ISOLATION AND CHARACTERIZATION OF THERMOSTABLE α-AMYLASE FROM AFAR REGIONAL STATE OF ETHIOPIA

By

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Nutrition

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June, 2013

Addis Ababa, Ethiopia

DECLARATION

This project is my original work except where due reference has been made in the acknowledgements. This work has not been submitted for a degree in any other university.

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June 2013

ACKNOWLEDGEMENT

I am thankful for my advisors Dr. Melese Abdisa and Prof. Nigussie Retta for providing me the opportunity to do this research under their supervision. I would also like to thank all staffs of the Food Science and Nutrition Center of Addis Ababa University.

My appreciation and heartfelt thanks goes to my dear friends, classmates, colleagues, and all concerned about me.

My sincere thanks and love goes to my family for their unconditional love and support .

ABSTRACT

Thermophilic microorganisms could be isolated from harsh environments where the environmental conditions are not favorable for most microorganisms. Enzymes isolated from these microbes could have higher activity and stability. Thermostable enzymes can be extracted from thermophilic microbes and be employed for industrial purposes. The isolation and characterization of these enzymes are crucial steps in biotechnology and enzyme sciences.

In this study, five (5) water samples and five (5) soil samples from Afar, a regional state in Ethiopia. Isolates in all the samples were grown at 70 ^{0}C to screen for thermophilic microorganisms. Among these thermophiles, thirty five (35) colonies from each sample which showed a positive amylolytic response were selected and the ratio of the clear halo created to the colony size (HCR) was compared. Based on the HCR, isolates from ALS and MS were selected for further characterization.

The optimum fermentation temperature was found to be 65 ^{0}C for both isolates with a fermentation period of 48 hours under the normal pH of the basal medium. The pH optima for the production of the enzymes were pH 6 for MS and pH 8 for ALS.

The enzymes isolated from both isolates were found to be very active at high temperature. Enzyme from ALS had a maximum activity at 90 ^{0}C while the enzyme from MS had its maximum activity at 85 ^{0}C. pH optimum for ALS was 8 and it was functional in a pH range of 6-11. The enzyme from MS had maximum activity at pH value of 6 and it had amylolytic activity in a pH range of 4-9.

Both enzymes showed good stability for pH and temperature. The enzyme from ALS retained 62% of its original activity (2.3817 ± 0.11 U/ml) after 40 minutes of incubation at 90^{0}C however, maintained 6% of its activity at 100 ^{0}C. The enzyme was deactivated after 30 minutes (retaining only 5% at 105 ^{0}C of its original activity). This enzyme was fairly stable for 30 minutes maintaining more than 50 % of its activity at pH values of 6-9, with no activity at pH values less than 6. The ion Zn^{2+} and higher concentrations (10 mM) of Mg^{2+} strongly inhibited enzyme

activity while 5mM concentration of Ca^{2+} and Ba^{2+} induced higher catalysis on the enzyme from ALS.

The enzyme from MS retained 65% of its original activity (2.5782 ± 0.56 U/ml) after 40 minutes of incubation at 85 ^{0}C however, maintained 4% of its activity at 100 ^{0}C. The enzyme was fairly stable for 40 minutes maintaining more than 50 % of its activity at pH values of 5-7, with no activity at pH values higher than 9. It had 69 % of its activity at pH value of 6 after 40 minutes of reaction time and maintained 53 % of its original activity at pH value of 4 after 30 minutes of reaction time. The ion Zn^{2+} and higher concentrations (10 mM) of Mg^{2+} strongly inhibited enzyme activity while 5mM concentration of Ca^{2+} and Ba^{2+} induced higher catalysis.

Considering the requirement of starch liquefaction process of an enzyme with a low pH optimum, the enzyme isolated from MS may offer an advantage since it functions well in acidic pH value of up to 4.

Even though the enzyme activities of ALS and MS improved with the addition of $CaCl_{2,}$ they had good amylolytic activities in the absence of Ca^{2+} which is a requirement in large-scale starch hydrolysis processes. Therefore, the enzymes from ALS and MS can be employed for starch hydrolysis in large-scale processes.

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LIST OF ABBREVIATIONS

ALS	Alelobad Soil
ALW	Alelobad Water
AWS	Awash Soil
AWW	Awash Water
BBS	Blen Buri Soil
BBW	Blen Buri Water
CFU	Colony Forming Unit
DNS	Dinitrosalicylic acid
DP	Degree of Polymerization
HCR	Halo to Colony Ratio
MS	Meteka Soil
MW	Meteka Water
OVAT	One Variable at a Time
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TS	Tendaho Soil
TW	Tendaho Water

1. INTRODUCTION

1.1 Background of the Study

Enzymes are globular proteins. They consist of one or more polypeptide chains and display properties that are typical of proteins. Like other proteins, enzymes consist of long chains of amino acids that fold to produce a three-dimensional product. Each unique amino acid sequence produces a specific structure, which has unique properties. Some enzymes require small non-protein molecules, known as cofactors in order to function as catalysts (Hiller *et al*., 1997).

Enzymes differ in function and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. They have unique ability to facilitate biochemical reactions without undergoing change themselves. This selective catalytic capability is what makes enzymes unique and useful in industries for production of foods, washing agents, textile manufacturing, pharmaceuticals, pulp and paper. Enzymes without being consumed in the process can speed up chemical processes that would otherwise run very slowly, or in some cases, not at all (Kirk *et al*., 2002). After the reaction is complete, the enzyme is released again and is ready to start another reaction. Nevertheless, most enzymes are used only once and discarded after their catalytic action. Currently, enzymes are becoming increasingly important in sustainable technology and green chemistry.

Enzymes, generally, are active at mild temperatures, otherwise, denatured. Enzymes have a characteristic pH at which their activity is maximal. For instance, alkalophilic α-amylase, protease and carboxymethylcellulase produced by alkalophilic *Bacillus* strains are active at alkaline pH (Prieto *et al*., 1995). Extreme pH values influence on the electrostatic interactions within the enzyme, leading to inactivation of enzyme. Other important factors that influence the effect of enzymatic processes are the concentration of enzyme, the time of treatment, additives like surfactants and chelators and mechanical stress (Hölker and Lenz, 2005).

Enzyme can break down particular compounds. The molecule that an enzyme acts on is known as its substrate, which is converted into a product or products. For each type of reaction in a cell, there is a different enzyme. Accordingly, they are classified into six broad categories namely

hydrolytic, oxidizing and reducing, synthesizing, transferring, lytic and isomerising. Some of the most common enzymes include amylases which break down starch into simple sugars, proteases which break down proteins, cellulases which break down cellulose, and lipases which split fats (lipids) into glycerol and fatty acids.

Amylases are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (Robyt and Ackerman, 1971; Reddy *et al*., 1999). Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes (Hebeda *et al*., 1990).

The amylase family of enzymes is of great significance due to its wide area of potential application. Amylases found potential applications in a number of industrial processes such as in the food fermentation, textiles and paper industries. Microbial amylases have successfully replaced the chemical hydrolysis of starch in starch-processing industries. The spectrum of amylase application has widened in many other fields, such as clinical, medical, and analytical chemistry (Pandey *et al*., 2000). Starch is widely used as a sizing agent, being readily available, relatively cheap and based on natural, sustainable raw materials (Mulimani and Ramalingam, 2000). About 75% of the sizing agents used worldwide is starch and its derivatives (Arnesen *et al*., 1998). Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques.

Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. The amylases of microorganisms (fungi and bacteria) have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal α- amylases (Pandey *et al*., 1999). The major advantage of using microorganisms for the production of *amylases* is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics.

Bacterial amylase, however, is generally preferred over fungal amylase due to several characteristic advantages that it offers. Strains of *Aspergillus* species and *Bacillus* species mainly *Bacillus subtilis*, *B. stearothermophilus, B.amyloliquefaciens and B. licheniformis* are known to be good producers of α-amylase and have been widely used for commercial production of the enzyme for various applications (Ma *et al*., 2006; Pandey *et al*., 2000). An extremely thermostable α-amylase is available from the mesophile *B. licheniformis* (Morgan and Priest, 1981).

Thermophiles are organisms which are adapted to grow optimally at high temperatures. Thermostable enzymes, which have been isolated from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Demirijan *et al*., 2001; Haki and Rakshit, 2003). Enzyme stability implies extremely important industrial and biotechnological implications due to the fact that such enzymes can be employed for use in harsh industrial conditions where their specific catalytic activity is retained.

Therefore, effort needs to be galvanized for the isolation of unique thermostable and thermoactive amylases from thermophilic and hyperthermophilic microorganisms in order to allow more industrial processes to run at higher temperatures.

1.2 Statement of the Problem

Amylases are hydrolyzing enzymes in function which cause hydrolysis of molecules. Notably uses include hydrolysis of starch to yield glucose syrup, amylase-rich flour and in the formation of dextrin during baking in food industries. Furthermore, in the textile industry, amylases are used for removal of starch sizing and as additives in detergents. The enzyme is produced and often used by developed nations. However, the cost of producing this enzyme is high. Also, the cost of procurement by developing countries might even be higher as a result of importation. Thermostability of enzymes is of considerable interest as their enhanced stability and activity could greatly reduce enzyme replacement costs or permit processes to be carried out at high temperature.

Harsh environments are known to be good sources of extremophilic microorganisms which could produce extremophilic enzymes that can withstand adverse conditions. Thermophilic

microorganisms, with the potential to produce thermophilic enzymes, for instance, have been isolated from high temperature terrestrial and marine habitats (Bertoldo and Antranikian, 2002). Bertoldo and Antranikian (2002) described that the most common habitats for isolation of thermostable enzymes are volcanically and geothermally heated hydrothermal vent systems such as hot springs and submarine hot vents. Such environments are available in Afar Regional State at specific sites such as Alelobad, Meteka, Blen Buri, Tendaho, and Awash.

However, sufficient published information is not available if thorough investigation has been made to isolate and characterize α-amylase producing organisms and the produced enzymes. Therefore, local sites of harsh environmental conditions, which are potential sources for thermophilic microorganisms, need to be investigated for thermostable enzymes.

1.3 Significance of the Study

Today a large number of microbial α-amylases are marketed with applications in different industrial sectors and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey *et al*., 2000; Gupta *et al*., 2003). Each application requires not only unique properties with respect to specificity and pH dependence, but also thermostability. For such applications, enzymes that have stable catalytic activity at higher temperature and thus in harsh industrial conditions are sought.

Because, there are many advantages of using thermostable enzymes as an increased reaction rate and decreased contamination risk through the use of high temperatures. Significant reduction of risk of contamination in biological processes where high operational temperatures above 60 ºC employed was reported by Niehaus *et al*., (1999). Furthermore, in large scale fermentations with heat sensitive microorganisms, extensive efforts for cooling the fermentation process cost as much as ten percent of the energy. The thermophilic fermentations are valuable as they need not to be cooled (Niehaus *et al*., 1999).

Therefore, screening of micro-organisms from high geothermal sites and harsh environments like Afar Regional State with possibility to discover thermophilic α-amylase for industrial application is highly significant. Therefore, the study was conducted with the following general and specific objectives.

1.4 Objectives of the Study

The general objective of this study was to isolate thermostable α-amylase producing microorganisms from selected areas in Afar, and to extract and characterize the enzyme from these microorganisms.

The specific objectives of the study were:

- Identify potential strains of α-amylase producing thermophilic microorganisms,
- Isolation of potential strains of α-amylase producing thermophilic microorganisms
- Screening of α-amylase producing microbes for α-amylase activity,
- Selection of α-amylase producing microbes for α-amylase activity,
- Optimizing conditions for α-amylase production, and
- Extracting α-amylase from the isolated strains of thermophilic microorganisms.
- Characterizing α-amylase from the isolated strains of thermophilic microorganisms.

2. LITERATURE REVIEW

2.1 History of Enzymes in Food Processing

In 2000 BC the Egyptians and Sumerians developed fermentation for use in brewing, bread-making and cheese-making. The history of amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff. This was followed by several reports of digestive amylases and malt amylases. It was much later in 1930, that Ohlsson suggested the classification of starch digestive enzymes in malt as α- and β-amylases according to the anomeric type of sugars produced by the enzyme reaction. In 1835, a Swedish chemist Jöns Jako Berzelius, carried out studies on organic catalysts. However, industrial enzymes date back to 1874 when Hansen manufactured chymosin from the stomach of calves for the manufacture of cheese.

In 1894 Jokichi Takamine, first manufactured an enzyme from a microbial source, which was a digestive enzyme taka-diatase from *Aspergillus*. The catalytic activity of enzymes was found in yeast (the word enzyme is ancient Greek for 'in yeast') and had been used for centuries. In 1926 James B. Sumner of Cornell University, made the first enzyme in pure crystalline form and was awarded a Nobel Prize in 1946 for his work on the enzyme urease, which was extracted from jack bean (Gupta *et al*., 2003; Chemical Heritage Foundation, 2002).

The existence of enzymes was associated with the history of ancient Greece where they used enzymes from microorganisms in baking, brewing, alcohol production and cheese making (Haki and Rakshit, 2003). With better knowledge and purification of enzymes, the number of applications has increased several fold and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged (Haki and Rakshit, 2003). These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to pulp and paper industries (Pandey *et al*., 2000), starch hydrolysis for the production of ethanol and high fructose corn syrup, starch soil removal in laundry washing powders and dish-washing detergents, textile de-sizing, the production of modified starches, baking, and hydrolysis of oil-field drilling fluids (Richardson *et al*., 2002). Since 1980, the most widely used enzyme for these applications has been α-amylases.

