGKGNVDT	SLVGQKGYKQ	FEYVNEELPD
251	LLALSDIVVS	RAGANAIFEL	LALRKPMLLI	PLSKAASRGD	QILNARSFEK
301	AGYAEVLMEE	EVTNESLPAA	IHRLYENKDR	YKKNMEK**SGS**	**SDPLQTLLAL**
351	**IQNTAR**				

Fig. 36 Amino acid sequence of from *Geobacillus thermodenitrificans*. Matched peptides of predicted enzyme are shown in bold red colour.

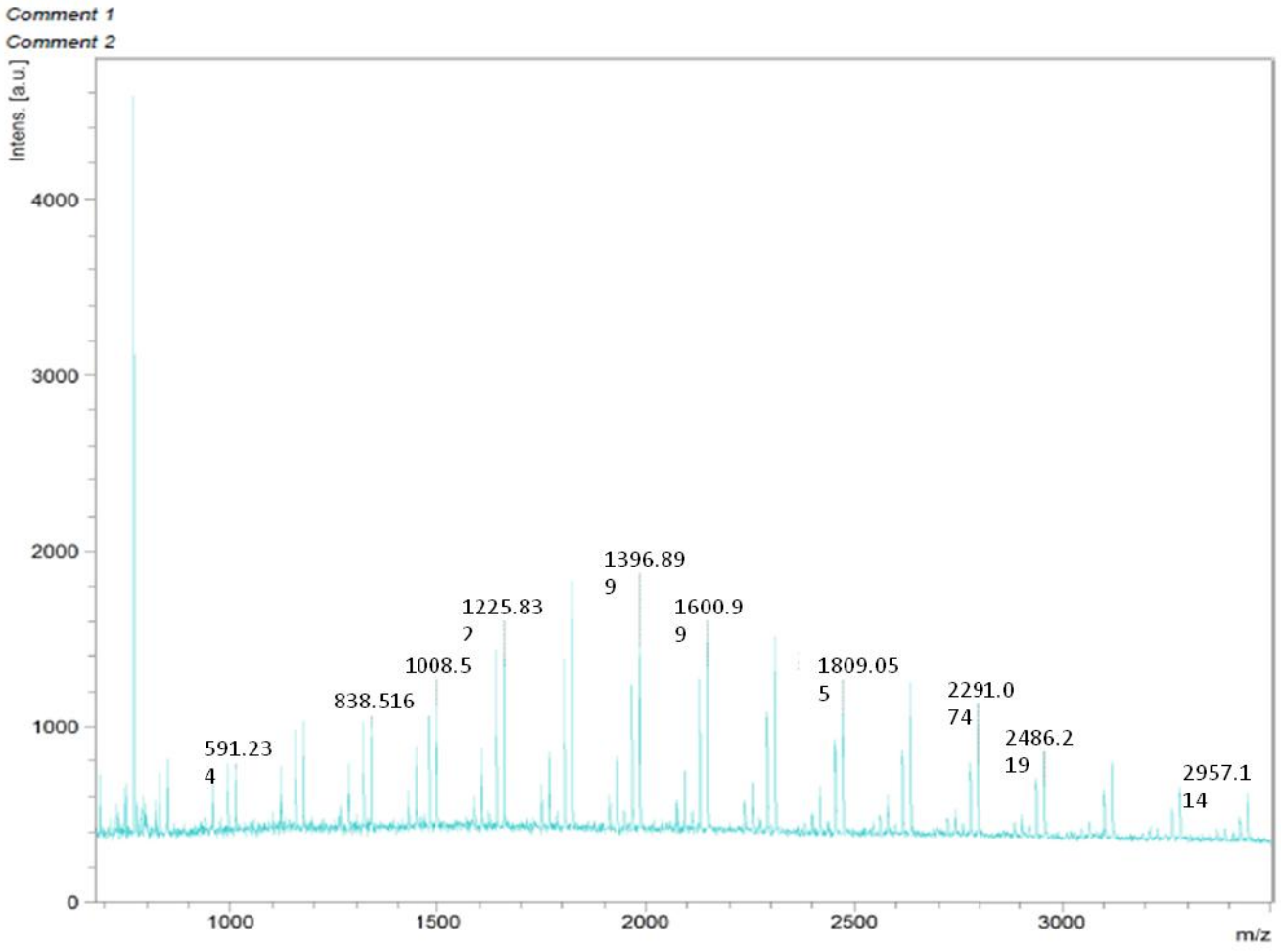


Fig. 37 MALDI-TOF mass spectrum of purified α-amylase from *B.sonorensis* GV2. The mass spectrum shows a series of protonated peptide molecular ions

```
  1 MEKEVWRFID SGNCSPAFNM ALDEALLEWH  SEGKIPPTIR FYGWNPPTLS
 51 IGYFQKVEKE IDMEAVKKYG LGFVRRPTGG  RGVLHDQELT YSVIVSELHP
101 AMPQTVTEAY RVISQGILEG FRFLGLDAYF  AVPKTEEEKA DLKNPRSAVC
151 FDAPSWYELV VEGRKVAGSA QTRQKGVILQ  HGSILLHLDE DMLFSLFKYP
201 NERVKERLRQ NFKNKAVAIN ELTDRNITIN  EAKEAFFRGF EKGLNVQLER
251 YELTDDELFY VQQLAKNKYE TDEWNFKR
```

Fig. 38 Amino acid sequence of from *Anoxybacillus flavithermus*. Matched peptides of predicted enzyme are shown in bold red colour.

Matrix-Science search indicated that this partial amino acid sequence was partially homologous by a score of 6 with UDP transferalyase. Amino acid sequence of amylase from *Anoxybacillus flavithermus* WK1 has been shown in Fig. 38 and the matched peptides have been shown in bold. The protein sequence coverage in this case is only 6%. The peptide sequence is Ala-Val-Ala-Ile-Asn-Glu-Leu-Thr-Asp-Arg-Asn-Ile-Thr-Ile-Asn-Glu-Ala-Lys.

Table 39.The MALDI TOF mass spectrum (peptide mass fingerprinting) analysis of the reduced and carboxymethylated *B. sonorensis* GV2 α-amylase digested with chymotrypsin

m/z	Resolution	Intensity	Area
566.087	2555	258.91	115
591.234	2183	388.81	753
614.117	489	233.23	670
742.442	2562	334.46	160
838.516	2932	1964.92	591
911.438	2294	428.13	358
1008.581	3060	384.3	286
1135.672	3935	632.16	393
1225.832	3771	2471.27	1799
1306.837	4652	413	349
1396.899	4325	3140.81	2459
1463	4376	1290.21	1074
1546.055	1703	1007.74	3169
1600.99	5791	1866.84	1844
1646.055	6325	617.21	456
1706.108	3743	359.59	529
1744.094	6585	386.42	337
1809.055	4319	642.24	812
1853.19	6494	433.4	476
1904	4725	804.08	1061
2003.332	6374	1333	355
2291.074	8803	861	510
2327.429	6059	132.7	201
2379.495	4444	174.82	340
2384.449	5301	125.79	215
2486.219	2391	158.96	591
2584.776	5670	2205.89	3439
2585.772	5241	273.93	532
2808.853	4517	227.75	523
2957.114	8890	651	465

*range from 700-3200m/z

Literature survey revealed that the Michelin *et al.* (2010) studied peptide sequencing by MALDI-TOF MS of purified α-amylase of *Paecilomyces variotii* and found 100% homology with *Bacillus* sp. amylase. On the other hand Kolawole *et al.* (2011) reported purified amylase from finger millet which had no homology with the reported amylases available in the literature and they concluded that their enzyme had unique amino acid sequence. In harmony to this finding, it is concluded here that in the present study a minimal similarity (5 to 6%) between the purified α-amylases from *B. aerius* GC6 and *B. sonorensis* GV2 and their respective counterparts in terms of their amino acid sequencing reveals these purified amylases as novel and unique.

4.7 IMMOBILIZATION OF PURIFIED α-AMYLASE USING VARIOUS HYDROGELS

Immobilization can be defined as the physical confinement or localization of intact enzyme or cells to a certain region of space with preservation of desired catalytic activity (Karel *et al.,* 1985). Immobilized enzymes are generally more stable compared to free enzymes, due to the curtailment of their degrees of freedom (of rotation). Enzymes may be immobilized by using different protocols i.e. physical or chemical fixation on pre-existing supports. Entrapment of enzymes renders them unable to move freely, preventing the unfolding of their structure which in turn preserves its function (Kumar *et al.,* 2006). The main goal of immobilization is their re-use for very long times. The fixation of enzymes on porous supports prevents them from aggregation, proteolysis and interaction with hydrophobic interfaces etc. (Guisan, 2008).

Numerous biotechnological processes are advantaged by immobilization of enzymes, and therefore, several techniques and support materials have been proposed for the purpose of immobilization (Kourkoutas *et al.*, 2004). Among the four techniques i.e. entrapment, cross linking, covalent binding and adsorption generally used for immobilization, entrapment has been reported to be the best as it imposes minimal constraints and is efficient (D'Souza, 1989; Ramakrishna and Prakasham, 1999; Kourkoutas *et al.*, 2004). Keeping in view these observations, entrapment technique was chosen for the immobilization of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase in the present study.

For the immobilization of enzymes, support material is also of utmost importance. Generally, natural hydrogels are the preferred carriers being cheaper and non-toxic. The immobilization procedures with these carriers are mild, fast and the beads formed can be reused without any considerable loss in the activity (Pandey *et al.,* 2000c). Among various natural hydrogels, alginate, agar, agarose and gelatin have been used in the present study for immobilization of purified enzymes.

Thus purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 was immobilized by using entrapment technique. Different natural carriers utilized for immobilization of enzyme were calcium alginate, agar, agarose and gelatin.

4.7.1 Immobilization of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 on calcium alginate

Alginate as an immobilization medium has been extensively studied mainly because of its ease of formulation, mild gelation conditions, biocompatibility and acceptability as a food additive (Hari *et al.*, 1996; Riaz *et al.*, 2009). It has been reported that the immobilization yield of enzyme depends on the concentration of sodium alginate (Dey *et al.,* 2003), the porosity of the calcium alginate beads depend upon the gelling agent concentration. Pore size of the bead should be such that substrate and product can easily diffuse in and out of the alginate gel matrix but the enzyme should retain in the micro environment of beads (Riaz *et al.*, 2009).

To determine the optimum concentration for enzyme immobilization, varying amounts of sodium alginate (0.5-4.0% w/v) were used to vary the relative degree of cross linking which would create different pore size. For *B. aerius* GC6 α-amylase, with increase in the concentration of sodium alginate from 0.5 to 3.0%, there was a subsequent increase in immobilization efficiency from 48.16% to 90.11%, due to an increased cross linking and decreased pore size which could make the beads hold the enzyme firmly. The immobilization yield was found to be highest (90%) for a final sodium alginate concentration of 3% (w/v) (Fig. 39). Further increase in the concentration of the gelling agent to 4% resulted in a considerably decreased immobilization yield up to 52.01%. It could be attributed

to the decreased porosity of gel beads, which caused diffusion limitation of the substrate.

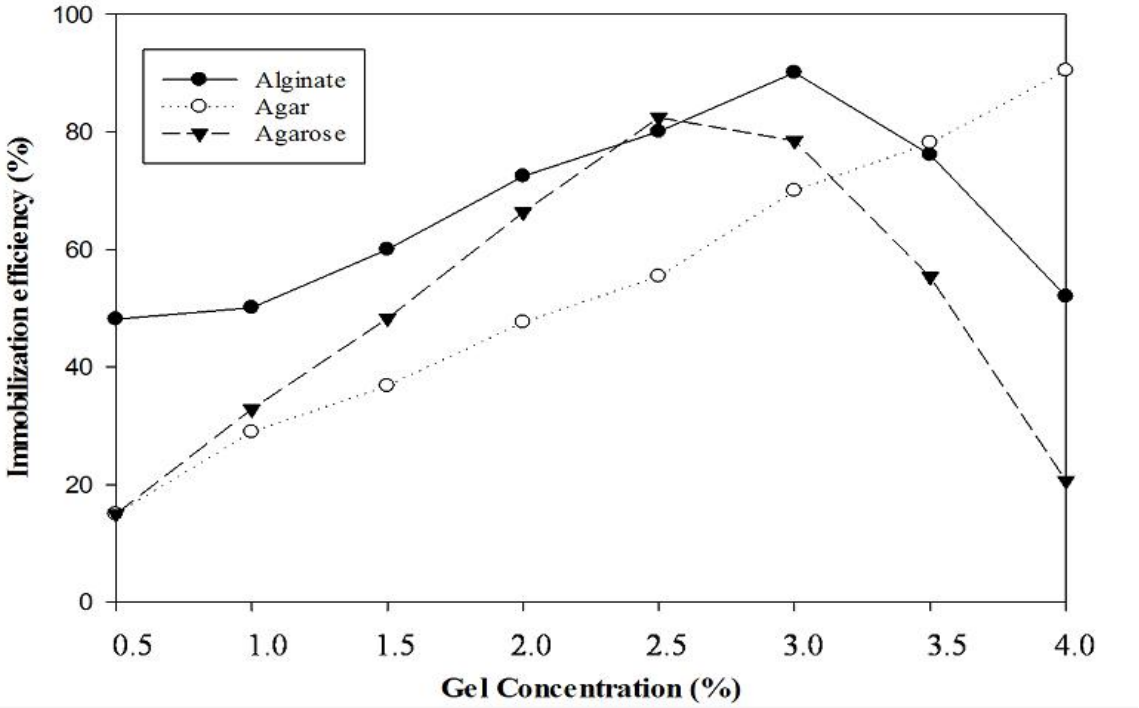


Fig. 39 Effect of gel concentration on immobilization efficiency of *B. aerius* GC6 α-amylase

Similar trend has been shown by *B. sonorensis* GV2 α-amylase, where an increase in sodium alginate concentration in the range spanning 0.5-3% has shown a consequent increase in immobilization yield from 56.23-92.89% and a further increase in the gel concentration upto 4% resulted in decreased immobilization efficiency to 49.06% (Fig. 40). In practice reducing the porosity can reduce leakage but some initial leakage of the enzyme molecule is certain to occur (Knezevic *et al.*, 2006). So 100% immobilization yield could not be attained. The lower immobilization yield in case of lower concentration of sodium alginate solutions might be due to larger pore size and consequently greater leakage of the enzyme from matrix. The more permeable beads showed activity losses due to a rapid leakage of the enzyme from the beads. It has been reported that capsules formed from sodium alginate solutions lower than 0.5% (w/v) had gelled poorly, produced sticky surfaces and were fragile and difficult to handle (Konsoula and Kyriakides, 2006). A higher concentration of the gelling

agent however forms rigid beads, which led to a decreased accessibility of entrapped enzyme to substrate for reaction thereby reducing the yield.

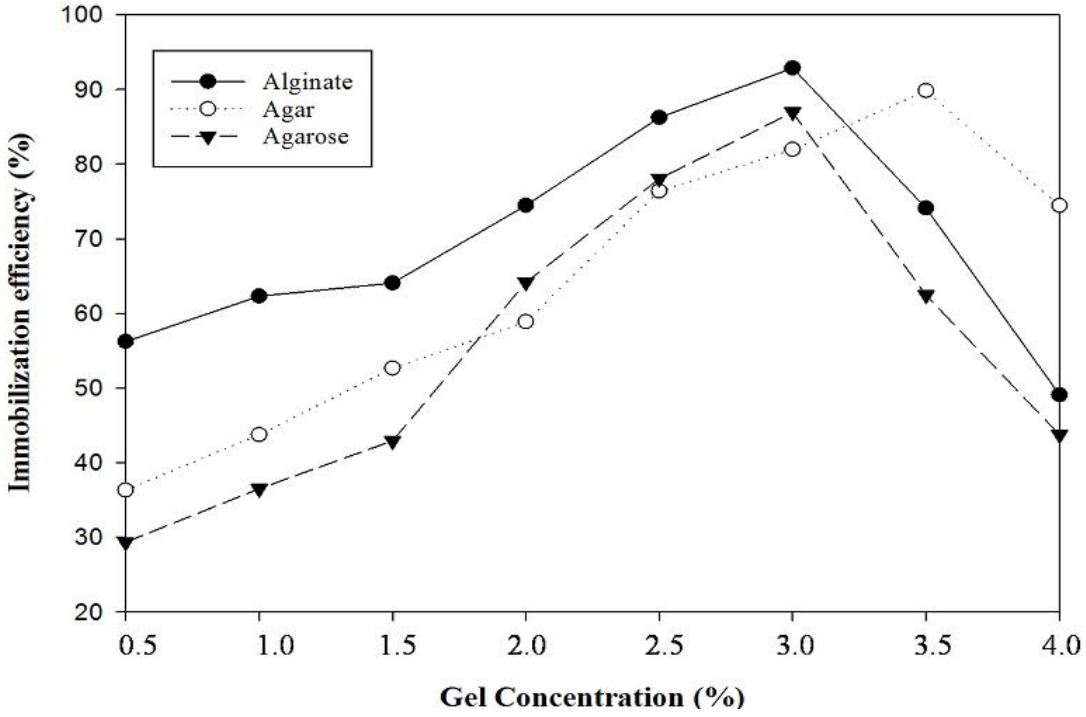


Fig. 40 Effect of gel concentration on immobilization efficiency of *B. sonorensis* GV2 α-amylase

Several workers have used calcium alginate for immobilization of amylase. In course of optimizing the concentration of sodium alginate to form appropriate beads, Dey *et al.* (2003) found 4% sodium alginate as best concentration for efficient immobilization of α-amylase produced by *B. circulans*. They also observed decreased immobilization efficiency at lowered gel concentration due to leakage of enzyme from the gel beads. Our findings are in consonance to these findings. Riaz *et al.* (2009), while immobilizing α-amylase from *B. subtilis* KIBGE-HAR reported 4% sodium alginate as optimum with highest immobilization efficiency and with an increase in alginate concentration, immobilization efficiency decreased. In this light, *B. aerius* GC6 and *B. sonorensis* GV2 with a respective immobilization efficiency of 90.11 and 92.89% at 3% sodium alginate concentration are cost effective.

As shown in the Plate 5, beads formed by immobilization of purified α-amylase in calcium alginate were spherical, smooth, shiny, and white-cream in

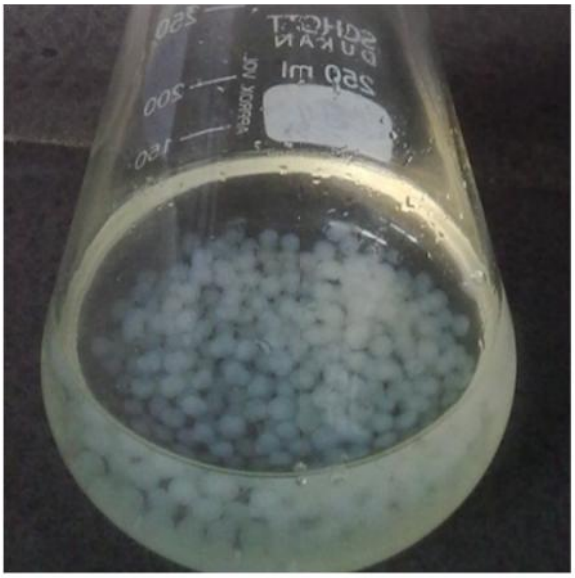
colour with an average diameter of 3.1 mm. Beads were formed immediately after the mixture of sodium alginate and enzyme was dripped into the cold solution of calcium chloride. Similar bead size and shape were reported by other workers (Williams and Munnecke, 1981; Yang *et al.,* 2002; Mariam *et al.,* 2009). The bead diameter has been reported to influence the fermentation rates. For better substrate and product diffusion, beads of smaller size (1-3 mm) are generally preferred (Kawakami *et al.,* 1990; Park and Chang, 2000). The bead size observed in the present study is almost within the limits suggested for good fermentation.

Concentration of calcium chloride was also varied from 0.1, 0.2... 0.5 M in order to acquire stable beads capable of securing maximum enzyme. It was found that beads cured in 0.2 M calcium chloride retained highest α-amylase for *B. aerius* GC6 with immobilization efficiency of 91% as well as for *B. sonorensis* GV2 with an immobilization efficiency of 92%. As the concentration of calcium chloride increased beyond 0.2 M, immobilization yield of α-amylase decreased. It might be due to the change in pH of calcium chloride solution with change in calcium chloride concentration. Effect of calcium chloride concentration on stability of beads has been studied by several workers. Talekar and Chavare (2012) while immobilizing a commercial α-amylase found 1 M $CaCl_2$ as best for stable beads with an immobilization efficiency of 89%. Increase in the concentration of $CaCl_2$ to 2 M decreased activity of 40% was observed. The results agree with our findings.

4.7.2 Immobilization of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 using Agar and Agarose as carrier

Another hydrogels used for comparison were agar and agarose. In order to determine the optimum concentration of agar for efficient entrapment of purified α-amylase of *B. aerius* GC6 and *B. sonorensis* GV2, 0.5, 1.0…, 4.0%, w/v of agar was used to vary the relative degree of cross linking of agar beads which would create different pore size.

For *B. aerius* GC6 α-amylase, with 0.5% agar an immobilization efficiency of 15% was obtained. With an increase in concentration of agar from 1.0 to 3.0%, the immobilization efficiency increased from 28.92-70.00%.



(a)



(b)



(c)



(d)

Plate 5. Natural carriers for the immobilization of purified α-amylase (a) beads of calcium alginate (b) chips of agarose (c) chips of agar (d) chips of gelatin

Immobilization yield was found to be highest (90.47%) for a final agar concentration of 4% (w/v) (Fig. 39). A lowered value for immobilization yield was observed at higher concentrations of sodium alginate. The lower immobilization yield in case of lower concentration of agar might be due to larger pore sized fragile beads which led to a greater leakage of the enzyme from matrix while at higher concentrations the rigidity of beads forbids the substrate to enter the beads. In case of *B. sonorensis* GV2 α-amylase, 0.5% agar gave an immobilization yield of 36.32%. Further increase in the concentration, increased the immobilization efficiency upto 3.5% (Fig. 40). Highest immobilization efficiency of 89.83% had been observed at 3.5% agar concentration which further declined to 74.46% at 4.0% agar concentration. Prakash and Jaiswal (2011) immobilized α-amylase isolated from soyabean seeds on agar matrix. A 4% agar resulted in 77% immobilization efficiency.

Similarly, varying concentrations of agarose from 0.5, 1.0... 4.0% was used for immobilization of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2. With increasing concentration of agarose from 0.5 to 2.0% (Fig. 39), *B. aerius* GC6 α-amylase exhibited an increase in immobilization efficiency from 15.00 to 66.36%. A highest yield of 82.48% was observed at 2.5% agarose, which further declined to 20.63% at an increased concentration of 4.0%. A 10% glutaraldehyde used as hardening agent has been reported to be used to crosslink the proteins. For *B. sonorensis* GV2 α-amylase, 0.5% agarose showed an immobilization efficiency of 29.33% which increased to a maximum of 86.99% at 3.0% agarose (Fig. 40). Subsequent increase in the gel concentration to 4.0% reduced the efficiency to 43.73%. Thus a similar trend has been followed for the immobilization efficiency of purified enzyme entrapped on agar and agarose beads. Prakash and Jaiswal (2011) attempted to immobilize α-amylase isolated from soyabean seeds on agarose gel and found 1% agarose as optimum with 75% immobilization efficiency.

4.7.3 Immobilization of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 using gelatin as carrier

Gelatin is a very useful immobilization matrix for biomolecules and microbial cells. It is advantageous due to its easy availability, abundance and less

expensive nature. In addition, it stabilizes the immobilized enzymes itself being a protein. A 10, 20..., 50% concentration of gelatin was used for immobilization of purified α-amylase of *B. aerius* GC6 and *B. sonorensis* GV2. 10% glutaraldehyde was used for hardening of beads. Maximum gelation resulting in highest immobilization efficiency of 84.46% was obtained at 20% gelatin concentration for *B. aerius* GC6 while for *B. sonorensis* GV2, highest immobilization efficiency of 82.59% was obtained at 30% gelatin concentration (Table 40). Increase and decrease in gelatin concentration has resulted in decreased immobilization efficiency due to the reasons mentioned above. Jaiswal *et al.* (2012) while optimizing an alpha amylase on gelatin matrix found a 20% gelatin concentration as best with an immobilization efficiency of 82.5%. All the values of response variables are the mean of triplicate values with standard deviation as calculated by Tukey Analysis for One Way ANOVA by GraphPad Prism software. Standard error of mean has been given at the end of the table.

Table 40. Effect of gelatin concentration on the immobilization efficiency of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Gelatin concentration (%)	Immobilization efficiency (%)	
	***B. aerius* GC6**	***B. sonorensis* GV2**
10	56.42±0.23[a]	61.42±0.24[a]
20	84.46±0.19[b]	74.28±0.18[b]
30	77.02±0.21[c]	82.59±0.21[c]
40	52.69±0.17[a]	57.28±0.17[a]
50	29.46±0.21[d]	38.46±0.09[d]
SE(m)	**0.15**	**0.18**

4.7.4 Effect of curing time on the stability of hydrogel beads

Curing time is the time required for the gel to set and get hardened. It is thus the time which affects the stability of the resulting gel beads prepared by using any of the matrices. It is an important parameter in immobilization as it produces stable gel beads which could reduce the enzyme leakage and increase the immobilization yield of the enzyme. So, the effect of curing time on the enzyme immobilization yield was evaluated. Curing of sodium alginate beads in 0.2M calcium chloride solution and agar, agarose and gelatin beads in 10% glutaraldehyde solution was optimized at a varying time of 30, 60..., 180 min. For

B. aerius GC6 α-amylase, curing time of 120 min was observed to be the best for alginate beads yielding 92.45% immobilization (Fig. 41). Similarly, for agar 150 min of curing time yielded 89.97% immobilization. With agarose, an immobilization yield of 88.93% was observed at 120 min of curing time while with gelatin highest immobilization efficiency of 81.65% was observed at 60 min of curing time. While for *B. sonorensis* GV2 α-amylase, curing time of 120 min was observed to be the best for alginate beads yielding 90.98% immobilization (Fig. 42). Similarly, for agar 150 min of curing time yielded 89.97% immobilization. With agarose, an immobilization yield of 87.46% was observed at 90 min of curing time while with gelatin highest immobilization efficiency of 86.78% was observed at 90 min of curing time.

Prolonged curing of the beads beyond the best yielding time within curing solution did not improve the immobilization yield (Dey *et al.*, 2003). It might be due to the constant hardness of the gel beads which is observed after particular curing time for particular gelation agent. This could have resulted in constant leakage of α-amylase above the particular curing time. At lower curing time, the beads were very soft and fragile resulting in more leakage and hence giving very low percent immobilization yield.

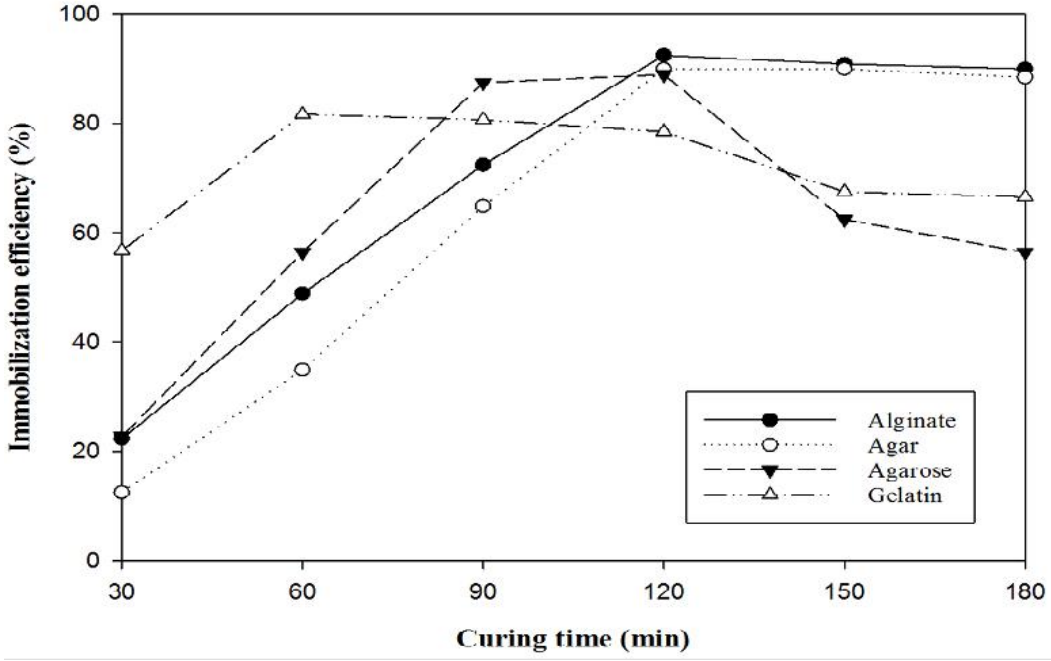


Fig. 41 Effect of curing time on the immobilization efficiency of *B. aerius* GC6 α-amylase

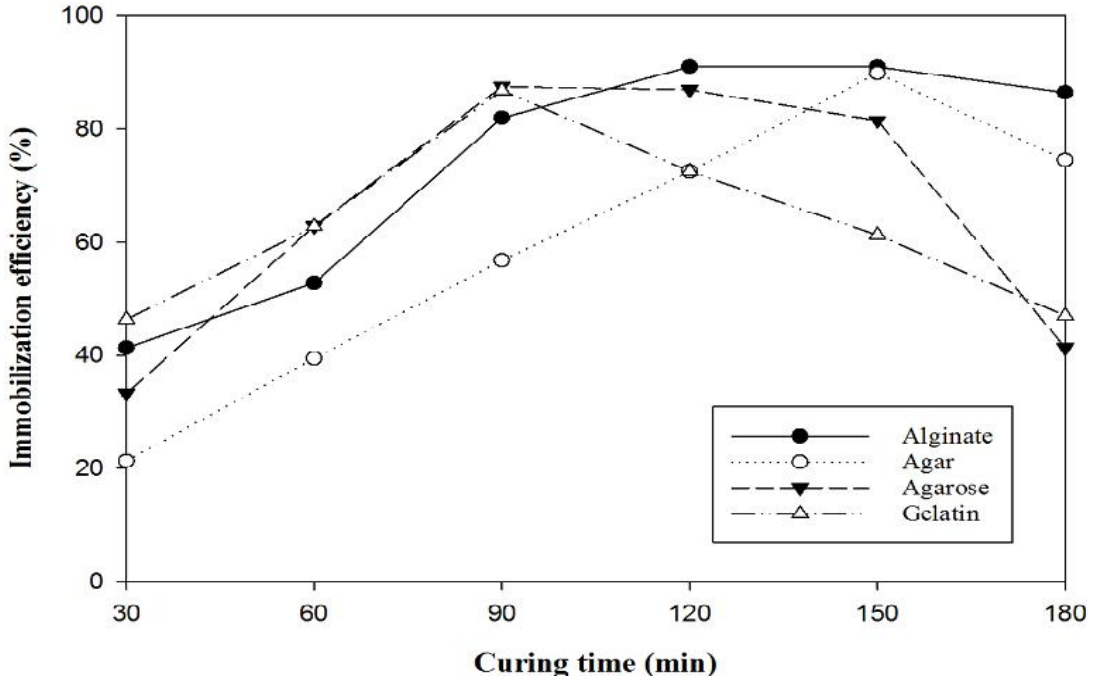


Fig. 42 Effect of curing time on the immobilization efficiency of *B. sonorensis* GV2 α-amylase

Literature review showed that our findings are in agreement with other workers. Dey *et al.* (2003) optimized curing time for α-amylase isolated from *B. circulans* GRS313 in terms of the hardness of beads. A curing time of 120 min produced beads with 85 g hardness which did not showed any further increase with increasing curing time. Similarly, Talekar and Chavare (2012) evaluated the effect of curing time on the stability of alginate beads, for immobilization of commercial α-amylase. 120 min of curing resulted in an immobilization efficiency of 90%. Increased curing time did not result in better efficiency.

4.7.5 Reusability of enzyme immobilized using different natural hydrogels

Several studies indicated that immobilized enzyme has shown increased catalytic activity, increased stability of enzyme (El-Batal *et al.*, 2005), easy recovery of enzyme, easy separation from product minimizing or eliminating protein contamination of product, repeated or continuous use of a single batch of enzyme (Varavinit *et al.*, 2002). To a large extent this procedure prevents enzyme losses due to washout and at the same time maintains biocatalyst at high concentrations (Baldino *et al.*, 2001) and also protects enzyme from microbial

contamination. The main advantage of immobilized enzyme is its ability to be reused for many batches of the process. Reusability of enzyme is a key factor for its cost effective industrial use. A carrier used for immobilization should be such, which can hold the enzyme for the longest period of time with minimum loss of enzyme. Thus the carriers used in the present study for entrapment of enzyme were compared with respect to their ability to be reused. The reusability of immobilized α-amylase onto various hydrogels was studied by assaying the activity in the beads several times for starch hydrolysis.

B. aerius GC6 purified α-amylase immobilized onto calcium alginate beads retained activity upto 8 cycles. Upto 4 cycles approximately 80% of the activity was retained. 5^{th} and 6^{th} cycle retained respective activity of 76.66 and 56.69% (Fig. 43). Till the end of 7^{th} cycle activity retained was 36.76%. However a considerable decline in activity to 22.59% was observed at the end of 8^{th} successive cycle. The activity of the first run was considered to be 100%. This successive decrease in activity may be due to the leakage of the entrapped enzyme from the gel bead.

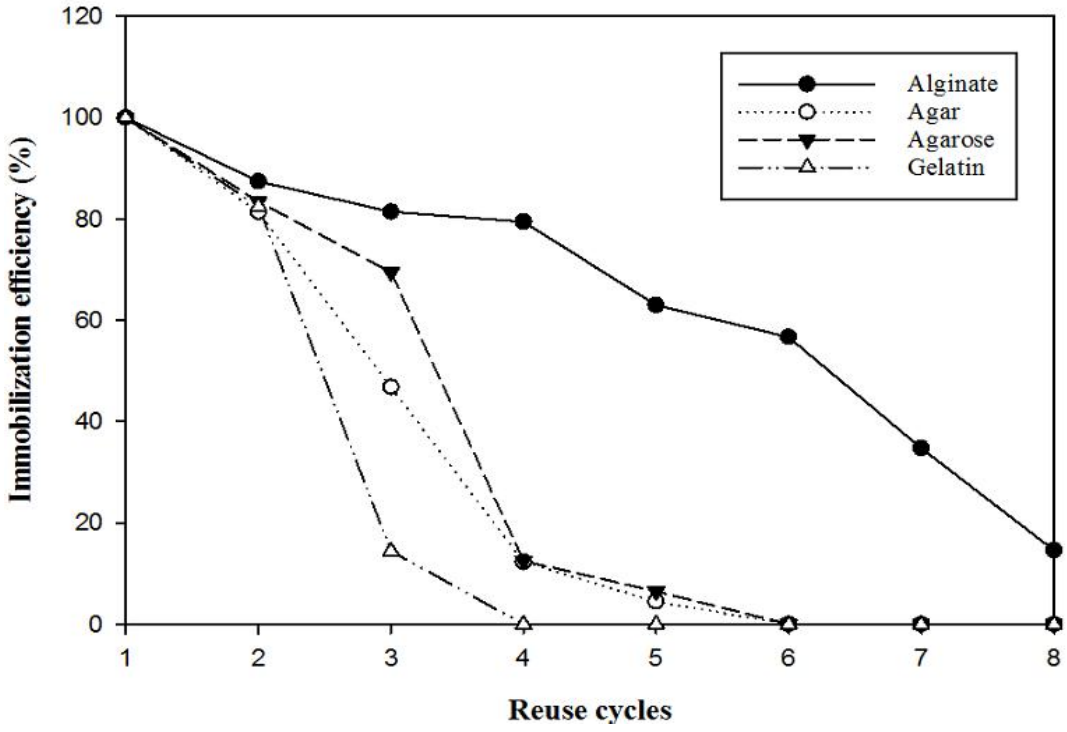


Fig. 43 Number of reuse cycles of hydrogel immobilized purified *B. aerius* GC6 α-amylase

A similar trend of decrease in activity was observed in agar, agarose and gelatin. Agar and agarose showed activity upto 5 cycles. More than 80% of the activity was retained till the end of 2nd cycle in both the carriers. However, at the end of 3rd cycle, 72.82% activity was retained by the enzyme entrapped onto agarose while only 51.77% activity was retained by the enzyme entrapped onto agar (Fig. 44).

In successive cycles for *B. aerius* GC6, there was an abrupt decrease in the activity in both the carriers. In case of gelatin, the beads could retain activity only upto 3 cycles. There was a considerable sudden decrease in the residual activity and the activity decreased abruptly from 82.47 to 14.43% (Fig. 43). Thus beads formed by calcium alginate for entrapment of *B. aerius* GC6 α-amylase retaining activity upto 8 cycles were adjudged as the best carrier for immobilization. In case of purified *B. sonorensis* GV2 α-amylase, more or less a similar trend was observed. Enzyme immobilized onto calcium alginate beads retained 22.78% activity till the end of 8th cycle. Till the end of five cycles, approximately 80% of activity was retained. For 6th and 7th cycle, a respective activity of 49.42% and 36.79% was observed (Fig. 44).