2.2 Thermophiles

Thermophiles are organisms which are adapted to grow optimally at high temperatures (Bertoldo and Antranikian, 2002). They have been isolated from high temperature terrestrial and marine habitats. The most common habitats are volcanically and geothermally heated hydrothermal vent systems such as hotsprings and submarine hot vents (Bertoldo and Antranikian, 2002). Ecological studies have showed that both aerobic and anaerobic species and many morphological and physiological types of microorganisms can exist in thermophilic environments (Brock, 2001; Madigan *et al*., 1997).

Extreme thermophiles are mostly distributed among the genera of *Bacillus, Clostridium, Thermoanaerobacter, Thermus, Thermotoga, Aquifex*. Most hyperthermophiles, on the other hand, include the two kingdoms of *Archaea, Crenarchacota* (*Sulfolobus, Pyrodictium, Pyrolobus.*), *Euryarchaeaota* (*Thermococcus, Pyrococcus), methanogenes* (*Methanococcus, Methanobacterium*), sulfate reducers and halophiles (Bertoldo and Antranikian, 2002).

2.3 Thermostable Enzymes

2.3.1 The evolution of thermostability

Improvements in stability, particularly thermostability, are commonly sought by protein engineers. In industrial processes, high temperatures impact such benefits as increased substrate solubility, decreased viscosity of the medium, lower risk of microbial contamination or higher rates of concurrent non-enzymatic reactions (Kuchner and Arnold, 1997).

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 fulfill these requirements. Within this context, directed evolution is very effective in closing these functional discrepancies (Huimin *et al*., 2002).

Thermophiles produce proteins called chaperonins that help to refold to their native form after denaturation. The cell membrane of thermophiles is composed of saturated fatty acids that provide hydrophobic environment for the cell. The DNA of thermophiles has also been reported to have a reverse DNA gyrase producing positive supercoils in the DNA. Since this difference

increases the melting point of DNA, the stability at high temperatures is achieved (Haki and Rakshit, 2003).

Alterations in the amino acid composition of proteins bring about additional electrostatic interactions, formation of hydrogen and disulfide bonds, and enhancement of hydrophobic interactions or compaction of the structure. Some thermophilic enzymes having proteolytic or amylolytic action are stabilized by Ca, Mg, Zn and other ions. The mechanism of stabilization is through the binding of cations to the labile parts of the globule. It is also known that some mesophilic enzymes are also stabilized by metal ions (Mozhaev and Martinek, 1984).

Differing the numbers of hydrogen bonds and salt bridges may also be another factor for stability. For example, 19 additional hydrogen bonds have been detected in a thermophilic protease which has not been present in its mesophilic counterpart (Mozhaev and Martinek, 1984). It has also been suggested that deletion or shortening of loops may increase the thermal stability and that oligomerisation can be another contributing factor (Kumar *et al*., 2000).

Thermostable enzymes, isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Dimirijan *et al*., 2001). Enzymes from thermophilic microorganisms are referred to as thermozymes, displaying unique characteristics such as temperature, chemical and pH stability. They can be used in several industrial processes, in which they replace chemicals or mesophilic enzymes (Bruins *et al*., 2001).

Advances in this area have been possible with the isolation of a large number of beneficial thermophilic microorganisms from different exotic ecological zones of the earth and the subsequent extraction of useful enzymes from them (Groboillot, 1997; Bharat and Hoondal, 1998; Bauer *et al.,* 1999; Kohilu *et al*., 2001).. The most widely used thermostable enzymes are the amylases in the starch industry (Poonam and Dalel, 1995; Crabb and Mitchinson, 1997; Emmanuel *et al*., 2000; Sarikaya *et al*., 2000). A number of other applications are in various stages of development. The study of hypethermophilic bacteria and Archaea, which grow optimally at temperatures greater than 80 ^{o}C, has resulted in the discovery of many thermostable

enzymes with industrial applications. These include a number of extracellular amylolytic enzymes from cultured microbes (Richardson *et al*., 2002).

Thermozymes are often used when the enzymatic process is compatible with existing (high-temperature) process conditions. The main advantages of performing processes at higher temperatures are reduced risk of microbial contamination, lower viscosity, improved transfer rates, and improved solubility of substrates (Bruins *et al*., 2001). Thermostable polymer-degrading enzymes such as amylases, pullulanases, xylanases, proteases and cellulases play an important role in food, chemical, pharmaceutical, paper, pulp and waste-treatment industries (Bruins *et al*., 2001). The application of these enzymes as biocatalysts is attractive because they are stable and active under conditions that were previously regarded as incompatible with biological materials. Furthermore, it is clear that some extremophiles, particularly those from the Archaea, have novel metabolic pathways and so might serve as a source of enzymes with novel activities and applications (Hough and Danson, 1999).

2.3.2 Stability of thermozymes

The stability of enzymes and proteins *in vitro* remains a critical issue in Biotechnology. Both storage and operational stabilities affect the usefulness of enzyme-based products. Storage stability, or shelf life, refers to an enzyme's maintaining its catalytic properties in the period between manufacture and eventual use. Operational stability describes the persistence of enzyme activity during a process, i.e., under conditions of use (Jaenicke, 1991).

The stability of proteins is the result of a delicate balance between large stabilizing and large destabilizing forces. Consequently, relatively small changes in either the stabilizing or destabilizing forces can result in large changes in stability. A small number of extra salt bridges, hydrophobic interactions, or hydrogen bonds can confer this extra degree of stabilization. There are, therefore, no systematic structural differences between extremely stable and normal proteins (Matthews, 1993).

The stability of enzymes is interesting from a fundamental as well as an industrial point of view. Thermozymes can function as examples for improved stability. A better understanding of the

stability of thermozymes could reveal ways to stabilise other enzymes. Engineering of mesophilic enzymes might be an option when specific functionality cannot be found among thermozymes (Bruins *et al*., 2001).

Conformational stability of proteins is the result of compromise between two opposing factors: flexibility, for the catalytic function of the enzyme, and rigidity, for conformational stability. Thermozymes are significantly more rigid than their mesophilic counterparts at room temperature. Their high rigidity protects them from unfolding and preserves their catalytically active structure. Therefore, they can be optimally active under more denaturing conditions (e.g. higher temperatures) (Bruins *et al*., 2001).

Many attempts have been made to understand the stability of extremozymes in terms of their three-dimensional structure. This approach requires high-resolution structural data for homologous enzymes from both mesophiles and extremophiles so that differences, which might result in enhanced stability of the extremozymes, can be identified by structural comparison (Hough and Danson, 1999). It is clear that there are many features that have been identified as possible contributors to increase protein thermostability. However, there are substantial differences between the conclusions reached with different proteins and there appears to be no universal rule for the structural basis of stability (Hough and Danson, 1999).

Sufficient experimental evidence i.e., sequences, mutagenesis, structure, and thermodynamics, has been accumulated on hyperthermophilic proteins in recent years to conclude that no single mechanism is responsible for the remarkable stability of hyperthermophilic proteins. Increased thermostability must be found, instead, in a small number of highly specific mutations that often do not obey any obvious rule (Vieille and Zeikus, 2001).

2.4 Amylases

Enzymes that participate in the hydrolytic degradation of starch are collectively referred to as amylolytic enzymes or amylases. Specific enzymes classified within this group include α-amylase, β-amylase, gluco-amylase (also known as amyloglucosidase), pullulanase and inso-amylase.

Amylases are, classified into two categories, endoamylases and exoamylases (Gupta *et al*., 2002). Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyse from the non-reducing end, successfully resulting in short end products. A large array of amylases, are involved in the complete breakdown of starch. Enzymatic degradation of starch yields glucose, maltose and other low molecular weight sugars. Also, enzymatically-mediated isomerisation of glucose yields high-fructose syrups.

Abundant supplies of starch may be obtained from seeds and tubers, such as corn, wheat, rice tapioca and potato. The widespread availability of starch from such inexpensive sources, coupled with large-scale production of amylolytic enzymes, facilitates the production of syrups containing glucose, fructose or maltose, which are of considerable importance in the food and confectionery industry. Furthermore, they may be produced quite competitively when compared with the production of sucrose, which is obtained directly from traditional sources such as sugar-beet or sugar-cane (Gupta *et al*., 2002).

Starch may be hydrolysed by chemical or enzymatic means. Chemical hydrolysis was used formerly and involves heating in the presence of acid. However, enzymatic hydrolysis generates fewer byproducts and produces higher yields of end product compared to the chemical method. The initial step in starch hydrolysis entails disruption of the starch granule (Gupta *et al*., 2002). Solubilisation of the granules, (the process of "gelatinisation"), facilitates subsequent catalytic degradation. Gelatinisation is normally achieved by heating the starch to temperatures often in excess of 100 ^{o}C for several minutes. α- Amylase may be added immediately prior to the heating step, in order to render more efficient the process of granule disruption. Once the granules have been disrupted, additional α-amylase is added in order to liquefy the starch slurry. This process reduces the viscosity of the starch solution (Pandey *et al*., 2000).

2.5 Microbial Sources of Amylases

Several amylase-producing bacteria, fungi and other microorganisms have been isolated and characterized over many decades. Bacteria and fungi secrete amylases outside their cells to carry out extra-cellular digestion.

Among mold species producing high levels of amylase, those of *Aspergillus niger*, *Aspergillus oryzae* (Baysal *et al*., 2003), *Thermomyces lanuginosus* (Arnesen *et al*., 1998) and *Penicillium expansum* (Haki and Rakshit, 2003) in addition to many species of the genus *Mucor* (Baysal *et al*., 2003). It was reported that four species of *Ganoderma* mushrooms could produce relatively weak amylase in sawdust medium (Kumar *et al*., 2000). Amylolytic yeasts differ strongly with regard to amylase secretion and the extent of starch hydrolysis (El-Helow *et al*., 2000). Strains of *Filobasidiuim capsuligenum* are capable of extensive starch hydrolysis (Brock, 2001).

Regarding to bacteria, *Bacillus* species and the related genera produce a large variety of extracellular enzymes, of which amylases are of particular significance to the industry e.g., *B. cereus* (Hayashida *et al*., 1988), *B. circulans* (Van Ee *et al*., 1992). Bacteria belonging mainly to the genus *Bacillus* have been widely used for the commercial production of thermostable α-amylase (Hebeda *et al*., 1990). However, most of the *Bacillus* liquefying amylases, such as the enzymes from *B. amyloliquefaciens* and *B. stearothermophilus* have pH optima of between 5 and 7.5 (Collins *et al*., 1993). Many alkaline amylases have been found in cultures of *Bacillus* sp. (Hayashi *et al*., 1988). These alkaline amylases are all of the saccharifying type, except for the enzymes from *Bacillus* sp. strain 707 (Zeikus *et al*., 1996).

Thermostable β-amylases have been isolated from *Bacillus* species (Chemical Heritage foundation, 2002). Also, *Lactobacillus plantarum* strain A6 was selected for its ability to synthesize large amounts of extracellular α-amylase (Giraud *et al*., 1993). Furthermore, a variety of ruminal bacteria exhibit the ability to utilize starch as a growth substrate and are present in the rumen in sufficient numbers to be of quantitative significance in the fermentation of this substrate. These species include *Bacteroides ruminicola*, *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium*, and *Streptococcus bovis* (Oliveira *et al*., 2006).

Genes encoding intracellular α-amylases have been reported for *Escherichia coli* and *Streptococcus bovis* (Gupta *et al*., 2002). Although there has been some characterization of these activities, no clear physiological role for intracellular α- amylase has been established for either *E. coli* or *Streptococcus bovis*. However, many hyperthermophilic microorganisms possess

starch-hydrolyzing enzymes in their genomes even though they live in environments where starch is rare (Aiyer, 2005).

Among the polysaccharide-degrading enzymes of *Thermotoga maritime* described so far are two α-amylases, one is an extracellular putative lipoprotein (AmyA) (Baysal *et al*., 2003) and one is located in the cytoplasm (AmyB) (Gupta *et al*., 2003). *Geobacillus thermoleovorans* has been found to produce hyperthermostable, high maltose-forming and Ca^{2+} independent α- amylase (Francis *et al*., 2003). Numerous hyperthermophilic Archaea, especially deep-sea *Thermococcale* and *Sulfolobus* species have been reported to produce α-amylases (Fernandes, 2010).

The industrial potential of high-maltose forming α-amylases from *Thermomonospora curvata* (Collins *et al*., 1993) is limited by their moderate thermostability and Ca^{2+} requirement. α-Amylases are secreted by several species of *Streptomyces*, for example *S. albus* (Baysal *et al*., 2003). Gene encoding extracellular α-amylase has been cloned from many *Streptomyces* species (Baysal *et al*., 2003). In addition, α-amylase activity of *Thermoactinomyces* species was first reported by Ma *et al*., (2006). After that, several α- amylases with different characters were found in other studies (Collar *et al*., 2000). Within actinomycetes, available reports on β-amylase production are scanty and refer mainly to nonthermostable enzyme (Chemical Heritage foundation, 2002).

2.6 Classification of Amylases

Enzymes belonging to amylases, endoamylases and exoamylases, are able to hydrolyse starch. These enzymes are classified according to the manner in which the glycosidic bond is attacked. The starch degrading enzymes are found in the numerous glycoside hydrolase (GH) families (13, 14 and 15), mainly in GH family 13 (Baysal *et al*., 2003).

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 endoamylase. It is found in a wide variety of microorganisms, belonging to the Archaea as well as the Bacteria (Pandey *et al*., 2000). The end products of α-amylase action are oligosaccharides with varying length with α-configuration and α-limit dextrins, which constitute branched oligosaccharides. α-amylases are

often divided into two categories according to the degree of hydrolysis of the substrate (Souza *et al.*, 2001). Saccharifying α-amylases hydrolyze 50 to 60% and liquefying α-amylases cleave about 30 to 40% of the glycosidic linkages of starch.

Enzymes belonging to the second group, the exoamylases, either exclusively cleave α,1-4 glycosidic bonds such as β-amylase (EC3.2.1.2) or cleave both α,1-4 and α,1-6 glycosidic bonds like amyloglucosidase or glucoamylase (EC3.2.1.3) and α-glucosidase (EC3.2.1.20). 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. β-amylase and glucoamylase also convert the anomeric configuration of the liberated maltose from α to β. 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.*, 2000).

2.7 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 (Lonsane and Ramesh, 1990). 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. The diameter of the area of hydrolysis, within limits, was always related to the potency of the amylase (Collins *et al.*, 1993).

2.7.1 Factors affecting production

The production and stability of α-amylase in the medium is affected by a variety of physicochemical factors. In spite of expression's possibility under a wide range of culturing conditions, α-amylase could be denatured under some conditions. Many proteins easily aggregate into so-called inclusion bodies during expression in bacterial systems (Sharma and Satyanarayana, 2006). Inhibition of protein aggregation during fermentation/expression can be achieved by adjusting the production conditions (Bragger *et al.*, 1989).

Regarding to the incubation period, many investigators have found that extracellular α- amylase production is growth associated (Andreu *et al*., 1999). The changes in productivity of extracellular enzymes can be attributed to the differences in the timing of induction of separate components of the enzyme system, the inhibition by products of substrate hydrolysis and differential inactivation by proteases and/or variation in the pH during cultivation conditions (Madigan *et al*., 1997). Among the physical parameters, the temperature and pH of the medium play an important role in α-amylase production and stability. Generally, the influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic, thermophilic or psycrophilic.

Among the fungi and actinomycetes, most amylase production studies achieved the optimum yields within the range 25°C- 40°C (Gupta *et al*., 2003). However, thermophilic fungi, such as *Thermomyces lanuginosus* (Mishra and Maheshwari, 1996), and actinomycetes, namely and *Thermoactinomyces vulgaris* (Andreu *et al*., 1999) have been reported to produce α-amylase optimally at 50 °C, 55 °C and 55 °C, respectively. On the other hand, it has been produced at a wider range of optimal temperature by bacteria reaching to 90 °C in *Thermococcale* and *Sulfolobus* species (Fernandes, 2010). Also, the pH values were reported to serve as an indicator of the initiation and end of enzyme synthesis because the change in pH affects α-amylase stability in the medium (Gupta *et al*., 2002). It is worth noting that the α-amylase active site consists of a large number of charged groups (Arnesen *et al*., 1998) which explain the fact that most α-amylases had optimum pH in the acidic to neutral range (Pandey *et al*., 2000).

In general, amylase activity is connected with the substrate utilization. The inducibility nature of α-amylase has been assured in different microorganisms (Andreu *et al*., 1999). α-amylase production is also appeared to be subjected to catabolite repression by maltose and glucose, like most other inducible enzymes that are affected by substrate hydrolytic products (Baysal *et al*., 2003). However, α-amylase synthesis by *Bacillus* strains was reported to not subject to catabolite repression by monosaccharides (Zayed and Mostafa, 1992). Gupta *et al.* (2003) have classified xylose and fructose as strongly repressive to α-amylase synthesis. Addition of starch to the medium has normally been employed for the production of α- amylase from various microorganisms as reported in the literature.

Nitrogen source as a basal component of the medium is a major factor affecting α-amylase production. Its effect was not only as a nitrogen source but also as a metal ion source and a pH controller as well. Many investigators had recorded that organic nitrogen sources supported maximum α-amylase production by various bacteria (Andreu *et al*., 1999). The increased α-amylase production by organic nitrogen sources could be attributed to the high nutritional amino acids and vitamins content. However, various inorganic salts have been reported to support better production in fungi (Gupta *et al*., 2003). As a metal ion source, ammonium chloride was found to enhance the production of the α-amylase by *T. vulgaris*, where chloride is a stabilizer, over that of other ammonium salts (Andreu *et al*., 1999). In addition, the same authors also reported different productivity of α-amylase by using sodium nitrate from potassium nitrate.

2.7.2 Activity measurement of enzyme

The diversity and heterogeneity of natural substrates coupled with the mixed specificities of individual enzymes presents a problem in the characterization of amylases. Furthermore, the enzymatic degradation of native insoluble substrates involves steps and mechanisms which are not yet understood at the molecular level. Therefore biochemical studies always use starch in some modified form to simplify analyses. There are basically four different types of substrates used for activity measurements: purified insoluble substrates approximated to a native substrate, modified insoluble substrates, soluble modified polysaccharides and soluble oligosaccharides. Catalytic activity is usually measured by quantifying formed soluble saccharides or chromophoric aglycon. The action of enzyme on insoluble substrates can also be assayed by other means. For example, a viscosimetric method has been used to measure α-amylase activity on starch pastes (Bragger *et al*., 1989).

The measurement of soluble products from insoluble or soluble polymeric substrates often means assaying the formed reducing sugars. One of the simplest and most widely used is the 3, 5-dinitrosalisylic acid (DNS) method (Gigras *et al*., 2002). However, the colour development in the reaction is not strictly proportional to the number of reducing sugars present, but also to the length of the oligosaccharides, leading to higher apparent reducing values with longer sugars (Robyt and Ackerman, 1971). DNS itself also breaks down the substrate. Several other reducing sugar determination methods have also been developed. In some cases dye groups have been

attached to the polymeric substrate, e.g. dyed amylose-and amylopectin (Ramesh and Lonsane, 1990) and dyed and cross-linked starch. The enzymatic assay is based on colour released from the substrate.

Starch forms a deep blue complex with iodine and with progressive hydrolysis of the starch, it changes to red brown. Several procedures have been described for the quantitative determination of amylase based on the reduction in blue colour intensity resulting from enzyme hydrolysis of starch (Zayed and Mostafa, 1992). This method determines the dextrinising activity of α-amylase in terms of decrease in the iodine colour reaction. Also, the coupled assay methods have been used for amylases, in which the concentration of released glucose is determined either by glucose oxidase/peroxidase (Bruins *et al*., 2001) or by hexokinase/glucose-6-phosphate dehydrogenase method (Bruins *et al*., 2001).

Generally, various available methods for the determination of α-amylase activity are based on decrease in starch–iodine colour intensity, increase in reducing sugars, degradation of colour-complexed substrate and decrease in viscosity of the starch suspension.

2.7.3 Industrial desirable aspects

The stability of biocatalysts is often a limiting factor in the selection of enzymes for industrial applications due to the elevated temperature or extreme pH of many biotechnological processes. Therefore, there is a continuing demand to improve the stability of the enzymes and thus meet the requirements set by specific applications.

As an example, the problem with traditional detergent enzymes is that they have to function in a washing machine under conditions that are very unfavorable for the stability of the enzyme. The pH is highly alkaline in washing conditions. The high temperature (55–60 °C) in a dishwasher requires thermostable enzymes. In addition, it is preferred to be resistant to various detergent ingredients, such as surfactants, chelating and oxidative agents (bleach).

In general, temperature has a complex effect on protein either directly or indirectly for both physical and chemical induced aggregation processes (Kirk, 2002). Therefore, it is the most

critical environmental factor for consideration when proteins are handled during the entire development and commercialization processes. The advantages for using thermostable α-amylases in industrial processes include the decreased risk of contamination, the increased diffusion rate and the decreased cost of external cooling. In short, almost all industries need thermostable enzymes. Besides thermostability and other factors such as activity with high concentrations of starch, i.e. more than 30% dry solids, or the protein yields of the industrial fermentation are important criteria for commercialization (Zeikus *et al*., 1996). Also, α-amylases with wide pH range is desired to satisfy all applications either acidic as glucose syrup production or alkali as detergents industries.

However, there is a recent trend to use intermediate temperature stable (ITS) α-amylases (Ahuja *et al*., 1998, as cited in Gupta *et al*., 2003). Olesen (1991) found that this feature render the enzyme to be useful for baking industry through avoiding stickiness in bread. Also, a modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At these lower temperatures, detergents with α-amylases optimally working at moderate temperatures and alkaline pH would be favourable (Pandey *et al*., 2000). Although a wide variety of microbial α-amylases is known, α-amylase with 'ITS' property has been reported from only a few microorganisms (Gigras *et al*., 2002).

Another important desirable feature is calcium independency. Most known α-amylases, with a few exceptions, contain a conserved Ca^{2+} binding site (Tolan, 1996) which makes calcium be important to the enzyme activity. In manufacture of fructose syrup, the Ca2+ ions inhibit the glucose isomerase enzyme used in the final step of the process (Hebeda *et al*., 1990) and may lead to the formation of inorganic precipitates which have deleterious effects on fermentation and downstream processing.Because the removal of these metal ions is both cost and time consuming to the overall industrial process, the use of stable and functional α-amylases in the absence of Ca^{2+} ions at high temperatures would be highly favored.

2.7.4 Production of amylase by solid state fermentation and submerged fermentation

Although the details of the specific fermentation processes adopted by different manufacturers vary, there remain two main methods for amylase production, submerged fermentation (SmF)

and solid-state fermentation (SSF). Solid-state fermentation has gained renewed interest from researchers for the production of these enzymes in view of its several economic and engineering advantages and has been often employed to produce amylases (Pandey, 1992). Some of the advantages of solid-state fermentation over that of submerged fermentation are due to SSF being a simple technique, low capital investment, lower levels of catabolite repression and better product recovery (Mulimani and Ramalingam, 2000). The SSF technique is mainly confined to process involving fungi (Souza *et al*., 2001). It is believed that this technique is not suitable for bacterial cultures because of higher water activity requirements (Lonsane and Ramesh, 1990). However, successful bacterial growth using the SSF technique is known in many natural fermentations (Lonsane and Ramesh, 1990). Lonsane and Ramesh (1990) reviewed the production of bacterial thermostable α-amylase in SSF by *B. amyloliquefaciens* and *B. licheniformis.* They referred to the SSF process as the potential tool for achieving economy in enzyme production and starch hydrolysis.

2.7.4.1 Solid state fermentation (SSF)

Commercial production of amylases are carried out in various stages, essentially because the environmental factors required for the optimum growth of the micro-organism being employed may differ from those required for the production of enzymes. These parameters include nutrient supplementation, pH of the medium, osmotic relationship, degree of aeration, temperature, inoculum size and the control of contamination during fermentation (Pandey *et al*., 2000). The commercial success of amylases is linked to the utilization of starchy biomass as an industrial raw material for solid-state fermentation. On a dry basis, agricultural substrates like corn, wheat, sorghum and other cereal grains contain around 10 – 70% (w/w) starch, hydrolysable to glucose with a significant weight increase, offers a good resource in many fermentation processes (Soni *et al*., 2003). A number of studies have been carried out to optimize various SSF conditions with the aim of increasing α-amylase yields.

Omidiji *et al*. (1997) developed a simple and cheap media based on cheesy whey, corn steep liquor and soya bean meal for α-amylase production. It was claimed that the medium could be exploited for the industrial production of α-amylase. Studies on the effects of different carbon sources on α-amylase production showed that lactose, dextrin and soluble starch to be suitable

for enzyme production, while the highest enzyme yield was reported for glucose. El Helow *et al*. (2000) compared α-amylase production in three different nutritional media. In their study different patterns of enzyme induction were obtained when beet pulp, corn cob, rice husk, wheat bran and wheat straw were used separately to partially replace the nutrient contents of the selected medium. α-amylase was maximally expressed in the presence of corn cob or wheat bran. Hiller *et al*. (1997) demonstrated the effect of lactose and nitrogen on cell physiology and α-amylase production. Results showed cell-growth and α-amylase production patterns to be similar regardless of the limiting nutrient. Oxygen-transfer conditions and especially the dissolved oxygen tension were reported as vital factors for α-amylase production by Milner *et al*. (1997).

High aeration rates were found to be essential for good enzyme yields. As a practice, single-stage inoculum is used for fermentation processes. Generally, it is carried out in a routine way without being given critical attention. Keeping this in mind, Milner *et al*. (1997), studied one-stage and two-stage inocula for α-amylase production and found that significantly better results were obtained using two-stage inoculum. Considerable work has also been carried out on the effect of moisture levels on bacterial α-amylase production technique (Ramesh and Lonsane, 1990). In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and the moisture level of the substrate are therefore most important (Baysal *et al*., 2003).

Industrially important enzymes including amylases have traditionally been obtained from submerged cultures because of ease of handling and greater control of environmental factors. Soccol and Vandenberghe (2003) were able to increase the protein content of husks by 10.6% by SSF and Pandey (2003) reported that cultivation in SSF resulted in 4-fold-higher enzyme production than in SmF. However, solid-state fermentation constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly.

2.7.4.2 Submerged fermentation (SmF)

SSF has developed in eastern countries over many centuries, and has enjoyed broad application in these regions. In western countries the SSF had to compete with classical submerged

fermentation and because of the increasing pressure of rationalization and standardization, SSF was widely superseded by classical submerged fermentation since the 1940s. This is mainly because of problems in engineering that appear when scaling up SSF (Hölker and Lenz, 2005).