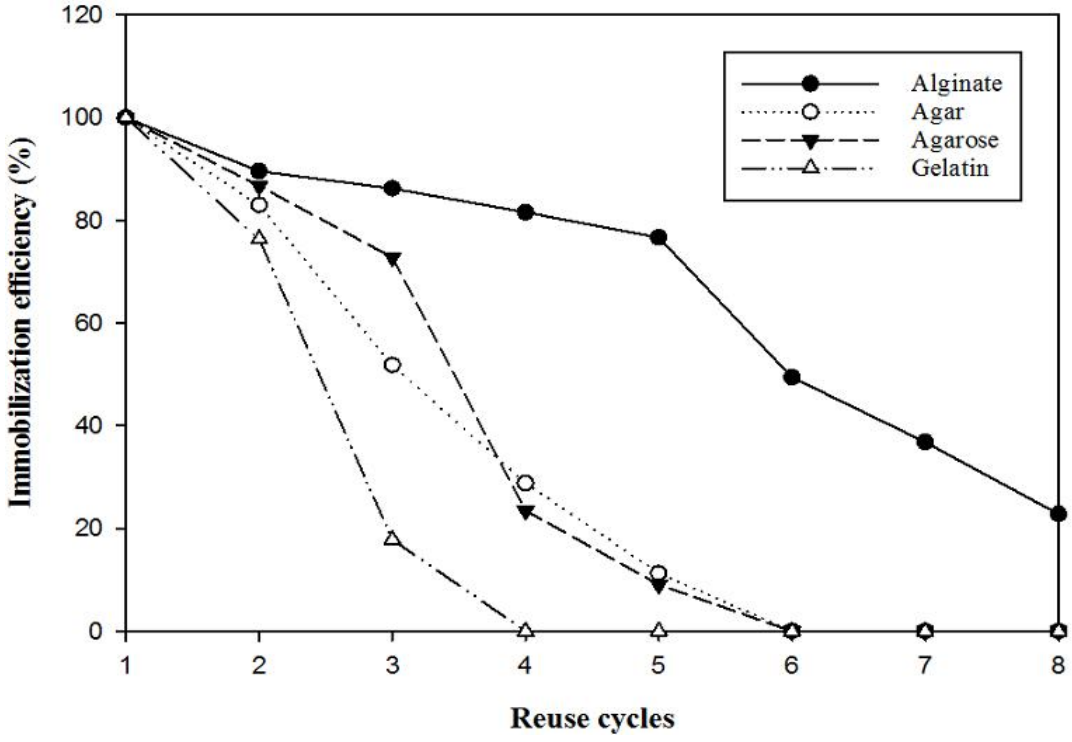


Fig. 44 Number of reuse cycles of hydrogel immobilized purified *B. sonorensis* GV2 α-amylase

In case of agar, agarose and gelatin, a similar successive decrease in activity was observed. Agar beads showed a decreased activity of 51.82% after the end of three cycles with 82.99% activity at the end of 2nd cycle. 4th and 5th cycle showed an abrupt decrease to 28.78 and 11.28% respectively. For agarose, 2nd and 3rd cycle showed a decline in activity with an immobilization efficiency of 86.73 and 72.82%. 4th cycle however showed a sudden dip in efficiency to 23.48% with 9.03% efficiency at the end of 5th cycle. Gelatin showed activity upto four cycles which showed an abrupt decline to 27.82% at the end of 3rd cycle from 76.34% at the end of 2nd cycle. End of 4th cycle marked and enzyme activity decline to 17.82% (Fig. 43). The enzyme in the present study is operationally more stable on calcium alginate beads than other carriers.

Decrease in the immobilization efficiency of enzyme with every successive cycle can be attributed to the leakage of enzyme from the matrix. Several workers studied reuse of entrapped enzyme on various matrices. Demirkan *et al.* (2011) reported 6 reuses of calcium alginate entrapped *B. amyloliquefaciens* α-amylase with 51% residual activity. They credited the denaturation of enzyme and its physical loss from the carrier because of the overuse of beads as a possible reason for decline in residual activity. Similarly, Talekar and Chavare (2012) reported 10 reuses of calcium alginate immobilized amylase with 35% residual activity. *B. circulans* GRS 313 α-amylase was immobilized onto calcium alginate by Dey *et al.* (2003) and observed 10 successful reuses with retention of 83% activity up to seven cycles. Prakash and Jaiswal (2011) immobilized soyabean α-amylase onto agar and agarose matrices and revealed reuses till 5 cycles for both the carriers with a successive loss in activity after every cycle.

Sodium salt of alginate had thus been chosen in the present study for being stable, possessing higher mechanical strength, lowest shrinkage rates and highest porosity among the other gels. It emerged as best carrier owing to its highest immobilization efficiency, consistent gel matrix at lowest concentration of the gel, stability of beads and reusability of immobilized enzyme. It was thus chosen for further characterization studies. Kumar *et al.* (2006) has however, reported the inability of the bacterial amylase to bind to alginate.

4.7.6 Characterization of immobilized *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase for enzymatic properties

The change in the enzymatic properties of the immobilized enzyme is due to the reaction of enzyme and the substrate in the microenvironment which is different from the enzyme substrate reaction in the bulk solution environment. The change is also due to the modification in the three dimensional conformation of the protein when linked with the support matrix. These conformational changes occurring in the enzyme systems leads to altered enzymatic properties of an immobilized enzyme than that of the free enzyme (Sharma *et al.*, 2008). Enzyme immobilization improves the operational stability as a result of number of bonds formed between the enzyme and the support matrix. Owing to the above facts, characterization of immobilized enzyme with respect to its behaviour to the external environment thus becomes indispensible.

4.7.6.1 Effect of pH on free and immobilized enzyme

pH of the medium is one of the major parameters capable of affecting the enzyme activity. A slight alteration in pH from optimum results in decreased enzyme activity. Immobilization of enzyme usually results in a shift of optimum pH due to conformational changes in enzymes. Optimum pH for free enzyme yielding highest α-amylase titres was considered as 100%.

Immobilized *B. aerius* GC6 α-amylase gave maximum activity of 107.25% at pH 9.0. At pH 10.0, 101.5% (Fig. 45) of the original activity was observed for immobilized enzyme which was 86.54% for the free enzyme. Unlike free enzyme, alginate immobilized α-amylase exhibited considerable relative activity of 31.47 and 12.66% respectively at pH 11 and 12 as well. Thus immobilized enzyme exhibited a wider range of pH tolerance than the free enzyme. Unlike immobilized *B. aerius* GC6 α-amylase, shift in the optimum pH for immobilized α-amylase of *B. sonorensis* GV2 was observed. It showed an increased relative activity of 108.19% at pH 9.0 while relative activity of 114.27% was observed at optimum pH 10.0 (Fig. 46). Wider pH tolerance range of pH 7.0-12.0 was observed with a relative activity of 57.80% at pH 11.0 and 19.05% at pH 12.0. Free enzyme showed no activity at pH 12.0.

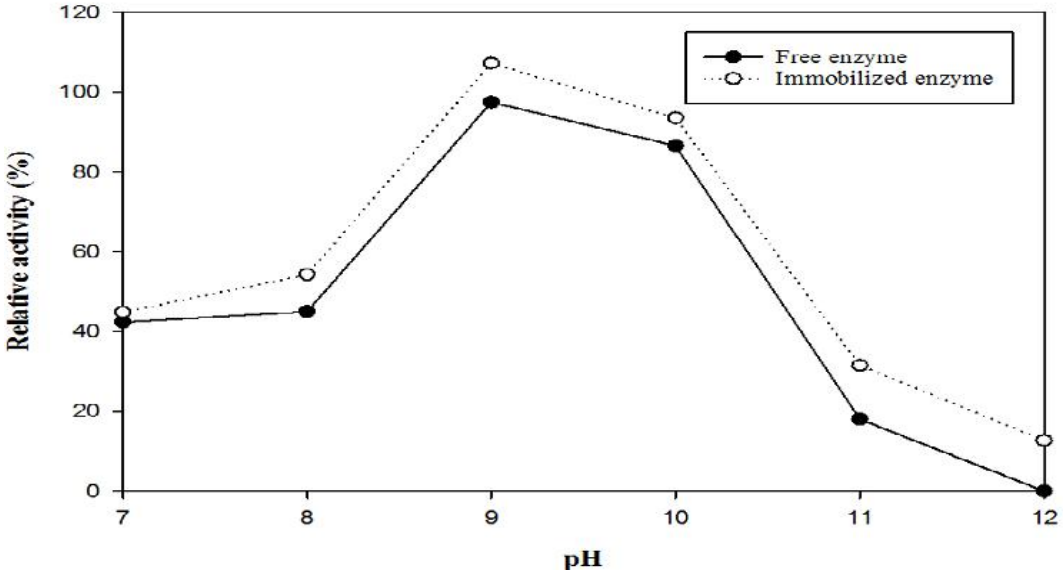


Fig. 45 Comprative account on the effect of pH on the activity of free and alginate immobilized α-amylase from *B. aerius* GC6

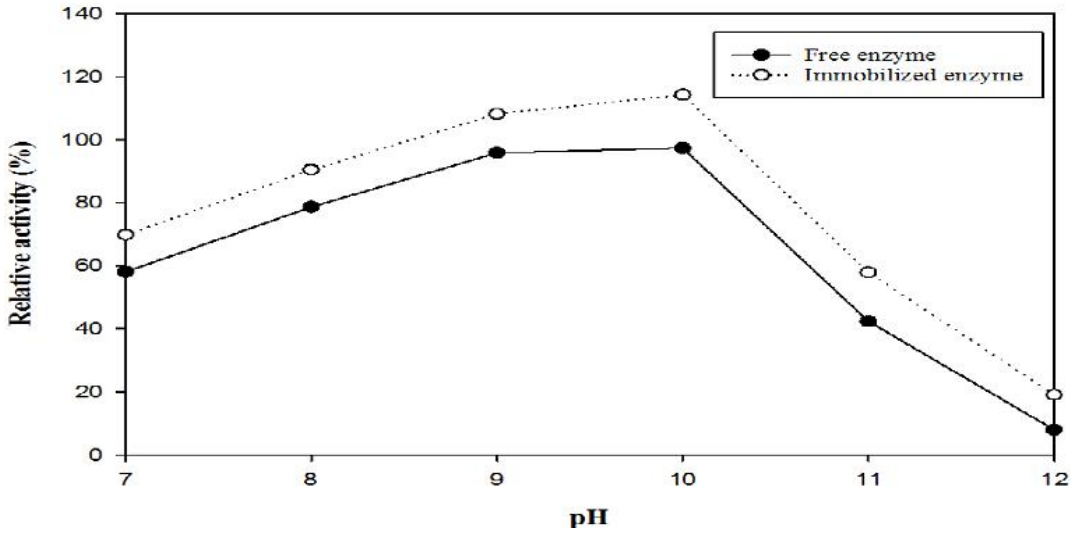


Fig. 46 Comprative account on the effect of pH on the activity of free and alginate immobilized α-amylase from *B. sonorensis* GV2

The results are in consonance with Varavinit *et al.* (2002), who reported an increase in the relative activity of dialdehyde cellulose immobilized amylase from *B. licheniformis* over a pH range spanning 8.0-12.0. Demirkan *et al.* (2011) reported an increased range of alkali tolerance for calcium alginate immobilized α-amylase isolated from *B. amyloliquefaciens*. At optimum pH also, an increased activity was observed for immobilized enzyme than the free enzyme. Dragomirescu *et al.* (2012) reported a shift from optimum pH of 4.6 to 3.0 thus widening the range of pH tolerance from pH 3.0-8.0 for *B. amyloliquefaciens*

DSM7 α-amylase immobilized on silica gel. However no considerable increase in the relative activity of free and immobilized enzyme was observed. This increase in enzyme activity even at higher pH could be attributed to lesser perturbations to which an immobilized enzyme is imposed. Moreover the immobilization matrix is able to protect the enzyme against denaturation at higher pH.

Talekar and Chavare (2012) reported a shift towards acidic pH in immobilized enzyme, showing highest activity at pH 5.5 which was 7.0 for the free enzyme. The formation of a dynamic pH-gradient during hydrolysis with liberation of protons has frequently been observed for immobilized enzymes (Trevan, 1980; Halwachs *et al.*, 1978). Static proton or substrate gradients caused by partition of charged groups near surfaces with stationary charges (Goldstein *et al.*, 1964; McLaren and Packer, 1970) has been observed as possible causes for the shift of pH optima. According to Talekar *et al.* (2010), the shift in optimum pH could be resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site. Similar shift in pH optima from 5.0 for free enzyme to 5.5 for silica gel entrapped commercial α-amylase has been reported by Park *et al.* (2005) and Riaz *et al.* (2009) from 7.0 to 7.5 for α-amylase from *B. subtilis* KIBGE-HAR.

4.7.6.2 Effect of temperature on free and immobilized enzyme

Enzyme activity also depends upon temperature. 50^{o}C has been observed as optimum temperature yielding highest enzyme titres with free enzyme for both the isolates. Considering this activity to be 100%, an alginate immobilized *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase exhibited an increased enzyme activity at the same temperature. Calcium alginate entrapped *B. aerius* GC6 α-amylase exhibited a relative activity of 119.48% and 106.24% at 50 and 60 ^{o}C respectively, which was 100 and 96.11% respectively, for the free enzyme (Fig. 47). Moreover, alginate entrapped α-amylase resulted in comparatively higher enzyme titres at increased temperatures than the free enzyme i.e. at 70^{o}C, 87.99% relative activity was observed which was 74% for the free enzyme. At 90^{o}C, relative activity of 44.21% was exhibited by the immobilized enzyme. Thus it is

evident from the data that the calcium alginate immobilized enzyme possessed greater temperature resistant as compared to the free enzyme.

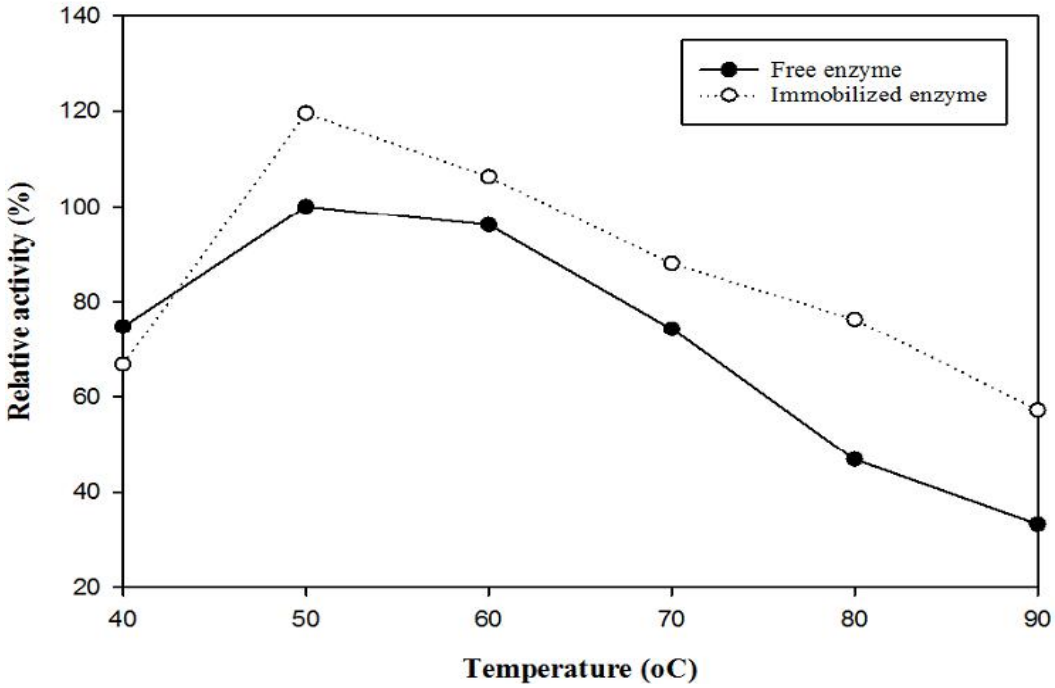


Fig. 47 Comparative account on the effect of temperature on the activity of free and alginate immobilized α-amylase of *B. aerius* GC6

Similarly, *B. sonorensis* GV2 α-amylase exhibited an increased relative activity at the optimum temperature of 50^{o}C. A relative activity of 121.45% and 106.04% was observed at 50 and 60^{o}C respectively. At a higher temperature of 70^{o}C, relative activity exhibited by free enzyme was 62.36% which was 79.34% for the alginate entrapped enzyme (Fig. 48). Similarly, at 90^{o}C, free enzyme showed a relative activity of 33.92% while the immobilized enzyme showed a raised activity of 43.07%.

In the present study, a remarkable increase in the relative activity of immobilized enzyme at optimum temperature of free enzyme has been observed. Moreover, the tolerance range for purified alginate entrapped α-amylase has become widened for both the strains exhibiting a considerable activity at raised temperatures up to 90^{o}C. In agreement to our findings, Varavinit *et al.* (2002) reported an increase in the relative activity of dialdehyde cellulose immobilized amylase from *B. licheniformis* over a temperature range of 30-80^{o}C.

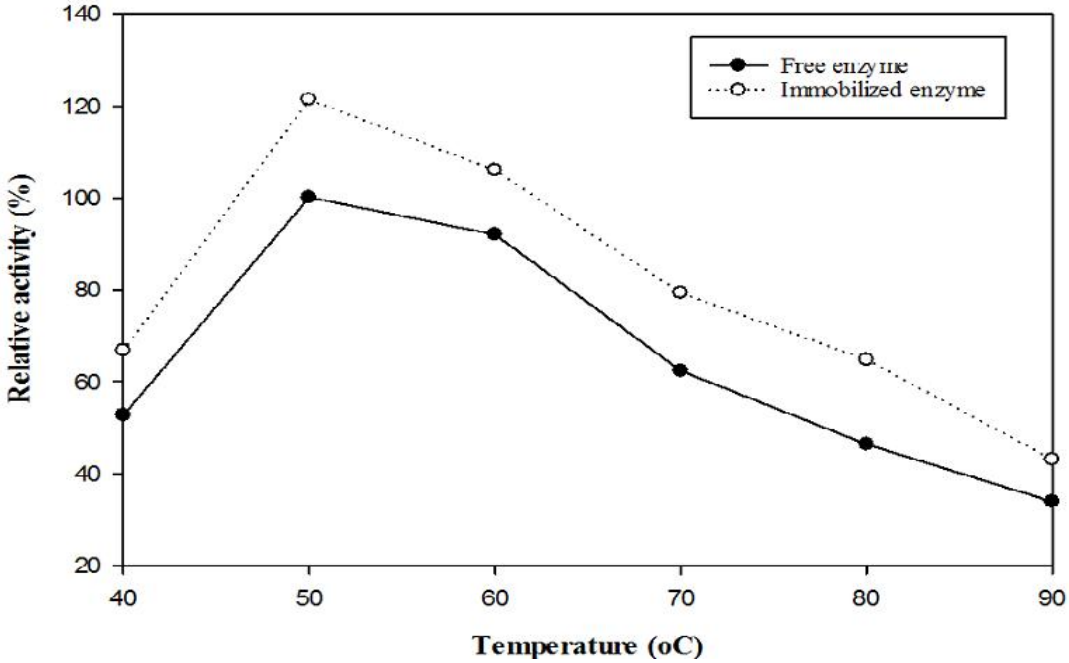


Fig. 48 Comparative account on the effect of temperature on the activity of free and alginate immobilized α-amylase of *B. sonorensis* GV2

However, unlike pH no shift in optimum temperature has been observed in the present study. Talekar and Chavare (2012) in their study of calcium alginate immobilized commercial alpha amylase, reported a shift in optimum temperature from 50 to 60^{o}C. In consonance to our findings, the immobilized enzyme was also found to be more temperature tolerant to the free enzyme. In contrary to our results, Dragomirescu *et al.* (2012) reported no much difference in the temperature tolerance potential of free and silica gel immobilized enzymes isolated from *B. amyloliquefaciens*.

4.7.6.3 Effect of divalent ions on the activity of free and immobilized enzyme

The effect of divalent ions on the activity of free and immobilized enzyme was investigated. For alginate immobilized *B. aerius* GC6 α-amylase, an increased enzyme activity was observed in case of $Mg^{2+,}$ Cu^{2+} and Ni^{2+} up to 119.24%, 99.48% and 107.67% as compared to the free enzyme (Fig. 49). Thus activation of enzyme activity, in the presence of these divalent ions became more pronounced in calcium alginate entrapped α-amylase. However, a decrease in the activity was observed in alginate immobilized enzyme in case on Mn^{2+}. It was also reported that immobilization prevented the inhibitory effect of Co^{2+}. Similarly for alginate immobilized *B. sonorensis* GV2 α-amylase, an increased

activity was observed for divalent ions Mn^{2+}, Mg^{2+} and Ni^{2+} up to 89.79%, 108.24% and 109.43% (Fig. 50). There was no effect of Cu^{2+} ions and the inhibitory effect of Co^{2+} has been reversed in the alginate entrapped enzyme.

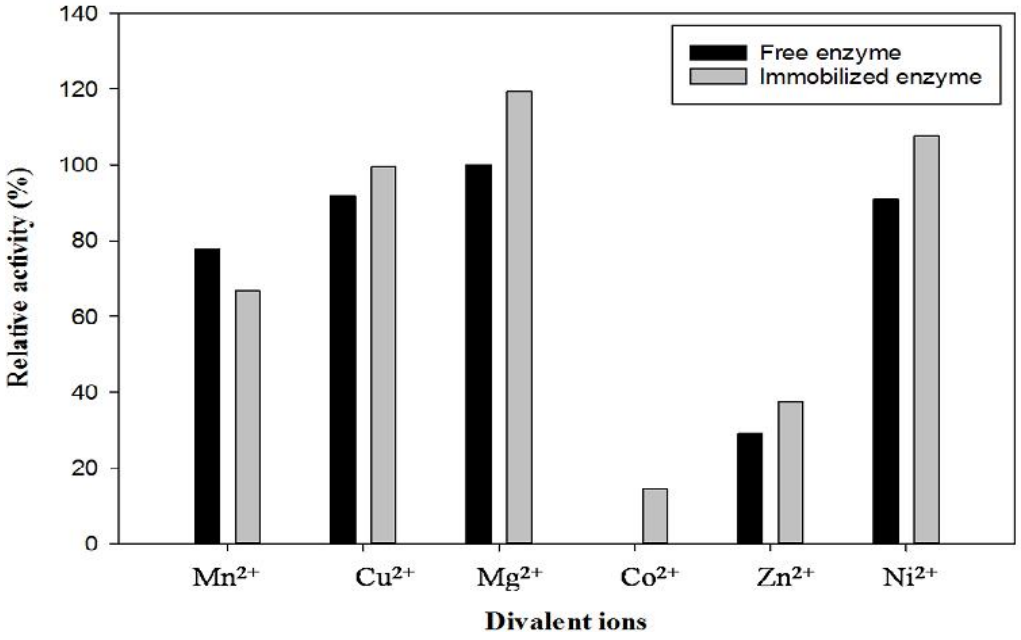


Fig.49 Comparative account on effect of divalent ions on the activity of free and alginate immobilized α-amylase from *B. aerius* GC6

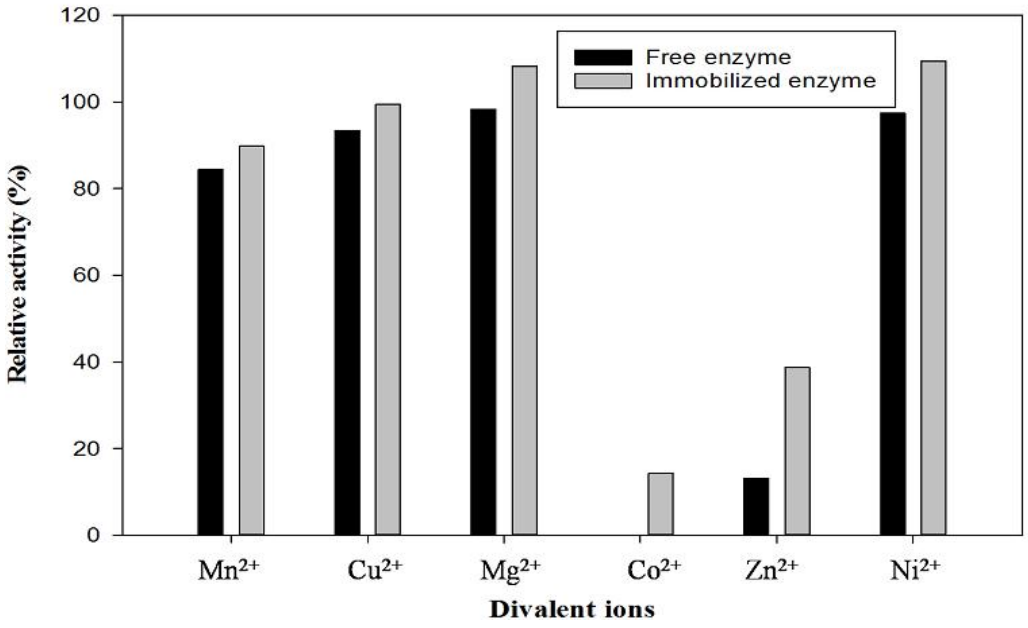


Fig.50 Comparative account on effect of divalent ions on the activity of free and alginate immobilized α-amylase from *B. sonorensis* GV2

Several workers have reported the influence of divalent ions on the activity of enzyme; however the nature of enzyme, source of isolation etc.

determines the action of divalent ions on a particular enzyme. In harmony to our findings Dey *et al.* (2003) reported an apparent increase in relative activity of calcium alginate entrapped *B. circulans* α-amylase in the presence of Mg^{2+} and Ca^{2+}. In contrary to our findings, Demirkan *et al.* (2011) reported an increase in relative activity in case of Ba^{2+} in calcium alginate immobilized *B. amyloliquefaciens* alpha amylase. However reduction in amylolytic potential of immobilized enzyme was observed in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}. In agreement to our findings however, a reversal of inhibition in case of Cu^{2+} have been observed.

4.8 IMMOBILIZATION OF PURIFIED α-AMYLASE FROM *B. aerius* GC6 AND *B. sonorensis* GV2 ONTO SYNTHETIC IRON OXIDE NANO PARTICLES

Natural carriers used for immobilization of enzyme are easy to handle and flexible but the retention and stability of enzyme in the beads or chips formed are the big lacunae in this technique. Nanoparticles are thus, attractive carrier for immobilization because of a higher specific surface area for loading a larger amount of enzyme, a lower mass transfer resistance for reacting substrates, non-toxicity and biocompatibility, and ease of separation from a reaction mixture by the application of magnetic field. Moreover their ability to retain enzyme for longer times make them even more beneficial as in enzyme technology, reuse of enzyme is of at most importance to reduce the process cost. However, it is difficult to directly immobilize enzymes onto the surface of pristine magnetite nanoparticles and maintain high catalytic activities. The surface modification of magnetite nanoparticles has therefore become indispensible for realizing their applications (Wang *et al.*, 2012).

Naked iron magnetic nanoparticles are generally unstable in strong acidic solutions and undergo leaching, which strongly limits the reusability and reduces the lifetime of such materials. The large ratio of surface area to volume brings in another limitation—aggregation of particles and thus a minimization in their surface energy occurs due to strong magnetic attractions between particles, limiting their dispersion in aqueous solutions and matrices. The exposition of the proteins/enzymes to such interfaces would result in the decrease or loss of the

activity. To overcome such limitations, various approaches are used to modify the surface via loading of other target chemicals or biological materials during or after the synthesis process. These techniques not only optimize the surface properties such as biocompatibility, dispersibility and biodegradability of iron magnetic nanoparticles, but also provide an environment for the transferring of hydrophobic iron oxide nanoparticles into a hydrophilic system. (Xu *et al.*, 2014).

Immobilization of *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase has been done by entrapment technique. Ascorbic acid modified iron oxide nano-particles were used for the immobilization of enzyme. Nano-particle immobilized α-amylase exhibited an immobilization efficiency of 121.48% for *B. aerius* GC6 and 122.66% for *B. sonorensis* GV2 as that of their counterparts.

4.8.2 Reusability of the nano-particle immobilized α-amylase

For the purified enzymes of *B. aerius* GC6 and *B. sonorensis* GV2 immobilized onto ascorbic acid modified iron oxide nano particles reuse efficiency was studied similarly by evaluating its starch hydrolysis efficiency. Purified *B. aerius* GC6 α-amylase showed a considerably good stability in nano-particles giving an immobilization efficiency of 87.48% at the end of 22nd cycle (Fig. 51). This showed that the ascorbic acid modified nano-particles are exceptionally good immobilization agent for *B. aerius* GC6 α-amylase. Similarly, for purified *B. sonorensis* GV2 α-amylase, the reuse studies showed a relative activity of 82.46% at the end of 20 cycles (Fig. 52). High retention of α-amylase for more than 20 cycles, on nano particles is one of the main highlights of this study suggesting a permanent mode of efficient immobilization for varied use in the relevant industry.

Several workers have reported immobilization of various enzymes on nanoparticles and have yielded results harmonious to our findings. Wang *et al.* (2012) immobilized lipase onto alkyl silane modified magnetic nanoparticles and observed 65% retention after 7 cycles. Ahmad *et al.* (2013) synthesized TiO_2 nanoparticles for immobilization of α-amylase and reported five reuses with 70% retention of activity. Kalburcu *et al.* (2014) reported six reuses with no apparent

loss in the enzymatic activity of immobilized α-amylase on p(HEMA-MAT)-Ni^{2+} nanospheres. However, this type of firm binding as shown in case of *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase for endless cycles has reported for the first time in the literature.

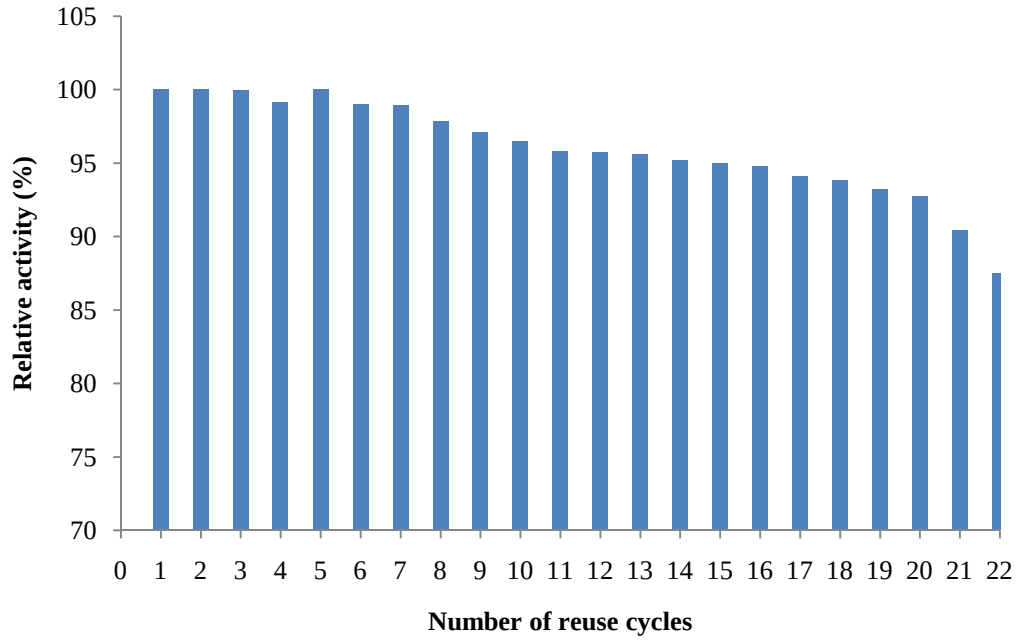


Fig. 51 Number of reuse cycles of nano particle immobilized purified *B. aerius* GC6 α-amylase

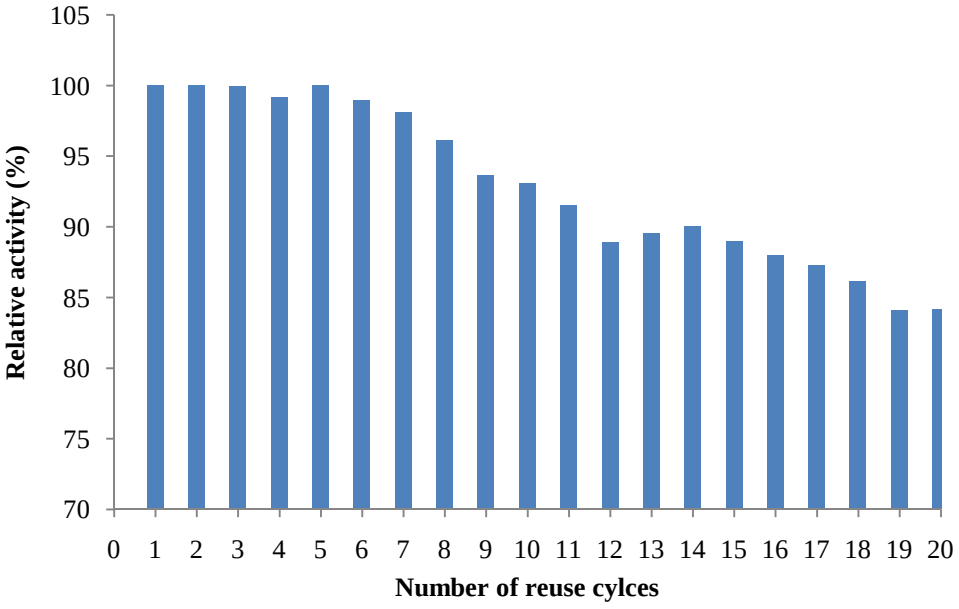


Fig. 52 Number of reuse cycles of nano particle immobilized purified *B. sonorensis* GV2 α-amylase

4.8.3 Characterization of nano particle immobilized *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase for enzymatic properties

4.8.3.1 Effect of pH on the activity of free and immobilized enzyme

The effect of pH on the activity of free and immobilized enzyme of *B. aerius* GC6 is given in Fig. 53. Optimum pH for free enzyme yielding highest α-amylase titres was considered as 100%. For purified α-amylase of *B. aerius* GC6 immobilized on magnetic nanoparticles, maximum relative activity of 119.24% was observed at pH 9.0. At pH 10.0 the relative activity of immobilized enzyme was 99.56% which was 86.54% for the free enzyme (Fig. 53). At higher pH of 11.0, comparatively very high relative activity i.e. 58.14% was observed. Thus a wider tolerance range of pH was observed for iron oxide nanoparticles immobilized enzyme. For *B. sonorensis* GV2 α-amylase, an increased relative activity of 124% was observed at pH 10.0 and 107.25% at pH 9.0. At higher pH i.e. 11.0, considerable activity of 78.14% was observed and at pH 12.0, 22.66% activity was observed (Fig. 54). In contrary to our findings, Jalal *et al.* (2011) immobilized α-amylase onto magnetic nanoparticles and observed that the immobilized enzyme was more sensitive to higher pH of 5-6 than the free enzyme.

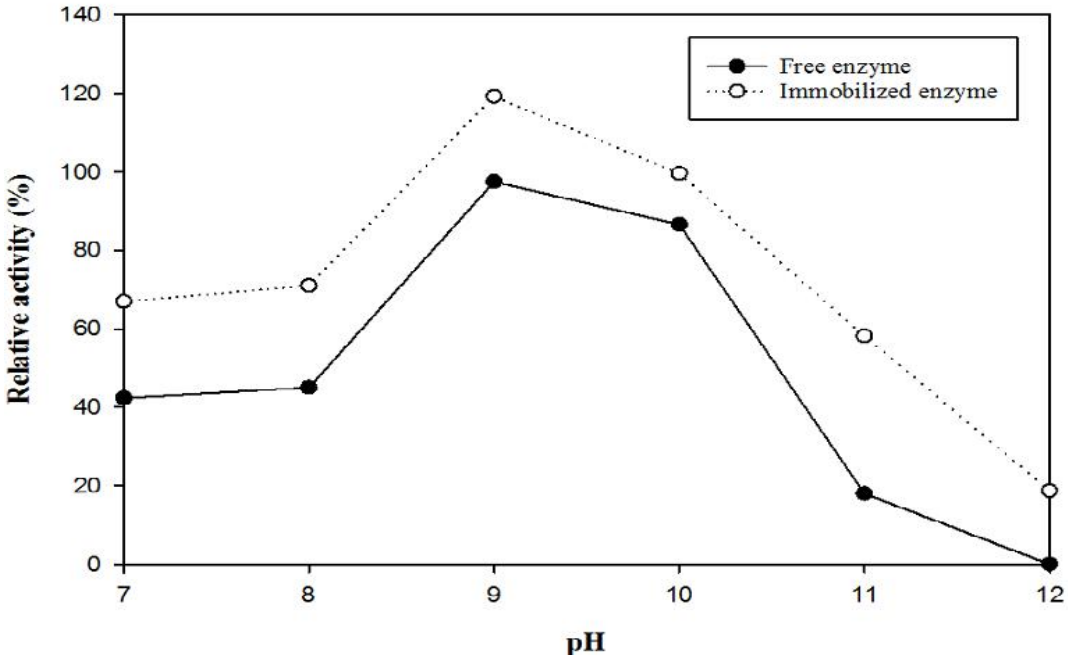


Fig. 53 Comparative account on the effect of pH on the activity of free and nano particle immobilized α-amylase of *B. aerius* GC6

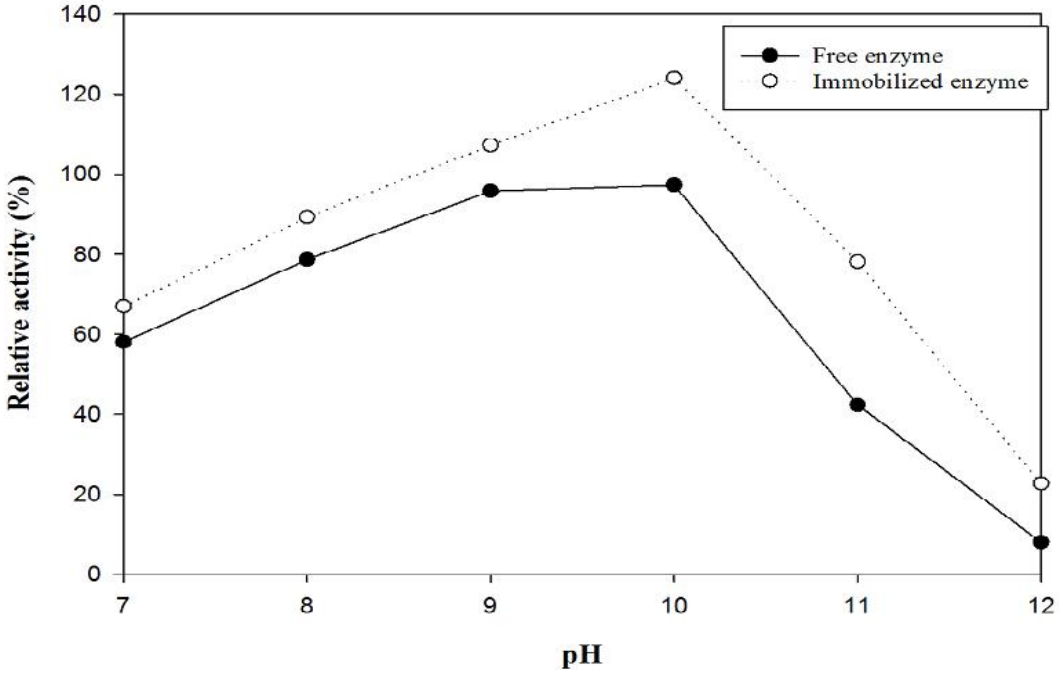


Fig. 54 Comparative account on the effect of pH on the activity of free and nano particle immobilized α-amylase of *B. sonorensis* GV2

4.8.3.2 Effect of temperature on the activity of free and immobilized enzyme

Enzyme activity also depends upon temperature. 50^{o}C has been observed as optimum temperature yielding highest enzyme titres with free enzyme. Considering this activity to be 100% (Fig. 55), nano particle immobilized *B. aerius* GC6 α-amylase exhibited an increased enzyme activity upto 121.26% at the same temperature. At an elevated temperature of 60^{o}C, 104.34% of the original activity was observed. Further increase in temperature to 70^{o}C revealed the activity as 87.98% of the original activity. The immobilized enzyme resulted in comparatively higher enzyme titres i.e. 44.67% at increased temperatures upto 90^{o}C than the free enzyme. Thus it is evident from the data that the iron oxide nano particle immobilized enzyme possessed greater temperature resistant as compared to the free enzyme.