The majority of enzyme manufacturers produce enzymes using SmF techniques with enzyme titers in the range of grams per litre (Harvey and McNeil, 1993). Such levels are prerequisite if specific compounds are to be considered as commodities because product recovery costs are inversely proportional to concentration in a fermentation broth (Morgan and Priest, 1981). The application of SmF has been mainly confined to bacterial species (Pandey *et al*., 2000), the industrial exploitation of SSF for enzyme production has been confined to processes involving fungi and is generally believed that these techniques are not suitable for bacterial cultivation (Gupta *et al*., 2003). It is often stated that growth of filamentous fungi in submerged culture are quite similar to those of unicellular organisms that reproduce by binary fission. Due to practical difficulties that hinder studies of filamentous organisms in submerged culture, growth is based mainly on studies with unicellular organisms. Attachment and growth on bioreactor walls, agitators, probes and baffles lead to a degree of heterogeneity within the biomass which is more pronounced in the case of pelleted growth. Areas of growing, and non-growing biomass inside the bioreactor influences the overall growth (Gupta *et al*., 2003). A number of processes have been reported for the production of enzymes, ethnol, and single-cell proteins using SmF (Zayed and Mostafa, 1992). Although, SSF offers numerous advantages over SmF such as simpler techniques and lower cost, there are few designs available for bioreactors operating in solid-state conditions. This is principally due to several problems encountered in the control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture. SSF lacks the sophisticated control mechanisms that are usually associated with SmF.

Production of α-amylase has generally been carried out using SmF. However, SSF systems appear to be a promising technology as was shown by Francis *et al*. (2003), using spent brewing grains in SSF for the production of α-amylase and determining that the supplementation with Tween-80 and calcium ions enhanced α-amylase activity. Gelatinization coupled with liquefaction, which is possible by the action of thermostable amylases have also been reported in SmF (Stamford *et al*., 2001). The production of frustosyl transferase derived from

microorganisms has been produced by SmF using *Aspergillus, Penicillium* and *Aureobasidium* species (Prapulla *et al*., 2000). There are several reports dealing with extracellular lipase production by fungus such as *Rizhopus* sp., *Aspergillus* sp. and *Penicillium* sp. on different substrates under submerged conditions (Cordova *et al*., 1998). Citric acid is one of the most commonly used organic acids in food and pharmaceutical industries and can be obtained by chemical synthesis, however, the cost is much higher than using fermentation. Citric acid is mainly produced by SmF, from filamentous fungus *A. niger* (Prado *et al*., 2004). Submerged production of α-amylase using synthetic media has been reported by many workers (Sharma and Satyanarayana, 2006). The contents of synthetic media such as nutrient broth, soluble starch as well as other components are very expensive (Haq *et al*., 2003).

The industrial enzyme production using SmF is often limited by the costs of substrates for the cultivation of the producer microorganisms. The use of low cost substrates, such as agricultural wastes has been suggested as an alternative to reduce the production costs (Oliveira *et al*., 2006). A study using sugarcane bagasse, and grass hydrolsates as carbon source for xylanase production by *B. circulans* was carried out by Oliveira *et al*. (2006). High production of enzyme was achieved using bagasses hydrolysates (8.4 U/ml) and in media with grass hydrolysates (7.5 U/ml), these results were higher than when xylan was used (7.0 U/ml) and demonstrated well that agro-industrial byproducts can be used as alternatives to reduce the cost of SmF (Harvey and McNeil, 1993). The utilization of both brewery and meat processing wastes for amylase production by *A. niger* under submerged culture conditions produced the highest amylase (70.29 and 60.12 U/ml) and protease (6.11 and 6.03 U/ml) yields respectively. The added advantage in using these food wastes also resulted in the COD in both wastes being reduced by more than 92%.

2.8 Purification and Characterization of Amylases

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.

2.9 Starch

Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. Amylases are capable of digesting these glycosidic linkages found in starch. Amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of α-amylase, microbial sources are used for the industrial production. This is due to their advantages such as cost effectiveness, consistency, less time and space required for production as well as ease of process modification and optimization.

In the present day scenario, α-amylases have applications in all the industrial processes such as in food, detergents, textiles and paper industry, for the hydrolysis of starch. They can also be of potential use in the pharmaceutical and fine chemical industries. In this light, microbial α-amylases have completely replaced chemical hydrolysis in the starch processing industry. Despite this, interest in new and improved α-amylase is growing and consequently, the research is intensified as well to meet requirements set by specific applications. Starch and starch-containing substrates are wide spread in nature and also in industrial praxis. They can predominantly find their application in many industrial processes.

2.9.1 Sources and utilization

Starch occurs mainly in the seeds, roots and tubers of higher plants. Some algae produce a similar reserve polysaccharide called phytoglycogen. Plants synthesize starch as a result of photosynthesis. It is synthesized in plastids as a storage compound for respiration during dark periods. It is also synthesized in amyloplasts found in tubers, seeds, and roots as a long-term storage compound. In these latter organelles, large amounts of starch accumulate as water-insoluble granules. The shape and diameter of these granules depend on the botanical origin.

Regarding to commercial starch sources, the granule sizes range from 2–30 µm (maize starch) to 5 – 100 µm (potato starch) (Robyt and Ackerman, 1971). A variety of different enzymes are involved in the synthesis of starch. Sucrose is the starting point of starch synthesis. It is converted into the nucleotide sugar ADP-glucose that forms the actual starter molecule for starch formation. Subsequently, enzymes such as soluble starch synthase and branching enzyme synthesize the amylopectin and amylose molecules (Tzanov *et al*., 2001).

Starch-containing crops form an important constituent of the human diet. Besides the direct use of starch-containing plant parts as a food source, starch is harvested and chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch or maltodextrin derivatives, or cyclodextrins. In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The major industrial sources are maize, tapioca, potato, and wheat.

2.9.2 Structure and properties

Starch is a polymer of glucose linked to one another through the C1 oxygen by a glycosidic bond. This glycosidic bond is stable at high pH but hydrolyzes at low pH. At the end of the polymeric chain, a latent aldehyde group is present. This group is known as the reducing end. Two types of glucose polymers are present in starch: (i) amylose and (ii) 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 number of glucose residues, also indicated with the term DP (degree of polymerization), varies with the origin. The relative content of amylose and amylopectin varies with the source of starch. The average amylose content in most common starches, e.g. in barley, corn and potato, is 20-30% (Pandey *et al*., 2000).

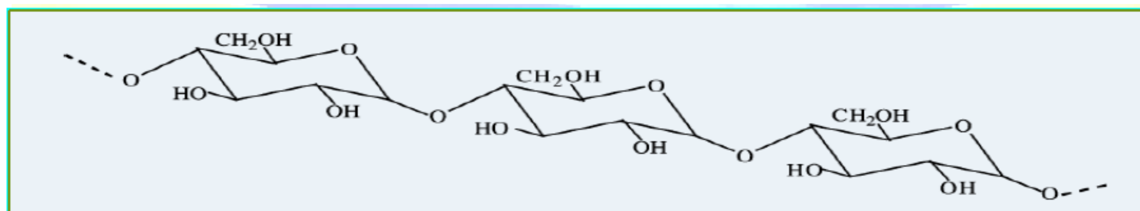

Figure 1. Structure of amylose

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% (Kuchner and Arnold, 1997), but varies with the botanical origin. The complete amylopectin molecule contains about 2000,000 glucose units, thereby being one of the largest molecules in nature (Pandey *et al.*, 2000). The most commonly accepted model of the structure of amylopectin is the cluster model, in which the side chains are ordered in clusters on the longer backbone chains (Kuchner and Arnold, 1997).

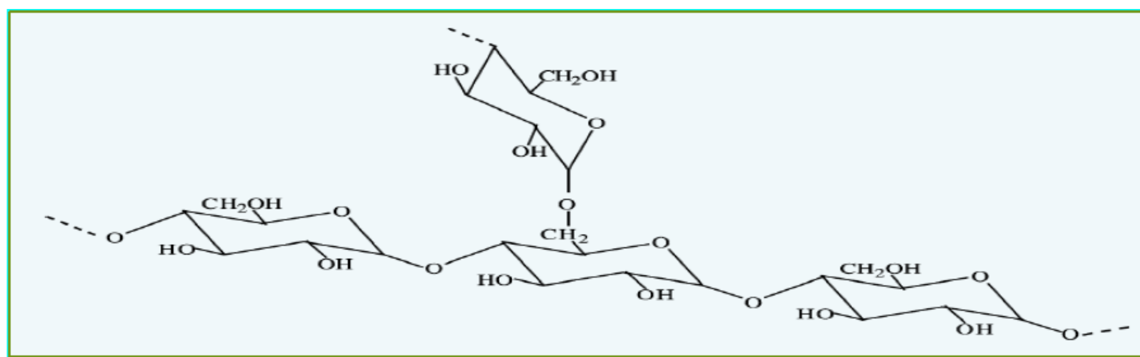

Figure 2. Structure of amylopectin

The orientation of the starch chains is thought to be perpendicular to the granule surface (Gupta *et al.*, 2002). Native starch is partly crystalline. The crystallinity of native starch varies between 15 and 45% depending on the origin and pretreatment (Gupta *et al.*, 2002). According to the currently accepted concept, amylopectin forms the crystalline component whereas amylose exists mainly in the amorphous form (Demirjian *et al.*, 2001). Structural studies have shown that native starch has crystalline polymorphism. In x-ray diffraction, cereal starch typically gives A-type patterns of monoclinic symmetry, and tuber starch gives B-type patterns of hexagonal symmetry (Giri *et al.*, 1990). The crystal lattice of B-type starch contains more water molecules than the A-structures, which is proposed to be the reason for higher stability of the A-structure. Both structures' molecular conformations are practically identical. They have left-handed double helices with parallel strands. Double helices contain six glucose units per turn in each chain and the glucose units are in a chair conformation. Within the double helix, there are inter-chain but no intra-chain hydrogen bonds. In addition, parallelly packed double helices are connected through a hydrogen bonding network.

2.9.3 Biodegradation of starch

The degradation of starch occurs mainly through the action of microorganisms in plant litter and soil. Since the native substrate is water-insoluble and cannot penetrate into cells, the biodegradation of starch occurs extracellularly. Amylases are mainly secreted into the medium or are found membrane-bound. Some microbial strains are known to produce intracellular amylases; the reason for this is unknown (Mozhaev and Martinek, 1984).

2.9.3.1 Enzymatic degradation of starch

The effective hydrolysis of starch demands the action of many enzymes due to its complexity, although a prolonged incubation with one particular enzyme can lead to (almost) complete hydrolysis. Few microorganisms produce a complete set of enzymes capable of degrading starch efficiently. There are basically four groups of starch-converting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases.

Endoamylases are able to cleave α,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. 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 (β-amylase).

The third group of starch-converting enzymes is the debranching enzymes that exclusively hydrolyze α,1-6 glycosidic bonds: isoamylase (EC3.2.1.68) and pullulanase type I (EC3.2.1.41). These enzymes exclusively degrade amylopectin, thus leaving long linear polysaccharides. There are also a number of pullulanase type enzymes that hydrolyze both α, 1-4 and α,1-6 glycosidic bonds. These belong to the group II pullulanase and are referred to as α-amylase–pullulanase or amylopullulanase. The main degradation products are maltose and maltotriose.

The fourth group of starch-converting enzymes are transferases that cleave an α,1-4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amylomaltase (EC2.4.1.25) and cyclodextrin glycosyltransferase (EC2.4.1.19) form a new α, 1-4 glycosidic bond while branching enzyme (EC2.4.1.18) forms a new α,1-6 glycosidic bond. Cyclodextrin

glycosyltransferases have a very low hydrolytic activity and make cyclic oligosaccharides with 6, 7, or 8 glucose residues and highly branched high molecular weight dextrins, the cyclodextrin glycosyltransferase limit dextrins. Amylomaltases are very similar to cyclodextrin glycosyltransferases with respect to the type of enzymatic reaction. The major difference is that amylomaltase performs a transglycosylation reaction resulting in a linear product while cyclodextrin glycosyltransferase gives a cyclic product.

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose, and glucose, etc. Dextrins are shorter, broken starch segments that form as the result of the random hydrolysis of internal glucosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on.

Most of the enzymes that convert starch belong to one family based on the amino acid sequence homology: the α-amylase family or family 13 glycosyl hydrolases according to the classification of Henrissat (1991). Other little enzymes that convert starch don't belong to family 13 glycosyl hydrolases like β-amylases that belong to family 14 glycosyl hydrolases (Rao *et al*., 1998); and glucoamylases which belong to family 15 glycosyl hydrolases (Baysal *et al*., 2003).

2.9.3.2 Catalytic mechanism and substrate binding

The α-glycosidic bond is very stable having a spontaneous rate of hydrolysis of approximately 2×10^{-15} s^{-1} at room temperature (Lonsane and Ramesh, 1990). Members of α- amylase family enhance this rate so enormously that they can be considered to belong to the most efficient enzymes known. The α-amylase family enzymes always carry strictly conserved three essential catalytic residues (Baysal *et al*., 2003). Of these three residues, the roles of Glu230 and Asp206 have been generally accepted as working for acid (proton donor) and base (nucleophile) catalyst, respectively (Henrissat, 1991). The catalytic mechanism has been discussed mostly on the basis of these two residues. However, the critical role of the third residue Asp297 seems to be still undefined and under dispute, except the facts that it plays an important role in the distortion of the substrate (Arnesen *et al*., 1998).