Similarly for *B. sonorensis* GV2 α-amylase an increased activity of 119.33% was observed at 50^{o}C. A further increase in temperature to 60^{o}C resulted in activity of 106.12%, which were 79.79, 48.66% at 70 and 80^{o}C respectively (Fig. 56). At 90^{o}C, a relative activity of 36.78% was observed which was far higher than the free enzyme. A similar increase in activity at higher

temperatures (50-90^{o}C) has been shown by Demir *et al.* (2012) while immobilizing α-amylase onto $CaCO_3$ nanoparticles. Ahmad *et al.* (2013) observed nano- TiO_2 immobilized amylase to be active at 60^{o}C for 60 min thus revealing the ability of immobilized amylase to withstand higher temperatures.

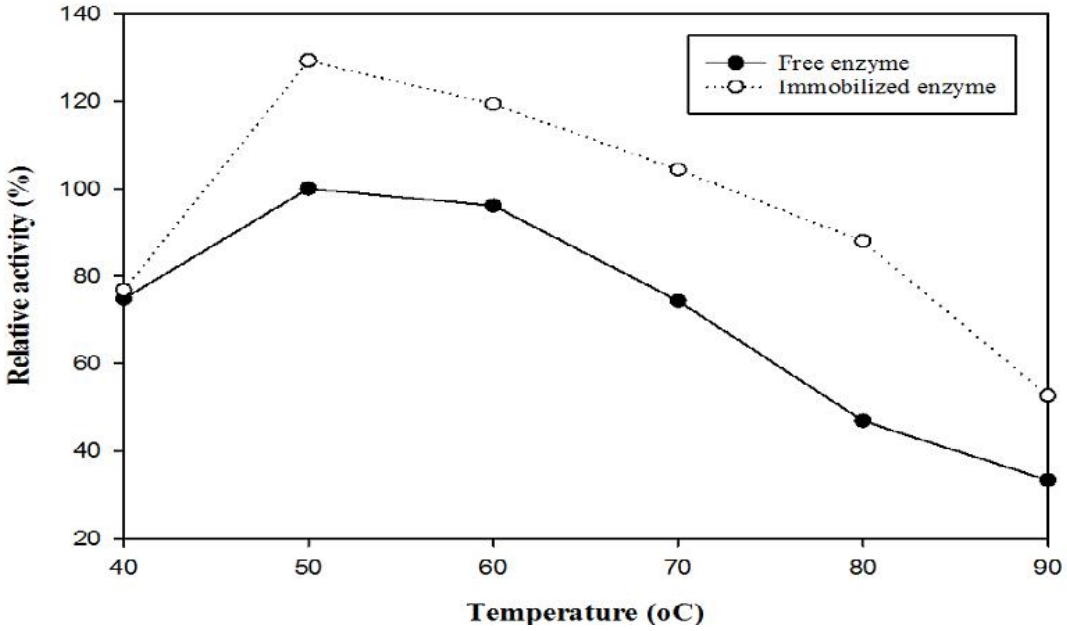


Fig. 55 Comparative account on the effect of temperature on the activity of free and nano particle immobilized α-amylase of *B. aerius* GC6

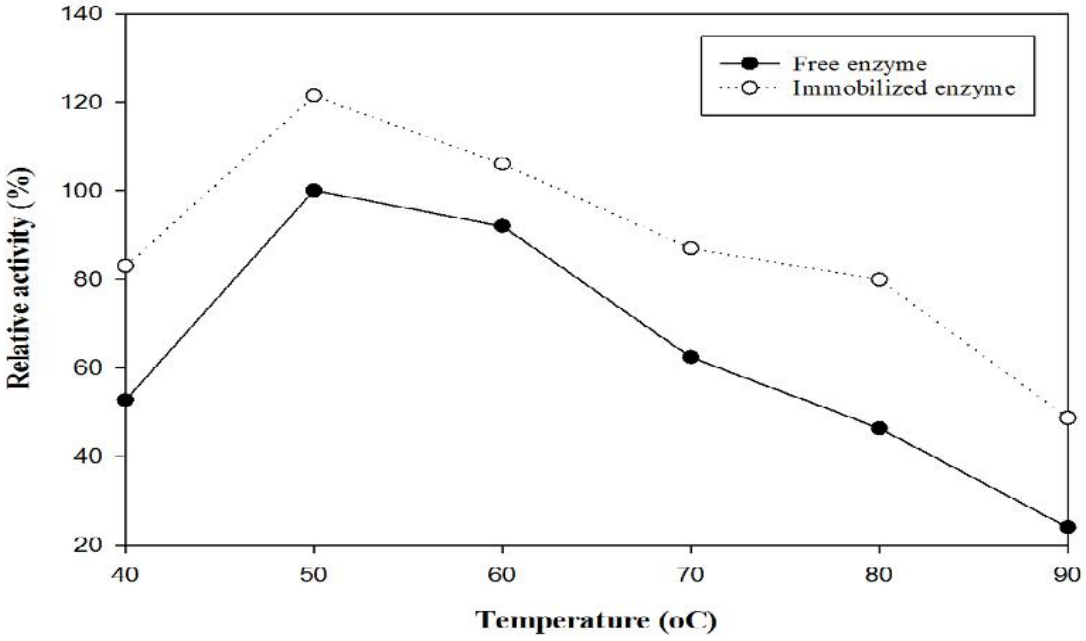


Fig. 56 Comparative account on the effect of temperature on the activity of free and nano particle immobilized α-amylase of *B. sonorensis* GV2

4.8.3.2 Effect of divalent ions on the activity of free and immobilized enzyme

The effect of divalent ions on the activity of free and immobilized enzyme was investigated. For nano particle immobilized *B. aerius* GC6 α-amylase, an increased enzyme activity was observed in case of Mn^{2+}, $Mg^{2+,}$ Cu^{2+}, Zn^{2+} and Ni^{2+} up to 96.79%, 112.24%, 109.48%, 43.22% and 123.64% as compared to the free enzyme (Table 41). It was also reported that immobilization prevented the inhibitory effect of Co^{2+}. Similarly for alginate immobilized *B. sonorensis* GV2 α-amylase, an increased activity was observed for divalent ions Mn^{2+}, Cu^{2+}, Mg^{2+} and Ni^{2+} up to 92.66%, 128.11%, 111.69% and 116.29% (Table 41). The inhibitory effect of Co^{2+} has been reversed in nano particle entrapped enzyme.

Table 41. Effect of metal salts on residual activity (%) of the free and the nano-particle immobilized *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Divalent ions	Relative activity (%)			
	***B. aerius* GC6**		***B. sonorensis* GV2**	
	Free enzyme	**Immobilized enzyme**	**Free enzyme**	**Immobilized enzyme**
Mn^{2+}	82.80±0.43[a]	96.79±0.23[a]	86.82±0.12[a]	92.66±0.22[a]
Cu^{2+}	92.02±0.23[b]	109.48±0.29[b]	93.27±0.20[b]	128.11±0.31[b]
Mg^{2+}	136.11±0.36[c]	112.24±0.17[c]	139.42±0.19[c]	111.69±0.29[c]
Co^{2+}	0.00±0.00[d]	14.34±0.04[d]	0.00±0.00[d]	17.46±0.11[d]
Zn^{2+}	2.91±0.16[d]	13.22±0.13[d]	16.92±0.32[e]	23.46±0.16[d]
Ni^{2+}	91.02±0.34[e]	123.64±0.65[e]	96.24±0.29[b]	116.29±0.26[c]
SE (m)	**0.25**	**0.25**	**0.18**	**0.22**

4.9 APPLICATION OF PURIFIED α-AMYLASE IN BIOETHANOL PRODUCTION USING ALGAL BIOMASS

The world is facing a decline of fossil fuel resources, petroleum, natural gas and charcoal while energy requirements are progressively growing up. According to a research report, the world's oil and gas supply will disappear within three decades and also other natural resources such as coal will extinct within a century (Raja *et al.,* 2008). Moreover, traditional sources of fossil fuels are coupled with accumulation of green house gases derived from their

combustion have been turning them into unsustainable pillars of energy supply. The overexploitation of fossil fuels and the increasing greenhouse gases are considered to be the forthcoming crisis for human's population. Especially, carbon dioxide, which is the main component of greenhouse gas emissions, poses great challenge to the world. The inexorable usage of fossil fuels has contributed to approximately 98% of carbon emission from its combustion into the environment. Hence the fossil fuel usage has to be reduced in order to decrease the amount of carbon dioxide and other pollutants being emitted (Demirbas *et al.*, 2011).

Currently, as an alternative, most of the countries have imposed regulations/ legislations to use alternate/ renewable sources such as wind, hydro and solar energy to replace fossil fuel usage. Ethanol has emerged as an attractive alternative to the liquid petroleum resulting in worldwide search for its higher yield to overcome its limited supply (EIA, 2002). It has a number of advantages such as high biodegradability, minimal toxicity, minimal hydrocarbon emission, free from sulphur and aromatic compounds (Demirbas *et al.,* 2011; Atabani *et al.*, 2012). Ethanol is thus the most sought after biofuel which is currently being produced on a large scale, as it can be easily mixed with gasoline and used in internal combustion engine vehicles (Haveren *et al*., 2008).

Since India is the fourth (energy data) largest global contributor to carbon emissions, GOI transport policy has targeted EURO-III and IV vehicle emission norms for vehicles, which in turn require adoption of clean and green fuel. The Government of India (GOI) has approved the National Policy on Biofuels on December 24, 2009. The policy encourages use of renewable energy resources as alternate fuel to supplement transport fuels and had proposed an indicative target to replace 20 percent of petroleum fuel consumption with biofuels (bioethanol and biodiesel) by end of 12^{th} Five-Year Plan (2017). Thus the steady demand for ethanol coupled with an expected rise in blending for Ethanol Blending Program (EBP) will push total ethanol consumption heavily.

Biofuels seek to supplement conventional energy resources for meeting rapidly increasing requirement of transportation fuels and meeting energy needs

of India's vast rural population by use of non-food feed stocks and as a result to reduce dependence on import of fossil fuels. The intent is to provide a higher degree of national energy security in an environmentally friendly, cost-effective and sustainable manner. Believing India to be endowed with significant potential for generating energy through renewable resources, the GOI is promoting and encouraging production and use of a) ethanol derived from sugar molasses/juice for blending with gasoline and b) biodiesel derived from inedible oils and oil waste for blending with diesel. Presently, production of biodiesel from jatropha seeds is commercially negligible. Slow progress made towards jatropha planting has resulted in lower availability of jatropha seeds to be used as feed-stock for biodiesel production. According to India Biofuel Annual (2013), domestic ethanol production for CY 2014 declined to 1.9 billion liters due to a downswing in India's sugar production cycle (October-September) for second consecutive year. Steady demand for ethanol coupled with an expected rise in blending for Ethanol Blending Program (EBP) will push total ethanol consumption in CY 2013 to 2.4 billion liters.

In view of the above facts, India poses a grim picture on international platform as far as biofuel production is concerned. The constraints imposed by the first and second generation biofuels have paved the way for use of algal biomass for bioethanol production as a third generation fuel. Therefore, development of this kind of third generation biofuel technology and its transfer definitely will help our technology starved industries immensely besides being highly eco friendly approach. Himachal Pradesh being a hilly state has a suitable climate as well as many sources of fresh water such as rivers, lakes, ponds and traditional bawris etc. which is a home for varied aquatic life and algae is one of the prominent forms. In addition to a large number of organic compounds such as vitamins, minerals, lipids and proteins, algae contains up to 60% carbohydrates. So far, the use of algae as an industrial raw material is rare compared with the diversity of algal species in HP. The chemical composition of algal cells renders it a potent industrial raw material and an alternative solution for the energy crisis. An extensive research is needed to obtain bioethanol from algae by fermentative processes.

4.9.1 Collection and identification of algal biomass

The fresh water green algae were collected from fresh water ponds of Solan, Himachal Pradesh. Algal biomass was identified on field using the Algal Identification Field Guide (Huynh and Serediak, 2006). It was found to have green hollow mesh or net like structure of its membrane like colonies. The single or multi layered free floating or secondarily attached net was the characteristic feature of the algae.

Further morphological examination under microscope revealed that the cells were typically arranged in pentagons or hexagons. The cells were cylindrical to oblong (Plate 6), upto 15 mm long. Adjacent cells (2-4) have connected through their edges forming 5-6 sided mesh. Microscopic examination of single cell revealed the presence of long cylindrical non-septate cells which were multinucleate in nature. Reticulate chloroplasts were visible. On the basis of morphological identification on field and under the microscope, it was identified as fresh water green algae *Hydrodictyon* sp. (Gupta, 1981)

4.9.2 Classification

Class: Chlorophyceae
Order: Cholorococcales
Sub-order: Zoosporineae
Family: Hydrodictyaceae
Genus: Hydrodictyon

Hydrodictyon sp. or 'Water Net' is an invasive species, which has been found erratically all around the globe. Water nets frequently form extensive surface and sub-surface mats in ponds and lakes in the summer months. They are sometimes considered weeds or pest organisms because they are so prolific that they can overwhelm lakes, ponds, aquacultures and even rice fields (Kelly *et al.*, 2003). Water net is a serious nuisance alga because it clogs water ways, irrigation ditches, taints potable water, causes economic losses to fresh water fisheries, aquatic plantation and has an adverse impact upon boating, fishing, swimming and tourism. It also smothers the beds of aquatic macrophytes causing their

collapse and decay. On beaches, water net accumulates in large drifts, decaying and causing localized anoxic conditions (Natural history museum, 2013).

Due to its negative impacts on the environment and biodiversity, its management presents a great environmental challenge. Thus, its use as a source of reducing sugars for production of ethanol seems promising. Its cultivation does not need any specific nutrient to be added in the medium, thus can be easily cultivated and collected from the fresh water bodies. This is the first report presented on the use of *Hydrodictyon* sp. as a substrate for the production of reducing sugars which in turn can be converted into bioethanol.

4.9.3 Biochemical characterization of algal biomass

Sufficient carbohydrate content is required to use algae as a feed stock biomass for ethanol production. Therefore, biochemical characterization of the biomass to be used for the production of ethanol is the first and foremost step. The quality of reducing sugars presents the most important aspect of the quantity of ethanol produced. Biochemical analysis of *Hydrodictyon* biomass revealed that it majorly (~ 85%) contained starch and cellulose (Table 42) which being easily hydrolysable by enzymatic action renders this biomass a preferred feedstock for biofuel industry. Additionally the complete absence of hemicelluloses is another advantage which makes this biomass easier to hydrolyse in the absence of lignin.

Table 42. Biochemical composition of *Hydrodictyon* biomass

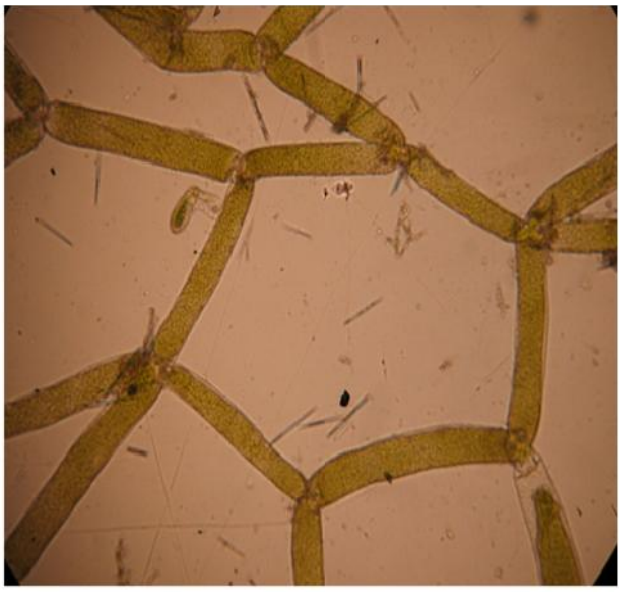
Constituent	Amount (%)
Starch	64.80
Cellulose	24.44
Hemicelluloses	0.00
Pectin	6.76
Protein	3.28

4.10 PHYSICOCHEMICAL PRETREATMENT OF ALGAL BIOMASS

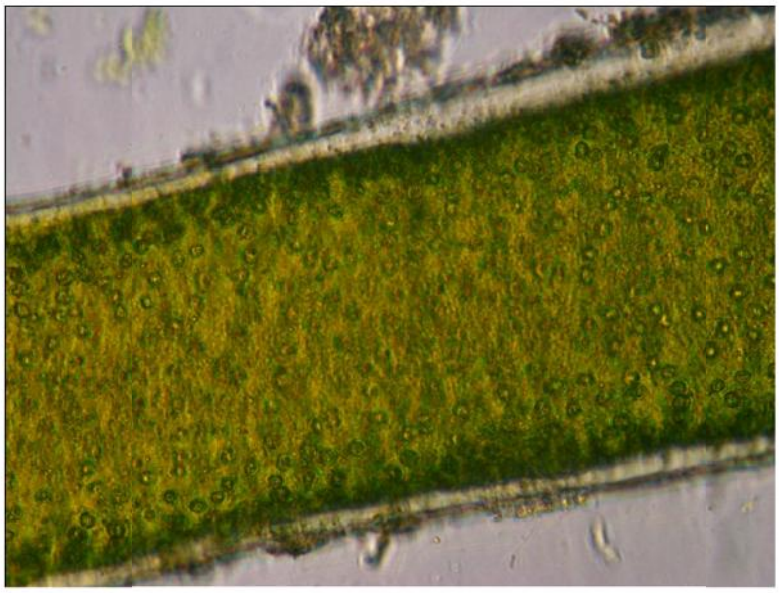
The carbohydrates in green algaemainly come from starch in chloroplasts and cellulose/ polysaccharides on cell walls (Domozych *et al.*, 2012; Richmond, 2004), which are not readily fermentable for ethanol production by microorganisms. Therefore, prior to ethanol fermentation, the polysaccharides of



(a)



(b)



(c)

Plate 6 **Identification of algal biomass (a) *Hydrodictyon* sp. growing in pond (b) Microscopic view (20X) of polygonal mesh of *Hydrodictyon* sp. (c) Microscopic view of single filament (45X)**

algae should be hydrolysed to fermentable sugars (Hahn-Hagerdal *et al.*, 2007). Biomasspretreatment is thus a crucial step because it is essential for the fermentable sugars to be released and made available for the fermentation process. However, since the pretreatment process contributes significantly to the cost of production, the most economical pretreatment must be selected besides its efficacy. An ideal biomass pretreatment procedure must be energy efficient and cost effective (Rabelo *et al.*, 2009).

The chemicals commonly applied in pretreatment process are either acid (HCl or H_2SO_4) or alkali (lime or NaOH), which are everyday industrial chemicals carrying minimal toxicity in their applied concentrations. Different physio-chemical pretreatments which have been standardized for lignocellulosic biomass in our research laboratory were tried for pretreating algae first handedly. Supernatant and residue both were taken as a source of reducing sugars.

Alkaline peroxide ($NaOH+H_2O_2$) pretreated algal biomass yielded 43.02 mg sugars per gram of the biomass. Acidic peroxide ($H_2SO_4+H_2O_2$) pretreated biomass yielded 64.69 mg/g sugars. NaOH+urea yielded 34.94 mg/g sugars (Table 43). Dilute acid i.e. 1% H_2SO_4 pretreatment yielded highest 76.32 mg/g sugars whereas dilute alkali i.e. NaOH (1%) pretreatment yielded 37.93 mg/g sugars. Acetic acid/ nitric acid yielded 29.59 mg/g sugars. Saturated steam under pressure when used as a means of pretreatment yielded50.70 mg/g sugars.

Table 43. Evaluation of different pretreatments for saccharification of *Hydrodictyon* biomass

Sr. No.	Pretreatment	Reducing sugars (mg/g biomass)		
		Supernatant	Residue	Total
1.	**$NaOH+H_2O_2$**	31.02±0.21[a]	12.00±0.24[a]	43.02±0.24[a]
2.	**$H_2SO_4+H_2O_2$**	56.45±0.13[b]	08.24±0.34[b]	64.69±0.33[b]
3.	**NaOH+ Urea**	27.94±0.09[c]	07.00±0.19[c]	34.94±0.19[c]
4.	**H_2SO_4**	**72.00±0.11[d]**	**04.32±0.23[d]**	**76.32±0.21[d]**
5.	**NaOH**	28.92±0.13[c]	09.01±0.12[e]	37.93±0.23[e]
6.	**Acetic acid/ Nitric acid**	20.47±0.12[e]	09.12±0.16[e]	29.59±0.32[f]
7.	**Saturated steam under pressure**	40.67±0.23[f]	10.03±0.24[f]	50.7±0.29[g]
	SE(m)	**0.14**	**0.21**	**0.25**

Alkaline peroxide pretreatment which was adjudged as best for *Populus deltoides* wood (Sharma and Sharma, 2013) yielded 43.02 mg/g sugars, best among the alkaline pretreated algal biomass. NaOH+urea (Tandon, 2014) pretreatment which was adjudged best for *Pinus roxburghii* wood yielded a maximum of 34.94 mg/g sugars. Alkaline treatment disrupts the cell wall of lignocellulosics dissolving hemicelluloses, lignin and silica by hydrolyzing uronic and acetic esters, and by swelling cellulose (Pandey *et al.*, 2000b). Crystallinity of cellulose is decreased due to swelling. As algal biomass did not consist of lignin or hemicellulose fractions thus alkali was unable to hydrolyse the biomass efficiently which made it unavailable as a compatible substrate for enzymatic action. The main constituent of *Hydrodictyon* sp. i.e. starch when reacts with NaOH leads to the formation of alkoxides. These alkoxides react with sodium and result in the surface degradation of the starch molecules which makes them unsuitable for the enzymatic action.

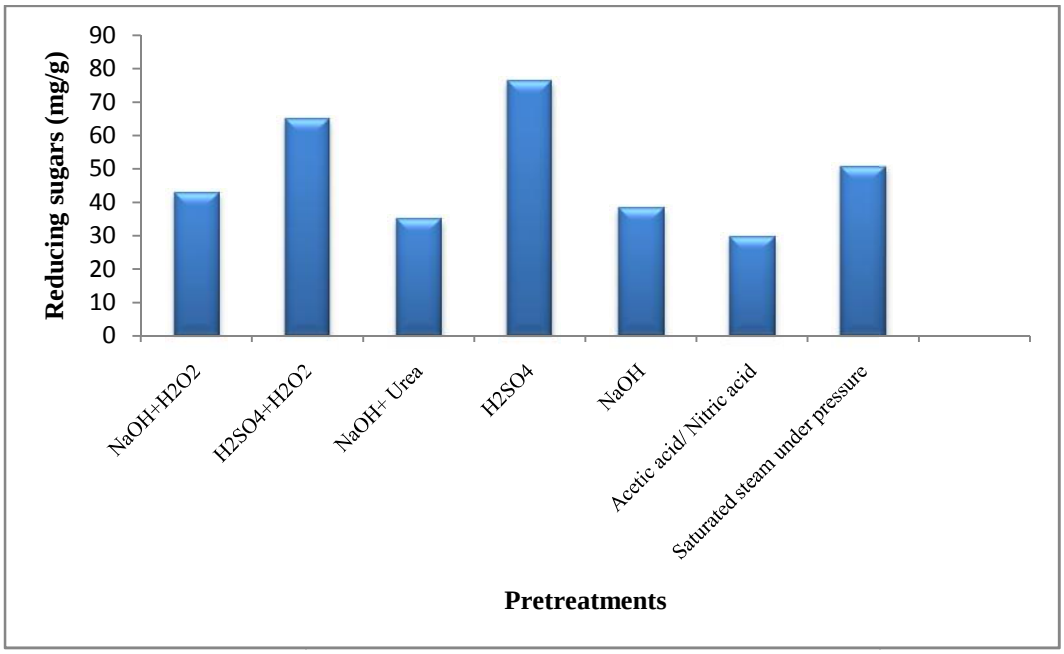


Fig. 57 Comparison of different physico-chemical pretreatments for the pretreatment of *Hydrodictyon* biomass

Acid pretreatment is usually carried out using mineral acids like H_2SO_4 and HCl. Dilute acid pretreatment is a simple single-stage process in which biomass is treated with dilute sulfuric acid at suitable acid concentrations and temperatures for a period of time. *Hydrodictyon* biomass when pretreated with

1% H_2SO_4 yielded 76.32 mg of sugars per gram of biomass which were comparatively very high when alkaline pretreatment was compared (Table 43). H_2SO_4+H_2O_2 pretreatment which was adjudged best for *E. teretecornis* and *P. deltoids* biomasswas found yielding 64.69 mg/g sugars (Fig. 57). Starch and cellulose, the main constituents of algal biomass when react with dilute acids, result in the formation of glucans which can be easily acted upon by appropriate enzymes for their further hydrolysis into simple hexoses. Thus, following dilute acid treatment, appropriate enzymes are needed for complete hydrolysis of the remaining carbohydrates in the treated biomass. Based on the highest reducing sugars produced, dilute H_2SO_4 pretreatment was selected for further hydrolysis of algal biomass.

The noticeably high sugar yield obtained from dilute acid pretreatment could be due to the chemical form of the microalgae-based carbohydrates. It is known that starch can be easily hydrolysed with dilute acids (John *et al.*, 2011), but for lignocellulosic material derived from terrestrial plants, dilute acid usually acts as a form of pretreatment rather, than hydrolysis due to their complex structure arising from the presence of lignin (John *et al.*, 2011). However, algal cellulose being lignin free is thus easily hydrolysable with dilute acid. This makes the sacccharification of microalgae biomass much easier than that of lignocellulosics and this is a clear advantage of using microalgae as the sugar substrate for fermentation compared to using lignocellulosic feedstock. However, acetic acid & nitric acid combination of pretreatment yielded only 29.59 mg/g sugars (Table 43). Low yield of hydrolysed sugars with this combination may be due to the weak acid combination which led to incomplete the ionization resulting in reduced hydrolysis efficiency.

So far there are scanty reports on physico-chemical pretreatment of algal biomass. Eshaq *et al.* (2011) chemically pretreated *Spirogyra* biomass with 1% NaOH for 2h whereas Sulfahri *et al.* (2011) pretreated the *Spirogyra* biomass at 100 ^oC for 2h followed by its enzymatic hydrolysis.In conformity to our findings, a few workers have reported dilute acid pretreatment for algal biomass. *Gracilaria salicornia* biomass when pretretated with 2% H_2SO_4 for 30 min at 120 ^oC, yielded 4.3 mg sugars/ g biomass as reported by Wang *et al.* (2011). Ho *et al.*

(2013) reported 1% H_2SO_4 as best pretreatment for efficient hydrolysis of *Chlorella vulgaris* biomass with 96% hydrolysis efficiency. Markou *et al.* (2013) investigated the effect of four acids viz. H_2SO_4, HNO_3, H_3PO_4 and HCl on the hydrolysis of *Arthrospira platensis* biomass and observed 0.5N HNO_3 as best pretreatment. However this is the first report on the pretreatment of *Hydrodictyon* biomass for reducing sugars production.

4.10.1 Optimization of selected (dilute acid) pretreatment of algal biomass

Dilute acids break down the starch and cellulose polymers in biomass to release individual sugars, which can be fermented into ethanol (Lee *et al.*, 1999; Lenihan *et al.*, 2010). Kinetic studies on the dilute acid hydrolysis of various starchy and cellulosic materials indicated that the hydrolysis kinetic parameters are strongly dependent on the substrate and acid concentration, temperature and reaction time (Malester *et al.*, 1988; Lenihan *et al.*, 2010). There are however, a very few reports available in literature regarding the acid pretreatment strategy for bioethanol production from green alga. Thus, studies on the effects and optimization of the process variables that influence the performance of the algal acid pretreatment process will be essential to the commercial outlook of bioethanol development from green algae.

Table 44. Optimization of H_2SO_4 pretreatment conditions for *Hydrodictyon* biomass

Time (min)	Reducing sugars (mg/g biomass)		
	0.5% H_2SO_4	**1.0% H_2SO_4**	**1.5% H_2SO_4**
30	30 ± 0.11^{a}	55 ± 0.21^{a}	50 ± 0.21^{a}
60	70 ± 0.09^{b}	60 ± 0.11^{b}	47.5 ± 0.17^{b}
90	80 ± 0.13^{c}	72.5 ± 0.4^{c}	52.5 ± 0.19^{c}
120	85 ± 0.06^{c}	75.5 ± 0.06^{c}	55 ± 0.20^{c}
150	97.5 ± 0.11^{d}	$67.5\pm.16^{d}$	27.5 ± 0.13^{d}
180	**120 ± 0.23^{e}**	62.5 ± 0.21^{e}	30 ± 0.22^{e}
210	112.5 ± 0.34^{f}	55 ± 0.22^{a}	30.25 ± 0.24^{e}
240	115 ± 0.23^{f}	42.5 ± 0.20^{f}	32.5 ± 0.11^{e}
SE(m)	**0.16**	**0.19**	**0.18**

Keeping in view these facts, conditions for dilute acid pretreatment i.e. time of acid hydrolysis and concentrations of dilute acid were optimized for the efficient degradation of algal biomass into simple sugars. Concentrations ranging from 0.5-1.5% were used for a time spanning 30 to 240 min at room temperature. The results obtained revealed the best combination as 0.5% H_2SO_4 for 180 min yielding maximum reducing sugars of 120 mg/g biomass (Table 44). At the same concentration, an increase in hydrolysis time did not result in net increase in the sugars concentration. The increased acid concentrations on the other hand, to 1% or 1.5% have resulted in decreased sugar yield to 72.5 mg/g biomass. A lowest yield of 27.5 mg/g sugars was obtained at 1.5% H_2SO_4 for 150 min. Theoretically and practically, the lowest acid concentration that can reach the highest hydrolysis performance would be the most favourable. Thus 0.5% H_2SO_4 for 180 min at room temperature was chosen for further studies (Plate 7b).

A review of the literature available reported a few studies with higher concentrations of H_2SO_4 ranging from 1-2.5% at a temperature exceeding 100^oC as best yielding. Kim *et al.* (2011) yielded a maximum of 9.4 mg/g reducing sugars from *Ulva lactuca* biomass with 1.0% H_2SO_4 for 30 min at 121^oC. Ho *et al.* (2013) reported a 96% hydrolysis at 121^oC from 1.0% H_2SO_4 from carbohydrate rich microalga *Chlorella vulgaris*; similarly, Trivedi *et al.* (2013) adjudged 1% H_2SO_4 at 100^oC for 1h to be the best yielding combination for macrophytic green alga *Ulva fasciata.* The pretreatment conditions reported in the present study are the most eco-friendly and cost effective. Such lower concentrations of acid would lead to the generation of least amount of inhibitors if any, moreover, the treatment being carried out at room temperature saves the energy cost for heating the preparations at very high temperatures thus making the pretreatment economically viable.

4.11 ENZYMATIC HYDROLYSIS OF ALGAL BIOMASS

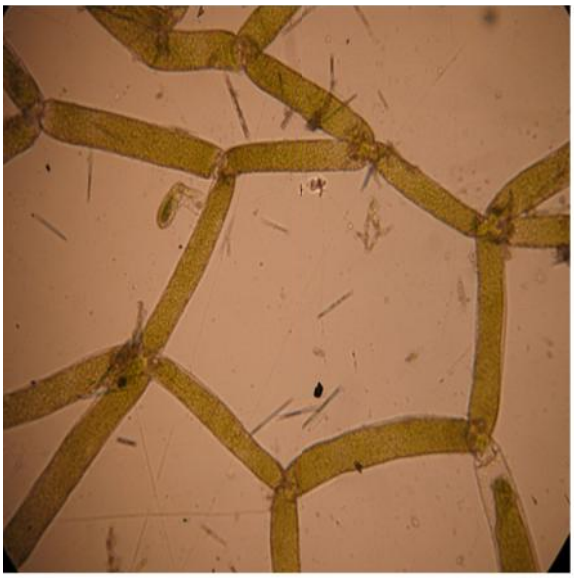
Mixture of inhouse enzymes was used for the enzymatic hydrolysis of dilute acid pretreated algal biomass. α-amylase, β-amylase, cellulase and pectinases were used for the hydrolysis of pretreated algal biomass to simple sugars. Behaviour of individual enzymes on the saccharification of *Hydrodictyon*

biomass was evaluated. 0.5% H_2SO_4 pretreatedbiomass was subjected to hydrolysis for 72h at 45^oC in the presence of hydrolytic enzymes obtained from inhouse microorganisms. α + β-amylase, yielded maximum reducing sugars of 472.22 mg/g biomass, followed by cellulase yielding 202.53 mg reducing sugars /g biomass (Table 45). Pectinases yielded least reducing sugars of 109.12 mg/g biomass. Total hydrolytic enzymes when used in a mixture yielded 783.87 mg/g biomass (Plate 7c). Same hydrolysis when carried out using commercial enzymes yielded 642.44 mg/g biomass, thereby proving use of inhouse enzymes to be more efficient.

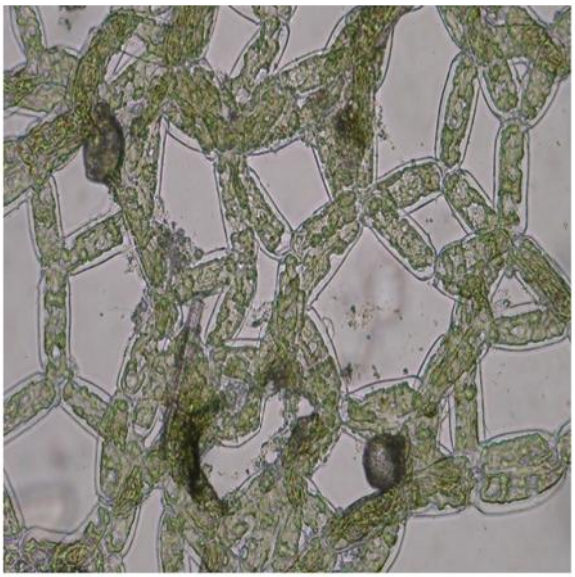
Different enzymatic ratios (per ml basis) for the mixture of enzymes were standardized for the hydrolysis (Table 46). Perusal of Table 46 revealed that maximum reducing sugars of 810 mg/g biomass were obtained with an enzymatic ratio of 4:3:2:1 comprising of α-amylase, β-amylase, cellulase and pectinases, followed by 790 mg sugars /g biomass with a ratio of 3:4:2:1. 780 mg/g of reducing sugars was produced at an enzymatic ratio of 3:3:3:1. Lowest reducing sugar yield of 600 mg/g has been obtained with an enzymatic ratio of 2:2:3:3. Reducing sugars produced with a particular enzymatic ratio revealed that the presence of α-amylase highest in the ratio led to the release of most reducing sugars, thereby strongly advocating the role these enzymes have in the hydrolysis of *Hydrodictyon* biomass owing to the amount of starch it contains.

Table 45. Evaluation of behaviour of individual enzyme on saccharification of *Hydrodictyon* sp. biomass

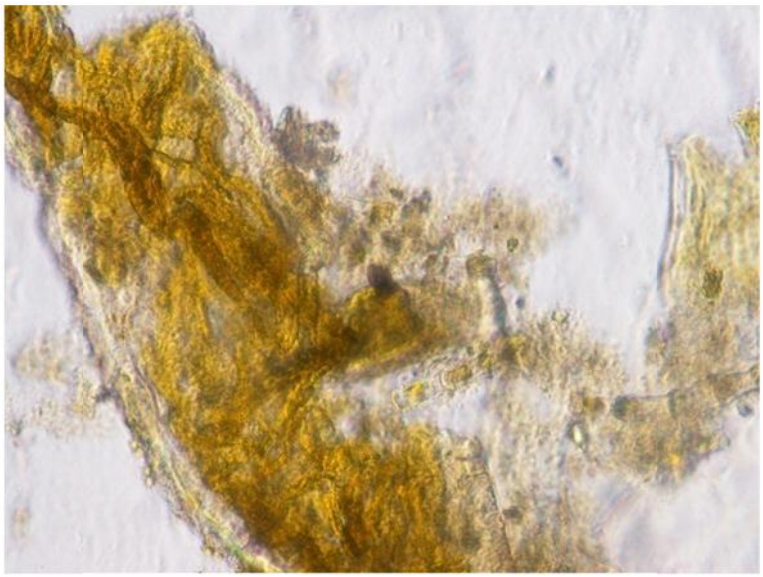
Enzyme	Reducing sugars (mg/g biomass)
α + β-amylase	472.22±0.39^a
Cellulase	202.53±0.23^b
Pectinase	109.12±0.33^c
Total enzyme (in house)	783.87±0.21^d
Synthetic enzyme	642.44±0.34^e
SE (m)	**0.3**



(a)



(b)



(c)

Plate 7. Surface analysis of *Hydrodictyon* biomass usinf imaging microscope at 45x magnification (a) Native algal cells (b) H_2SO_4 (0.5%) pretreated algal cells (c) Enzymatically hydrolysed algal cells

Table 46. Optimization of different ratio of enzymes for enzymatic hydrolysis of *Hydrodictyon* sp. biomass

Enzymatic ratios	Reducing sugars (mg/g)
3:3:3:1	780±0.56[a]
2:2:4:2	660±0.34[b]
2:3:4:1	640±0.23[b]
3:4:2:1	790±0.21[c]
2:2:2:1	600±0.19[d]
4:3:2:1	**810±0.22[a]**
SE (m)	**0.29**

A few workers have used acid pretreatment followed by enzymatic hydrolysis as a means for efficient saccharification of algal biomass. Ho *et al.* (2013) used cellulase and amylase enzymes obtained from *Pseudomonas* sp. CL3 whereas Trivedi *et al.* (2013) utilized commercial cellulase enzymes for the complete hydrolysis of *C. vulgaris* and *U. fasciata* biomass respectively. Mollers *et al.* (2014) used a mixture of enzymes viz. lysozyme, alpha-glucanases and amyloglucosidase for complete hydrolysis of *Synechococcus* biomass. A mixture of enzymes composed of amylase, cellulase, pectinases, β-glucosidase, chitinase, lysozyme and sulfatase was used for hydrolysis of *C. vulgaris* biomass by Kim *et al.* (2014).

4.11.1 Optimization of enzymatic hydrolysis conditions by using CCD of RSM

The enzymatic ratio yielding highest reducing sugars was chosen for the further optimization studies. Other parameters standardized were enzyme loading, incubation period and biomass to liquid ratio. The optimizations were done by applying CCD of RSM.

4.11.1.1 Regression model of response

Table 6 (Section 3.10.2) shows the maximum and minimum levels of variables chosen for trials (Run) in the CCD. For RSM based on the Central Composite Design, used for the optimization of independent variables for amylase production, 20 experimental runs with different combinations of three

factors were carried out. The independent variables used for the factorial analysis were biomass (X_1), enzyme loading (X_2) and incubation period while the dependent variable selected was reducing sugars. The complete experimental design with coded factors and response values has been given in Table 47, which shows considerable variation in the amount of reducing sugars production depending upon the interaction of various levels of three independent variables in the medium.

Table 47. Complete experimental design with independent and response variables for optimization of conditions for enzymatic hydrolysis of algal biomass

Std	Run	Block	Factor 1: Biomass (X_1)	Factor 2: Enzyme loading (X_2)	Factor3: Incubation period (X_3)	Response: Reducing sugars (mg/ml)
14	1	1	17.50	12.50	36	840
3	2	1	5.00	25.11	6.00	90
17	3	1	17.50	5.00	24.00	172.5
5	4	1	5.00	5.00	48.00	360
8	5	1	30.00	10.00	42.00	856
10	6	1	10.00	12.50	42.00	360
2	7	1	30.00	5.00	6.00	280
12	8	1	17.50	20.00	30.00	670
20	**9**	**1**	**25.00**	**12.50**	**42.00**	**940**
18	10	1	17.50	10.00	36.00	840
4	11	1	30.00	20.00	42.00	600
1	12	1	20.00	5.00	6.00	203
19	13	1	10.00	25.11	24.00	534
15	14	1	25.00	20.00	18.00	220
16	15	1	20.00	25.11	30.00	732
9	16	1	10.00	10.00	24.00	386
7	17	1	5.00	20.00	48.00	480
6	18	1	20.00	12.50	36.00	880
13	19	1	25.00	10.00	12.00	425
11	20	1	17.50	25.11	18.00	554

By applying multiple regression analysis on the experimental data, a quadratic model was generated for the response of amylase activity. The

significant model terms were evaluated by analysis of variance (ANOVA) (Table 48) in the optimization study (p<0.05) and were identified as X_1, X_2, X_3, X_1^2.

Reducing sugars (Y) = 443.69+181.55 * X_1+57.21* X_2+301.65* X_3

Eq.3

where Y is the reducing sugars (mg/g biomass), X_1 is biomass X_2 is enzyme loading and X_3 is incubation period. By optimizing the above parameters by using RSM, maximum reducing sugars obtained were 940 mg/g biomass at 25g/l biomass with enzyme loading of 12.5 ml/g biomass for 42 h.

Table 48. Analysis of variance ANOVA table for response surface linear model for enzymatic hydrolysis of *Hydrodictyon* sp. biomass

Source	Sum of squares	Degrees of freedom	Mean square	F-value	Prob > F
Model	7.456E+005	3	2.485E+005	6.78	0.0037*
X_1	2.554E+005	1	2.554E+005	6.96	0.0009*
X_2	57345.00	1	57345.00	1.56	0.2291
X_3	5.758E+005	1	5.758E+005	15.70	0.0001*
Residual	5.868E+005	16			
Cor Total	1.332E+006	19			

*Significant values

The statistical significance of Eq.3 was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model has been shown in Table 48. It is evident that the model is highly significant, as suggested by the model *F*- value and a low probability value (P_{model}>F= 0.0001). The coefficient of determination (R^2) was calculated as 0.9607 for production of reducing sugars, indicating that the statistical model can explain 96.07% of variability in the response. Normally, a regression model having an R^2 value > 0.9 is considered to have a very high correlation. The closer the R (correlation coefficient) is to 1.0, the stronger the model and the better it predicts the response (Haaland 1989). The value of R= 0.9151 for Eq. 3 indicates a close agreement between the experimental results and the theoretical values predicted by the model equation. An adequate precision of 19.064 for reducing sugars production was recorded. The coefficient of variation (CV) indicates the degree of precision

with which the treatments were compared. Usually the higher value of CV, the lower the reliability of the experiment is (Gangadharan *et al.*, 2008). A lower value of CV (3.75) indicated a better precision and reliability of the experiment.

The student *t*-distribution and the corresponding *P*-value, along with the parameter estimate have been given in Table 48 The *P*-values are used as a tool to check the significance of each of the coefficients which are necessary to understand the pattern of the mutual interactions between the variables. The smaller the *P*-values, the bigger the significance of the corresponding coefficient.

4.11.1.2 Localization of optimum conditions

The 3D response surface plots (Fig. 58, 59) described by the regression model were drawn to illustrate the effects of the independent variables on the response variable. The shape of the corresponding contour indicates whether the mutual interactions between the independent variables are significant or not. An elliptical contour plot indicates that the interactions are significant. Fig. 58 and 59 shows the curve between biomass concentration at X-axis and enzyme loading and time at Y- axis respectively, while keeping all other variables at their 'O' central levels. The plots depicted a moderate interaction between the considered variables towards an optimized production of reducing sugars.

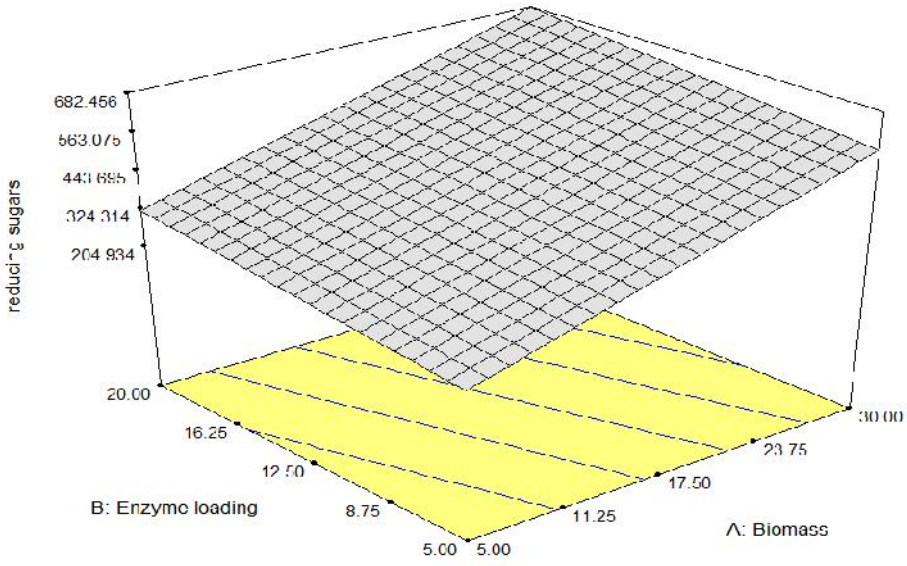


Fig. 58 Response surface curve representing interaction between enzyme loading and biomass

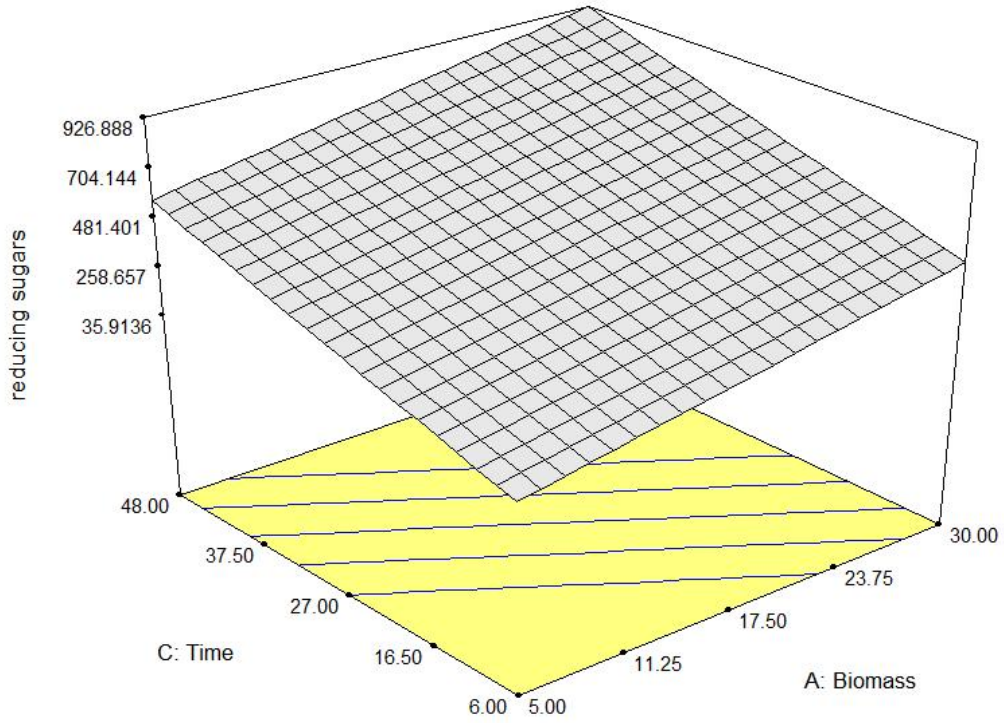


Fig. 59 Response surface curve representing interaction between time and biomass

4.11.1.3 Comparison of observed and predicted amylase activity

A regression model can also be used to predict future observations on the response Y (reducing sugars) corresponding to the particular values of the regression variables. Fig. 60 shows observed amylase activity versus those from the empirical model Eq. 3. The predicted R^2 of 0.3389 is in reasonable agreement with the adjusted R^2 of 0.4770. This indicated good agreement between the experimental and predicted values for amylase production. The adjusted R^2 corrects the R^2 value for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the predicted R^2. The model F-value of 6.78 and values of prob > F (<0.05) indicated that the model terms are significant. The figure proves the predicted data of the response from the empirical model is in agreement with the observed ones in the range of the operating variables.

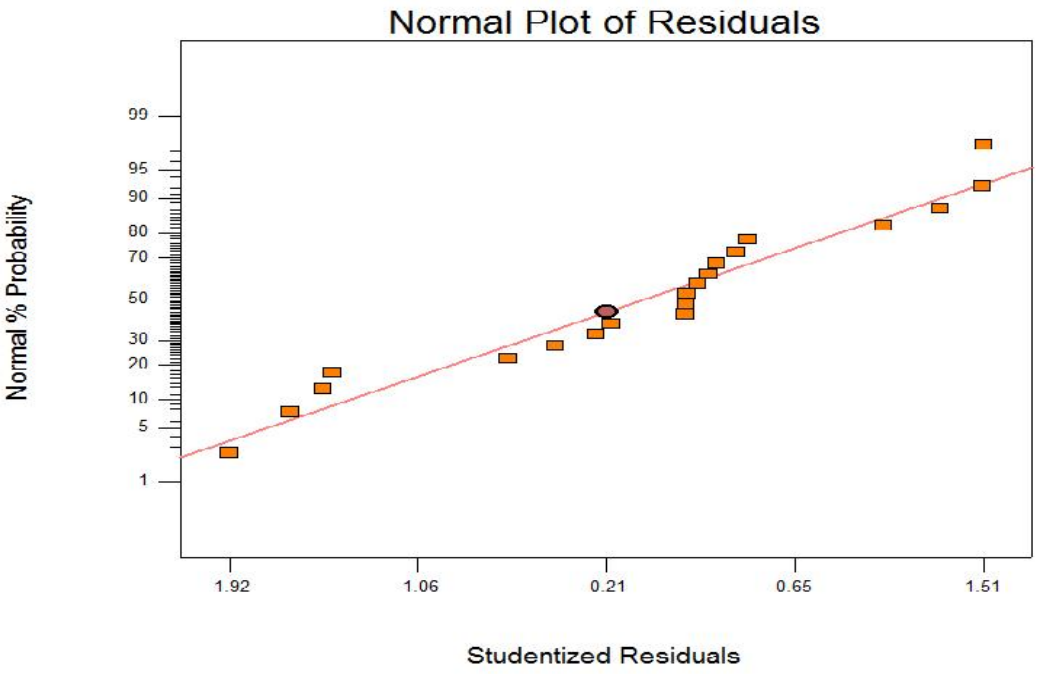


Fig. 60 Normal probability of internally studentized residuals

4.11.1.4 Model adequacy checking

Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit,

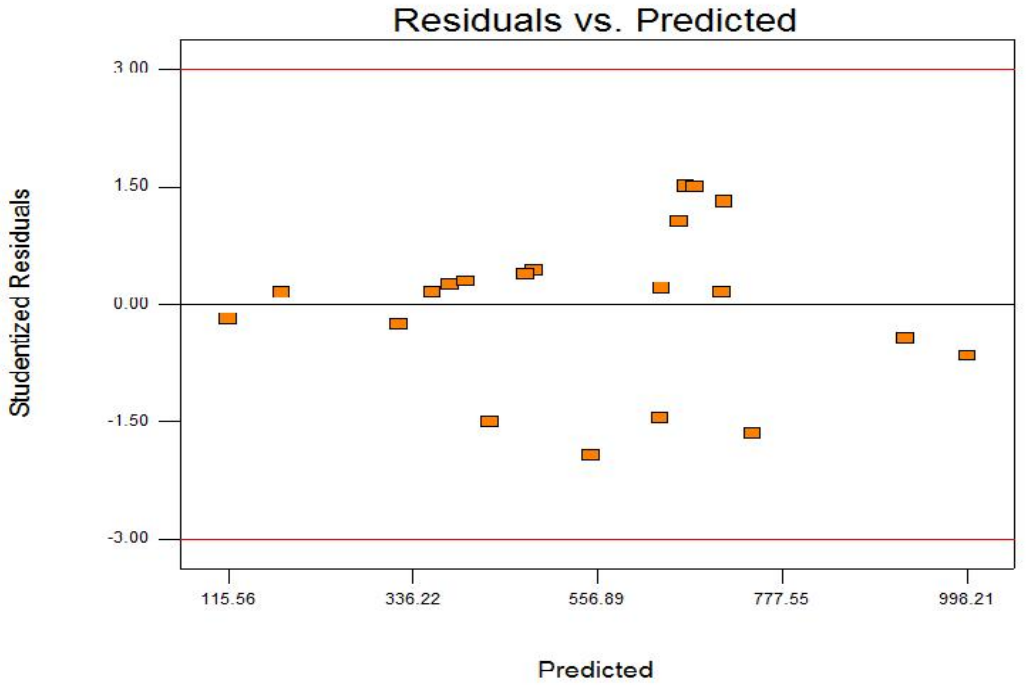


Fig. 61 Parity plot showing distribution of experimental and predicted values of sugars production by enzymatic hydrolysis

proceeding with the investigation and optimization of the fitted response surface likely give poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy. By constructing a normal probability plot for the residuals, a check was made for the normality assumption, as given in Fig. 61 the normality assumption was satisfied as the residual plot approximated along a straight line. The general impression is that the residuals scatter randomly on the display, suggesting that the variance of the original observation is constant for all values of predicated response (Y). Fig. 58 is satisfactory, so we conclude that the empirical model is adequate to describe the amylase activity by response surface.

4.11.1.5 Validation of the model

The statistically optimal values of variables were obtained when moving along the major and minor axis of the contour, and the response at the centre point yielded maximum amylase production. These observations were also verified from canonical analysis of the response surface. The canonical analysis revealed a minimum region for the model. The stationary point presenting maximum reducing sugars had the critical values as 940 mg sugars /g biomass at 25g/l biomass with 12.50 ml/ g of enzyme loading for 42 h. Although higher concentrations of biomass are expected to release higher amounts of fermentable sugars during the pretreatment and hydrolysis process, it has been reported that higher biomass concentrations reduce bioethanol production levels due to overburdened metabolism and the toxicity of high ethanol concentrations towards yeast cells (Cromie and Doelle, 1988). In consonance to these findings, a biomass concentration of 30g/ l resulted in a decreased reducing sugars yield.

In literature there are a very few reports available regarding the statistical optimization of enzymatic hydrolysis conditions. Ho *et al.* (2013), observed 20g/ l biomass with an enzyme loading of 20 ml/g biomass for 50 h as the best combination for the generation of highest reducing sugars from *C. vulgaris*. Trivedi *et al.* (2013) found 2% enzyme dosage for 36 h at 1:20 (w/v) biomass: liquid ratio as optimum for enzymatic hydrolysis of *U. fasciata* biomass.

4.12 FERMENTATION OF ETHANOL IN A SHAKE FLASK EXPERIMENT

Photosynthetic biomass is a promising resource for the generation of biofuels. Plant-derived lignocellulosic biomass is abundant but, due to the

recalcitrant nature of this material, significant challenges have to be solved if this biomass is to be used for the microbial production of biofuels (Jorgensen *et al.*, 2007; Limayem and Ricke, 2012). Photosynthetic microorganisms constitute an appealing alternative source of biomass for many reasons such as they grow much faster than terrestrial plants, have a higher efficiency in using the energy of light, and can be cultivated in areas and in a manner that do not compete with plant based food and feed production (Dismukes *et al.*, 2008; Larkum *et al.*, 2012; Parmar *et al.*, 2011; Jones and Mayfield, 2012). The most abundant photosynthetic organisms in nature are cyanobacteria and eukaryotic algae (Graham *et al.*, 2009). However, the direct use of biomass from algae as a feedstock for the generation of biofuels is underexplored.

In the present study, fresh water green alga *Hydrodictyon*, after subjecting to various physical and chemical pretreatments was used for the first time for ethanol production. A 0.5% H_2SO_4 pretreated dried algal powder was enzymatically hydrolysed to produce reducing sugars using mixture of inhouse hydrolytic enzymes. The reducing sugars thus obtained were fermented into ethanol by utilizing ethanologen *S. cerevisiae*-I (MTCC3089) under different modes of fermentation.

4.12.1 Bioconversion of pretreated algal biomass under Separate Hydrolysis and Fermentation (SHF) mode

Separate hydrolysis and fermentation (SHF) refers to a process inwhich enzymatic hydrolysis (saccharification) of polysaccharides and the microbialfermentation are performed sequentially in separate units. The major advantageof this method is that the enzymatic hydrolysis and fermentation can be carried out at theirown optimum conditions of temperature and pH. However, the drawbacks of SHF process include feedback inhibition of the enzymes by released sugars and a high probability of contamination since separate vessels are used forhydrolysis and fermentation. The hydrolysis process is long and the released sugars as carbonsource provide the opportunity of contamination with naturally-occurring microbes. Apossible source of contamination could be the enzyme preparation. In practice, it is difficultto sterilize enzymes in large scale, since it must be filter-sterilized owing to the denaturationof enzymes in an

autoclave (Taherzadeh and Karimi, 2007). In addition, it is not feasible toadd antibiotics in hydrolysis reactor because antibiotics may affect the growth andfermentation of microorganisms in the subsequent fermentation step.

Pretreatment and enzymatic hydrolysis of the biomass was carried out by following the protocol as standardized earlier. Parameters for efficient SHF procedure have been standardized.

Inoculum size for *S. cerevisiae*-I in the range spanning 10, 15…30% was studied. An 18h old second generation culture has been used as an inoculum, after an incubation of 72 h at 30^{o}C. Among the inoculum sizes tested, highest ethanol yield of 13.26 g/l has been observed at 20% inoculum size with 0.87 g/g ethanol yield co-efficient and 0.37 g/l/h specific rate of product formation (Table 49). Fermentation efficiency was found to be 61.34% with 91.97% sugar consumption. Lower inoculum size of 10 and 15% resulted in a lower ethanol yield of 7.6 g/l and 9.2 g/l respectively with lesser ethanol yield co-efficient of 0.72 g/g and 0.54 g/g respectively. This could be attributed to the fact that comparatively lesser cells were available for the degradation of reducing sugars which resulted in lesser consumption of sugars (71.08%) and thus lesser product yield.

Table 49. Optimization of inoculum size for ethanol production from *Hydrodictyon* biomass under Separate Hydrolysis and Fermentation (SHF)

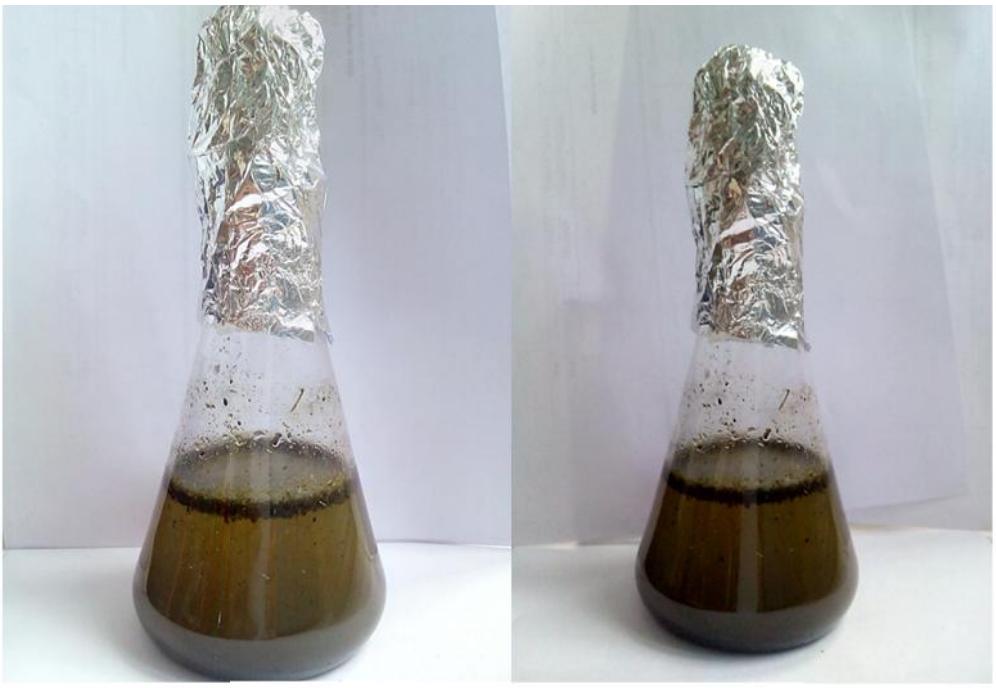
Inoculum size (%)	Ethanol (g/l)	Ethanol (g/g)	*$Y_{p/s}$	**Q_{pmax}	***Fermentation efficiency	****Theoretical yield
10	7.60±0.21^{a}	0.30±0.08 ^{a}	0.72±0.12 ^{a}	0.21±0.06 ^{a}	36.01±0.11^{a}	0.74±0.09^{a}
15	9.20±0.16^{b}	0.36±0.11 ^{a}	0.54±0.11 ^{a}	0.25±0.04 ^{a}	53.89±0.34^{b}	0.76±0.03^{a}
20	**13.26±0.13^{c}**	**0.53±0.14 ^{a}**	**0.87±0.16 ^{a}**	**0.37±0.11 ^{a}**	**61.34±0.24^{c}**	**1.27±0.07^{b}**
25	9.40±0.09^{d}	0.37±0.12 ^{a}	0.53±0.13 ^{a}	0.26±0.09 ^{a}	45.08±0.21^{d}	0.92±0.11^{c}
30	8.60±0.11^{e}	0.34±0.07 ^{a}	0.51±0.17 ^{a}	0.24±0.08 ^{a}	43.14±0.19^{e}	0.84±0.14^{d}
SE(m)	**0.14**	**0.10**	**0.13**	**0.07**	**0.21**	**0.08**

*$Y_{p/s}$:Ethanol yield co-efficient (g/g)
**Q_{pmax}: Specific rate of product formation (g/l/h)
*** Fermentation efficiency: Ethanol produced (g/g) / Theoretical yield of ethanol x100
****Theoretical yield: Ethanol produced (g/g) / Standard ethanol produced per g

Increase in inoculum size however, has shown an increase in ethanol yield upto an optimum of 20%, further increase beyond which has resulted in a decreased product yield. This decrease might be due to the inhibition of fermenting organism because of accumulated product. The literature survey revealed no concrete optimization of inoculum size for the fermentation of algal biomass to ethanol. An inoculum size ranging from 5-20% has however been used by researchers for fermentation experiments. Kim *et al.* (2011); Kim *et al.* (2014) and Mollers *et al.* (2014) reported a respective inoculum size of 10%, 5% and 10% as optimum for conversion of marine algal biomass, *C. vulgaris* biomass and *Synechococcus* biomass into ethanol.

Fermentation period in the range spanning 24 to 72 h was studied at regular intervals of 12h. Maximum ethanol yield of 13.66 g/l with ethanol yield co-efficient of 1.23 g/g has been observed at 36^{th} h followed by yield of 13.26 g/l, 12.89 g/l and 12.34 g/l at 48^{th}, 60^{th} and 72^{nd} h respectively with an ethanol yield co-efficient of 0.72 g/g, 0.41 g/g and 0.42 g/g respectively(Table 50). A maximum specific rate of product formation of 0.38 g/l/h has been observed at 36^{th} h of fermentation with 96.17% consumption of sugars. It was thus finalized as optimum fermentation time. Similarly, Ho *et al.* (2013) observed 36 h as best fermentation time yielding 4.27g/l ethanol from microalga *C. vulgaris.* Optimizing fermentation period for ethanol production by green alga *U. fasciata,* Trivedi *et al.* (2013) observed 12 h time as optimum giving 100% theoretical yield. A higher fermentation time has also been found to be optimum by several workers. Eshaq *et al.* (2011) observed 5^{th} day as best for ethanol fermentation from *Spirogyra* biomass. Harun and Danquah (2011) observed maximum ethanol yield at 24^{th} h of incubation utilizing *C. humicola* which declined on further incubation up to 30 h with consumption of 80% reducing sugars.

Thus conversion of reducing sugars into ethanol by following SHF approach yielded a maximum ethanol of 13.66 g/l (0.54g/g) after 36 h of fermentation with 20% inoculum of *S. cerevisiae*-I (MTCC 3089). Fermentation efficiency was 63.24% with a theoretical yield of 1.33.



a



b

Plate 8. Conversion of reducing sugars to bioethanol from potential *Hydrodictyon* biomass in (a) shake flask and (b) bioreactor

Table 50. Evaluation of optimum fermentation period for ethanol production from *Hydrodictyon* biomass under Separate Hydrolysis and Fermentation (SHF)

Time (h)	Ethanol (g/l)	Ethanol (g/g)	*$Y_{p/s}$	**Q_{pmax}	***Fermentation efficiency	****Theoretical yield
24	7.60±0.21[a]	0.30±0.09 [a]	0.4±0.09 [a]	0.32±0.09 [a]	36.01±0.21[a]	0.58±0.09[a]
36	**13.66±0.11[b]**	**0.54±0.11 [a]**	**1.23±0.21[b]**	**0.38±0.11 [a]**	**63.24±0.32[b]**	**1.05±0.11[b]**
48	13.26±0.09[c]	0.53±0.13 [a]	0.72±0.12 [a]	0.28±0.12 [a]	62.84±0.12[c]	1.03±0.05[c]
60	12.89±0.13[d]	0.51±0.17 [a]	0.41±0.11 [a]	0.21±0.08 [a]	57.03±0.24[d]	1.00±0.04[d]
72	12.34±0.15[e]	0.49±0.11 [a]	0.4±0.13[a]	0.17±0.04 [a]	54.12±0.19[e]	0.96±0.11[e]
SE(m)	**0.13**	**0.06**	**0.12**	**0.08**	**0.21**	**0.08**

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same as given in Table 49

4.12.2 Bioconversion of pretreated algal biomass under SSF (Simultaneous Saccharification and Fermentation) mode

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, 1999; 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.

For carrying out SSF in a shake flask, temperature, pH, inoculum size of fermenting organism and fermentation time has been standardized. Fermentation pH ranging from 5.2-6.0 has been investigated. A maximum of 10.25 g/l ethanol at pH 5.6 has been obtained with ethanol yield co-efficient of 1.46 g/g and 0.28 g/l/h as specific rate of product formation (Table 51). Fermentation efficiency was found to be 44.95% with a theoretical yield of 0.80. pH 5.6 was thus considered as optimum and was selected for further optimization studies. Inoculum size and time for fermentation were then standardized simultaneously. A 15% inoculum of *S. cerevisiae*-I at 28th h of incubation yielded 12.77g/l ethanol. An increased fermentation period upto 40th h resulted in retained ethanol with a final yield of 12.39 g/l (Table 52). Increased inoculum to 30% however resulted in a decreased ethanol yield upto 4.56 g/l. Thus, pH 5.6 and 28 h of fermentation period with 15% inoculum was utilized for evaluation of fermentation temperature for conversion of reducing sugars to ethanol under SSF. An ethanol yield of 13.16 g/l was obtained at an optimum temperature of 35 ^{o}C (Table 53). 13.16 g/l ethanol corresponded to 0.41 g/g ethanol yield co-efficicent and 1.46 g/l/h specific rate of product formation. Fermentation efficiency was observed to be 61.05% and 0.80 theoretical yield was observed.

Table 51. Evaluation of optimum pH for ethanol production from *Hydrodictyon* biomass under Simultaneous Saccharification and Fermentation (SSF)

pH	Ethanol (g/l)	Ethanol (g/g)	*$Y_{p/s}$	**Q_{pmax}	***Fermentation efficiency	****Theoretical yield
5.2	3.80±0.12^{a}	0.15±0.06 ^{a}	0.25±0.07 ^{a}	0.10±0.06 ^{a}	22.64±0.06^{a}	0.37±0.04^{a}
5.4	6.40±0.21^{b}	0.25±0.09 ^{a}	0.24±0.06 ^{a}	0.17±0.05 ^{a}	29.97±0.17^{b}	0.50±0.11^{b}
5.6	**10.25±0.24^{c}**	**0.41±0.27^{b}**	**1.46±0.19^{b}**	**0.28±0.08 ^{b}**	**44.95±0.34^{c}**	**0.80±0.13^{c}**
5.8	6.56±0.13^{d}	0.26±0.04 ^{a}	0.09±0.02 ^{a}	0.18±0.04 ^{a}	31.09±0.21^{d}	0.49±0.14^{d}
6.0	5.13±0.17^{e}	0.20±0.07 ^{a}	0.03±0.01 ^{a}	0.14±0.05 ^{a}	34.18±0.28^{e}	0.40±0.17^{e}
SE(m)	**0.17**	**0.10**	**0.07**	**0.07**	**0.21**	**0.11**

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same as given in Table 49

Table 52. Evaluation of optimum inoculum size and fermentation period for ethanol production from *Hydrodictyon* biomass under Simultaneous Saccharification and Fermentation (SSF)

Time (h) / Inoculum size (%)	Ethanol (g/l)							
	12	**16**	**20**	**24**	**28**	**32**	**36**	**40**
10	-	-	-	3.55±0.11	4.89±0.05	4.75±0.12	4.64±0.10	3.01±0.04
15	6.60±0.12	8.28±0.14	9.34±0.11	9.74±0.09	**12.77±0.08**	12.77±0.11	12.50±0.11	12.39±0.11
20	6.60±0.13	7.10±0.12	9.09±0.21	10.30±0.12	11.30±0.12	10.85±0.13	10.56±0.12	10.52±0.10
25	5.25±0.17	5.75±0.11	6.49±0.18	6.75±0.07	6.75±0.14	6.00±0.09	6.00±0.11	5.86±0.09
30	5.36±0.15	6.49±0.13	8.28±0.12	4.56±0.08	4.56±0.11	4.50±0.09	4.25±0.07	4.25±0.08

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******** } same as given in Table 49

Table 53. Evaluation of optimum temperature for ethanol production from *Hydrodictyon* biomass under Simultaneous Saccharification and Fermentation (SSF)

Temperature (^oC)	Ethanol (g/l)	Ethanol (g/g)	*$Y_{p/s}$	**Q_{pmax}	***Fermentation efficiency	****Theoretical yield
25	3.44±0.12^a	0.15±0.06 ^{a}	0.25±0.07 ^{a}	0.10±0.06 ^{a}	22.64±0.06^a	0.37±0.04^a
30	6.4 0±0.21^b	0.25±0.09 ^{a}	0.24±0.06 ^{a}	0.17±0.05 ^{a}	29.97±0.17^b	0.50±0.11^b
35	**13.16±0.24^c**	**0.41±0.27^b**	**1.46±0.19^b**	**0.28±0.08 ^{b}**	**61.05±0.34^c**	**0.80±0.13^c**
40	6.56±0.13^d	0.26±0.04 ^{a}	0.09±0.02 ^{a}	0.18±0.04 ^{a}	31.09±0.21^d	0.49±0.14^d
45	2.13±0.17^e	0.20±0.07 ^{a}	0.03±0.01 ^{a}	0.14±0.05 ^{a}	34.18±0.28^e	0.40±0.17^e
SE(m)	**0.17**	**0.10**	**0.07**	**0.07**	**0.21**	**0.11**

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******** } same as given in Table 49

Asada *et al.* (2012) observed a maximum of 19.4 g/l ethanol with a fermentation efficiency of 68.8% using *Chlamydomonas fasciata* under SSF at 5.0 pH and 40^{o}C temperature, using commercial enzymes for hydrolysis. Ho *et al.*, (2013) obtained 4.17 g/l ethanol from *C. vulgaris* biomass under SSF at 30^{o}C temperature, pH 6.0 and a biomass concentration of 20 g/l.

The results of the shake flask experiments revealed almost at par ethanol yields of 13.66 g/l and 13.16 g/l from SHF and SSF respectively. For SHF, hydrolysis time required was 42h at 45 ^{o}Cfollowed by fermentation period of 36 h at a temperature of 30 ^{o}C and 5.6 pH with 20% inoculum. However, for SSF, a 28 h of hydrolysis and fermentation period at 35^{o}C and 5.6 pH was found to be optimum at 15% inoculum. In this view it can be concluded that the SSF process seems to be a better one for enzymatic hydrolysis based bioethanol production, since it not only simplifies the reaction procedures and shortens the reaction time required to reach the maximum ethanol production, but also achieves ethanol yields which are at par to the SHF.

4.13 SCALE UP OF ETHANOL PRODUCTION IN A STIRRED TANK BIOREACTOR

4.13.1 Bioconversion of pretreated algal biomass under Separate Hydrolysis and Fermentation (SHF) mode

Scale-up of bioethanol production was done utilizing the conditions standardized in shake flask experiments under SHF mode. A 7.5 l volume bioreactor was inoculated with 3.0 l of 0.5% H_2SO_4 pretreated and enzymatically hydrolysed *Hydrodictyon* biomass. This preparation was seeded with 20% *S. cerevisiae*-I inoculum (18h old culture) at pH 5.6 and 30 ^{o}C temperature. Fermentation started after 6h of inoculation, which marked for the growth and multiplication of yeast cells. At 12th h of fermentation, 6.4 g/l of ethanol was obtained with 0.52 g/g product yield co-efficient, 0.06 g/h/l specific rate of product formation. At 18th h of fermentation, an ethanol yield of 13.86 g/l was observed with a fermentation efficiency of 64.36% (Table 54), which reached maxima at 24 h with 13.93 g/l ethanol with a fermentation efficiency of 64.84% and theoretical yield of 1.06. Gas chromatogram showing ehanol yield at 24h has been shown in Fig. 62. Sampling however was done up to 42 h at regular

intervals of 6h. Upto 42nd h, a reduced ethanol of 11.39 g/l was obtained. At this stage of fermentation, pH of the fermentation broth has started to increase thereby suggesting that the yeast has been superseded by some other synergistic microbial community which has taken up the process. This marked the end of alcoholic fermentation and hence the batch.