The generally accepted catalytic mechanism of the α-amylase family is that of the α- retaining double displacement (Van Dam and Hille, 1992). The mechanism involves two catalytic residues in the active site; a glutamic acid as acid/base catalyst and an aspartate as the nucleophile. It involves five steps: (i) after the substrate has bound in the active site, the glutamic acid in the acid form donates a proton to the glycosidic bond oxygen, i.e. the oxygen between two glucose molecules at the subsites −1 and +1 and the nucleophilic aspartate attacks the C1 of glucose at subsite −1; (ii) an oxocarbonium ion-like transition state is formed followed by the formation of a covalent intermediate; (iii) the protonated glucose molecule at subsite +1 leaves the active site while a water molecule or a new glucose molecule moves into the active site and attacks the covalent bond between the glucose molecule at subsite −1 and the aspartate; (iv) an oxocarbonium ion-like transition state is formed again; (v) the base catalyst glutamate accepts a hydrogen from an incoming water or the newly entered glucose molecule at subsite +1, the oxygen of the incoming water or the newly entered glucose molecule at subsite +1 replaces the oxocarbonium bond between the glucose molecule at subsite −1 and the aspartate forming a new hydroxyl group at the C1 position of the glucose at subsite −1 (hydrolysis) or a new glycosidic bond between the glucose at subsite −1 and +1 (transglycosylation). Studies with cyclodextrin glycosyltransferase have shown that the intermediate indeed has a covalently linked bond with the enzyme (Arnesen *et al*., 1998).

Other conserved amino acid residues e.g. histidine, arginine, and tyrosine play a role in positioning the substrate into the correct orientation into the active site, proper orientation of the nucleophile, transition state stabilization, and polarization of the electronic structure of the substrate (Arnesen *et al*., 1998).

2.10 Industrial Enzyme Applications

The effective catalytic properties of enzymes have already promoted their introduction into several industrial products and processes. Recent developments in biotechnology, particularly in areas such as protein engineering and directed evolution, have provided important tools for the efficient development of new enzymes. This has resulted in the development of enzymes with improved properties for established technical application and in the production of new enzymes

tailor-made for entirely new areas of application where enzymes have not previously been used (Kirk *et al*., 2002).

The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries. Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent and baking industries, represent the second largest group (Godfrey and West, 1996). The fastest growth over the past decade has been seen in the baking and animal feed industries, but growth is also being generated from applications established in a wealth of other industries spanning organic synthesis to paper and pulp and personal care (Kirk *et al*., 2002).

Natural microorganisms have over the years been a great source of enzyme diversity. The developments in bioinformatics and the availability of sequence data have increased immensely the efficiency of isolating an interesting gene from nature (Kirk *et al*., 2002). Rational protein engineering and the possibility of introducing small changes to proteins, on the basis of their structure and the related biochemical and biophysical properties, introduced a new valuable tool to enzyme optimisation in the 1980's. Directed evolution is the latest addition to the toolbox (Tobin *et al*., 2000). New exciting technology is predicted to out-compete the existing technologies, but we expect that time will demonstrate how the combined use of rational design, directed evolution and nature's diversity will be far superior to any technology (Kirk *et al*., 2002).

2.10.1 The detergent industry

Enzymes now comprise one of the ingredients of modern compact detergents. The main advantage of enzyme application in detergents is due to much milder conditions than with enzyme free detergents (Gupta *et al*., 2003). Their use as detergent additives still represents the largest application of industrial enzymes, both in terms of volume and value. The use of these enzymes in detergents affords numerous advantages, such as energy saving because they require lower temperature, and the reduction or replacement of other components that may be more harmful to the environment (Rodrígiez *et al*., 2006). The major component of these enzymes, are

proteases, but other and very different hydrolases are introduced to provide various benefits, such as the efficient removal of specific stains (Kirk *et al.*, 2002).

Constantly, new and improved engineered versions of the "traditional" detergent enzymes, proteases and amylases, are developed. These new second and third generation enzymes are optimized to meet the requirements for performance in detergents, the composition of which is also constantly developed (Kirk *et al.*, 2002). The earlier detergents were very harsh, caused injury when ingested and were not compatible with delicate china and wooden dishware. This forced the detergent industries to search for milder and more efficient solutions (Van Ee *et al.*, 1992). Some of these enzymes are obtainable from renewable sources, are biodegradable and act without risking aquatic life or having a negative effect upon residual water treatments (Rodrígiez *et al.*, 2006).

Some of the limitations of α-amylase in detergents are that the enzyme shows sensitivity to calcium and stability is severely compromised in a low calcium environment (Gupta *et al.*, 2003). One of the main problems that effects enzyme reactions is that they might undergo thermal deactivation under the conditions used for washing with the detergent (Rodrígiez *et al.*, 2006).

2.10.2 Starch liquefaction and saccharification

The major market for α-amylase lies in the production of starch hydrolysates such as glucose and fructose (Gupta *et al.*, 2002). The enzymatic conversion of starch to high fructose corn syrup is a well-established process and provides a beautiful example of a bioprocess in which the consecutive use of several enzymes is necessary. Because of their high sweetening property, these are used in huge quantities in the beverages industry as sweeteners for soft drinks. Enzymes used in the starch industry are also subjected to constant improvements (Van der Maarel *et al.*, 2002).

The first step in the process is the conversion of starch to oligomaltodextrins by the action of α-amylase. The co-comitant injection of steam puts extreme demands on the thermostability of the enzyme. Using traditional α-amylases, the pH has to be adjusted to an undesirable high level and

calcium must be added to stabilize the enzyme. New α-amylases with optimized properties, such as, enhanced thermal stability, acid tolerance, and ability to function without the addition of calcium, have recently been developed offering obvious benefits to the industry (Pandey *et al*., 2000).

2.10.3 Textile applications

This industry is under considerable environmental pressure owing to its large energy and water consumption and subsequent environmental pollution. One of the most energy and water consuming steps in the processing of cotton is the scouring step, the removal of various remaining cell-wall components on the cellulose fibres performed at high temperature and under strong alkaline conditions (Kirk *et al*., 2002). As an alternative, Tzanov *et al*. (2001) developed a successful substitution of traditional chemical processes by introducing an enzyme-based process performed at much lower temperatures and using less water. Following this, enzymes have now been introduced into most steps in the manufacturing of cotton textiles (Kirk *et al*., 2002). In textile weaving, a starch paste is applied for warping. This gives strength to the textile at weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of string due to laid down warp (Aiyer, 2005). After weaving the cloth the starch has to be removed, it is at this point of the process that α-amylase is introduced to help with the removal of the starch (Kirk *et al*., 2002). Kirk *et al*. (2002) reported that enzymes are used in at least seven various unit operations in textile wet processing and the manufacturing of denim.

2.10.4 Paper industry

The use of α-amylase for the production of low viscosity, high molecular weight starch for coating of paper has been reported (Bruinenberg *et al*., 1996). The use of amylases in the pulp and paper industry is in the modification of starches for coated paper. As for textiles, sizing of paper is performed to protect the paper against mechanical damage during processing. This process is said to improve the quality of the finished paper, and enhances the stiffness and the strength in paper.

Starch is subsequently used as the sizing agent, which is added to the process as slurry. The temperature of this process is between 40 ^{o}C to 60 ^{o}C. In the process a constant viscosity of the starch is required. To maintain the viscosity of the natural starch it is adjusted by partially degrading the polymer with α-amylase in a batch or continuous process. The conditions depend on the source of the starch and the α-amylase used (Tolan, 1996).

2.10.5 Enzymes for the food industry

The applications of enzymes in the food industry are many and diverse, ranging from texturising to flavouring. In most food applications the enzymes are applied to processed food products as processing agents upstream from the final product. Advances have been made in the optimization of enzymes for existing applications and in the use of recombinant protein production to provide efficient mono-component enzymes that do not have potential detrimental side-effects (*Kirk et al*., 2002).

2.10.5.1 Baking industry

Enzymes such as malt and microbial α-amylases have been widely used in the baking industry (Hamer, 1995). Enzymes are used in bread and rolls to give these products a higher volume, better colour, improve flavour and as an anti-staling agent. Malt preparations have led the way and created opportunities for many enzymes to be used commercially in baking. Enzyme preparations such as proteases, lipases, xylanases, pullulanases, pentosanases, cellulases, glucose oxidase, and lipoxygenases are applied in the bread industry for various purposes, but none have been able to replace α-amylase (Prieto *et al*., 1995). Currently, the α-amylases used in baking have been cereal enzymes from barley malt and microbial enzymes from both fungi and bacteria (Hebeda *et al*., 1990).

Supplementation of flour with exogenous fungal α-amylase has a higher activity and enhances the rate of fermentation and reduces the viscosity of the dough. There is also an improvement in the volume and texture of the product, which also generates additional sugar in the dough, subsequently improving the taste, crust color and toasting qualities of the bread (Van Dam and Hille, 1992).

There is an increasing focus on lipolytic enzymes (Collar *et al*., 2000). Recent findings suggest that (phospho) lipases can be used to substitute or supplement traditional emulsifiers, as the enzymes degrade polar wheat lipids to produce emulsifying lipids *in situ*. Currently the use of enzymes in the baking industry is moving towards the further understanding of bread staling and the mechanisms behind the enzymatic prevention of staling when using α-amylases and zylanases (Andreu *et al*., 1999).

Water-binding capacity and water retention in the starch and hemicellulose fractions of the bread, which are the substrates of α-amylases and zylanases, respectively, are important for maintaining softness and elasticity. The three-dimensional structure of amylases applied for anti-staling, provides an insight into the mechanism of enzyme action (Dauter *et al*., 1999). An excess of α-amylases used will result in an undesirable sticky texture to bread. Therefore, intermediate temperature stable α-amylases are used to prevent this from occurring. They are active after starch gelatinisation and become inactive before the completion of the baking process (Gigras *et al*., 2002).

2.10.5.2 Novel food applications

As a food component, fat contributes to some key sensory and physiological properties in products. The production of low-fat products, are normally associated with some technical problems such as poor texture, flavor and mouth feel (Keeton, 1994). Therefore, modification of food products using fat repacers or fat mimetics is often viewed as an effective way to overcome these problems due to the reduction in fat content (Giese, 1996). The mouth feel for fat in a product is a phenomenon of rheology, as unlike sweetness and saltiness, there is no sensor in the mouth for fats. Instead, the sensation of fattiness is a complex interrelated phenomenon involving viscosity and flowability poperties of a food product (Ma *et al*., 2006). Lucca and Tepper (1994) found that particles less than 3 μm in diameter were too small for human tongue to distinguish as individual particles. Instead the particles were perceived as a creamy and smooth fluid.

The hydrolysis of corn starch into fine particles with enzymes is suitable for use as a fat mimetic in low fat foods. Hydrolyzed corn starch is added to produce low fat mayonnaise reducing the fat

content by 60% in mayonnaise. Mayonnaise manufactured with fat mimetics, have a similar sensory quality as compared with the high fat mayonnaise (Ma *et al*., 2006).

2.10.5.3 Beverage alcohol and fuel ethanol production

For large-scale processing, the bioconversion of starchy materials to ethanol is very useful because it can be used as a biofuel and as the starting material for various chemicals. The production of ethanol from starchy biomass commonly involves three-step processes: liquefaction of starch by an endoamylase such as α-amylase to reduce the viscosity of the gelatinized starch produced after the cooking of the grains, enzymatic saccharification of the low-molecular-weight liquefaction products to produce glucose, and fermentation of glucose.

However, the present process for ethanol production from starchy materials via fermentation requires improvement of cost reduction. Although noncooking and low temperature- cooking fermentation systems (Prado *et al*., 2004) have succeeded in reducing energy consumption by approximately 50% (Prado *et al*., 2004), it is still necessary to add large amounts of amylolytic enzymes to hydrolyze the starchy materials.

Many researchers have reported attempts to resolve this problem by using recombinant glucoamylase-expressing yeasts with the ability to ferment starch to ethanol directly (Matthews, 1993). Also, a non-cooking fermentation system using a cell surface-engineered yeast strain promises to be very effective in reducing the production costs of ethanol (Pandey, 2003).

In beer industries microbial amylases are used to aid cereal amylase in the production of fermentable sugar. Ethanol is the most utilized liquid biofuel. Over the past decades, there has been an increasing interest in fuel ethanol as a result of increased environmental concern and higher crude oil prices. Ethanol fuels can be derived from renewable resources such as agricultural crops and by products. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world (Giraud *et al*., 1993). 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 (Arnesen *et al*., 1998). Enzymes such as α-amylase, glucoamylase and cellulases are important to produce fermentable sugars to produce ethanol (Kirk *et al*., 2002).

2.10.6 Treatment of starch processing waste water

Starch is also present in waste produced from food processing plants. Starch waste causes pollution problems. Biotechnological treatment of food processing waste water can produce valuable products such as microbial biomass protein and also purifies the effluent (Bruins *et al*., 2001).

2.10.7 Miscellaneous applications

Besides amylases' use in the saccharification or liquefaction of starch, these are also used for the clarification of formed haze in fruit juices, the pretreatment of animal feed to improve the digestibility (Pandey *et al*., 2000). α-amylase is used for the production of low viscosity, high molecular weight starch for coating of paper. Starch is a good sizing agent for the finishing of paper. It 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 °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. Also, good desizing of starch sized textiles is achieved by the application of α-amylases, which selectively remove the size and do not attack the fibers. It also randomly cleaves the starch into dextrins that are water soluble and can be removed by washing.