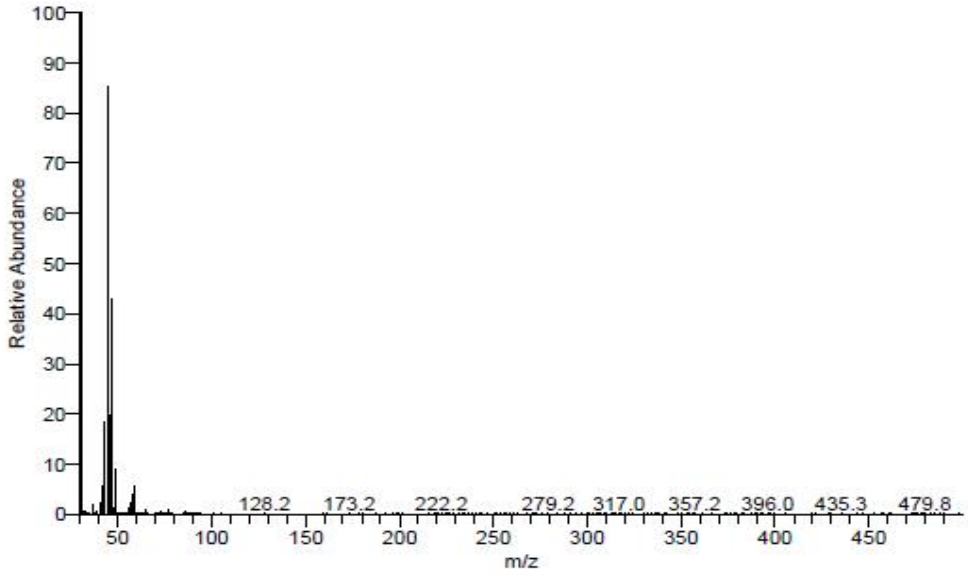


Fig. 62 GC MS profile of ethanol produced under Separate Hydrolysis and Fermentation (SHF) in a stirred tank bioreactor

In comparison to shake flask conditions, an increased ethanol yield of 2.56% has been observed in a bioreactor at reduced fermentation time of 18h, thus making the bioconversion process economical.

Quantitative estimation of sugars in the fermentation broth at various stages of hydrolysis and fermentation was carried out using high performance liquid chromatography (HPLC). Fermentation broth was subjected to HPLC at various stages of the process in bioreactor. Initial sample after 42 h of hydrolysis at 45 ^{o}C (Fig. 63b) depicted 940 mg/g glucose. Second sample at which highest ethanol production has been shown, i.e. at 24 h of fermentation (Fig. 63c) showed 75.65 mg/g glucose with 90.65% conversion efficiency. This corresponded to 13.93 g/l ethanol with fermentation efficiency of 64.84%. Third sample at 42 h of fermentation, which marked the end of fermentation showed 28.56 mg/g glucose with a conversion efficiency of 96.88% (Fig. 63d). Kim *et al.* (2011) have reported the quantification of reducing sugars from marine algae by employing HPLC.

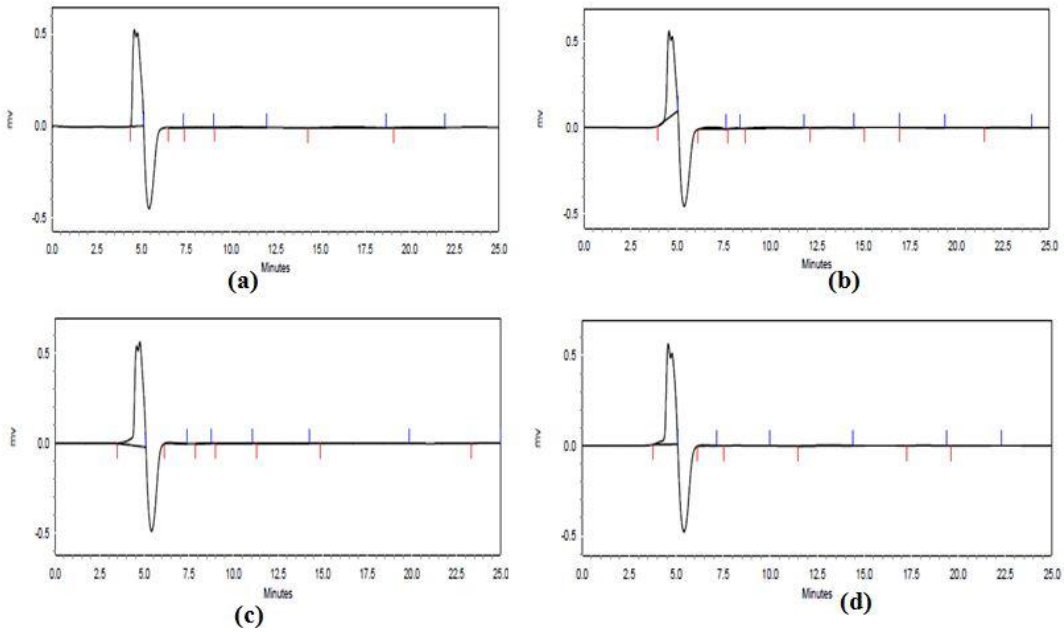


Fig. 63 Chromatograms of sugars during different stages of hydrolysis under Separate Hydrolysis and Fermentation (SHF) in a stirred tank bioreactor (a) Standard (b) Enzymatically hydrolysed biomass (42 h) (c) 24 h (d) 42 h

Table 54. Ethanol production from pretreated and enzymatically hydrolysed *Hydrodictyon* biomass under Separate hydrolysis and fermenatation (SHF)

Time (h)	Ethanol (g/l)	Ethanol (g/g)	*$Y_{p/s}$	**Q_{pmax}	***Fermentation efficiency	****Theoretical yield
0	0±0.00 [a]	0±0.00 [a]	0±0.00 [a]	0±0.00 [a]	0±0.00 [a]	0±0.00 [a]
3	0±0.00 [a]	0±.00 [a]	0±0.00 [a]	0±0.00 [a]	0±0.00 [a]	0±0.00 [a]
6	3.8±0.08[b]	0.15±0.04[b]	0.31±0.11[a]	0.04±0.09[a]	18.02±0.21[b]	0.29±0.11[b]
12	6.4±0.12[c]	0.26±0.06[c]	0.52±0.28[b]	0.06±0.05[a]	32.64±0.19[c]	0.51±0.18[b]
18	13.86±0.09[d]	0.55±0.09[d]	3.3±0.11[c]	0.77±0.12[b]	64.36±0.33[d]	1.08±0.09[c]
24	**13.93±0.11[e]**	**0.56±0.11[d]**	**9.34±0.31[d]**	**0.58±0.14[c]**	**64.84±0.27[d]**	**1.09±0.11[c]**
30	13.96±0.22[d]	0.56±0.08[d]	9.30±0.27[d]	0.46±0.21[d]	64.48±0.31[d]	1.09±0.09[c]
36	12.46±0.15[f]	0.50±0.11[e]	8.56±0.19[e]	0.34±0.19[d]	54.37±0.29[e]	0.98±0.07[d]
42	11.39±0.09[g]	0.46±0.12[f]	7.79±0.29[f]	0.27±0.17[e]	45.45±0.27[f]	0.90±0.06[d]
SE (m)	**0.43**	**0.33**	**0.15**	**0.28**	**0.38**	**0.26**

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same as given in Table 49

4.13.2 Bioconversion of pretreated algal biomass under Simultaneous Saccharification and Fermentation (SSF) mode

As in case of SHF, in SSF also scale up of ethanol production has been done from lab scale to pilot scale in a 7.5 l capacity bioreactor. Fermentation conditions optimized for shake flask experiment were utilized for the scale up. 0.5% H_2SO_4 pretreated 25 g/l *Hydrodictyon* biomass was subjected to enzymatic hydrolysis. Fermentation broth was inoculated with 15.0% *S. cerevisiae*-I inoculum (18h old culture) and fermentation was started at 5.6 pH and 35^oC temperature. Samples were drawn at regular intervals. Fermentation started after 6 h of inoculation. At 12th h of fermentation, 14.41 g/l ethanol was observed with a fermentation efficiency of 56.22% which reached its maxima at 16th h with 16.67 g/l with a fermentation efficiency of 66.68% and theoretical yield of 1.31. gas chromatogram showing ethanol yield at 16h has been shown in Fig. 64. Specific rate of product formation was 1.48 g/l/h and ethanol yield co-efficient was 4.48 g/g. Sampling was done till 36 h, at 36th h, a reduced ethanol yield

Table 55. Ethanol production from pretreated and enzymatically hydrolysed *Hydrodictyon* biomass under Simultaneous Saccharification and Fermenatation (SSF) mode

Time (h)	Ethanol (g/l)	Ethanol (g/g)	*$Y_{p/s}$	**Q_{pmax}	***Fermentation efficiency	****Theoretical yield
0	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a
3	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a
6	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a	0±0.00^a
12	14.41±0.32^b	0.58±0.21^b	2.68±0.18^b	1.20±0.08^a	56.22±0.18^b	1.17±0.09^b
16	**16.67±0.27^c**	**0.67±0.17^c**	**4.48±0.28^c**	**1.48±0.29^a**	**66.68±0.31^c**	**1.31±0.25^c**
24	16.23±0.14^c	0.65±0.14^c	4.26±0.16^c	0.67±0.13^b	64.12±0.32^d	1.27±0.19^d
30	16.03±0.16^c	0.64±0.16c	3.39±0.17^d	0.53±0.11^c	64.38±0.26^d	1.24±0.21^d
36	15.09±0.19^d	0.60±0.14^c	3.34±0.21^d	0.42±0.13^d	60.36±0.15^e	1.17±0.22^e
SE(m)	**0.16**	**0.23**	**0.29**	**0.33**	**0.31**	**0.11**

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******** } same as given in Table 49

of15.09 g/l has been observed. At this stage, the pH of the fermentation broth has started to increase which marks the end of fermentation and hence the batch (Table 55).

In comparison to shake flask conditions, an increased ethanol yield of 26.67% has been observed in a bioreactor at reduced fermentation time of 16 h, thus making the bioconversion process economical.

Quantitative estimation of sugars in the fermentation broth at various stages of hydrolysis and fermentation was carried out using high performance liquid chromatography (HPLC). Fermentation broth was subjected to HPLC at various stages of the process in bioreactor. Initial sample after 6 h of fermentation at 35^{o}C (Fig. 65b) depicted 386 mg/g glucose. Second sample at which highest ethanol production has been shown, i.e. at 16 h of fermentation (Fig. 65c) showed 186 mg/g glucose. This corresponded to 16.67 g/l ethanol with fermentation efficiency of 66.68 %. Third sample at 42 h of fermentation, which marked the end of fermentation, showed 28 mg/g glucose with a conversion efficiency of 96.88% (Fig. 65d). Asada *et al.* (2012) have reported the quantification of reducing sugars from micro algae *C. fasciata* by employing HPLC.

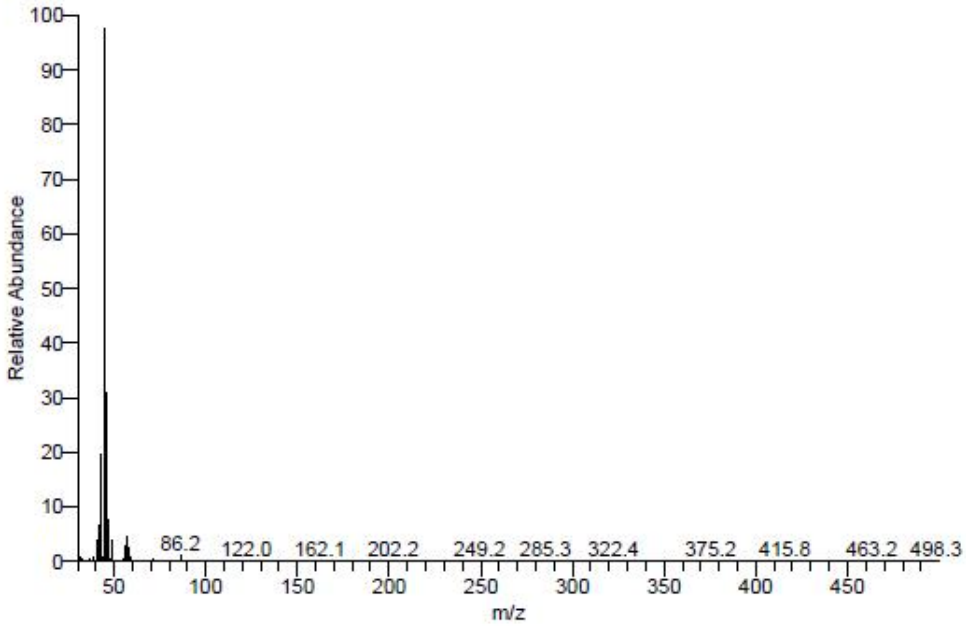
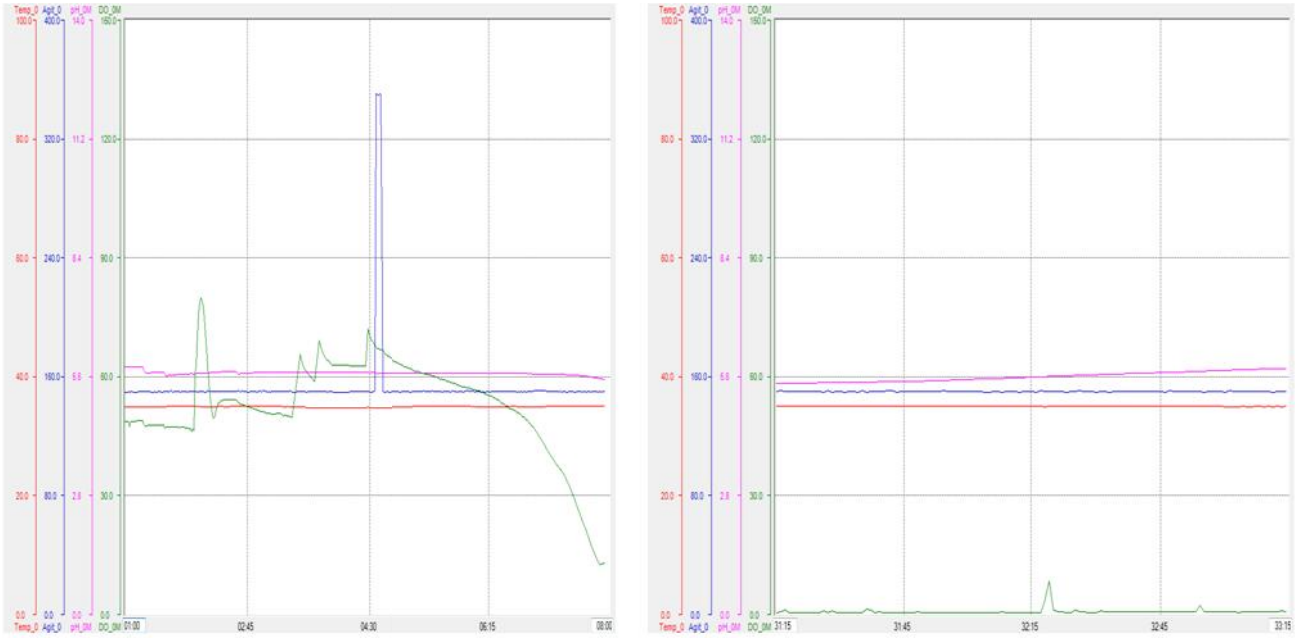
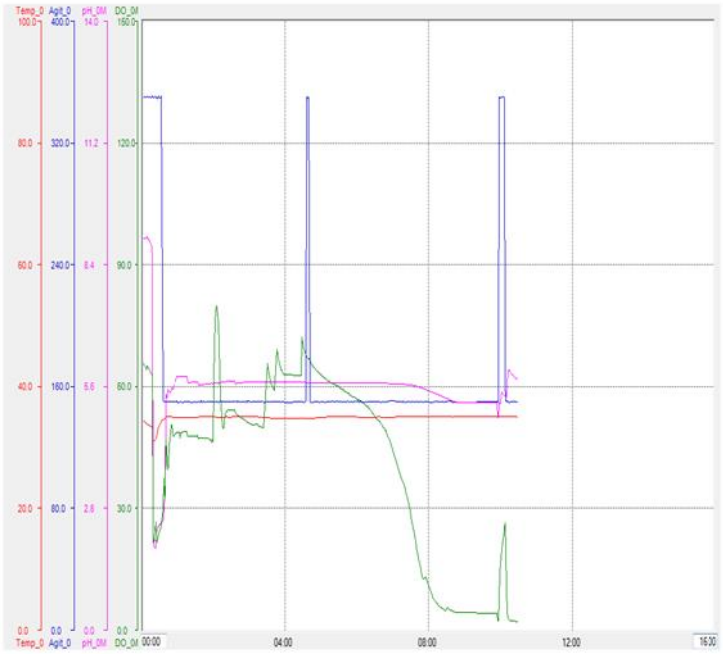


Fig. 64 GC MS profile of ethanol produced under Simultaneous Saccharification and Fermentation (SSF) in a stirred tank bioreactor



0 h 24 h



36 h

Fig. 66 Physiological behaviour of *S. cerevisiae* under separate hydrolysis and fermentation (SHF) in a stirred tank bioreactor

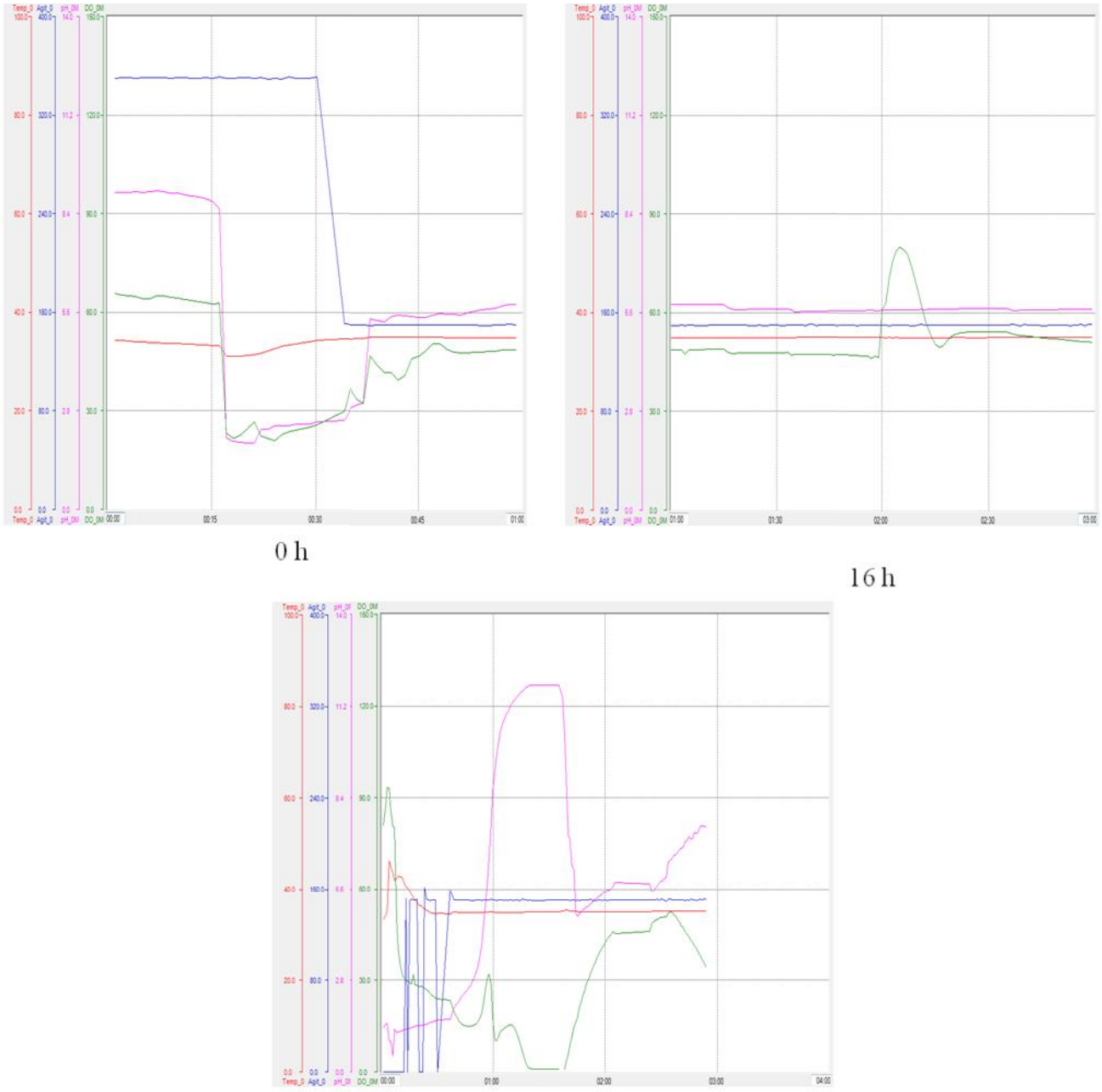


Fig. 67 Physiological behaviour of *S. cerevisiae* under simultaneous saccharification and fermentation (SSF) in a stirred tank bioreactor

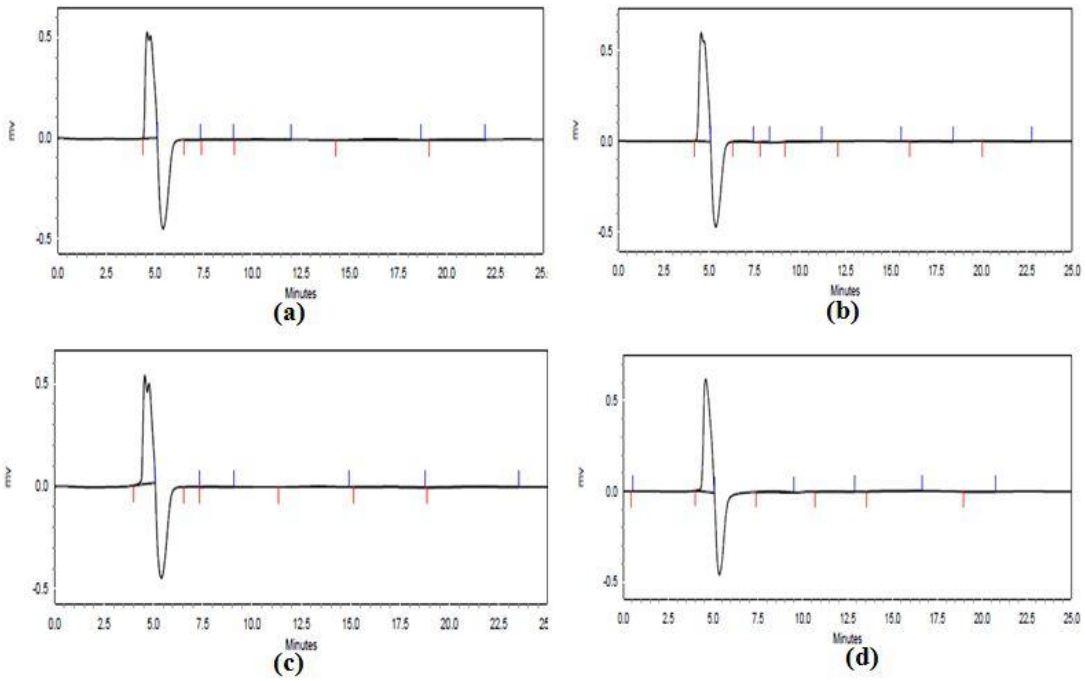


Fig. 65 Chromatograms of sugars during different stages of hydrolysis under Simultaneous Saccharification and Fermentation (SSF) in a stirred tank bioreactor (a) Standard (b) 6h (c) 16 h (d) 42 h

4.13.3 Mathematical Modelling for Cell Growth

Most of the growth processes are explained in terms of Monod or logistic equations (unstructured models). Logistic equation is simple for calculation of fermentation parameters related to biological and geometrical significance using sigmoid profiles independent of substrate concentration (Garcia-Ochoa *et al.* 1995). The idea of microbial growth kinetics has been dominated by an empirical model originally proposed by Monod (1942). Fig. 66 and 67 reveal the physiological behaviour of *S. cerevisiae* in a stirred tank bioreactor under SHF and SSF respectively.

According to Musatto *et al.* (2010) the carbon source consumption is divided by the yeast into biomass and ethanol production. This behaviour of yeast is strongly influenced by available oxygen. This metabolic behaviour can be observed clearly with a comparison between $Y_{(P/S)}$ and $Y_{(X/S)}$ values. In the present work, $Y_{(P/S)}$ was significantly higher than $Y_{(X/S)}$ which reflected the amount of distributed oxygen was satisfactory for the yeast metabolism.

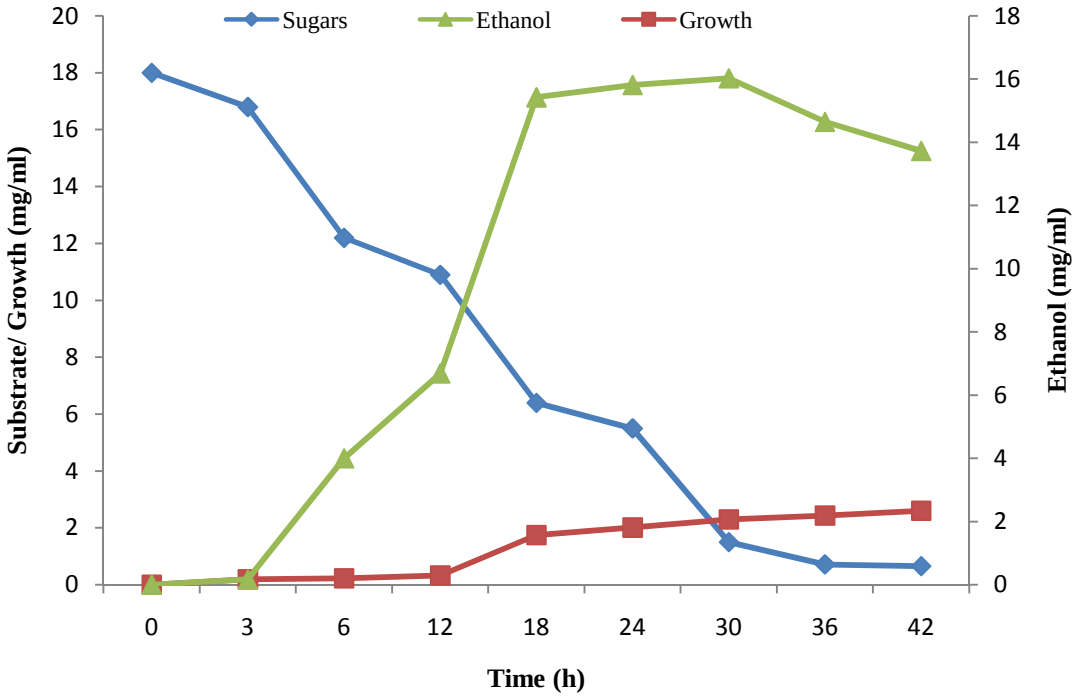


Fig. 68 Growth kinetics for ethanol production under Separate Hydrolysis and Fermentation (SHF) in a stirred tank bioreactor

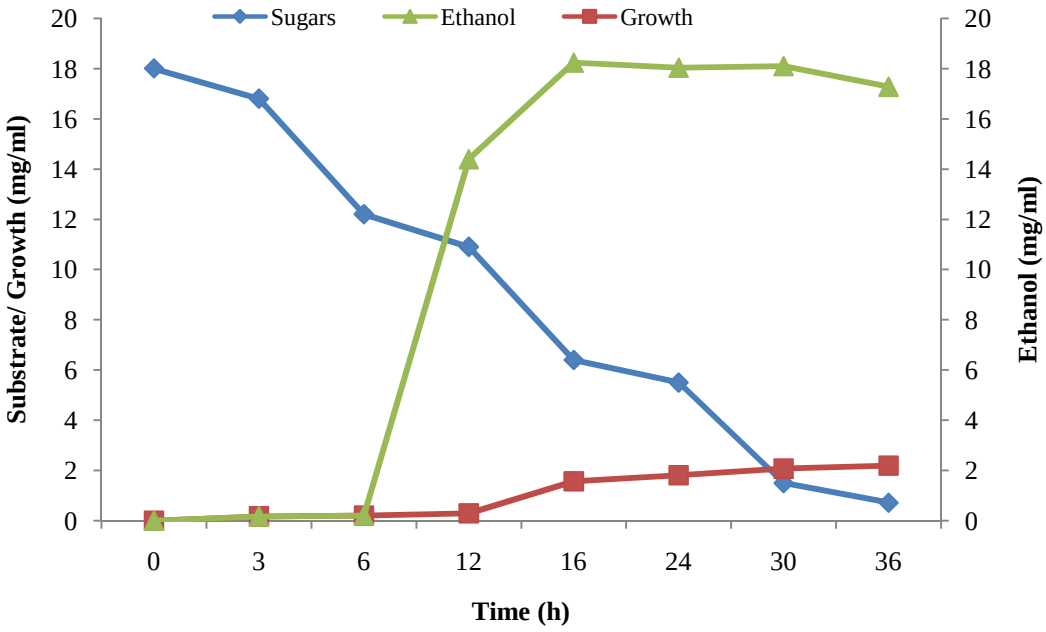


Fig. 69 Growth kinetics for ethanol production under Simultaneous Saccharification and Fermentation (SSF) in a stirred tank bioreactor

The sigmoidal growth pattern of *S. cerevisae* (MTCC-3089) had been analyzed using logistic equation for its variation against time during log phase. A conventional growth pattern was observed during batch fermentation in separate hydrolysis and fermentation where exponential phase lasted for 30 h and was followed by stationary phase upto 42 h. The production of ethanol had started at 6h and obtained maximum of 13.93 g/l at 24 h (Fig. 68). In case of simultaneous saccharification and fermentation, ethanol production started from 6^{th} h which attained maxima at 16^{th} h (16.67 g/l) (Fig. 69).

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters μmax and Ks. In addition to this, Monod also related the yield coefficient ($Y_{x/s}$) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (Q) (Fig. 70 and Fig. 71)

$$Y_{x/s} = dx/ds$$

$$Y_{x/s} = X_m - X_0/S_0\text{-}S_m$$

In separate hydrolysis and fermentation (SHF), model prediction for cell growth was followed with the experimental results with coefficient of determination, which revealed that this kinetic model was appropriate for predicting the experimental cell growth i.e.

$Y_{x/s}$	=	2.39
$Y_{P/S}$	=	9.34
μ_{max}	=	3.42 h^{-1}
Q_{pmax}	=	0.58 g/l/h
Ks	=	121.06 mg/ml

Model prediction for cell growth was followed with the experimental results with coefficient of determination in case of simultaneous saccharification and fermentation (SSF) model was predicted as:

$Y_{x/s}$	=	1.44
$Y_{P/S}$	=	4.48
μ_{max}	=	6.04 h^{-1}
Q_{pmax}	=	1.48 g/l/h
Ks	=	162.06 mg/ml

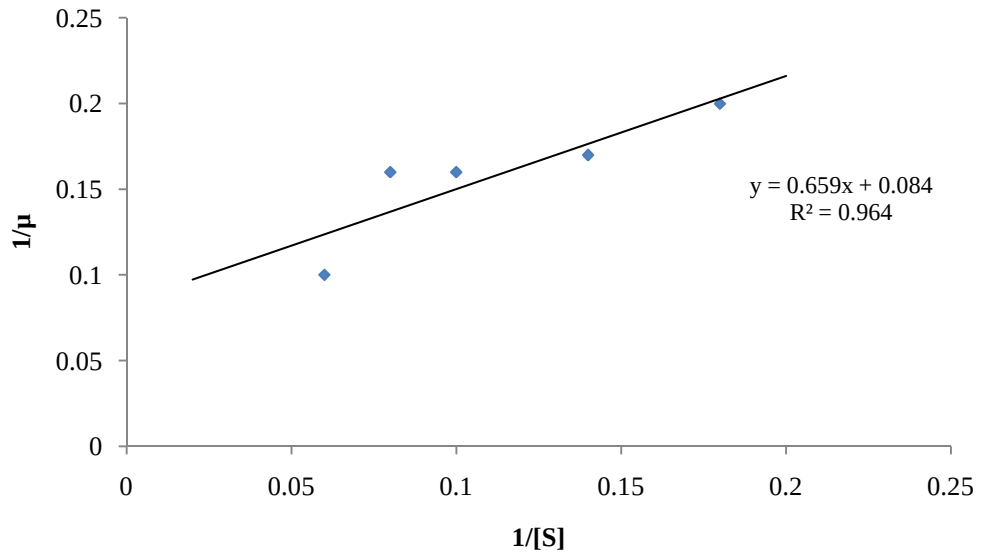


Fig. 70 Comparison between experimental results of specific growth rate and substrate utilization and model prediction for Separate Hydrolysis and Fermentation (SHF)

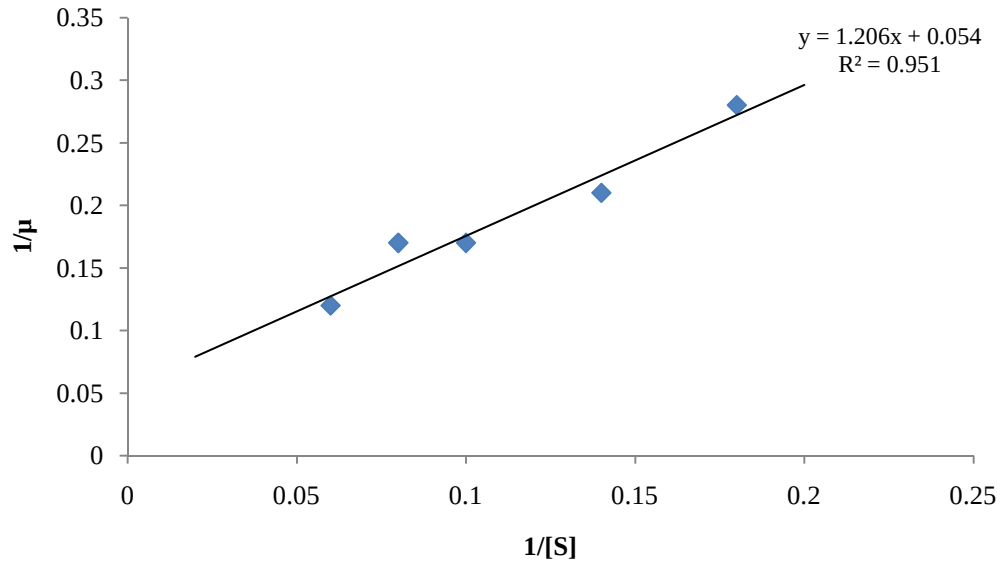


Fig. 71 Comparison between experimental results of specific growth rate and substrate utilization and model prediction for Simultaneous Saccharification and Fermentation (SSF)

The maximum specific growth rate μ_{max} is a characteristic of all organisms and it is related to their ability to reproduce themselves in nutritional conditions of plenty. It is simply defined as the increase of biomass per unit time under optimal feeding conditions, in which there are not limiting nutrients. While

the interpretation of μ_{max} as the maximum specific growth rate is straight forward. The empirical constant K_S is the substrate concentration at which organisms are substrate limited to a growth rate of half the prevailing maximum value. This constant is closely related to the mechanism of transport of the substrate over the cell membrane, so it depends on cell membrane properties and intracellular conditions, on the type of transporter proteins in the cell and on the substrate properties. For this reason, the value of K_S can be interpreted as a reflection of the affinity of the cell towards the substrate (S) (Luederking and Piret, 1959)

Because of the adaptive ability of organisms, both μ_{max} and K_S can vary with the environmental conditions, for example with the nature of the growth medium and the duration of exposure to it, with temperature and pressure. For these reasons the values of μ_{max} and K_S have to be estimated for each specific couple cell-substrate and under constant conditions of temperature, pressure and medium composition.

4.13.4 Kinetic Analysis for Ethanol Production

The Luedeking-Piret equation (Luedeking and Piret, 1959) was applied for the kinetic modeling of ethanol production. Table 56 depicts the attributes in the stirred tank bioreactor during fermentation under SHF and SSF mode. The Luedeking-Piret model indicates that the rate of ethanol production (Q_p) is linearly proportional to cell growth rate and cell biomass in both cases of separate hydrolysis and fermentation (Fig. 72) and simultaneous saccharification and fermentation (Fig. 73).

The results indicated that this model evidently was very appropriate for describing the product formation rate under both modes of fermentation. The coefficient of determination, R^2 for SHF was 0.943 and for SSF was 0.907.

On comparing the ethanol yield and working conditions of SHF and SSF, it was concluded that SSF was more efficient for ethanol production with 16.67 g/l ethanol, 66.68% fermentation efficiency and 1.31 theoretical yield as compared to SHF with 14.01 g/l ethanol, with a fermentation efficiency of 65.04

% and theoretical yield of 1.09. Moreover the fermentation time for SSF is just 16 h which is less than half of what is required in SHF.