Furthermore, high molecular weights amylases were found in culture supernatants of an environmentally derived microbial mixed culture selected for its ability to utilize starch containing plastic films as sole carbon sources. This suggests a new application for amylases in biodegradation. 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 (Bertoldo and Antranikian, 2002).

A modern trend is to use starch for production of a more efficient and specific degradation products through a particular combination of activities. Amylase from *Aspergillus niger*, a saccharifying enzyme which produces maltose, maltotriose and some glucose, is capable of alcoholysis for the synthesis of methyl-glucosides from starch in the presence of methanol.

As these products are a series of methyloligosaccharides, from methyl-glucoside to methylhexomaltoside, the biotechnological applications of using starch as substrate for the production of alkyl-glucosides is analyzed (Francis *et al*., 2003). Moreover, it becomes possible to produce lactic acid directly from starch by an efficient simultaneous saccharification and fermentation from soluble starch by recombinant *Lactobacillus* strains (Van Ee *et al*., 1992). Finally, α-amylase is suggested as an enzyme that contributes to the reduction of AuCl4- to gold nanoparticles (Au-NPs) which makes it ideal for the production of Au-NPs (Zayed and Mostafa, 1992).

2.10.8 Analysis in medical and clinical chemistry

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 (Gupta *et al*., 2003). There are several processes in the medicinal and clinical areas that involve the application of amylases. Some other processes that have been developed using amylases are for example, detection of higher oligosaccharides (Giri *et al*., 1990) and biosensors with an electrolyte ioltor semiconductor capacitor transducer for process monitoring (Menzel *et al*., 1998).

3. MATERIALS AND METHODS

3.1 Experimental Area

The study was conducted on samples collected from Afar Regional State of Ethiopia. It has the lowest point in Africa. The southern part of the region consists of the valley of the Awash River, which empties into a string of lakes along the Ethiopian-Djibouti border. The Afar Depression, a plate tectonic triple junction is found in the Afar Regional State. This geologic feature is one of Earth's great active volcanic areas. Due to this volcanic activity the floor of the depression is composed of lava, mostly basalt. There are promising geothermal energy sources and hot springs in different areas of the region.

Locations from where the samples were collected are Awash, Blen Buri, Meteka, Tendaho and Alelobad.

3.2 Experimental Design and Data Analysis

Completely Randomized Design is used in all the experiments. One way analysis of variance (ANOVA) was followed for the temperature and pH data of the sampling sites. Significant statistics were reported at $p=0.05$. Other characterization assay data were generated and reported in figure. Enzymes assay were carried out in triplicates and the mean values and standard deviations were calculated and reported. Relationships among studied factors were presented using appropriate graphs and tables.

3.3. Materials and Equipment

3.3.1. Chemicals and media used

Chemicals and media used in the experiment include, Iron sulphate hydrated, Magnisium sulphate hydrated, different buffers, Calcium chloride, Magnisium chloride, Zinc chloride, Potassium iodide, Iodine, Soluble starch, Sodium hydroxide, DNS, Nutrient agar, Meat extract agar, bacteriological peptone, buffered peptone water, stains and indicators, and stock solutions.

3.3.2 Equipment used

List of equipment used include Spectrophotometer (UV-780C, England), Autoclave (LS –B50L, Germany), Incubator (Electrothermal Incubator, Model DNP, England), Shaking Water bath (YCW-012S, China), Hot plate (Wagtech, B212 model, Germany), Centrifuge (centrifuge 80-2, China), pH meter (MP511 Lab pH meter, England), Digital Caliper, Balance (Ohaus, adventurer model), Refrigerator (Cooltech, Prestige model), Vortex mixer (Vortex, VM-300P model), Constant temperature heating shaker (ZHWY, model103B, China), Drying oven (DHG, model 9055A, China), etc.

3.4 Sampling and Sample Management

Water (sample size of one liter) and Soil/Mud samples (sample size of one kilogram) from each geothermal site under study were taken aseptically. Water samples from the hot springs were taken by a long sterile pipe by pressing to 10 - 20 cm into the water source. Soil/ mud samples were collected by deep pressing up to 20 cm using a metal pipe of 50 cm diameter. The temperature and pH of the samples were measured at the spot using portable thermometer and a pH meter. The temperature of the water samples at the actual sampling area varies as we go down the source. Therefore, the temperature recorded at the spots may not be representative of the whole. The samples were coded as ALS (Alelobad Soil), ALW (Alelobad water), AWS (Awash soil), AWW (Awash water), BBS (Blen Buri Soil), BBW (Blen Buri Water), MS (Meteka Soil), MW (Meteka Water), TS (Tendaho Soil), and TW (Tendaho Water).

Table 1. Location of sample sites and some physical characteristics at the stage of sample collection

Study area	Temperature (^{o}C)		P^{H}		Location
	Soil	Water	Soil	Water	
Meteka	65.1	82.5	6.50	6.43	9^{0}52’N40^{0}31’E
Blen Buri	32.4	42.3	9.31	7.51	25 km from Awash
Tendaho	24.3	26.4	8.20	8.40	11^{0}44’13N40^{0}59’39”E
Alelobad	57.3	88.2	8.70	7.50	15 km from Logia
Awash	36.6	46.0	8.00	8.70	90^{0}8’N40^{0}E

The sample collection areas lie within the Ethiopian rift system. The temperature of the water and soil samples varied between 26.4 ^{o}C at Tendaho to 88.2 ^{o}C at Alelobad and 24.3 ^{o}C at Tendaho to 65.1 ^{o}C at Meteka respectively. Significant statistical difference ($p>0.05$) was observed among the temperature of the samples due to location except for Alelobad and Meteka. There was no significant difference in temperature among water and soil samples from Blen Buri and Awash, and also, between Meteka and Alelobad.

The pH values of both soil and water samples from Meteka were significantly ($p>0.05$) lower than pH values from other sites. Consequently, the pH of these samples fall in the range of acidity while samples from the other sites indicated alkalinity.

The water and soil/mud samples were collected into sterilized PE bottles and immediately transported to Microbiology laboratory of the Food Science and Nutrition Center of Addis Ababa University. Once in the laboratory, the samples were aseptically split into two categories. Category one was kept in a refrigerator and the other was shelved at room temperature until analysis. The possible impact of the storage condition was examined by performing a comparative analysis on the total number of viable microorganisms of both categories and the result was examined for significant difference.

3.5 Methods

For the isolation and characterization of thermophilic microorganisms and the enzyme they produce, the following methods were employed.

3.5.1 Incubation period determination using growth curve estimation

One hundred (100) ml of nutrient starch broth was prepared in a 250 ml flask and was pre-incubated at 65 ^{o}C for 30 minutes. One (1) ml of thoroughly mixed sample aliquot of the soil and water samples was added to the flask containing nutrient starch broth and was mixed till homogenized. Three milliliters of the suspension was pipetted out of the flask into a cuvette and absorbance was read at 660 nm for every 12th hour of incubation until 72 hours at 660 nm. The absorbance versus the time of incubation was plotted and the time for maximum cell

concentration was determined, at the absorbance level where the optical density started to show no rapid change.

3.5.2 Total microbial count

Total microbial load was determined as per ISO 6222 and as follows. A well mixed 25 g soil sample / 25 ml water sample was aseptically transferred into a 500 ml bottle containing sterile 225 ml buffered peptone water stock solution. The mixture was thoroughly mixed until a homogenized suspension is obtained. The suspension was allowed to settle down large particles before sub-samples were taken for microbial analysis. This mixture was considered as 10^{-1} of the original dilution. The original solution was serially diluted by taking 1ml of the dilution into nine (9) ml of buffered peptone water. Appropriate aliquots (1ml) from the dilutions were transferred into sterile Petri dishes and Plate Count Agar (about 15ml) was pour plated and were left to solidify. The tampered culture medium was then incubated at 65 ^{0}c for 48 hours. Colonies grown on the plates were counted and the result was reported as the number of colony forming units/ml for the water samples and colony forming units per gram for soil samples using the formula

$$N = \Sigma \mathrm{C}/[\mathrm{V}(\mathrm{n1} + \mathrm{n2})\mathrm{d}]$$

Where

N is the number of colony forming units;

ΣC is the sum of the colonies counted on all the dishes retained from two successive dilutions, at least one of which contains 15 colonies;

V is is the volume of inoculums applied to each dish, in milliliters;

n1 is the number of dishes retained at the first dilution;

n2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution retained.

3.5.3 Screening and isolation of thermophilic microorganisms

Thermophilic microorganisms were isolated from the samples using Nutrient-starch-agar medium (Nutrient agar and soluble starch dissolved in distilled water) by serial dilution method

(Holt and Krieg, 1994). Briefly, one (1) g of the soil sample was dissolved in 100 ml of sterilized saline water (0.85%) and suitable dilutions (10^{-1}-10^{-6}) were prepared. Ten (10) ml of the diluted suspension was taken in a test tube and given heat shock at 90 °C for 15 min and then cooled to room temperature. One (1) ml of each diluted suspension (in triplicates) was transferred to petri dishes and nutrient starch agar was pour plated in to the Petri dishes. The Petri dishes were gently rotated clockwise and anticlockwise to facilitate a uniform spreading of diluted suspension on nutrient starch agar and were incubated at different temperature (45, 50, 55, 60, 65, and 70°C) in an incubator for 48 hrs. The plates were covered with cooking bags to prevent drying of the agar. Colonies grown on the plates were considered as thermopiles yet those grown at the maximum incubation temperature (70 ^{o}C) were selected as highly thermophilic and were sub-cultured to get pure colonies. The pure colonies isolated were stored in a refrigerator at four (4) ^{0}C for further investigation.

3.5.4 Isolation of amylase producers

Thirty five (35) colonies with largest colony sizes grown at the highest incubation temperature (70 ^{o}C) were picked by a loop and transferred to nutrient starch agar (Nutrient agar and soluble starch dissolved in distilled water) plates. The plates were then incubated at 65°C for 48hours and were flooded with Lugol's Iodine [I_2 (1%W/V) and KI (2%W/V) dissolved in 100 ml distilled water] solution. The isolates which showed clear halo zones were taken as potential sources of α-amylase (Bragger *et al.*, 1989). The selected isolates were then transferred to nutrient starch agar slants and stored at five (5) °C in a refrigerator for further analysis.

3.5.5 Selection of best amylase producers

Among the isolates which showed positive response for the Iodine test, two samples were selected for further investigation. Selection was based on measurement of the average diameter of the colonies and the average halo diameter formed during the Iodine tests and there-by comparing the ratio of the halo diameter to the colony size. The measurements were taken by using a digital calipper.

3.5.6 Biochemical characterization of the selected amylase producers

Biochemical tests catalase and oxidase tests and oxygen requirement were studied. Finally the genus of the selected isolate was identified (Pandey *et al*., 1994). Further biochemical tests such as citrate utilization test, indole test, Voges-Proskauer (VP) test, nitrate reduction test were carried.

3.5.6.1 Gram Reaction

A loop full of the overnight culture was spread onto the microscope slides until a thin film was formed. After drying, they were fixed by passing the slide through flame for approximately 2 seconds. The heat fixed smear was first stained with crystal violet for 1 min. After rinsing under the tap water, the slides were transferred into iodine solution and were kept for 1 min. And then, slides were washed in 95% alcohol for 10 - 15 seconds. They were then stained with safranin for 30 seconds. After staining, the slides were left to dry on paper towels and the cells were examined under light microscope. Gram positive cells appeared purple.

3.5.6.2 Catalase Test

Isolates were grown on nutrient starch agar for 48 hrs at 65 ^{0}C. 3% hydrogen peroxide was poured onto the colonies. Formation of air bubbles indicated the presence of catalase activity.

3.5.6.3 Oxidase Test

Isolates were grown on nutrient starch agar for 48 hrs at 65 ^{0}C. A filter paper was placed into a petri dish and wetted with 1% solution of tetramethyl-p-phenylenediamine. One large colony was taken with a loop and placed onto the wet filter paper. The presence of oxidase activity was confirmed by the formation of blue-purple color.

3.5.7 Enzyme assay

α-Amylase activity was determined according to the method described by Gigras *et al*. (2002) and Bailey *et al*. (1992). Briefly, the enzyme activity was determined at specific temperatures by mixing 0.5 ml of the enzyme with 0.5 ml of a 1% (w/v) soluble starch solution made up in specific buffer solutions. After ten (10) min, the reaction was terminated with the addition of one (1) ml of dinitrosalicylic acid solution (DNS). The final reaction volume was adjusted to 200 µl.

One unit of amylase activity was defined as the amount of enzyme that releases 1 mg/ml of reducing sugars (glucose equivalents) under the assay conditions.

3.5.8 Preparation of maltose standard curve

Standard maltose solution (1 mg/ml) was pipetted out in the range of 0.2, 0.4, 0.6, 0.8 and 1 ml, into 5 separate test tubes. A test tube containing two (2) ml blank solution (distilled water) was also prepared. Using distilled water, the volume was brought up to two (2) ml in each test tube. One (1) ml of DNS reagent was added to each tube and the test tubes were covered with aluminum foil. The contents in the test tubes were heated in a boiling water bath for five (5) minutes. The test tubes were cooled to room temperature and nine (9) ml distilled water was added to each test tube and mixed well. Three (3) ml of the solution was taken from each test tube into different cuvettes to measure its absorbance and placed in a colorimeter at 540 nm. The amount of maltose versus absorbance at 540 nm was plotted and the equation for the curve was calculated (Appendix B). The slope was taken as the extinction coefficient.

3.5.9 α-amylase activity calculation

The enzyme activity was determined using the formula

$$Enzyme\ activity\ (U/ml) = \Delta\varepsilon \times Vf/(\Delta t \times \Sigma \times Vs \times d)$$

Where, $\Delta\varepsilon$ is absorbance at 540 nm

Vf is final volume including DNS

Δt is time of hydrolysis

Σ is extinction coefficient

Vs is volume of the enzyme used (in ml)

d is diameter of the cuvette (1cm for a standard cuvette)

3.5.10 Enzyme production and isolation

Following the method of Soni *et al*. (2003), a liquid culture medium (basal medium) containing 0.5% meat extract, 1% polypeptone and 1% soluble starch was prepared. One hundred milliliters of medium in 500 ml baffled flasks was inoculated with four (4) ml of an overnight culture and

incubated at different temperatures (45, 50, 55, 60, 65, and 70 °C) with rotary shaking speed of 130 rpm. The cultures were harvested at different fermentation times (12, 24, 48, 60, and 72 hrs) and cells were separated by centrifugation at 2500 rpm for 30 minutes. The cell free culture supernatant was used as the enzyme source.

3.5.11 Optimization of enzyme production process parameters

Various process parameters influencing enzyme production during submerged fermentation were optimized for maximal enzyme production. These were applied to submerged fermentation as follows: incubation period (12, 24, 48, 60, and 72 hrs); incubation temperature (45, 50, 55, 60, 65, and 70 ^{o}C); initial pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0), and substrate concentration (0.2 - 2% w/w starch). The fermentation was carried out by applying one variable at a time (OVAT) for each of the parameters tested.

3.5.12 Production of amylase under optimum conditions

On the basis of the results obtained with all the optimum parameters, viz., substrate concentration, pH, incubation temperature, and incubation period, the enzyme was produced by submerged fermentation and assayed.

3.5.13 Enzyme extraction

The fermentation product was filtered using filter paper. The filtrate was collected and centrifuged at 2500 rpm and 30 minutes. The clear supernatant was analyzed for amylase activity as described above in section 3.5.7.

3.5.14 Enzyme characterization

The extracted enzymes were further characterized as follows.

3.5.14.1 Assay of enzyme activity under varied temperature condition

The optimal temperature for α-amylase activity was determined by assaying the enzyme activity between 45 – 115 ^{o}C for 10 min at optimum pH. Standard assay conditions as described in section 3.5.7 were followed.

3.5.14.2 Assay of enzyme activity under varied pH condition

The effect of pH on α-amylase activity was performed at optimum temperature using different buffers in a range of 4-11. These included [sodium acetate buffer (pH 4 - 5), phosphate buffer (pH 6 - 7), Tris–HCl buffer (pH 8 - 9), glycine/NaOH buffer (pH 10.0) and Na_2HPO_4/NaOH buffer (pH 11.0)]. The concentrations of all the buffers were 50mM. The reaction mixture included 0.5 ml of enzyme solution, 1 ml of buffer and 0.5 ml of 1% starch solution. The assay mix was left undisturbed for 10 minutes. The amylase activity was determined as described in section 3.5.7.

3.5.14.3 Assay of enzyme activity under different metal ions and chemical compounds

The effect of metal ions on the α-amylase activity was determined by adding 1 mM, 5 mM and 10 mM of each ion ($ZnCl_2$, $MgCl_2$, $BaCl_2$, $CaCl_2$), and chemical compounds (1 mM EDTA and urea). Activity was determined by pre-incubating 0.5 ml of the enzyme solution and 0.5 ml of the salt solution for 30 min at optimum temperature. Thereafter, residual enzyme activity was determined as described in section 3.5.7. The activity of the enzyme assayed in the absence of a cation was taken as 100%.

3.5.14.4 Assay of enzyme stability under varied pH

pH stability of the enzyme was determined after keeping 0.5 ml of the enzyme at 4 ^{o}C for 1 hour in 1 ml of different buffers [sodium acetate buffer (pH 4 - 5), phosphate buffer (pH 6 - 7), Tris–HCl buffer (pH 8 - 9), glycine/NaOH buffer (pH 10.0) and Na_2HPO_4/NaOH buffer (pH 11.0)] ranging from pH 4-11. The α-Amylase activity was determined every 10 minutes as described in section 3.5.7 and compared to the initial enzyme activity.

3.5.14.5 Assay of enzyme stability under varied temperature

Thermostability of α-amylase was assessed by incubating 0.5 ml enzyme solution at 45 – 115 ^{o}C in 1ml 20mM appropriate buffer [sodium acetate buffer (pH 4 - 5), phosphate buffer (pH 6 - 7), Tris–HCl buffer (pH 8 - 9), glycine/NaOH buffer (pH 10.0) and Na_2HPO_4/NaOH buffer (pH 11.0)] for 0, 10, 20, 30, 40, 50, and 60 minutes. The remaining activity was then measured and compared to the initial enzyme activity (which was considered as 100% active level). The initial

enzyme activity was assayed as described in section 3.5.7 under optimum temperature conditions but without heating the enzyme.

4. RESULT AND DISCUSSION

4.1 Incubation Time from Growth Curve Estimation

Enzyme production is growth associated with growth and is induced by the presence of the substrate in the medium. As depicted in Fig. 3, the optical density (OD) measurements taken every 12th hour showed that the cell mass reached its maximum within 36-48 hrs. Maximum OD values (0.69) were exhibited starting from the 36th hour till 48th hours. However, 48 hour was considered end of the stationary phase and as the optimum period for the production of enzyme. Therefore, it is plausible to assume the period of maximum cell growth match with maximum level of enzyme production.

The microbes on ALS were in a lag phase up to 10 hrs of incubation. They grew exponentially up to 36 hrs and then entered and stayed a stationary phase for a duration of nearly 12 hrs. This was finally followed by the decline phase. Similar growth curves were fitted for several other *Bacillus* species i.e. *Bacillus amyloliquefaciens* (Pandey, 2000), and *B. subtilis (*Swain *et. al*., 1996). In the reported findings of these authors, the stationary phase (36-48 hrs) was considered as the duration for maximum cell growth and enzyme production.

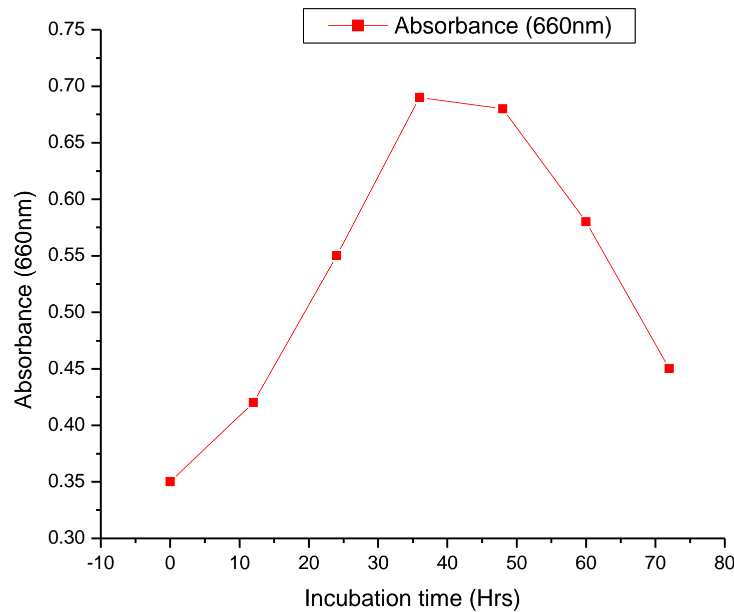

Figure 3. Growth curve for isolates from Alelobad Soil

4.2 Total Microbial Count

The aerobic plate count is designed to provide an estimate of the total number of aerobic organisms in the samples. In all the samples, thermophilic microorganisms were able to grow. The soil samples, generally, had higher number of microorganisms when compared to the water samples. Maximum number of colonies was observed in the soil sample collected from Meteka (MS) with 22.80×10^6 CFUs/gr and the least number of colonies were observed in the water sample taken from Blen Buri (BBW) with 3.00×10^6 CFUs/ml.

Growth condition of thermophilic microorganisms under room and refrigerated storage before analysis were not significantly affected by the storage conditions (Appendix A Table A.1).

4.3 Isolation and Screening of Thermophiles

The primary criterion used to isolate thermophilic microorganisms was based on incubation temperature. For this, the study was conducted on different incubation temperatures (45- 70 ^oC) at which thermophilic microorganisms are expected to grow were used. Thermophilic microorganisms from both sample categories (soil and water) were grown from all the samples at all incubation temperatures. Maximum number of colonies was observed at incubation temperature of 65^oC in all the samples. Soil sample from Alelobad had the maximum number of thermophiles at this temperature while the least was observed for the water sample from Blen Buri (Table 2). The effect of the incubation temperature on the number of thermophiles for each sample was tested for significance ($p<0.05$) and accordingly, there was no significant difference among the different incubation temperatures and the number of thermophilic microorganisms grown for each sample.

Table 2. Number of thermophilic microorganisms grown at incubation temperatures ranging from 45-70 °C for 48 hours

Sample	Temperature (°C)					
code	45	50	55	60	65	70
ALS	245	260	274	229	287	210
ALW	185	198	204	173	176	133
AWS	197	184	227	213	244	179
AWW	88	110	117	170	156	93
BBS	96	127	166	73	137	59
BBW	83	76	84	36	55	17
MS	218	198	201	176	229	149
MW	118	167	183	143	153	99
TS	94	118	103	67	84	52
TW	77	51	68	49	58	46

4.4 Screening for Amylase Producers

Thermophilic microorganisms grown at 70 °C, 35 CFUs from each water and soil sample, were selected and tested for amylolytic activity using Lugol's Iodine test. Most of the selected thermophiles from each sample showed amylolytic activity (Fig. 4). The amylolytic activity ranged from 13 (37.1%) for TW to 31 (88.6%) for MS.

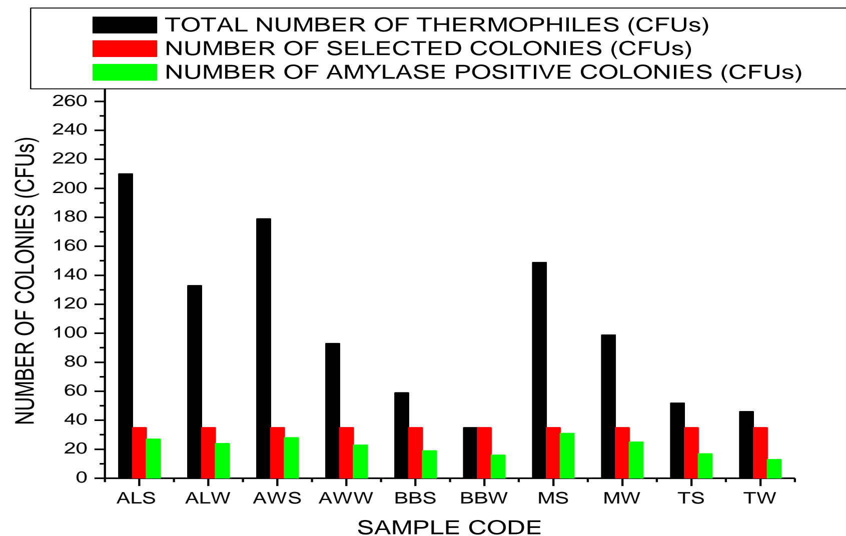

Figure 4. Number of thermophilic microorganisms and amylase positive isolates

4.5 Selection of Best Amylase Producers

Colonies showing amylolytic activity were selected and the diameter of the clear halo zone formed and the size of the colonies were measured using digital calliper. Halo diameter to colony size ratio (HCR) was used to select the best amylase producers among the different samples. ALS with HCR of 5.707, and MS with HCR of 5.638 had the highest HCR when compared to others (Table 3). Therefore, these samples were selected as the best amylase producers for further characterization. These isolates were preserved in a starch nutrient agar slants in a refrigerator at 4 ^{o}C.

Table 3. Halo diameter to colony ratio of amylase positive isolates

Sample Site	Colony diameter (mm)	Halo diameter (mm)	HCR*
ALS	0.964 ± 0.087	5.502 ± 0.285	5.707
ALW	0.944 ± 0.099	4.055 ± 0.182	4.296
AWS	0.920 ± 0.068	5.154 ± 0.271	5.602
AWW	0.913 ± 0.074	3.658 ± 0.310	4.007
BBS	0.821 ± 0.086	3.015 ± 0.397	3.672
BBW	0.803 ± 0.052	2.630 ± 0.246	3.275
MS	1.003 ± 0.047	5.655 ± 0.462	5.638
MW	0.937 ± 0.023	4.751 ± 0.227	5.070
TS	0.962 ± 0.053	4.908 ± 0.188	5.102
TW	0.834 ± 0.063	2.453 ± 0.251	2.941

Column 2 and 3 show mean of 35 selected isolates ± SD

HCR* is Halo diameter to colony ratio

4.5 Biochemical Characterization of the Selected Best Amylase Producing Isolates

The isolates ALS and MS were gram positive, rod shaped, motile, and gave positive results for catalase test and negative results for oxidase test (Table 4). Both isolates produced acid slant, acid butt, no gas and no H_2S in the KIA reaction. Based on the results, the strains ALS and MS were identified as belonging to the Genus *Bacillus*.