Table 56. Operation parameters in a stirred tank bioreactor during fermentation under SHF and SSF mode

Time (h)	SHF				SSF			
	Agitation (rpm)	Temperature (^oC)	pH	DO	Agitation (rpm)	Temperature (^oC)	pH	DO
0	150	30	6.0	0.8	150	35	6.0	0.8
3	150	30	5.63	0.6	150	35	5.63	0.6
6	150	30	5.55	0.6	150	35	5.55	0.5
12	150	30	5.50	0.4	150	35	5.40	0.4
18	150	30	5.50	0.2	150	35	5.40	0.2
24	150	30	5.50	0.1	150	35	5.40	0.1
30	150	30	5.59	0.0	150	35	5.48	0.0
36	150	30	5.86	0.0	150	35	5.98	0.0
42	150	30	7.04	0.0	150	35	6.46	0.0

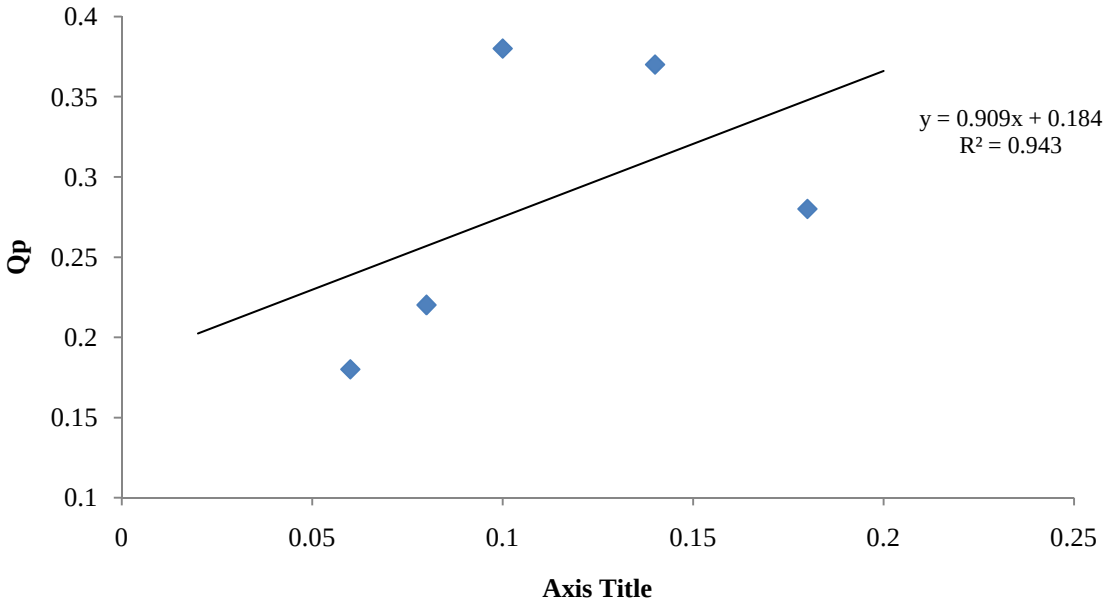


Fig. 72 Comparison between experimental results of specific rate of product formation and model prediction for separate hydrolysis and fermentation (SHF)

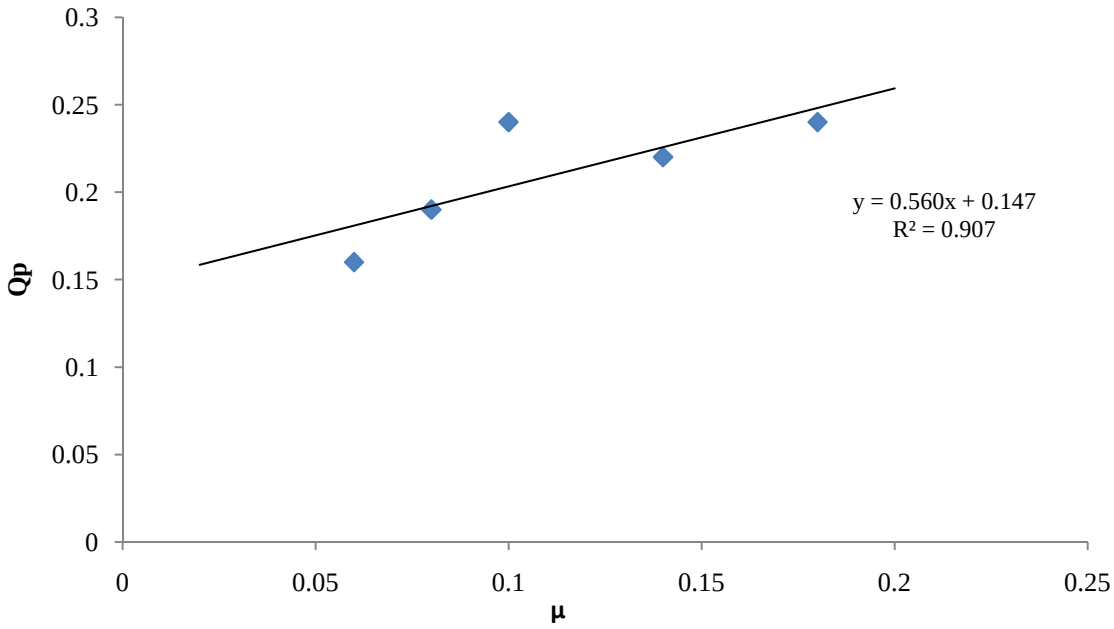


Fig. 73 Comparison between experimental results of specific rate of product formation and model prediction for simultaneous saccharification and fermentation (SSF)

This research has been designed with an objective of purifying α-amylase from extremophiles for its industrial usage. α-amylase extracted and purified from *B. aerius* GC6 and *B. sonorensis* GV2 owing to their unique and highly cherished properties make them a potential candidate in various industries. Application of purified α-amylase for the development of third generation biofuel, which has been undertaken in the present study strongly advocates the role of amylase in biofuel industry.

Chapter-5

SUMMARY AND CONCLUSIONS

Starch is a major reserve carbohydrate of all higher plants. In some cases it accounts for as high as 70% of the undried plant material. It is a heterogeneous polysaccharide composed of two high molecular weight entities called amylose and amylopectin. The hydrolysis of starch may be carried out using either acid or enzyme as catalyst. Enzymatic hydrolysis being more specific produces fewer byproducts and hence yields are higher. Starch-degrading amylolytic enzymes are thus, of great significance in industry ranging from food, fermentation, biofuel, textile to paper industries. Processing of starch is being carried out at higher temperatures therefore thermostable amylases are of particular interest in starch processing industries. Keeping in view all these facts, present investigation entitled **"Purification and characterization of amylase produced from potential extremophiles for industrial application"** was aimed to isolate most efficient amylogenic extremophiles from mushroom compost samples procured from Directorate of Mushroom Research, Chambaghat, Solan (H.P.). Hyper enzyme producers have been screened and identified by utilizing molecular techniques and their enzyme production potential has been enhanced by exploiting statistical tools. The enzyme thus produced has been purified to homogeneity by applying sequential purification steps and has been characterized with respect to various parameters. Immobilization of enzymes using various natural and synthetic carriers has been done and the enzymatic properties of free and immobilized enzymes were characterized. To assess the industrial application of purified α-amylase, algal bioethnaol production was conducted and its scale up in a bioreactor was carried out. The salient achievements of the research work conducted have been discussed as follows:

A total of 13 amylogenic bacteria had been isolated from mushroom compost. Physiological, metabolic and biochemical fingerprinting were performed for these bacterial isolates for their tentative identification. All the

isolates were found to be gram +ve, 61% were rod shaped, 15% were small rods while 23% were coccobacillus in shape. Tentative identification by using ABIS-online software (http://www.tgw1916.net/bacteria_abis.html) revealed isolates showed 92% homology to *Bacillus* sp.

Qualitative screening based on the zone of hydrolysis formed and quantitative screening based on the enzyme titers produced was conducted for all the isolates in order to identify the best enzyme yielding strains. Among all the strains, GC6 and GV2 showed biggest zone of hydrolysis. Maximum activity of 74.82 IU was possessed by GC6 followed by 68.24 IU by GV2. These two hydrolytic enzyme producing strains were thus selected for further studies. Identification of selected bacterial strains GC6 and GV2 was done by using 16S rRNA gene technique and these were identified as *Bacillus aerius* GC6 and *Bacillus sonorensis* GV2 respectively. The sequences has been submitted to NCBI and has been registered vide accession number *B. aerius* GC6 |KJ775810.1|, *B. sonorensis* GV2 |KJ775811.1|.

To enhance the amylogenic potential of the selected isolates, various process parameters and medium components viz. media types, incubation time, temperature, inoculum size, pH, substrate concentration, carbon sources, nitrogen sources and media additives etc. were first optimized by utilizing One Variable at a Time (OVAT) approach. Significant variables thus obtained were statistically optimized for an enhanced enzyme production by utilizing Central Composite Design (CCD) of Response Surface Methodology (RSM). For *B. aerius* GC6, enhanced α-amylase titers of 84.65 IU has been obtained at 50 $^{\circ}$C temperature and 9.0 pH @ 8.75% inoculum using 1.75% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration. Thus an increase of 11.61% was recorded in amylogenic potential of *B. aerius* GC6. Similarly, for *B. sonorensis* GV2, an enhanced enzyme titers of 82.78 IU/ml were observed with an increase of 17.55% at 50 $^{\circ}$C temperature and 9.0 pH @ 8.75% inoculum using 1.0% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration

Enzyme in purified form is a prerequisite in its studies of structure-function relationships and biochemical properties. Thus to concentrate enzyme

and to make it pure in order to increase its efficacy, sequential purification of extracted protein in three steps has been done. Ammonium sulphate precipitation was firstly done to concentrate the protein of interest from the bulk of proteins present in the supernatant. *B. aerius* GC6 α-amylase activity increased form 84.49 IU to 92.51 IU after ammonium sulphate precipitation with 1.38 purification fold and recovery of 54.74%. *B. sonorensis* GV2 α-amylase on the other hand exhibited an increase from 82.77 IU to 88.95 IU with a purification fold of 1.47 and 53.73% recovery. Concentrated α-amylase was then dialyzed which further concentrated the protein by forcing out the salt and other unwanted proteins from the membrane. Enzyme activity for *B. aerius* GC6 rose to 95.61 IU with 1.67 purification fold and 48.97% recovery, while that of B. *sonorensis* GV2 rose to 92.99 IU with 2.00 fold purification and 44.93% recovery. Gel exclusion chromatography was then performed to further purify the enzyme. Enzyme showed an increased respective enzyme activity of 103.46 IU for *B. aerius* GC6 with a purification fold of 15.16 and 45.26% recovery and 99.46 IU for *B. sonorensis* GV2 α-amylase with 11.46 fold purification and 36.04% recovery. Ion exchange chromatography was then carried out, yielding 112.89 IU of α-amylase for *B. aerius* GC6 with a final purification fold of 17.86 and 26.72% recovery and 106.76 IU for *B. sonorensis* GV2 with a final purification of 14.52 fold with 25.79% recovery. Molecular weight of *B. aerius* GC6 α-amylase was found to be 43kDA while that of *B. sonorensis* GV2 was found to be 45kDa.

Purified α-amylases from both the organisms were then characterized with respect to various physical and chemical characteristics. Both the enzymes showed a wider tolerance range for temperature and pH. Optimal pH range for purified *B. aerius* GC6 α-amylase has been found to be 7.0-10.0, retaining more than 85% activity at pH 9.0 and 10.0. *B. sonorensis* GV2 α-amylase showed an optimal activity ranging from pH 7.0-11.0. More than 75% of the activity was retained at pH 8.0-10.0 while at pH 11.0, 42% activity was retained. Thus the alkaline nature of the enzymes renders them potential candidate in detergent, textile, paper and biofuel industry. Behaviour of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 at varying temperatures ranging from 35-121°C was explored. Purified α-amylase from *B. aerius* GC6 and *B. sonorensis*

GV2 showed highest activity at optimum temperature of 50^{o}C. At a temperature of 60^{o}C, a respective activity of 96 and 92% was observed. At elevated temperatures upto 70^{o}C, 74 and 63% respective activity was retained. And upto 90^{o}C considerable activity was retained. The enzyme was even active at 121^{o}C with 20% relative activity. Thermostability of purified α-amylase from both the isolates is thus a striking feature which makes this enzyme a potential candidate to be used in starch processing industry.

Thermal inactivation parameters were studied in a temperature range spanning 35-121^{o}C. Half life of purified α-amylase at a temperature of 45-60°C was 90 min while it was 60 min at 70°C. Enzyme activity reduced to its half in just 15 min at higher temperatures of 90 and 121°C for both *B. aerius* GC6 and *B. sonorensis* GV2. Shelf life of the enzymes was studied for three months. For *B. aerius* GC6 α-amylase, 64.47% activity was retained after 70 days of storage at 4 ^{o}C and for *B. sonorensis* GV2 α-amylase, 58.47% activity was retained at 4^{o}C.

Various metal ions showed a varied effect on the behaviour of purified α-amylase from both the strains. In case of *B.* aerius GC6 α-amylase, an enhanced activity of 152.89% and 136.66% respectively was observed with Ca^{2+} and Mg^{2+} and for *B.* sonorensis GV2 an increased activity of 146.48% has been shown in the presence of Ca^{2+} in the medium, while 138.67% activity has been shown in the presence of Mg^{2+}, suggesting the enzyme to be a metalloprotein needing a cofactor for its maximum activity. The variable behaviours of enzymes towards different metal ions could be attributed to the source and nature of enzyme.

Various media additives were tested for their effect on purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase. Anionic detergent SDS retained more than 90% of the original activity; however, EDTA strongly inhibited the enzyme activity proving it to be a metalloenzyme. SDS stability of the enzyme is a rare feature which is very important, considering the applicability of amylase in industry. Other non-ionic surfactants such as Tween 20, Tween 80 and Triton X stabilized the enzyme activity. Purified enzymes from both the isolates were

found to be tolerant to ethanol, acetone and methanol however; they were comparatively less tolerant towards benzene and chloroform.

Purified enzymes showed specificity towards soluble starch as substrate. Corn starch, amylose and amylopectin however have yielded considerable enzyme activity. Raw starch hydrolysing efficiency of the enzymes was studied and it was found that purified *B. aerius* GC6 α-amylase showed 94% and 68% hydrolysis for potato and corn starch respectively, whereas *B. sonorensis* GV2 α-amylase showed 46% hydrolysis for corn starch. This is a very rare feature of bacterial α-amylase which makes it eligible for direct hydrolysis of raw starch thus rendering it a potential candidate for starch processing industry.

Immobilization of purified α-amylase from *B. aerius* GC6 and *B. sonorensis* GV2 was done by using different natural hydrogels viz. alginate, agar, agarose and gelatin. Different parameters for immobilization were studied viz. concentration of hydrogel, curing time, enzyme retention in terms of reusability. Among all the hydrogels, sodium alginate beads at a concentration of 3% were found to be most efficient for immobilization of purified α-amylase of *B. aerius* GC6 and *B. sonorensis* GV2. The entrapped enzyme in the form of beads was cured for 120 min in $CaCl_2$ solution and could be successfully reused till 8 cycles for *B. aerius* GC6 and till 7 cycles for *B. sonorensis* GV2.

Further characterization of alginate immobilized enzyme of both the strains was done for varying pH, temperatures and divalent ions. It was observed that the purified α-amylase, entrapped in alginate beads was more tolerant to the perturbations of temperature and pH at elevated levels, thereby suggesting the utility of immobilization for enhancing the stability and thus efficiency of enzyme.

However, the natural hydrogels have a constraint of being unable to retain the enzyme for long, thereby restricting its multiple reuses. Synthetic carriers have thus been introduced to get rid of this problem. These days, nano particles are gaining ample attention as a carrier for entrapment of enzymes as they are inert, easy to handle and can retain enzymes for long. Keeping in view the advantages offered by nano particles, purified enzymes of *B. aerius* GC6 and *B.*

sonorensis GV2 were immobilized using ascorbic acid modified iron oxide nano particles. These hydrogels showed a great retention of enzyme without any apparent loss in activity till 22 and 20 cycles respectively in case of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase. Effect of pH, temperature and divalent ions was studied on the nano particle immobilized enzyme from both the strains and it was found that the immobilized enzyme was more tolerant to the extremes or temperature and pH than their free counterparts.

Application of purified α-amylase in bioethanol production was studied using algal biomass as substrate for the production of reducing sugars which were further converted into ethanol using ethanologen *S. cerevisiae*-I (MTCC3089). Fresh water green algae *Hydrodictyon* sp. was used as a source of reducing sugars. Biochemical analysis of the constituents of the algal biomass was done in order to interpret the required enzymes for the hydrolysis of biomass. It was found that the *Hydrodictyon* sp. majorly consisted starch and cellulose (~ 90%) along with a little (~ 6%) pectin and rest proteins and other minerals.

Different physico-chemical pretreatments viz. saturated steam, acid and alkali etc. were first studied with an aim to render the *Hydrodictyon* biomass more accessible for enzymes for sugar production. Among the pretreatments tested, dilute H_2SO_4 pretreatment was adjudged as the best. Conditions for dilute H_2SO_4 pretreatment were then standardized and was found that 0.5% H_2SO_4 for 180 min at room temperature yielded highest reducing sugars. Following the pretreatment, enzymatic hydrolysis conditions were standardized. Different ratios for hydrolysis were standardized. Enzyme mixture comprising of α-amylase, β-amylase, cellulase and pectinases in a ratio of 4:3:2:1 yielded highest sugars of 810 mg/ g biomass. CCD of RSM was employed for the standardization of parameters for enzymatic hydrolysis. A maximum reducing sugars of 940 mg/g was obtained at 25 g/l biomass concentration, an enzyme loading of 12.50 ml/g biomass and for 42 h of incubation period at 45^{o}C, maximum saccharification of *Hydrodictyon* biomass was observed.

Following these conditions of pretreatment and enzymatic hydrolysis, the reducing sugars obtained were converted into bioethanol in a shake flask

experiment under different modes of fermentation viz. Separate hydrolysis and fermentation (SHF) and Simultaneous saccharification and fermentation (SSF).

During bioconversion studies in a shake flask under Separate hydrolysis and fermentation (SHF) mode, different conditions for fermentation viz. fermentation period, inoculum size of fermenting organism and were studied. 20% inoculum at 36^{th} h of fermentation period yielded 13.66g/l ethanol with a fermentation efficiency of 63.24% and theoretical yield of 1.33. Similarly, under Simultaneous saccharification and fermentation (SSF) mode, temperature, pH, inoculum size of fermenting organism were optimized and studied. A maximum ethanol yield of 10.25 g/l at pH 5.6 was observed. With 15% inoculum of *S. cerevisiae*-I, at 28 h of incubation period, 12.77g/l ethanol was produced exhibiting a fermentation efficiency of 60.48%. 13.16 g/l ethanol was obtained with a fermentation efficiency of 61.05% and 0.80 theoretical yield at 35^{o}C temperature.

Scale up of ethanol production was done in a 7.5 l stirred tank bioreactor under both SHF and SSF modes. The conditions which have been standardized for shake flask experiment were utilized for bioreactor operation. In case of SHF operation, 13.93 g/l ethanol was observed after 24 h of fermentation with fermentation efficiency of 64.84% and theoretical yield of 1.09. In case of SSF, 16.67 g/l of ethanol was obtained after 16 h of fermentation with a fermentation efficiency of 66.68% and theoretical yield of 1.31. On comparing the ethanol yield and working conditions of SHF and SSF, it was concluded that SSF was more efficient for ethanol production with 16.67 g/l ethanol, 66.68% fermentation efficiency and 1.31 theoretical yield. Moreover the fermentation time for SSF is just 16 h which is less than half of what is required in SHF.

Extraction and purification of α-amylase isolated from extremophiles *B. aerius* GC6 and *B. sonorensis* GV2 to homogeneity and its characterization has fulfilled the main aim of present study. Furthermore the development of technology for the successful conversion of algal biomass into bioethanol utilizing purified α-amylase signifies the industrial use potential of enzyme. The study strongly advocates the use of algae as a feedstock for the production of third generation biofuel as it is an environmental friendly approach.

Chapter-6

REFERENCES

Abdel-Fattah Y R, Soliman, Nadia A, El-Toukhy N M, El-Gendi H and Ahmed R S. 2013. Production, purification, and characterization of thermostable α-amylase produced by *Bacillus licheniformis* isolate AI20. *Journal of Chemistry* 1-11.

Abouzied M M and Reddy. 1986. Direct fermentation of potato starch to ethanol by cocultures of *Aspergillus niger*and *Saccharomyces cerevisiae. Applied and Environmental Microbiology* **52**: 1055-1059.

Adiguzel A, Ozkan H, Baris O, Inan K, Gulluce M and Sahin F. 2009. Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *Journal of Microbiological Methods* **79**: 321-328.

Adinarayana K and Ellaiah P. 2002. Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus* sp. *Journal of Pharmcy and Pharmaceutical Science* **5**: 272-278.

Agrawal P K. 2014. Microbial ecology of compost ecosystem: with special reference to mushroom compost. *Journal of Biological and Scientific Opinion* **2**(1): 45-50.

Ahmad R, Khatoon N and Sardar M. 2013.Biosynthesis charcaterization and application of TiO_2 nanoparticles in bicatalysis and protein folding. *Journal of Proteins and Proteomics* **4**(2):115-121.

Ahuja S K, Ferreira G M and Morreira A R. 2004.Application of Placket Burman design and response surface methodology to achieve exponential growth of aggregated shipworm bacterium. *Biotechnology and Bioengineering* **85**(6): 666-675.

Akin D E, Rigsby L L, Sethuraman A, Morrison W H, Gamble G R, Eriksson K E. 1995. Alterations in structure, chemistry, and biodegradability of grass lignocellulose treated with the white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus. Applied and Environmental Microbiology* **61**:1591–1598.

Alkando A A and Ibrahim H M. 2011.A potential new isolate for the production of a thermostable extracellular α-amylase. *Journal of Bacteriology Research* **3**(8): 129-137.

Allah M A G, Ghazy E A, Bayoumi, El-louboudy S S and Elsoud M M A. 2011. Optimization of acidic isoamylase production by isolated *Bacillus coagulans.Insight Microbiology* **1**(3): 34-38.

Al-Qodah A, Daghstani H, Geopel P and Lafi W. 2007.Determination of kinetic parameters of α-amylase producing thermophile *B. sphaericus*.*African Journal of Biotechnology* **6**(6): 699-706.

Alrumman S A, Mostafa Y S, Eifan S A, Alamri S A, Hesham A L. 2014. Isolation of thermoalkalophilic α- amylase producing bacteria and optimization of potato waste water medium for enhancement of α-amylase production. *Advances in Life Science and Technology* **20**(3): 41-51.

Al-ZaZaee M M A, Neelgund S, Gurumurthy D M and Rajeshwara A N. 2011. Identification, characterization of a novel halophilic *Bacillus cereus* Ms6: a source for extracellular α-amylase. *Advances in Environmental Biology* **5**(5): 992-999.

Amutha K and Priya K J. 2011.Effect of pH, temperature and metal ions on amylase activity from *Bacillus subtilis* KCX 006. *International Journal of Pharma & Bio Sciences* **2**(2): 407.

Andualem B. 2014. Isolation and screening of amylase producing thermophilic spore forming *Bacilli* sp. from starch rich soil and characterization of their amylase activities using submerged fermentation. *International Food Research Journal* **21**(2): 831-837.

Aneja K R. 2003. Experiments in microbiology, plant pathology and biotechnology.New Age International (P) Limited Publishers, New Delhi.

Ansari S A and Hussain Q. 2012. Potential applications of enzymes immobilized on/in nano materials: A review. *Biotechnology Advances* **30**(3): 512-523.

Arikan B. 2008. Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. *Bioresource Technology* **99**: 3071–3076.

Asad W, Maria A and Sheikh R A. 2010.Extracellular enzyme production by indigenous thermophilic bacteria: partial purification and characterization of α-amylase by *Bacillus* sp. Wa21. *Pakistan Journal of Botany* **43**: 1045–1052.

Asada C, Doi K, Sasaki C and Nakamura Y. 2012.Efficient extraction of starch from microalgae using ultrasonic homogenizer and its conversion into ethanol by simultaneous saccharification and fermentation. *Natural Resources* **3**: 175-179.

Asgher M, Asad M J, Rahman S U and Legge R L. 2007.A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *Journal of Food Engineering* **79:** 950–955.

Ashwini K, Kumar G, Karthik L and Bhaskara Rao K V. 2011. Optimization, production and partial purification of extracellular α-amylase from *Bacillus* sp.*marini*. *Archives of Applied Science Research* **3**(1): 33-42.

Atabani A E, Silitonga A S, Badruddin I A, Mahlia T M I and Masjuki H H. 2012.A comprehensive review on biodiesel as an alternative energy resource

and its characteristics. *Renewable and Sustainable Energy Reviews* **16**: 2070-2093.

Atanasijevic T, Shusteff M, Fam P, Jasanoff A. 2006. Calcium-sensitive MRI contrast agents based on superparamagnetic iron oxide nanoparticles and calmodulin. *Proceedings of National Academy of Sciences USA* **103**:14707–14712.

Ausubel F M, Brett R, Kingston R E, Moore D D, Seidman J G, Smith J A and Struhl L. 1991.*Current Protocols in Molecular Biology*, Vol. 1.Wiley. New York.

Avwioroko O J, Tonukari N J and Asagba S O. 2015.Biochemical characterization of crude α-amylase of *Aspergillus* spp. associated with the spoilage of cassava (M*anihot esculenta*) tubers and processed products in Nigeria. *Advances in Biochemistry* **3**(1): 15-23.

Awoyinka O A, Daini O, Satisha K and Chatterji D. 2014. Mass spectrometric determination of the primary structure of β amylase isolated from amylolytic Nigerian Maize cultivar. *British Biotechnology Journal* **4(6)**: 740-750.

Aygan A, Arikan B, Korkmaz H, Dincer S and Colak O. 2008.Highly thermostable and alkaline α-amylase from halotolerant alkaliphilic *Bacillus* sp. AB68. *Brazilian Journal of Microbiology* **39**: 547-553.

Azad M A K, Bae J H, Kim SJ, Lim JL, Song K S, Shin B S and Kim H R. 2009. Isolation and characterization of a novel thermostable alpha amylase from Korean pine seeds. *New Biotechnology* **26**(3): 143-149.

Babu K R and Satyanarayana T. 1993.Parametric optimization of extracellular alpha-amylase production by thermophilic *Bacillus coagulans*. *Folia Microbiologia* **38**: 77-80.

Bacovsky D, Mabee W and Worgetter M. 2010. How close are second-generation biofuels? *Biofuels Bioproducts and Biorefining* **4**:249–52.

Bai Y, Huang H, Meng K, Shi P, Yang P, Luo H, Luo C. Feng Y, Zhang W and Yao B. 2012. Identification of an acidic α-amylase from *Alicyclobacilllus* sp. A4 and assessment of its application in the starch industry. *Food Chemistry* **131**: 1473-1478.

Bajpai P, Verma N and Bajpai P. 1990. Culture conditions for production of thermostable α-amylase by *Bacillus* sp.25A. *Journal of Microbiology and Biotechnology* **5**: 39-52.

Bakri Y, Magali M and Thonart P. 2009.Isolation and identification of a new fungal strain for amylase biosynthesis. *Polish Journal of Microbiology* **58**: 269–273.

Baldino A, Macias M and Cantero D. 2001. Immobilization of glucose oxidase with calcium alginate gel capsules. *Process Biochemistry* **36**:601-606.

Bano S, Qader S A U, Aman A and Azhar A. 2009. Partial purification and some properties of α-amylase from *B. subtilis* KIBGE-HAS. *Indian Journal of Biochemistry and Biophysics* **46**: 401-404.

Bansode S D. 2010. Screening of nutritional components for α-amylase production in submerged fermentation by bacteria isolated from soil using Placket Burman design. *International Journal of Pharmacy and Pharmaceutical Sciences* **2**(1): 93-98.

Barsar B, Shamzi M M, Rosfarizan M, Puspaningsih N N T and Ariff A B. 2010. Enhanced production of thermophilic xylanase by recombinant *Escherichia coli* DH5α through optimization of medium and dissolved oxygen level. *International Journal of Agriculture and Biology* **12**(3): 321-328.

Bauer M, Driskil L, Callen W, Snead M, Mathur E and Kelly R. 1999. An endoglucanase EglA, from the hyperthermophilic archaeon *Pyrococcus furiosus* hydrolyzes a-1,4 bonds in mixed linkage (1-3), (1-4)-b-D-glucans and cellulose. *Journal Bacteriology* **181:** 284–290.

Becks S, Bielawaski C, Henton D, Padala R, Burrows K, Slaby R. 1995. Application of a liquid stable amylase reagent on the Ciba Corning Express clinical chemistry system. *Clinical Chemistry* **41**:186.

Berg B. 2003. Extremophiles as a source for novel enzymes.*Current Opinion in Microbiology* **6**: 213-218.

Stryer L, Berg J M, Tymoczko J L. 2002 Biochemistry. 5th edition.WH Freeman New York.

Bhabla T C and Chtanta D K. 2000.*In*: Food processing biotechnological applications (Marwich SS & Horra JK) eds. Assisted publishers, New York. pp 123-132.

Bharat B and Hoondal G. 1998.Isolation, purification and properties of thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. *Biotechnology Letters* **20:** 157–159.

Bihari V. 1985. Characterization and reactor performance studies with immobilized glucose oxidase. *Indian Journal of Microbiology* **25**(1): 11-17.

Bollag D M and Edelstein S J. 1991a.Concentrating protein solutions.*In*: Protein methods. A John Wiley Sons, INC, Publications, USA.

Bollag D M and Edelstein S J. 1991b.Protein methods.Wiley Liss, John Wiley and Sons, Inc. New York.

Box G and Wilson K B. 1951.On the experimental attainment of optimum conditions. *Journal Royal Statistics* **13**(1):1-45.

Bozic N, Ruiz J, Lopez-Santin J and Vujcic Z. 2011.Production and properties of the highly efficient raw starch digesting α-amylase from a *Bacillus licheniformis* ATCC 9945a. *Biochemical Engineering Journal* **53:** 203-209.

Brena B M and Viera F B. 2006.Immobilization of Enzymes and Cells, In: *Methods in Biotechnology*. J. M. Guisan eds. 2nd edition.Humana Press Inc., Totowa, NJ.

Brennan L and Owende P. 2010.Biofuels from microalgae-a review of technologies for production, processing and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews***14**: 557-577.

Bucke C.1987. Methods in Enzymology, In: *Immobilized Enzymes and Cells*. 2nd edition, Moshbach (eds.). Academic Press, Orlando **135**: 175-189.

Burgess-Cassler A and Imam S. 1991.Partial purification and comparative characterization of α-amylase secreted by *Lactobacillus amylovorous*.*Current Microbiology* **23:** 207-213.

Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, Osman G. 2003. Enzymatic properties of a novel thermostable, ther-mophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. *Process Biochemistry* **38**: 1397-1403.

Bury K, Hendrickson R, Brewer M, Dien B, Dreschel R and Ladisch M. 2000. Incremental ethanol yields from processing corn fiber by thermal pretreatiment and enzymatic hydrolysis. National Meeting of American Chemical Society. San Francisco, CA.

Calik P and Ozdamar T H. 2001. Carbon sources affect metabolic capacities of *Bacillus* species for the production of industrial enzymes theoretical analyses for serine and neutral proteases and alpha-amylase. *Biochemical Engineering Journal* **8**:61-81.

Caputi A, Ueda M and Brown J. 1969. Spectrophtometric determentaion of ethanol in wine. *American Journal of Enology and Viticulture* **19**: 160-165.

Castro G R, Baigori M D and Sineriz F. 1999.Studies on α-amylase production by *Bacillus licheniformis* MIR-61. *Acta Biotechnologica* **19**: 263-272.

Chakraborty S A, Khopade C, KokareK, Mahadik and Chopade B. 2009.Isolation and characterization of novel α-amylase from marine *Streptomyces* sp.D1. *Journal ofMolecular Catalysis B: Enzymatic* **58(**1–4): 17–23.

Chaudhary A B and Chincholkar S B. 1996.Cell Immobilization: A critical approach to ethanol production by *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Indian Journal of Microbiology* **36**: 75-83.

Chauhan B and Gupta R. 2004.Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp.RGR-14.*Process Biochemistry* **39**: 2115-2122.

Chavan S and Wadatkar C M. 2014.Isolation, purification and characterisation of alpha amylase from *Phaseolus aconitifolius*. *Journal of Advanced Drug Delivery* **1**(3): 114-121.

Chi M, Chen Y, Wu T, Lo H and Lin L. 2009.Engineering of a truncated α-amylase of *Bacillus* sp. strain TS-23 for the simultaneous improvement of thermal and oxidative stabilities. *Journal of Bioscience and Bioengineering* xx, xxx-xxx.

Chibata I, Tosa T and Fujimura F. 1983.Immobilized living microbial cells. In: G.T. Tsao ed. Annual Reports on Fermentation Processes, Academic Press, London 6:1-22.

Chisti Y. 2007. Biodiesel from microalgae. *Biotechnology* **25**:294–306.

Chung Y C, Kobayashi T, Kanai H, Akiba T, Kudo T. 1995. Purification and properties of extracellular amylase from the hyperthermophilic archeon *Thermococccus profundus* DT5432. *Applied and Environmental Microbiology* **61**: 1502-1506.

Coleman G and Elliot W H. 1962.Studies onα-amylase formation by *B. subtilis. Biochemistry journal* **83**(2): 256-263.

Collins B S, Kelly C T, Fogarty W M and Doyle E M. 1993. The high maltose producing α-amylase of the thermophilic actinomycete *Thermomonospora curvata*. *Applied Microbiology and Biotechnology* **39**:31-35.

Coman G and Bahrim G. 2011.Optimization of xylanase production by *Streptomyces* sp. P12-137 using response surface methodology and central composite design. *Annals of Microbiology* **61**: 773-779.

Coninck J D, Bouquelet S, Dumortier V, Duyme F and Denantes V I. 2000. Industrial media and fermentation process for improved growth and protease production by *Tetrahymena thermophila* BIII. *Journal of Industrial Microbiology and Biotechnology* **24**: 285-290.

Couto S R and Sanromán M A. 2006.Application of solid-state fermentation to food industry- A review.*Journal of Food Engineering* **76:** 291-302.

Creighton T E. 1984. Proteins, Freeman, Oxford, UK.

Cutler P. 2005.Methods in molecular biology:protein purification protocols. Humana Press Inc.,Totowa, NJ.

D'Souza S F. 1989.Immobilized Cells: Techniques and Applications.*Indian Journal of Microbiology* **29**(2): 83-87.

Dakhmouche D S, Gheribi-Aoulmi Z, Meraihi Z and Bennamoun L. 2006. Application of statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste-powder. *Journal of Food Engineering* **73**: 190-197.

De Azeredo L A I, Freire D M G, Soares R M A, Leite S G F and Coelho R R R. 2004. Production and partial characterization of thermophilic proteases from *Streptomyces* sp. isolated from Brazilian cerrado soil. *Enzyme and Microbial Technology* **34**:354–358.

Deb P, Talukdar S A, Mohsina K, Sarker P K and Sayem S M A. 2013.Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001.*SpringerPlus* **2**:154. doi: 10.1186/2193-1801-2-154.

Dehkordi M M and Javan F A. 2013.Application of alpha-amylase in biotechnology. *Journal of Biology and Today's World* **1**(1): 39-50.

Demir S, Gök S B and Kahraman M V. 2012. α-amylase immobilization on functionalized nano $CaCO_3$ by covalent attachment. *Starch – Stärke* **64**(1):3–9.

Demirbas M F, Balat M and Balat H. 2011.Biowastes-to-biofuels. *Energy Conversion and Management* **52**(4): 1815-1828.

Demirijan D, Moris-Varas F and Cassidy C. 2001.Enzymes from extremophiles. *Current Opinion in Chemistry and Boilogy* **5:** 144–151.

Demirkan E, Dincbas S, Sevinc N and Ertan F. 2011.Immobilization of *B. amyloliquefaciens* α-amylase and comparison of some of its enzymatic properties with the free form. *Romanian Biotechnological Letters* **16**(6): 6690- 6701.

Deutscher M.P. 1990. Methods in Enzymology. *In*: A Guide to Protein Purification. Academic Press, New York.

Devi B, Unni B G, Wann S B and Samanta R. 2012. Immobilization of partially purified alpha-amylase enzyme produced by a soil born *Bacillus sp. Advances in Applied Science Research* **3**(5):2739-2744.

Dey G, Mitra A and Banerjee M B R. 2001.Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. *Biochemical Engineering Journal* **7**: 227-231.

Dey G, Singh B and Banerjee R. 2003.Immobilization of α-amylase produced by *B. circulans* GRS313. *Brazilian Archives of Biology and Technology* **46**(2): 167-176.

Dhanasekaran D, Sivamani P, Rajkumar G, Panneerselvam A and Thajuddin N. 2006.Studies on free and immobilized cells of *Bacillus* species on the production of alpha-amylase. *The Internet Journal of Microbiology***2**(2).

Dhawale M R, Wilson J J, Khachatourians G G and Ingledew W M. 1982.Improved method for detection of starch hydrolysis. *Applied and Environmental Microbiology* **44**:747-750.

Dheeran P, Kumar S, Jaiswal Y K and Adhikari D K. 2010.Characterization of hyperthermostable *α*-amylase from *Geobacillus* sp. IIPTN. *Applied Microbiology and Biotechnology* **86**:1857–1866.

Diane W, Stephan R and Wolfgang Z. 1997.Application of thermostable enzymes for carbohydrate modification.*In*: Contribution of the Fourth International

Workshop on Carbohydrate as Organic Raw Materials, March 20–21, 1997. WUV-Universitatverlag, Vienna.

Dismukes G C, Carrieri D, Bennnette N, Ananyev G M and Posewitz M C. 2008. Aquatic phototrrops: efficient alternatives to land based crops for biofuels. *Current opinions in biotechnology* **19**: 235-240.

Domozych D S, Ciancia M, Fangel J U, Mikkelsen M D, Ulvskov P, Willats W G T. 2012. The cell walls of green algae: a journey through evolution and diversity. *Frontiers in Plant Science* **3:** 82.

Doran-Peterson J, Jangid A, Brandon S K, DeCrescenzo-Henriksen E, Dien B, Ingram L O. 2009. Simultaneous saccharification and fermentation and partial saccharification and co-fermentation of lignocellulosic biomass for ethanol production. *Methods in Molecular Biology* **581:** 263-280.

Dragomirescu M, Vintila T, Jurcoane S and Preda G. 2010. Sol-gel entrapment of *B. licheniformis* CMIT 1.33 proteases in silica gels. *Romanian Biotechnology Letters***15**: 5125-5133.

Eisenthal R, Danson M J and Hough D W. 2007. Catalytic efficiency and k_{car}/k_m: a useful comparator. *Trends in Biotechnology* **25**(6): 247-249.

Eklund R and Zacchi G. 1995. Simultaneous saccharification and fermentation of steam pretreated willow. *Enzyme and Microbial Technology* **17**(3): 255-259.

El-Batal A I, Atia K S and Eid M A. 2005. Stabilization of α-amylase by using anionic surfactant during the immobilization process. *Radiation Physics and Chemistry* **74**:96-101.

El-Ghaffar M A A and Hashem M S. 2009. Immobilization of α-amylase onto chitosan and its amino acid condensation adducts. *Journal of Applied Polymer Science* **112**: 805 – 814.

Elias M, Wieczorek G, Rosenne S and Tawfik D S. 2014.The universality of enzymatic rate- temperature dependency. *Trends in Biochemical Sciences* **39**(1): 1-7.

Elibol M. 2004. Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor*A3(2) with response surface methodology. *Process Biochemistry* **39**: 1057-1062.

Emmanuel L, Stefan J, Bernard H and Abdel B. 2000.Thermophilic archaeal amylolytic enzymes. *Enzyme and Microbial Technology* **26:** 3–14.

Energy Information Agency (EIA 2002) Renewable Energy Annual 2002 (DOE/EIA-0603)

Eshaq F S, Ali M N and Mohd M K. 2011. Production of bioethanol from next generation feed stock alga *Spirogyra* species. *International Journal of Engineering Science and Technology* **3**(2): 1749-1755.

Everly C and Alberto J. 2000. Stressors, stress and survival: overview. *Frontiers in Biosciences* **5**: 780–786.

Feller G, Le Bussy O and Gerday G. 1998.Expression of psychro-philic genes in mesophilic hosts: Assessment of the folding state of a recombinant a-amylase, *Applied and Environmental Microbiology* **64**(3): 1163-1165.

Ferreira S, Duarte A P, Ribeiro M H L, Queiroz J A and Domingues F C. 2009.Response surface optimization of enzymatic hydrolysis of *Cistus ladanifer* and *Cystis striatus* for bioethanol production. *Biochemical Engineering Journal* **45**: 192-200.

Fincan S A, Enez B, Özdemir S and Matpan Beklera F M. 2014.Purification and characterization of thermostable α-amylase from thermophilic *Anoxybacillus flavithermus*. *Carbohydrate Polymers* **102**: 144– 150.

Flores M E, Perez R and Huitron C. 1997.β-xylosidase and xylanase characterization and production by *Sreptomyces* species CH-M-1035. *Letters of Applied Microbiology* **24**: 410-416.

Fooladi J and Sajjadian A. 2010.Screening of thermophilic and hyperthermophilic bacterial population of three Iranian hot-springs to detect the thermostable α-amylase producing strain. *Iranian Journal of Microbiology* **2**(1): 49-53.

Francis F, Sabu A, Nampoothiri K M, Szakacs G and Pandey A. 2002.Synthesis ofα-amylase by *A. oryzae* in solid state fermentation. *Journal of Basic Microbiology* **42**: 320-326.

Fredrich, A and Antrakian G. 1996.Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilic anaerobic species of the order *Thermotogales*. *Applied and Environmental Microbiology* **62:** 2875–2882.

Fukui S and Tanaka A. 1982.Immobilized microbial cells. *Annual Review of Microbiology* **36**: 145-172.

Fumi M D, Trioli G and Colagrande O. 1998.Immobilization of *Saccharomyces cerevisiae* in calcium alginate gel and its application in bottle-fermented sparkling wine production. *American Journal of Enology and Viticulture* **39**: 267-272.

Gangadharan D, Sivaramakrishnan S, Nampoothiri K M, Sukumaran R K, Pandey A. 2008. Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens. Bioresource Technology* **99**: 4597–4602.

Garcia-Ochoa F, Gomez E and Santos V E. 1995. Oxygen transfer and uptake rates during xanthan gum production. *Enzyme Microbiology and Technology* **27**:680–690

Gavrilescu M and Chisti Y. 2005.Biotechnology-a sustainable alternative for chemical industry. *Biotechnology Advances* **23**: 471-499.

Ghollasi M, Ghanbari-Safari M, Khajeh K. 2013. Improvement of thermal stability of a mutagenised α-amylase by manipulation of the calcium-binding site. *Enzyme and Microbial Technology* **53**: 406–413.

Ghorai S, Banik S P, Verma D, Chowdhury S, Mukherjee S and Khowala S. 2009. Fungal biotechnology in food and feed processing. *Food Research International* **42:**577–587.

Gigras P, Sahai V and Gupta R. 2002.Statistical media optimization and production of its alpha-amylase from *Aspergillus oryzae* in a bioreactor.*Current Microbiology* **45**:203-208.

Gimbi, D M and Kitabatake D. 2002.Changes in α-amylases activities during seed germination of African finger millet. *International Journal of Food Science* **6**:481–488.

Giri N Y, Mohan A R, Rao L V, Rao C P. 1990. Immobilization of α-amylase complex in detection of higher oligosaccharides on paper.*Current Science* **59**:1339-1340.

Godia F, Casas C and Sola C. 1991. Application of immobilization yeast cells to sparkling wine fermentation. *Biotechnology Progess* **7**(5): 468-470.

Goldstein L, Levin Y and Katchalski E. 1964.A water-insoluble polyanionic derivative of trypsin and effect of the polyelectrolyte carrier on the kinetic behavior of the bound trypsin. *Biochemistry* **3**:1913-1919.

Goto C E. 1998. Production of amylase by *Aspergillus fumigatus* utilizing α-methyl-D-glycoside, a synthetic analogue of maltose as substrate.*FEMS Microbiology Letters* **167:** 139–142.

Goyal N, Gupta J K, Soni S K. 2005. A novel raw starch digesting thermostable a-mylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch, *Enzyme and Microbial Technology* **37:** 723-734.

Graham J, Wilcox L and Graham L. 2009.Algae.2nd edition. San Francisco: Benjamin Cummings (Pearson).

Groboillot A. 1994. Immobilization of cells for application in the food industry.*Critical Reviews in Biotechnology* **14:** 75–107.

Guangrong H, Dehui D, Weilian H and Jiaxin J. 2008.Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 witha statistical method. *African Journal of Biotechnology* **7**(8): 1115-1122.

Guisan J. 2008. Improvement of enzyme properties via immobilization techniques.*SciTopics*.http://www.scitopics.com/improvement_of_enzyme_properties_via_immobilization_techniqueshtml.

Gupta A K and Gupta M. 2005.Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* **26**: 3995–4021.

Gupta A, Gupta V K, Modi D R and Yadava L P. 2008. Production and characterization of α-amylase from *Aspergillus niger.Biotechnology* **7**: 551-556.

Gupta J S. 1981. Textbook of algae. Oxford & IBH Publ. Co. New Delhi. pp. 82-95

Gupta R, Biswas K, Mishra I and Suthindhiran K. 2012.Ethanol production from marine algae using yeast fermentation. *Research Desk* **1**(1): 17-22.

Gupta R, Gigras P, Mohapatra H, Goswami V K and Chauhan B. 2003. Microbial alpha amylase: a biotechnological perspective. *Process Biochemistry* **38**: 1599-1616.

Gupta A and Khare S. 2006.A protease stable in organic solvents from solvent tolerant strain *Pseudomonas aeruginosa*. *Bioresource Technology* **97**: 1788-1793.

Haaland PD (ed). 1989. *Experimental design in biotechnology.*Marcel Dekker, Inc., New York.

Hahn-Hagerdal B, Galbe M, Gorwa-Grauslung MF, Liden G, Zacchi G. 2007. Bioethanol-the fuel of tomorrow from the residues of today. *Trends in Biotechnology* **24:** 249-254.

Hailemariam A T, Gezmu T B and Haki G D. 2013.Thermostable alpha-amylase from geothermal sites of Ethiopia (Afar region): isolation, purification and characterization. *Greener Journal of Biological Sciences* **3(2):** 061-073.

Haki G D and Rakshit S K. 2003. Developments in industrially important thermostable enzymes: a review. *Bioresource Technology* **89**(1): 17-34.

Halling P J and Dunnill P 1980. Magnetic supports for immobilized enzymes and bioaffinity adsorbents. *Enzyme and Microbial Technology* **2:** 2–10.

Halwachs W, Wandrey C and Schügerl K. 1978. Immobilized α-chymotrypsin: pore diffusion control owing to pH gradients in the catalyst particles. *Biotechnology and Bioengineering* **20**:541-554.

Hamilton L M, Kelly C T, Fogarty W M. 1999. Production and properties of the raw starch-digesting amylase of *Bacillus* sp. IMD 435. *Process Biochemistry* **35**:27-31.

Hamza H M. 2004. Purification and immobilization of amylase enzyme produced from local isolate of *B. subtilis*. *Journal of Zankoy Sulaimani*.**7**(1): 61-68.

Hanfeld U, Gardossi L and Magner E. 2009. Understanding enzyme immobilisation. *Chemical Society Reviews* **38:** 453–468.

Haq I U, Ashraf H, Omar S and Qadeer M A. 2002. Biosynthesis of amyloglucosidase by *Aspergillus niger*using wheat bran as substrate. *Pakistan Journal of Biological Sciences* **5**: 962–864.

Hari P R, Chandy T and Sharma C P. 1996. Chitosan/ calcium alginate microcapsules for intestinal delivery nitrofurantion. *Journal of Microencapsulation* **13**: 319-329.

Harun R and Danquah M K. 2011. Influence of acid pre-treatment on microalgal biomass for bioethanol production. *Process Biochemistry* **46**:304-309.

Haseltine C, Rolfsmeier M and Blum P. 1996. The glucose effect and regulation of α-amylase synthesis in the hyperthermophilic archeon *Sulfolobus solfataricus. Journal of Bacteriology* **178**: 945-950.

Hashemi M, Razavi S H, Shojaosadati S A, Mousavi S M, Khajeh K and Safari M. 2010. Development of solid-state fermentation process for production of an alpha amylase with potentially interesting properties. *Journal of Biosciences and Bioengineering* **110**(3): 333-337.

Hassan S A, Ali S A, Abbasi A and Kamal M. 2011.Purification and biochemical characterization of a Ca^{2+} independent, thermostable and acidophilic α-amylase from *Bacillus* sp. RM16. *African Journal of Biotechnology* **10**(32): 6082-6089.

Haveren J S E L and Sanders J. 2008. Bulk chemicals from biomass.*Biofuels Bioproducts and Biorefining***2**:41–57.

Hayashida S, Teramoto Y and Inoue T. 1988.Production and characteristics of raw starch digesting α-amylase from *B. subtilis* 65. *Applied and Environmental Microbiology* **54**: 1615-1622.

Herbert R and Sharp R. 1992.Molecular Biology and Biotechnology of Extremophiles. Chapman and Hall, NewYork.

Hernandez M S, Rodrıguez M R, Guerra N P and Roses R P. 2006. Amylase production by *Aspergillus niger*in submerged cultivation on two wastes from food industries. *Journal of Food Engineering* **73**: 93–100.

Hewitt C J and Solomons G L. 1996.The production of α-amylase by *B. amyloliquefaciens* in a complex and totally defined synthetic culture medium. *Journal of Industrial Microbiology* **17**: 96-99.

Hmidet N, El-Hadj Ali N, Haddar A, Kanoun S, Alya S and Nasri M. 2008. Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive. *Biochemical Engineering Journal* **47**: 71-79.

Ho S H, Huang S W, Chen C Y, Hasunuma T, Kondo A and Chang J S. 2013. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresource Technology* **135**: 191-198.

Horisberger M. 1976. Immobilization of protein and polysaccharide on magnetic particles: selective binding of microorganisms by concanavalin A-magnetite, *Biotechnology and Bioengineering* **18**: 1647–1651.

Hu X, Wang C, Wang L, Zhang R and Chen H. 2014. Influence of temperature, pH and metal ions on guaiacol oxidation of purified laccase from *Leptographium qinlingensis. World Journal of Microbiology and Biotechnology* **30**(4):1285-1290.

Huynh M and Serediak N. 2006.Algae Identification Field Guide.Agriculture and Agri-Food Canada.http://www.planta.cn/forum/files_planta/oe_409.pdf

Iefuji H, Chino M, Kato M and Imura Y. 1996. Raw starch digesting and thermostable α-amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization and cloning and sequencing. *Journal of Biochemistry* **318**: 989-996.

Ikura Y and Horikoshi K. 1987. Effect of amino compounds on alkaline amylase production by alkaliphilic *Bacillus* sp. *Journal of Fermentation Technology* **65**: 707-709.

Industrial Enzymes Market by types, applications & geography-Global Trends & Forecasts to 2018. 2015. http://www.marketsandmarkets.com/Market-Reports/industral-enzymes-market-237327836.html.

Jaiswal N, Prakash O, Talat M, Hasan S H and Pandey R K. 2012.α-amylase immobilization on gelatin: Optimization of process variables. *Journal of Genetic Engineering and Biotechnology* **10**: 161–167.

Jalal R, Mortazavi Z Milani and Goharshadi E K. 2011.Immobilization of α-amylase onto magnetic nanoparticles by shaking method.Proceedings of 1st National Iranian New Chemistry Congress. 5-6 May 2011.

Janarthanan R, Prabhakaran P and Ayyasamy P M. 2014.Bioremediation of vegetable wastes through biomanuring and enzyme production. *International Journal of Current Microbiological and Applied Sciences* **3**(3): 89-100.

Jayaramu M, Rafik M, Manjula, Kumar M, Siddalingeshwara K G, Dayananda K S, Jayasimha G B and Dwarakanath V. 2011. Amylase synthesis in elevated level by optimizing fermentation parameters from *Aspergillus versicolor*. *International Journal of Research and Reviews in Pharmacy and Applied Sciences* **1**(4): 341-346.

Jen A C, Wake M C and Mikos A G. 1996. Review: Hydrogels for cell immobilization. *Biotechnology and Bioengineering* **50**: 357-364.

Jin F, Li Y, Zhang Ch, Yu Y. 2001. Thermostable α-amylase and β-galactosidase production from the thermophilic and aerobic *Bacillus* sp.JF strain. *Process Biochemistry* **36**: 559-564.

John R P, Anisha G, Namapoothiri K M, Pandey A. 2011. Micro and macroalgal biomass: a renewable resource for bioethanol. *Bioresource Technology* **102**: 186-193.

Johnson A K, Zawadzka A M, Deobald, Crawford R L and Paszczynski A J. 2008.Novel method for immobilization of enzymes to magnetic nanoparticles. *Journal of Nanoparticle Research* **10**(6): 1009-1025.

Jones C S and Mayfield S P. 2012. Algae biofuels: versatility for the future of bioenergy. *Current Opinion in Biotechnology* **23**: 346-351.

Jorgensen H, Kristensen J B and Felby C. 2007. Enzymatic conversion of lignocelluloses into fermentable sugars: challenges and opportunities. *Biofuels Bioproduction and Biorefining* **1**: 119-134.

Jyoti J, LalN, Lal R and Kaushik A. 2011.Partial purification and characterization of an acidophilic extracellular α-amylase from *B. lichemiformis* JAR-26. *International Journal of Advanced Biotechnology and Research* **2**(3): 315-320.

Kalburcu T, Tuzmen M N, Akgol S and Denizli A. 2014. Immobilized metal ion affinity nanospheres for α-amylase immobilization. *Turkish Journal of Chemistry* **38**: 28-40.

Kanwal B, Zia B A, Yasin M, Rahman K and Sheikh M A. 2004. Purification and Characterization of α-Amylase from Apple (*Malus pumila)*. *International Journal of Agriculture & Biology*. **15**: 8530-8536.

Karel S F, Libicki S B and Robertson C R. 1985.The immobilization of whole cells-engineering principles.*Chemical Engineering Science* **40**(8): 1321-1354.

Kathiresan K and Manivannan S. 2006.α-amylase production by *P. fellutanum* isolated from mangrove rhizospheric soil. *African Journal of Biotechnology* **5(**10): 829-832.

Kaur A, Kaur M, Samyal M L and Ahmed Z. 2012.Isolation, characterization and identification of bacterial strain producing amylase. *Journal of Microbiology and Biotechnology Research* **2**(4): 573-579.

Kaushal R. 2014. Evaluation of different physico-chemical and biological pretreatments for enzymatic hydrolysis of hardwood for ethanol production. Ph. D. Thesis. Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, HP, India.

Kawakami K, Tsuruda S and Miyagi K. 1990. Immobilization of microbial cells in a matrix of silicone polymer and calcium alginate gel: Epoxidation of 1-octene by *Nocardia corallina* B-276 in organic media. *Biotechnology Progress* **6**(5): 357-361.

Kelly C D, Fellers T J and Davidson M W. 2003. Molecular expressions: Science optics and you. Available at: http://micro.magnet.fsu.edu/hydrodictyon.html.

Kelly C T, McTigue M A, Doyle E M and Fogarty W M. 1995.The raw starch degrading alkaline amylase of *Bacillus* sp. IMD 370. *Journal of Industrial Microbiology and Biotechnology***15**: 446-448.

Khalil A. 2011. Isolation and characterization of three thermophilic bacterial strains (lipase, cellulose and amylase producers) from hot springs of Saudi Arabia. *African Journal of Biotechnology* **10**(44): 8834-8839.

Kharkrang K and Ambasht P K. 2012.Purification and characterization of alpha amylase from seeds of pearl millet (*Pennisetum typhoides). Journal of Proteins and Proteomics* **3**(1): 47-60.

Kierstan M and Bucke C. 1977.The immobilization of microbial cells, subcellular organelles and enzymes in calcium alginate gel. *Biotechnology and Bioengineering* **19**(3): 87-89.

Kılıc D, Apar F H and Ozbek B. 2005.α-amylase inactivation during rice starch hydrolysis. *Process Biochemistry* **40**: 1367–1379.

Kim K H, Choi I S, Kim H M, Wi S G and Bae H J. 2014. Bioethanol production from the nutrient stress-induced microalga *Chlorella vulgaris* by enzymatic hydrolysis and immobilized yeast fermentation.*Bioresource Technology* **153**: 47-54.

Kim N J, Li H, Jung K, Chang H N, Lee P C. 2011. Ethanol production from marine algal hydrolysates using *E. Coli* KO11.*Bioresource technology* **102:** 7466-7469.

Kiran KK and Chandra TS. 2008.Production of surfactant and detergent-stable, halophilic and alkalitolerant alpha-amylase by a moderately halophilic *Bacillus* sp. Strain TSCVKK. *Biotechnologically Relevant Enzymes and Proteins* **77**: 1023-1031.

Kirk O, Borchert T V and Fuglsang C C. 2002.Industrial enzyme applications.*Current Opinions in Biotechnology* **13:** 345-351.

Kitabatake N, Gimbi D M and Oi Y. 2003. Traditional non-alcoholic beveragr Togwa, in East Africa, produced from maize flour and germinated finger millet. *International Journal of Food Science and Nutrition* **54**(6): 447-455.

Kiyohara P K, Lima U A, Santos H S and Santos P S. 2003. Comparative studies between yeast immobilized on alumina beads and on membrane prepared by two routes. *Brazilian Journal of Microbiology* **34**: 129-137.

Knezevic Z, Milosavic N, Bezbradica D, Jakovljevic Z and Prodanovic R. 2006.Immobilization of lipase from *Canidida rugosa* on Eupergit support by covalent attachment.*Biochemical Engineering Journal* **30**(3): 269-278.

Koch R, Spreinat K, Lemke K, Antranikian G. 1991. Purification and properties of a hyperthermoactive α-amylase from the archaeobaterium *Pyrococcus woesei*. *Archives in Microbiology* **155**: 572-578.

Kohilu U, Nigam P, Singh D and Chaudhary K. 2001. Thermostable, alkaliphilic and cellulase free xylanases production by *Thermoactinomyces thalophilus* subgroup C. *Enzyme and Microbial Technology* **28**: 606–610.

Kokab S, Asghar M, Rehman K, Asad M J and Adedyo O. 2007.Bio-Processing of banana peel for α-amylase production by *Bacillus subtilis. International Journal of Agricultural Biology* **5**: 36–39.

Kolawole A O, Ajele J O and Sirdeshmukh R. 2011. Purification and characterization of alkaline stable β-amylase in malted African finger millet (*Eleusine coracana*) seed. *Process Biochemistry* **46**: 2178-2186.

Konsoula Z and Liakopoulou-Kyriakides M. 2006. Starch hydrolysis by the action of an entrapped in alginate capsules a-amylase from *Bacillus subtilis. Process Biochemistry* **41**: 343–349.

Koteswara, Rao Y, Lu S C, Liu B L and Tzeng Y M. 2006.Enhanced production of an extracellular protease from *Beauveria bassiana* by optimization of cultivation processes.*Biochemical Engineering Journal* **28**: 57-66.

Kourkoutas Y, Bekatorou A, Marchant R, Banat I M and Koutinas A A. 2004. Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiology* **21**: 377-397.

Krahe M, Antranikian G and Markel H. 1996.Fermentation of extremophilic microorganisms.*FEMS Microbiology Reviews***18:** 271– 285.

Kumar H D and Swati S. 2001.Modern Concepts of Microbiology, 2nd ed. Vikas Publishing House Pvt. Ltd., New Delhi.

Kumar L, Awasthi G and Singh B. 2011. Extremophiles: A Novel Source of Industrially Important Enzymes. *Biotechnology* **10**: 121-135.

Kumar R K, Venkatesh V K and Kumar U K. 2004. Evidence that cleavage of the precursor enzyme by autocatalysis caused ecretion of multiple amylases by *Aspergillus niger*. *FEBS Letters* **557**: 239-242.

Kumar S. 2012. Purification of cellulase and xylanase enzymes produced from potential microorganisms isolated from compost for industrial application. Ph. D. Thesis. Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, HP, India.

Kumar S and Nussinov R. 2011. How do thermophilic proteins deal with heat? A review.*Cellular and Molecular Life Sciences* **58**: 1216–1233.

Kunamneni A, Kumar S K and Singh S. 2005.Response surface methodological approach to optimize the nutritional parameters for enhanced production of amylase in solid state fermentation by *Thermomyces lanuginosus.African Journal of Biotechnology* **4**: 708-716.

Kwak Y S, Akeba T and Kudo T. 1998. Purification and characterization from hyperthermophilic archaeon *Thermococcus profundus*, which hydrolyzes both a-1,4 and a-1,6 glucosidic linkages. *Journal of Fermentation and Bioengineering* **86**: 363-367.

Kwan S H, So K H, Chan K Y and Cheng S C. 1993.Production of thermotolerant alpha amylases by *Bacillus circulans*.*World Journal of Microbiology and Biotechnology* **9**:50-52.

Laemmli U K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage.*Nature***227:** 680-685.

Lardon L., Hélias A., Sialve B, Steyer J P and Bernard O. 2009.Life-cycle assessment of biodiesel production from microalgae. *Environment Science and Technology* **43**: 6475–6481.

Larkum A W, Ross I L, Kruse O and Hankamer B. 2012.Selection, breeding and engineering of microalgae for bioenergy and biofuel production. *Trends in Biotechnology* **30**: 198-205.

Lee J and Parulekar S J. 1993.Enhanced production of α-amylase in fed batch cultures of *B. subtilis* TN 106. *Biotechnology and Bioengineering* **42**: 1142-1150.

Lee J T, Kanai H, Kobayashi T, Akiba T, Kudo T. 1996. Cloning, nucleotide sequence and hyperexpression of a-amylase gene from an archaeon, *Thermococcus profundus*. *Journal of Fermentation and Bioengineering* **82**: 432-438.

Lee Y Y, Iyer P and Torget R W. 1999. Dilute acid hydrolysis of lignocellulosic biomass. *In:* Advances in Biochemical Engineering and Biotechnology Recent progress in bioconversion of lignocllulosics, pp. 92-115.

Leenan E J T M, Santos V A P M S, Grolle K C F, Tramper J and Wijffels R H. 1996. Characteristics and selection criteria for support materials for cell immobilization in wastewater treatment. *Water Resources* **30**: 2895-2896.

Lei Z and Jiang Q. 2011. Synthesis and properties of immobilized pectinase onto the macroporous polyacrylamide microspheres. *Journal of Agricultural and Food Chemistry* **59(**6): 2592–2599.

Leloup V, Colona P and Buleon A. 1994.*In:* B.Godon (Ed.). Bioconversion of cereal products, New York.pp.79-127.

Lenihan P, Orozco A, O'Neill E, Ahmad M N M, Rooney D W and Walker G M. 2010. Dilute acid hydrolysis of lignocellulosic biomass. *Chemical Engineering Journal* **156**: 395-403.

Leuschner C and Antranikan G. 1995.Heat stable enzymes from extremely thermophilic and hyperthermophilic micoroorganisms.*World Journal of Microbiology and Biotechnology* **11**: 95–114.

Leveque E, Janeck S, Haye B and Belarbi A. 2000.Thermophilic archael amylolytic enzymes.*Enzyme and Microbial Technology* **26**: 3-14.

Li H, Chi Z, Wang X, Duan X, Ma L and Gao L. 2007. Purification and characterization of extracellular amylase form the marine yeast

Aureobasidium pullulans N13d and its raw potato starch digestion. *Enzyme and Microbial Technology* **40**:1006-1012.

Li Y, Horaman M, Wu N and Lan C Q. 2008. Biofuels from microalgae. *Biotechnology Progress* **24**: 815-820.

Lily M K, Bahuguna A, Bhatt K K and Dangwal K. 2012.Production, partial purification and characterization of α-amylase from high molecular weight polycyclic aromatic hydrocarbons degrading *Bacillus subtilis* BMT4i.*Turkish Journal of Biochemistry* **37**(4): 463-470.

Limayem A and Ricke S C. 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. *Progress in Energy Combustion and Science* **38**:449–467.

Lin L L, Chyau C C and Hsu W H. 2007.Production and properties of a raw starch degrading amylase from a thermophilic and alkalophilic *Bacillus* sp. TS-23. *Biotechnology and Applied Biochemistry* **28**: 61-68.

Liu B L and Tzeng Y M. 1998.Optimization of growth medium for production of spores from *Bacillus thuringienesis* using RSM. *Bioprocess Engineering* **18**: 413-418.

Liu J and Yang J. 2007.Cellulase production by *Trichoderma koningii* A83.4262 in solid state fermentation using lignocellulosic waste from the vinegar industry.*Food Technology and Biotechnology* **45**(4): 420-425.

Liu W T. 2006. Nanoparticles and their biological and environmental applications. *Journal of Bioscience and Bioengineering* **102**:1–7.

Lopez G. 1999. DNA supercoiling and temperature adaptation: a clue to early diversification of life. *Journal of Molecular Evolution* **46**: 439–452.

Lowry O H, Rosebrough N J, Farr A L and Randall R J. 1951.Protein measurement with the Folin-phenol reagent.*Journal of Biology and Chemistry* **193**: 265-275.

Luedeking R and Piert E L. 1959. A kinetic study of lactic acid fermentation: batch process at controlled pH. *Journal of Biochemistry, Microbiology and Technology Engineering* **1**(12): 393-431

Mahmood S and Rehman R S. 2008.Production and partial purification of extracellularα-amylase by *Trichoderma viride. Bangladesh Journal of Microbiology* **25**(2): 99-103.

Malester A I, Green M, Kimchie S and Shelef G. 1988. The effect of the neutralizing capacity of cellulosic materials on the kinetics of cellulose dilute acid hydrolysis. *Boil. Wastes* **26:** 115-124.

Manjula C, Rajaguru P and Muthuselvam M.2009. Screening for antibiotic sensitivity of free and immobilized actinomycetes isolated from India. *Advances in Biological Research* **3**: 84-88.

Marc J E, van der Maarel C, Joost B V, Uitdehaag C M, Leemhuis H and Dijkhuizen L. 2002. Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology* **94(**2): 137-155.

Marhuenda-Egea F C, Piere-Velazquez S, Cadenas C, Cadenas E. 2002. An extreme halophilic enzyme active at low salt in reversed micelles. *Journal of Biotechnology* **93**:159-164.

Mariam I, Manzoor K, Ali S and Haq I U.2009.Enhanced production of ethanol from free and immobilized *Saccharomyces cerevisiae* under stationary culture. *Pakistan Journal of Botany* **41**(2): 821-833.

MarketsandMarkets
2015.http://www.marketsandmarkets.com/PressReleases/industrial-enzymes.asp.

Markou G, Angelidaki I, Nerantzis E and Georgakakis D. 2013.Bioethanol production by carbohydrate-enriched biomass of *Arthrospira* (Spirulina) *platensis.Energies* **6**: 3937-3950

Martinsen A, Skjak-Braek G and Smidsrod O. 1989. Alginate as immobilization material: Correlation between chemical and physical properties of alginategel beads. *Biotechnology and Bioengineering* **33**: 79-89.

Maton A, Jean H and Charles W. 1993.Human Biology and Health, Prentice Hall, Englewood Cliffs, NJ, USA.

McLaren A D and Packer L. 1970.Some aspects of enzyme reactions in heterogeneous systems. *Advances in Enzymology* **33**:245

McNabb A, Shuttleworth R and Behme R. 1997.Fatty acid characterization of rapidly growing pathogenic aerobic actinomycetes as a means of identification. *Journal of Clinical Microbiology* **35**: 1361-1368.

Mendu D R, Ratnam B V V, Purnima A and Ayyanna C. 2005.Affinity chromatography of α-amylase from *Bacillus licheniformis. Enzyme and Microbial Technology* **37***:* 712-717.

Menzel C, Lerch T, Schneider K, Weidemann R, Tollnick C, Kretymer G, Scheper T, Schugert K. 1998. Application of biosensors with an electrolyte isolator semiconductor capacitor (EIS-CAP) transducer for process monitoring.*Process Biochemistry* **33**:175-180.

Michelin M, Silva T M, Benassi V M, Peixoto-Nogueira, Moraes L A B, Leao J M, Jorge J A, Terenzi H F, Polizeli M de L T M. 2010. Purification and characterization of thermostable α-amylase produced by fungus *Paecilomyces variotii.Carbohydrate Research* **345**: 2348-2353.

Miller G L.1959.Use of dinitrosalicylic acid reagent for determination of reducing sugars.*Analytical chemistry* **31**: 426-428

Mishra S, Noronha S B and Suraishkumar G K. 2005. Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data. *Process Biochemistry* **40**:1863-1870.

Mithu D, Kali P D and Chakrabartty P K. 2005.Purification and characterization of α-amylase from *Bacillus amyloliquefaciens* NCIM 2829.*Indian Journal of Biochemistry and Biophysics* **42**(5):287-294.

Mitidieri S, Martinelli S, Schrank A H, Vainstein A. 2006. Enzymatic detergent formulation containing amylase from *Aspergillus niger*: a comparative study with commercial detergent formulations. *Bioresource Technology* **97:** 1217-1224

Mohandas B S, Prabhakar A, Koteswara Rao R R, Madhu M M and Rao G H. 2010. Statistical optimization and neural modelling of amylase production from banana peel using *Bacillus subtili* MTCC 441. *International Journal of Food Eng*ineering **6**:1-6.

Mollers K B, Cannella D, Jogersen H and Frigaard N U. 2014.Cyanobacterial biomass as carbohydrate and nutrient feedstock for bioethanol production by yeast fermentation.*Biotechnology for Biofuels* **7**: 64.

Monod J. 1942. Recherches sur la croissance des cultures bacteriennes. Ed. Hermann and Cie

Mosier N, Wyman C, Dale B, Elander R, Lee Y Y, Holtzapple M and Ladish M. 2005. Features of promising technologies for pretreatments of Lignocellulosic biomass. *Bioresource Technology* **96**: 673-686.

Mozhaev V. 1993.Mechanism-based strategies for protein thermostabilization.*Trends in Biotechnol*ogy **11**: 88–95.

Mukherjee A K, Kumar T S, Raj S K and Roy J K. 2010.Statistical optimization of *Bacillus alcalophilus* α-amylase immobilization on iron-oxide magnetic nanoparticles.*Biotechnology and Bioprocess Engineering* **15**(6): 984-992.

Mukherjee J, Solanki K and Gupta M N. 2013.Immobilization of *Candida rugosa*lipase on superparamagnetic fe_3o_4 nanoparticles for biocatalysis in low-water media. *Immobilization of Enzymes and CellsMethods in Molecular Biology* **1051:** 117-127.

Mukhopadhyay A, Redding A M, Rutherford B J and Keasling J D. 2008. Importance of systems biology in engineering microbes for biofuel production.*Current Opinion in Biotechnology* **19**: 228-234.

Mullai P, Fathima N S A and Rene E R. 2010.Statistical analysis of main and interaction effects to optimize xylanase production under submerged cultivation conditions. *Journal of Agriculture Sci*ence **2**(1):144-153.

Muralikrishna G and Nirmala M. 2005. Cereal α-amylases- an overview. *Carbohydrate Polymer* **60**: 163–173.

Mussatto S I and Teixeira J A. 2010. Lignocellulose as raw material in fermentation processes. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* **2**: 887-907

Nagasaka Y, Kurosawa K, Yokota A and Tomita F. 1998.Purification and properties of the raw starch digesting glucoamylases from *Corticium rolfsii*.*Applied Microbiology and Biotechnology* **50**: 323-330.

Nahak S, Nahak G, Pradhan I and Sahu R K. 2011. Bioethanol from Marine Algae: A Solution to Global Warming Problem. *Journal of Applied and Environmental Biological Sciences* **1**(4): 74-80.

Najafi M F, Deobagkar D and Deobagkar D. 2005.Purification and characterization of an extracellular α-amylase from *Bacillus subtilis* AX20.*Protein Expression and Purification*.**41**:349-354.

Natural history museum.2013. http://www.nhm.ac.uk/nature-online/hydrodictyon.

Neelima D and Reddy B I. 2015. Isolation and charcaterization of amylase producing *B. subtilis* strain ss7 from flour mill waste dump soil and optimization for amylase production. *International Journal of Recent Scientific Research.*

Nguyen Q D, Rezessy-Szabó J M and Hoschke A. 2000.Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626. *Food technology and Biotechnology* **38**(3): 229–234.

Niazi M, Tehreema I, Romana T, Muhammad J S, Humaira S, Syed Q A and Ikram H. 2010. α-amylase production by *Bacillus licheniformis* under Solid State Fermentation conditions and its cross linking with metalosalts to confer thermostability. *International Journal of Agricultural Biology* **12**: 793–795.

Nigam P S and Singh A. 2010.Production of liquid biofuels from renewable resource.*Progress in Energy and Combustion Science* DOI: 10.1016/j.pecs.2010.01.003

Nisha S, Karthick A S and Gobi N. 2012. A Review on Methods, Application and properties of immobilized enzyme. *Chemical Science Review and Letters* **1(3):** 148-155.

Ochi K. 1992. Polyacrylamide gel electrophoresis analysis of ribosomal protein: a new approach for actinomycete taxonomy. *Gene* **115**: 261-265.

Okolo B N, Ezeogu LI, Mba C N. 1995. Production of raw starch digesting amylase by *Aspergillus niger* and *Bacillus alvei* grown on native starch sources. *Journal of the Science of Food and Agriculture* **69**:109-15.

Olufunke F O T and Azeez I I. 2013.Purification and characterization of beta-amylase of *Bacillus subtilis* isolated from kolanut weevil. *Journal of Biology and Life Science* **4(**1): 68-78.

Palmer T. 2001. *Enzymes: Biochemistry, Biotechnology and Chemical Chemistry*, Horwood Publication, Chichester, UK, 2001.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D and Mohan R. 2000a. Advances in microbial amylases: Review acrticle. *Biotechnology and Applied Biochemistry* **31**: 135-152.

Pandey A, Soccol C R, Nigam P and Soccol V T. 2000b. Biotechnological potential of agro-industrial residues I: sugarcane bagasse. *Bioresource Technology* **74**: 69-80.

Pandey A, Soccol CR and Soccol VT. 2000c.Biopotential of immobilized amylases. *Indian Journal of Microbiology* **40**: 1-14.

Park J K and Chang H N. 2000.Microencapsulation of microbial cells. *Biotechnology Advances* **18**: 303-319.

Parmar A, Singh N K, Pandey A, Gnansounou E and Madamwar D. 2011. Cyanobacteria and microalgae: a positive prospect for biofuels. *Bioresource Technology* **102:** 10163-10172.

Patel J B. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Journal of Molecular Diagnostics* **6:** 313-321.

Pathak S and Narula N. 2013.Optimization of pH for the production of amylase by soil mycotic flora of Jabalpur Region.Research and Reviews: *Journal of Microbiology and Biotechnology* **2(**1): 17-22

Pederson H and Nielson J. 2000. The influence of nitrogen sources on the alpha amylase productivity of *Aspergillus niger*in continues cultures. *Applied Microbiology and Biotechnology* **3**: 278–281.

Pilkington P A, Margaritis A and Mensour N A. 1998. Mass transfer characteristics of immobilized cells used in fermentation processes. *Critical Reviews in Biotechnology* **18**:237-255.

Pires-Cabral P, da Fonseca M M R and Ferreira-Dias D. 2010. Esterification activity and operational stability of *Candida rugosa* lipase immobilized in polyurethane foams in the production of ethyl butyrate. *Biochemical Engineering Journal* **48(**2): 246–252.

Prabakaran D and Hewitt C J. 2009.The production of amylase by *Bacillus* sp. in a complex and a totally defined synthetic culture medium. *Journal of Industrial Microbiology* **17**: 96-99.

Prajapati V S, Trivedi U B and Patel K C. 2014. A statistical approach for the production of thermostable and alklophilic alpha-amylase from *Bacillus amyloliquefaciens* KCP2 under solid-state fermentation. *Biotech*nology DOI: 10.1007/s13205-014-0213-1.

Prakash O and Jaiswal N. 2011. Immobilization of a thermostable α-amylase on agarose and agar matrices and its application in starch stain removal. *World Applied Sciences Journal* **13** (3): 572-577.

Premila J S and Dhandayuthapani K. 2013.Optimization of α - amylase production of *Bacillus stearothermophilus* KDP from sago industry waste. *International Journal of Applied BioResearch* **16**: 17-21.

Qader S A, Bano S, Aman A, Syed N and Azhar A. 2006.Enhanced production and extracellular activity of commercially important amylolytic enzyme by a newly isolated strain of *Bacillus* sp AS-1. *Turkish Journal of Biochemistry* **31:** 135-140.

Rabelo S C, Filho R M and Costa A C. 2009. Lime pretreatment of sugarcane bagasse for ethanol production. *Applied Biochemistry and Biotechnology* **153**: 139-150.

Raharjo S, Ardiansyah, Endang and Tien. 2010. Isolation of thermostable α-amylase from local thermophilic bacteria for liquefaction. *In:* Proceedings of third international conference on Mathematics and natural sciences. pp. 342-354.

Raja R, Hemaiswarya S, Kumar N A, Sridhar S and Rengasamy R. 2008. A perspective on the biotechnological potential of microalgae.*Critical Reviews in Microbiology* **34**: 77-88.

Rajagopalan G and Krishnan C. 2008. Immobilization of maltooligosaccharide forming α-amylase from *B. subtilis* KCC103: properties and application in starch hydrolysis. *Journal of Chemical Technology and Biotechnol*ogy **83**: 1511-1517.

Rajput I R and Li W F. 2012.Potential role of probiotics in mechanism of intestinal immunity. *Pakistan Veterinary Journal* **32**: 303–308.

Rajput IR, Li W F, Li Y L, Jian Land Wang M Q. 2013.Application of probiotic (*Bacillus subtilis*)to enhance immunity, antioxidation, digestive enzymes activity and hematological profile of Shaoxing duck. *Pakistan Veterinary Journal* **33**: 69–72.

Ramachandran S, Patel A K, Nampoothiri K M, Chandran S, Szakacs G, Soccol C R and Pandey A. 2004. Alpha amylase from a fungal culture grown on oil cakes and its properties. *Brazilian Archives of Biology and Technology* **47**(2): 309-317.

Ramakrishna S V and Prakasham R S. 1999.Microbial Fermentation with Immobilized Cells. *Current Science* **77**: 87-100.

Ramesh M V and Lonsane B K. 1989.*Process Biochemistry* **24:** 176–178.

Ranjan A, Patil C and Moholkar V S. 2010. Mechanistic assessment of micro algal lipid extraction. *Industrial and Engineering Chemistry Research* **49**: 2979–2985.

Rao J L U M and Satyanarayana T. 2007.Purification and characterization of a hyperthermostable and high maltogenic α-amylase of an extreme thermophile *Geobacillus thermoleovorans. Applied Biochemistry and Biotechnology* **142**: 179-193.

Rao K V B, Bose H, Richa K, Singh K, Karthik L and Kumar G. 2013. RSM mediated optimization of amylase production from marine*Bacillus* sp. VITRKHB. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **4(4):** 523-536.

Rasiah I A and Rehm B H. 2009.One-step production of immobilized alpha-amylase in recombinant *E. coli. Applied and Environmental Microbiology* **75**(7): 2012-2016.

Rasooli I, Astaneh S D A, Borna H and Barchini K A. 2008. A thermostable α-amylase producing natural variant of *Bacillus* spp. isolated from soil in Iran. *American Journal of Agricultural and Biological Sciences* **3:**591-596.

Reda A I. 2007. Characterization and 16S rDNA identification of thermo-tolerant bacteria isolated from hot springs. *Journal of Applied Science Research* **3**: 994–1000.

Reddy P R M, Ramesh B, Mrudula S, Reddy G and Seenayya G. 2003. Production of thermostable β-amylase by *Clostridium thermosulfurogenes* SV2 in solid state fermentation: optimization of nutrient levels using reponse surface methodology. *Process biochemistry* **39**: 267-277.

Reese E T and Mguire A. 1969.Surfactants as stimulants of enzyme production by microorganisms. *Applied Microbiology* **17**: 273-283.

Regina M, Broetto F, Giovannozzi-Sermanni, Marabatini R and Peranni C. 2008. Influence of stationary and bioreactor cultivation on *Lentinula edodes* (bark) Pegler lignocellulolytic activity. *Brazilian Archives of Biology and Technology* **51**: 223-233.

Riaz Qader S A U, Anwar A and Iqbal S. 2009. Immobilization of a thermostable α-amylase on calcium alginate beads from *Bacillus subtilis* KIBGE-HAR. *Australian Journal of Basic and Applied Sciences* **3**(3): 2883-2887.

Richmond A. 2004. Handbook of microalgal culture: biotechnology and applied phycology. Blackwell science limited.

Robinson P J, Dunnill P and Lilly M D. 1973. The properties of magnetic supports in relation to immobilized enzyme reactors. *Biotechnology and Bioengineering* **15**: 603–606.

Roy A, Khanra K, Mishra A and Bhattacharyya N. 2014. Partial purification and characterization of amylase from a newly isolated *Bacillus megaterium* strain KAN1 from fermented rice handia. *American Journal of Current Microbiology* **2**:1-5.

Rusetski A N and Ruuge E K. 1990. Magnetic fluid as a possible drug carrier for thrombosis treatment. *Journal of Magnetism and Magneric Materials* **85**: 299– 302.

Saboury A A, Ghasemi S and Dahot M U. 2005.Thermodynamic study of magnesium ion binding to α-amylase. *Indian Journal of Biochemistry and Biophysics* **42**: 326-329.

Sadasivam S and Manickam A. 1991.Biochemical methods. New Age International Ltd. New Delhi, India.

Safarikova M, Roy I, Gupta MN, Safarik I. 2003. Magnetic alginate microparticles for purification of α-amylases, *Journal of Biotechnology* **105**: 255–260.

Sahin N, Ozturk E and Isik K. 2002.Selective isolation and numerical classification of novel thermophilic *Streptomycetes*. *Turkish Journal of Biology* **26**: 13-24.

Sahnoun M, Bejar S, Sayari A, Triki M A, Kriaa M and Kammoun R. 2012. Production, purification and characterization of two α-amylase isoforms from a newly isolated *Aspergillus oryzae* strain S2. *Process Biochemistry* **47**: 18-25.

Sambrook J and Rusell D W. 1989.*In*: Molecular cloning- A laboratory manual. 3rd edn.Cold spring harbor laboratory press, Cok Spring Harbor New York.

Sander K and Murthy G S. 2010.Life cycle analysis of algae biodiesel.*International Journal of Life Cycle Assessment* **15**: 704–714.

Sani I, Abdulhamid A, Bello F, Yahaya M and Bagudo A I. 2014.Isolation, partial purification and characterization of α-amylase from *Bacillus subtilis. Journal of Microbiology and Biotechnology Research* **4**(1):49-54.

Sankaralingam S, Shankar T, Ramasubburayan R, Prakash S and Kumar C. 2012. Optimization of culture conditions for the production of amylase from *Bacillus licheniformis* on submerged fermentation. *American-Eurasian Journal of Agricultural & Environmental Science* **12**(11): 1507-1513.

Santos E O and Martins M L L. 2003. Effect of medium composition on formation of amylase by *Bacillus* sp. *Journal of Brazilian Archives of Biology and Technology* **46**: 129-134.

Saroja N, Shamala T R and Tharanthan R N. 2000.Biodegradation of starch-g-polyacrylonitril a packaging material by *Bacillus cereus. Process Biochemistry* **36**: 119–125.

Savage N and Diallo M S. 2005. Nanomaterials and water purification: opportunities and challenges. *Journal of Nanoparticle Research* **7**:331–342.

Saville B A, Khavkine M, Seetharam G, Marandi B and Zuo Y L. 2004.Characterization and performance of immobilized amylase and cellulose. *Applied Biochemistry and Biotechnology* **113**: 251–259.

Saxena R K, Dutt K, Agarwal L and Nayyar P. 2007. A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. *Bioresource Technology* **98**: 260-265.

Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J, Posten C, Kruse O and Hankamer B. 2008. Second generation biofuels: high efficiency microalga for biodiesel production. *Bioenergy research* **1**: 20-43.

Sellek G A and Chaudhuri J B. 1999.Biocatalysis in organic media using enzymes from extremophiles. *Enzyme and Microbial Technology* **25**:471-482.

Senthilkumar P K, Uma C and Saranraj P. 2012. Amylase production by *Bacillus* sp. using cassava as substrate. *International Journal of Pharmaceutical & Biological Archives* **3**(2):300-306.

Shafiei M, Ziaee A A and Amoozegar M A. 2010. Purification and biochemical characterizatio of a novel SDS and surfactant stable, raw starch digesting and halophilic α-amylase from a moderately halophilic bacterium, *Nesterenkonia* sp. strain F. *Process Biochemistry* **45**: 694-699.

Shafique S, Rukhsana B and Shazia S. 2009. Screening of *Aspergillus niger*and *Aspergillus flavus* strains for extra cellular α-amylase activity. *Pakistan Journal of Botany* **41:** 897–905.

Shah A R and Madamwar D. 2005.Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. *Process Biochemistry* **40**: 1763-1771.

Sharma M, Kumar V and Pundir C S. 2008. Immobilization of porcine pancreatin lipase onto free and affixed arylamine glass beads and its application in removing oil stains. *Indian journal of Biotechnology* **7:** 328-332.

Sharma N. 2013. Bioconversion of cellulosic waste into bioethanol as biofuel.Ph. D. Thesis. Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, HP, India.

Sharma N and Sharma N. 2013.Cost effective production of cellulase and xylanase enzymes by *Myceliophthora thermophila* SH1 using lignocellulosic forest waste and bioconversion of alkaline hydrogen peroxide pretreated *P. deltoides* wood to bioethanol under SHF. *Journal of Agroalimentary Processes and Technologies* **19**(4): 419-428

Sharma N, Vamil R, Ahmad S and Agarwal R. 2012. Effect of different carbon and nitrogen sources α-amylase production from *B. amyloliquefaciens. International Journal of Pharmaceutical Science and Research* **3**(4):1161-1163.

Sharma V and Singh P K. 2012. Strain improvement of *Bacillus coagulans* and *Geobacillus stearothermophilus* for enhanced thermostable cellulase production and the effect of different metal ions on cellulase activity. *International Journal of Engineering Science and Technology* **4**(11): 4704-4709.

Shaw J F, Lin F P, Chen S and Chen H C. 1995.*Botanical Bulletin Academia Sinica* **36**: 195–200.

Shiau R and Hung J H. 2003.Improving the thermostability of raw-starch-digesting amylase from a *Cytophaga* sp. by site-directed mutagenesis.*Applied Environmental Microbiology* **69**: 2383–2385.

Shigechi H, Koh J, Fujita Y, Matsumoto T, Bito Y, Ueda M, Satoh E, Fukuda H and Kondo A. 2004. Direct production of ethanol from raw corn starch via fermentation by a novel surface engineered yeast strain co-displaying glucoamylase and α-amylase. *Applied and Environmental Microbiology* 5037-5040.

Silva C J S M and Roberto I C. 2001.Optimization of xylitol production by *Candida* guilliermondii FTI 20037 using response surface methodology. *Process Biochemistry* **36**: 1119-1124.

Sindhu G S, Sharma P, Chakrabarti T and Gupta J K. 1997. Strain improvement for the production of thermostable alpha amylase. *Enzyme and Microbial Technology* **21**: 525-530.

Singh P, Gupta P, Singh R and Sharma R. 2009. Factors affecting alfa amylase production on submergedfermentation by *Bacillus* sp. *International Journal of Pharmacy and Life Sciences* **3**(12): 2243-2246.

Singh R K, Mishra S K and Kumar N. 2010.Optimization of α-amylase production on agriculture byproduct by *Bacillus cereus* MTCC1305 using solid state fermentation. *Research Journal of Pharmaceutical, Biological and Chemical Sciences***1**(4): 867-876.

Singh R, Kumar V and KapoorV. 2014.Partial purification and characterization of a heatstable α-amylase from a thermophilic actinobacteria,*Streptomyces* sp. MSC702. *Enzyme Research* DOI: http://dx.doi.org/10.1155/2014/106363.

Singhania R R, Sukumaran R K and Pandey A. 2007. Improved cellulase production by *Trichoderma reesei* RUTC30 under SSF through process optimization. *Applied Biochemistry and Biotechnology* **142**(1): 60-70.

Sivakumar T, Shankar T, Vijayabaskar P, Muthukumar J and Nagendrakannan E. 2012. Amylase production using *Bacillus cereus* isolated from a vermicompost site. *International Journal of Microbiological Research* **3** (2): 117-123.

Sivaramakrishnan S, Gangadharan D, Nampoothiri K M, Soccol C R and Pandey A.2006. α-amylases from microbial sources – an overview on recent developments. *Food Technology and Biotechnology* **44**(2): 173–184.

Smidsrod O and Skjak-Braek G. 1990.Alginate as immobilization matrix for cells. *Trends in Biotechnology* **8:** 71-78.

Sodhi H K, Sharma K, Gupta J K and Soni S K. 2005.Production of a thermostable α-amylase from *Bacillus* sp. PS-75 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. *Process Biochemistry* **40**: 525-534.

Sohail M, Ahmad A, Shahzad S and Khan S A. 2005.A survey of amylolytic bacteria and fungi from native environmental samples. *Pakistan Journal of Botany* **37**(1): 155-161.

Sohn O J, Kim C K and Rhee J I. 2008.Immobilization of glucose oxidase and lactate dehydrogenase onto magnetic nanoparticles for bioprocess monitoring system. *Biotechnology and Bioprocess Engineering* **13**: 716-723.

Soniyamby A R, Praveesh B V, Nandhini B, Lalitha S and Palaniswamy M. 2011. Enhancement of amylase production by *Bacillus* sp. isolated from marine water. *World Applied Sciences Journal* **14**(3): 449-455.

Struch T, Neuss B, Bringer-Mayer S, Sahm H. 1991.Osmotic adjustment of *Zymomonas mobilis* to concentrated glucose solutions. *Journal of Applied Microbioogy and Biotechnology* **34**: 518-523.

Sulfahri, Mushlihah S, Sunarto E, Irvansyah M Y, Utami R S, and Mangkoedihardjo S. 2011. Ethanol production from algae *Spirogyra* with fermentation by *Zymomonas mobilis* and *Saccharomyces cerevisiae. Journal of Basic and Applied Science Research* **1**(7): 589-593**.**

Sumrin A, Waqar A, Bushra I, Muhammad T S, Sana G, Humera S, Imran S, Shah J, Sultan A, Mureed H and Sheikh R. 2011. Purification and medium optimization of α-amylase from Ba*cillus subtilis*168. *African Journal of Biotechnology* **11**: 2119–2129.

Sun Y and Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource technology* **83**: 1-11.

Sundarram A and Murthy T P K. 2014.α-amylase production and applications: a review. *Journal of Applied and Environmental Microbiology* **2**(4): 166-175.

Swain M R, Kar S, Padmaja G and Ray R C. 2006. Partial characterization and optimization of production of extracellular α-amylase from *B. subtilis* isolated from culturable cowdung microflora. *Polish journal of microbiology* **55**: 289-296.

Syed D G, Agasar D and Pandey A. 2009. Production and partial purification of α-amylase from a novel isolate *Streptomyces gulbargensis*. *Journal of Industrial Microbiology & Biotechnology* **36**(2):189–194.

Taherzadeh M J and Karimi K. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *International Journal of Molecular Science* **9:** 1621–1651.

Talekar S and Chavare S. 2012. Optimization of immobilization of α-amylase in alginate gel and its comparative biochemical studies with free α-amylase. *Recent Research in Science and Technology* **4**(2): 1-5.

Talekar S, Ghodake V, Kate A, Samant N, Kumar C and Gadagkar S. 2010. Preparation and characterization of cross-linked enzyme aggregates of *S. cerevisiae* invertase. *Australian Journal of Basic and Applied Sciences.***4:** 4760-4765.

Tamilarasan K, Muthukumaran C and Kumar M D. 2012.Application of response surface methodology to the optimization of amylase production by *A. oryzae* MTCC1847. *African Journal of Biotechnology* **11**(18): 4241-4247.

Tamura K, Dudley J, Nei M and Kumar S. 2007. MEGA4: Molecular Evolution Genetic Analysis (MEGA) version 4.0. *Molecular Biology and Evolution* **24**: 156-159.

Tandon D. 2014. Improvement of bioethanol production from enzymatic degradation of softwood biomass by standardizing fermentation processes. Ph. D. Thesis. Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, HP, India.

Tanyildizi M S, Ozer D and Elibol M. 2005.Optimization of α-amylase production by *Bacillus* sp. using response surface methodology. *Process Biochemistry* **40**: 2291-2296.

Teodoro C E S and Martins M L L. 2000. Culture conditions for the production of thermostable amylase by *Bacillus* sp. *Brazilian Journal of Microbiology* **31**: 298-302.

Thippeswamy S, Girigowda K and Mulimani V H. 2006. Isolation and identification of alpha-amylase producing *Bacillus* sp. from dhal industry waste. *Indian Journal of Biochemistry and Biophysics* **43**(5): 295-298.

Thompson D B. 2000. On the non-random nature of amylopectin branching. *Carbohydrate Polymers* **43(**3): 223–239.

Thys R C S, Guzzon S O, Olivera F C and Brandelli A. 2006.Optimization of protease production by *Microbacterium* sp. in feather meal using response surface methodology. *Process Biochemistry* **41**: 67-73.

Tischer W and Wedekind F.1999.Immobilized Enzymes: Methods and Applications. *In*: Topics in Current Chemistry. Springer Verlag Berlin Heidelberg pp. 95-126

Tiwari S, Shukla N, Mishra P, and Gaur R. 2014. Enhanced production and characterization of a solvent stable amylase from solvent tolerant *Bacillus tequilensis* rg-01: thermostable and surfactant resistant. *The Scientific World Journal*http://dx.doi.org/10.1155/2014/972763.

Tonkova A. 2006. Microbial starch converting enzymes of the α-Amylase family.*In*: Microbial Biotechnology in Horticulture, R.C. Ray & O.P. wards

(Ed.), pp. (421-472), , Science Publishers, Enfield, ISBN 9781578084173, New Hampshire, USA

Trevan M D. 1980. *In:* Immobilized enzymes – an introduction and applications in biotechnology. Wiley,New York, pp 11–55.

Trivedi N, Gupta V, Reddy C R K and Jha B. 2013.Enzymatic hydrolysis and production of bioethanol from common macrophytic gren alga *Ulva fasciata* Delile. *Bioresource Technology* **150**: 106-112.

Tunga R, Banerjee R and Bhattacharya V C. 1999.Optimization of n-variable biological experiments by evolutionary operation-factorial design technique. *Journal of Bioscience and Bioengineering* **87**: 224-230.

Ul-Haq I, Muhammad M J, Uzma H and Fazal A. 2010.Kinetics and Thermodynamic Studies of Alpha Amylase from *Bacillus licheniformis* Mutant. *Pakistan Journal of Botany* **42**: 3507–3516.

Usha B, Krishna V G, Muni K D and Hemalatha K P J. 2011.Partial characterization of α-amylase from germinating little millets (*Panicum sumatrense*). *Journal of Phytology* **3**(1): 01–08.

van der Maarel M J E C, vander Veen B, Uitdehaag J C M, Leemhuis H and Dijkhuizen L. 2002. Properties and applications of starch converting enzyme of alpha amylase family. *Journal of Biotechnology* **94**: 137-155.

Varalakshmi K N, Pallavi N U, Banerjee A, Bhuvaneshwari S, Prabhakar M and Gogoi P. 2012. Production and partial purification of α-amylase from *Pseudomonas* sp. under solid-state fermentation. *Turkish Journal of Biochemistry* **37**(1): 21–28.

Varavinit S, Chaokasem N and Shobsngob S. 2002.Immobilization of a thermostable α-amylase. *Science Asia* **28**: 247-251.

Vaseekaran S, Balakumar S and Arasaratnam V. 2010.Isolation and identification of a bacterial strain producing thermostable α-amylase.*Tropical Agricultural Research* **22**(1): 1– 11.

Veille C and Zeikus G J. 2001. Hyperthermophilic enzymes: sources, use and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews* **65**: 1–43.

Vengadaramana A. 2013. Industrial Important Microbial alpha-Amylase on Starch-Converting Process.*Scholars Academia Journal of Pharmarcy* **2**(3): 209-221

Ververis C, Georghiou K, Danielidis D, Hatzinikolaou D Z, Santas P, Santas R, Corleti V. 2007. Cellulose, hemicelluloses, lignin and ash content of some organic materials and their suitability for use as paper pulp supplements. *Bioresource Technology* **98**: 296–301.

Vidyalakshmi R, Paranthaman R and Indhumathi J. 2009. Amylase production on submerged fermentation by *Bacillus* sp. *World Journal of Chemistry* **4**(1): 89-91.

Vieille K, Zeikus G J. 2011. Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms of thermostability. *Microbiology and Moleular Biology Reviews* **65**(1):1-43.

Vijayabaskar P, Jayalakshmi D and Shankar T. 2012.Amylase production by moderately halophilic *Bacillus cereus* in solid state fermentation. *African Journal of Microbiology Research* **6**(23):4918-4926.

Vullo D L and Waschman M B. 2005. A simple laboratory exercise for ethanol production by immobilized Bakery Yeasts (*Saccharomyces cerevisiae*). *Journal of Food Science Education* **4**:53-55.

Wada M, Kato J and Chibata I. 1980. Continuous production of ethanol using immobilized growing yeast cells. *European Journal of Applied Microbiology and Biotechnology* **10**: 275-287.

Wang J, Meng G, Tao K, Feng M, Zhao X, Li Z, Xu Z, Xia D, Lu J R. 2012. Immobilization of lipases on alkyl silane modified magnetic nanoparticles: effect of alkyl chain length on enzyme activity. *PLoS ONE* **7**(8): e43478

Wang X, Liu X and Wang G. 2011.Two-stage hydrolysis of invasive algal feedstock for ethanol fermentation. *Journal of Integrative Plant Biology* **53**(3): 246-252.

Wenster-Botz D. 2013. Experimental design for fermentation media development: Statistical design or global random search? *Journal of Bioscience Bioengineering* **90** (5): 473–483.

Willey J M, Sherwood L M and Woolverton C J. 2008. Prescott, Harley and Kleins Microbiology, 7thEdn., McGraw Hill Co. Inc., Boston.

Williams D and Munnecke D M. 1981. The production of ethanol by immobilized yeast cells. *Biotechnology and Bioengineering* **23**: 1813-1825.

Wilson K. 2010. Chromatographic techniques.*In:* Biochemistry and molecular biology. 7th ed. Wilson K and Walker J (eds.). Cambridge University Press, New York. pp. 433-462

Wind R D, Buitelaar R M, Eggink G, Huizing H J and Dijkhuzen L. 1994. *Applied Microbiology and Biotechnology*.**41**: 155-162.

Wolfenden R, Lu X and Young G. 1998.Spontaneous hydrolysis of glycosides. *Journal of the American Chemical Society* **120**: 6814-6815.

Xavier A M R B, Correia M F, Pereira S R and Evtuguin D V. 2010. Second-generation bioethanol from eucalypt sulphite spent liquor. *Bioresource Technology* **101**:2755–61.

Xiao Z, Storms R and Tsang A 2006.A quantitative starch-iodine method for measuring alpha amylase and glucoamylase activities. *Analytical Biochemistry* **351**:146-148.

Xie F, Quan S, Liu D, Ma H, Li F, Zhou F, Chen G. 2014. Purification and characterization of a novel α-amylase from a newly isolated *Bacillus methylotrophicus* strain P11-2. *Process Biochemistry* **49**: 47–53.

Xie W and Ma N. 2010. Enzymatic transesterification of soybean oil by using immobilized lipase on magnetic nano-particles. *Biomass and Bioenergy* **34(**6): 890–896.

Xu J, Sun J, Wang Y, Sheng J, Wang F and Sun M. 2014. Application of iron magnetic nanoparticles in protein immobilization.*Molecules.***19**:11465-11486.

Yang S X, Qiang Y, Shan M L and Yuan Y S. 2002. Fermentation of ethanol by immobilized cells of *Pichia stipitis. Journal of Nanjing Forestry University* **26**(3): 1-4.

Yokotsuka K, Mizuo Y and Toshihide M. 1997. Production of bottle fermented sparkling wine using yeast immobilized in double layer gel beads or strands. *American Journal of Enology and Viticulture* **48**(4): 471-481.

Yucel Y, Demir C, Dizge N and Keskinler B. 2011. Lipase immobilization and production of fatty acid methyl esters from canola oil using immobilized lipase. *Biomass and Bioenergy* **35(**4): 1496–1501.

Zambare V P. 2011. Optimization of amylase production from *Bacillus* sp. using statistics based experimental design. *Emirates Journal of Food and Agriculture* **23**(1): 37-47.

Zeikus J, Vielle C and Savachenko A. 1998. Thermozymes: biotechnology and structure-function relationships. *Extremophiles* **2:** 179–183.

Zhang D H, Yuwen L X and Peng L J. 2013. Parameters affecting the performance of immobilized enzyme. *Journal of Chemistry.* DOI: http://dx.doi.org/10.1155/2013/946248.

Zhang D H, Yuwen L X, Li C, and Li Y O. 2012.Effect of poly (vinyl acetate-acrylamide) microspheres properties and steric hindrance on the immobilization of *Candida rugosa* lipase.*Bioresource Technology* **124**: 233–236.

Zhang W. 2003. Nanoscale iron particles for environmental remediation: an overview. *Journal Nanoparticles Research* **5**:323–332.

Zohra R R and Ahmad M. 2012. Optimization of cultural conditions for production of amylase from thermophilic *Bacillus* sp. *Pakistan Journal of Biochemistry and Molecular Biology* **45**(2): 99-103.

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Title of thesis	:	"**Purification and characterization of amylase produced from potential extremophiles for industrial application"**
Name of student	:	Gitanjali
Admission No.	:	F-2011-12-D
Name of Major Advisor	:	**Dr. Nivedita Sharma**
Major field	:	Microbiology
Minor field(s)	:	i) Molecular Biology and Biotechnology ii) Biochemistry
Degree awarded	:	Ph.D.
Year of award of degree	:	2015
No. of pages in thesis	:	294+I
No. of words in abstract	:	420

ABSTRACT

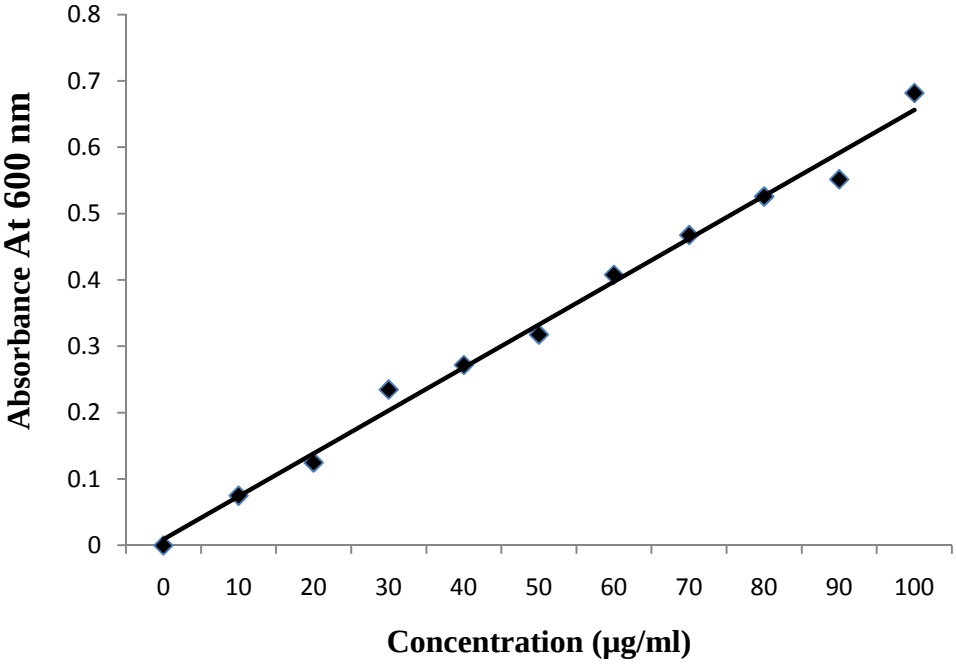
Mushroom compost being a highly probable source for amylogenic microorganisms was utilized as a source for isolation of amylolytic microorganisms. In total, 13 amylolytic bacteria have been screened from mushroom compost. Among them, alkalothermophilic hyperamylogenic strains GC6 and GV2 were selected and identified as *B. aerius* GC6 |KJ 775810.1| and *B. sonorensis* GV2 |KJ775811.1|. Cultural conditions and process parameters viz. media types, pH, temperature, inoculum size, incubation time, substrate concentration, divalent ions and surfactants etc. were optimized firstly through classical one variable at a time (OVAT) followed by statistical optimization by employing central composite design of response surface methodology. The enzymes obtained from both the strains were purified to homogeneity by following a sequential purification approach. *B. aerius* GC6 α-amylase was purified to a final purification fold of 17.86 and had a molecular weight of 43 kDa whereas *B. sonorensis* GV2 α-amylase was purified to a final purification fold of 14.52 and had a molecular weight of 45 kDa. α-amylase activity was found to be maximum at 50 ^{o}C and pH 9.0 for *B. aerius* GC6 and at 50 ^{o}C and pH 10.0 for *B. sonorensis* GV2. α-amylase from both the strains was quite thermostable with retention of more than 50% activity after incubation of 90 min at 45-60 ^{o}C. Kinetic characteristics of α-amylase from both the strains showed that the enzyme was very efficient qualitatively as well as quantitatively. Raw starch adsorption and hydrolysis ability shown by α-amylase from both the strains is a rare feature of bacterial α-amylase making it a potential candidate for starch processing industry. Immobilization of purified α-amylase from both the strains onto natural hydrogels showed a considerable retention upto 8 cycles however immobilization using iron oxide nano particles proved to be quite stable with apparently no loss in activity even after 20 cycles. Applicability of purified α-amylase from *B. aerius* GC6 was assessed by utilizing it for the hydrolysis of *Hydrodictyon* biomass for the production of bioethanol. Mixture of α-amylase, β-amylase, cellulase and pectinase from potential inhouse microorganisms was used for saccharification of biomass followed by its fermentation to ethanol using *S. cerevisiae*-I (MTCC3089). A final ethanol concentration of 13.93 g/l and 16.67 g/l with a fermentation efficiency of 64.84% and 66.68% was obtained in a stirred tank bioreactor under separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) mode respectively. This is the first report on α-amylase from *B. aerius* and *B. sonorensis*. Thermal and alkaline stability of this enzyme makes them a potential candidate for use in starch processing industry.

Signature of Major Advisor **Signature of student**

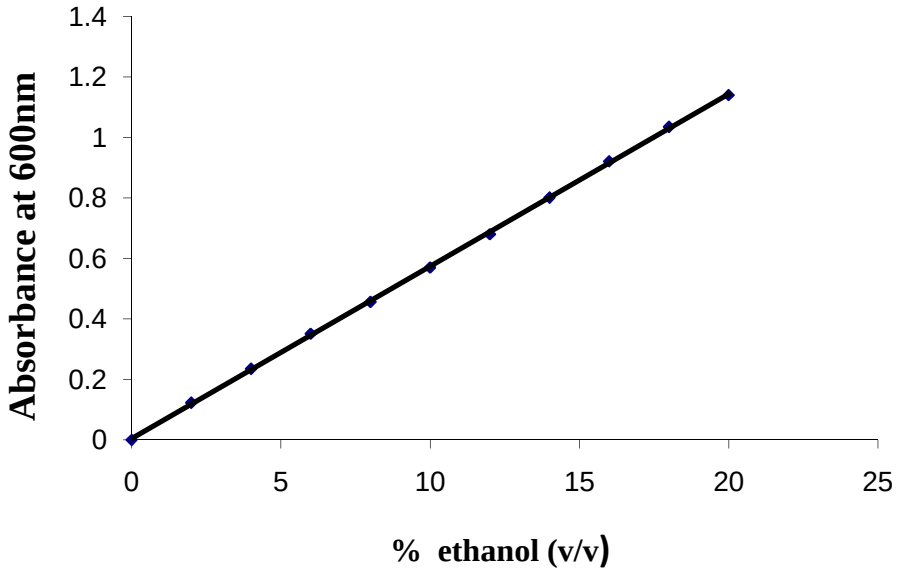
Countersigned

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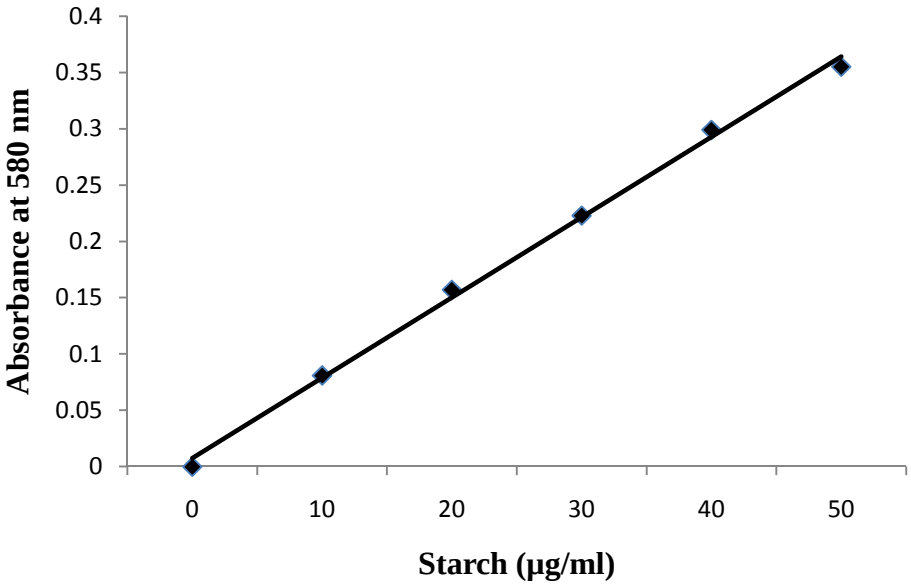
Appendix – I



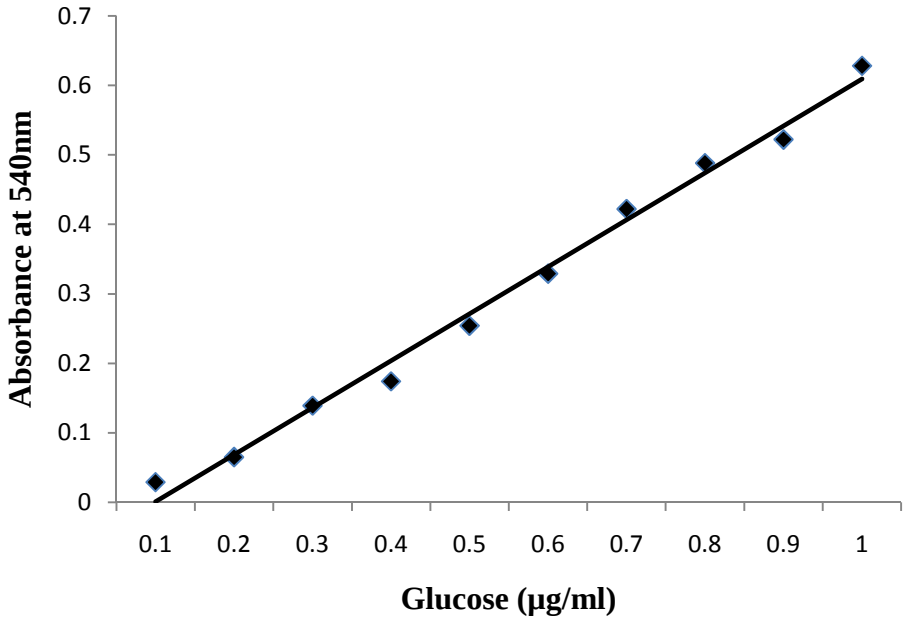
Standard curve for Bovine Serum Albumin (BSA)



Standard curve for ethanol



Standard curve for starch



Standard curve for glucose

CURRICULUM VITAE

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M.Sc. Microbiology	First	CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, HP	2010

Whether sponsored by some state/Central Govt./ Univ./SAARC : No

Scholarship/Stipend/ Fellowship, any other financial assistance received during the study period : Yes

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