Table 4. Biochemical characterization of the selected best amylase producing isolates

Biochemical test	Response	
	ALS	MS
Growth in air	Positive	Positive
Anaerobic growth	Negative	Negative
Indole production	Negative	Negative
Vogus-proskaeur	Positive	Positive
Catalase test	Positive	Positive
Citrate production	Positive	Positive
Oxidase production	Negative	Negative
Nitrate reduction	Positive	Positive
Gram's test	Positive	Positive

4.6 Effect of Fermentation Period on Enzyme Production

The suitable fermentation period at which highest α-amylase activity production takes place was studied for ALS and MS. Both ALS and MS isolates produced highest α-amylase activities (U/ml) of 2.5214 ± 0.12, and 2.5507 ± 0.0.12 at 48 hrs of fermentation time while growth of both strains reached maximum at 48-60 hrs.

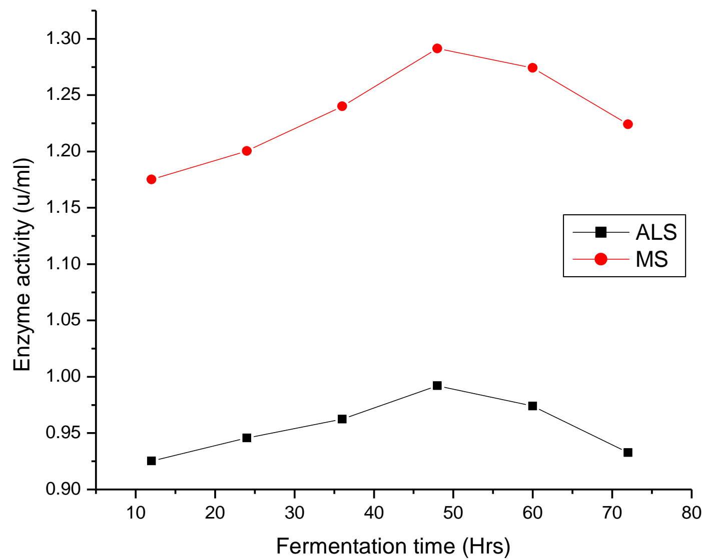

Figure 5. Effect of fermentation time on enzyme activity (at pH 7 and 45 ^{o}C)

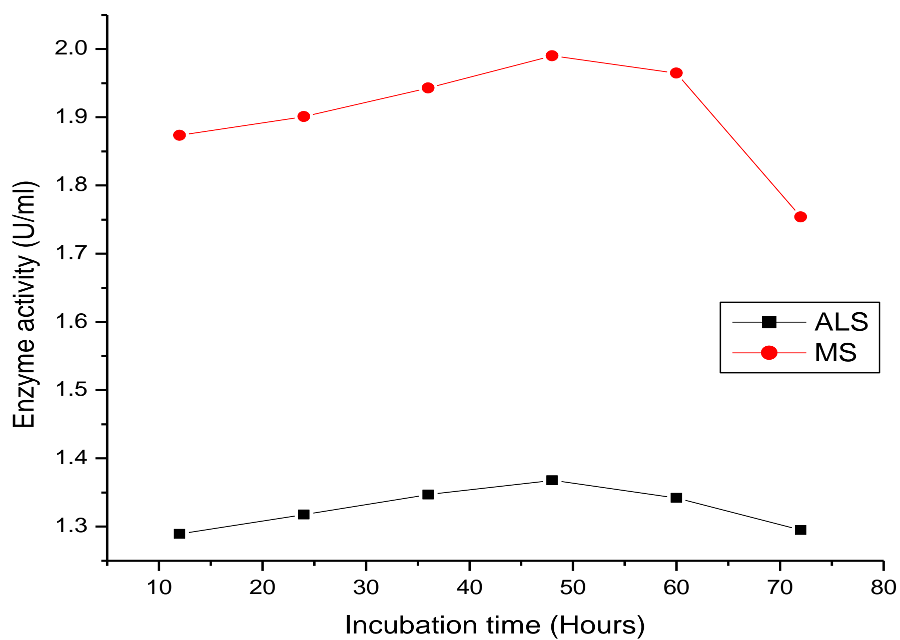

Figure 6. Effect of incubation time on enzyme activity (at pH 7 and 50 ^{o}C)

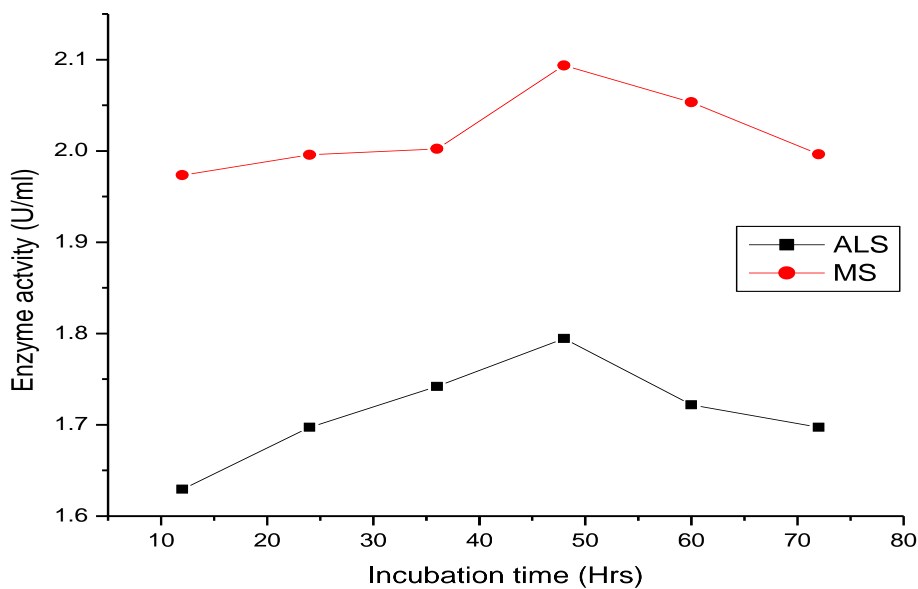

Figure 7. Effect of incubation time on enzyme activity (at pH 7 and 55 ^{o}C)

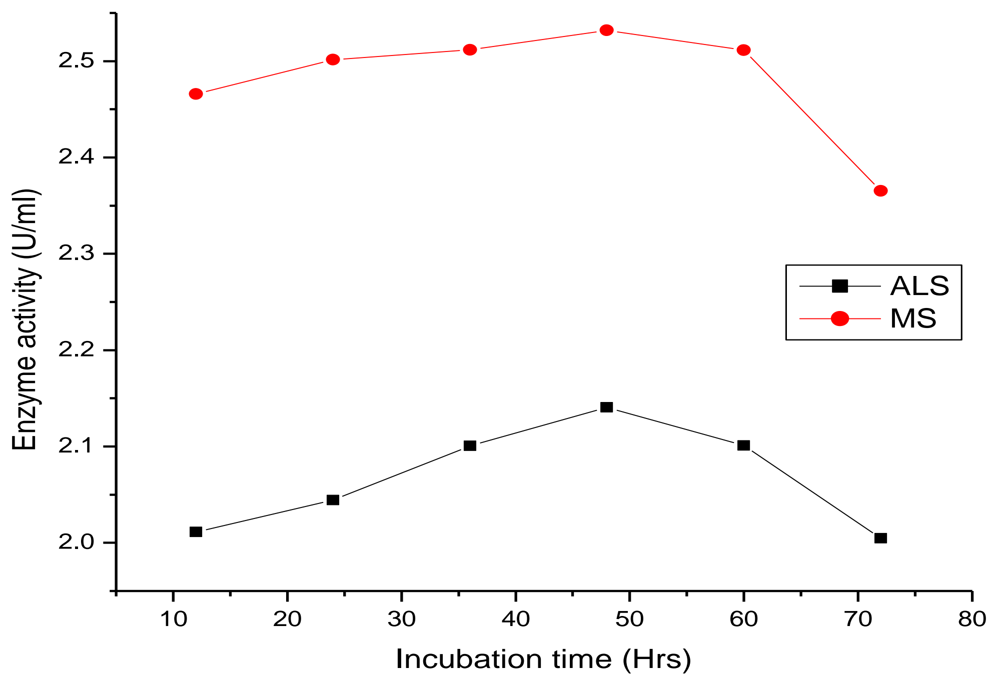

Figure 8. Effect of fermentation time on enzyme activity (at pH 7 and 60 °C)

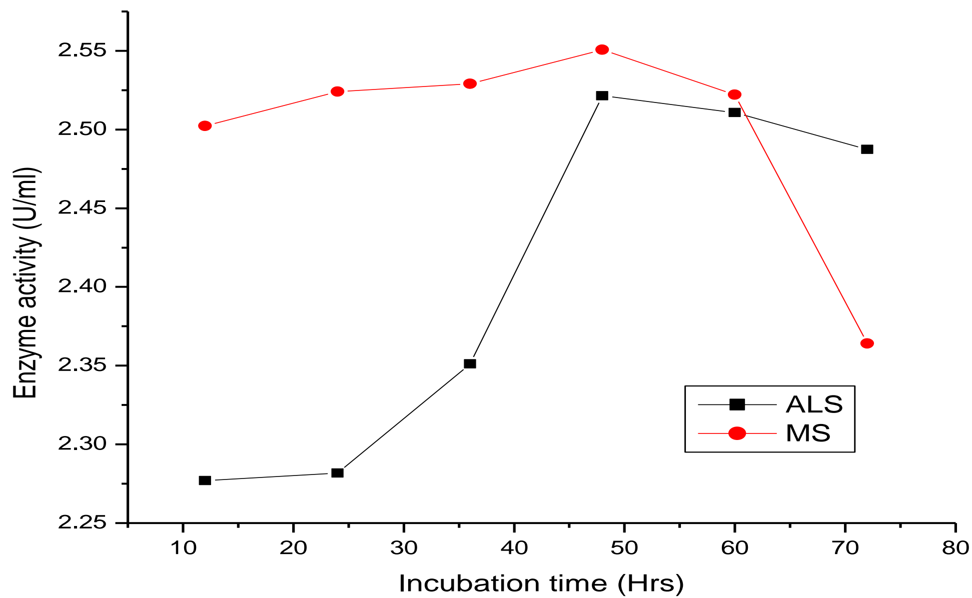

Figure 9. Effect of fermentation time on enzyme activity (at pH 7 and 65 °C)

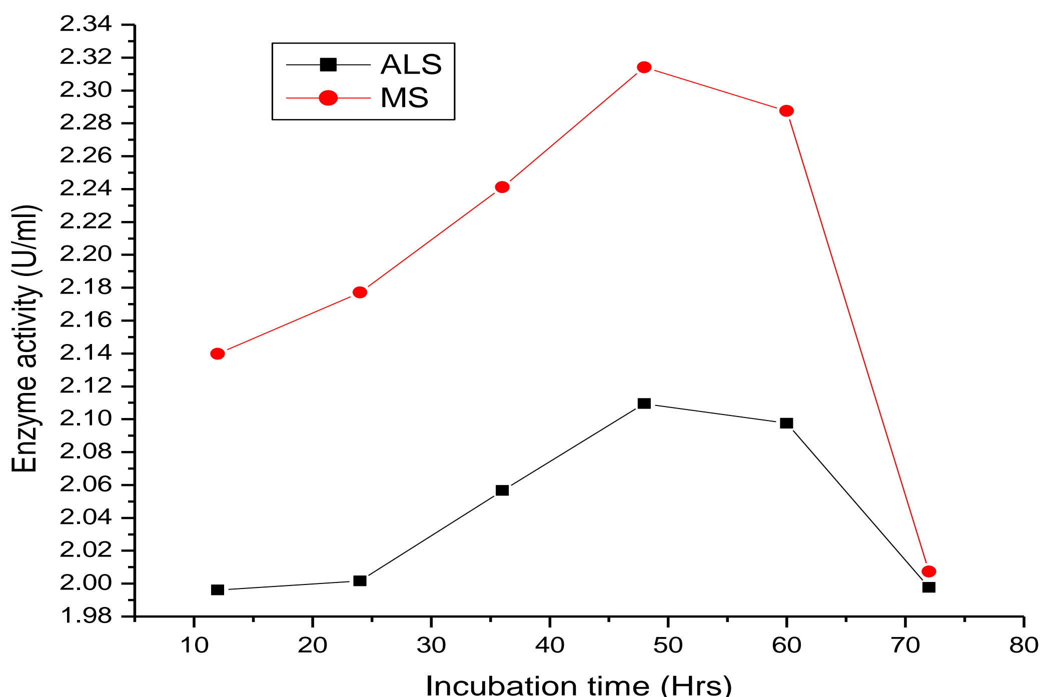

Figure 10. Effect of incubation time on enzyme activity (at pH 7 and 70 ^{o}C)

4.7 Effect of Incubation Temperature on Enzyme Production

Long term exposure to higher temperatures alters growth of microorganisms. Extreme heat may also have a lethal effect. Thus, microorganisms should not be exposed, even for few minute, to a temperature higher than their growth maximum (Giese, 1996) as it causes coagulation of proteins, disruption of nucleic acid base pair interactions and melting away of lipids on the cell membrane resulting a pronounced decrease in the potential of the microorganisms in liberating enzymes and other metabolites.

The influence of temperature on the production of amylase was investigated by using various incubation temperatures (45, 50, 55, 60, and 70 °C). Production of amylase by ALS (2.5214 ± 0.12 U/ml) and MS (2.5507± 0.12 U/ml) was optimum at 65°C production temperature. When the temperature was increased or decreased, there was gradual decrement in enzyme activity for MS yet the enzyme activity sharply dropped for ALS. At 45 °C temperature, the production of amylase was extremely low. It has been reported that thermostability increased with the temperature at which cultures were grown (Hough and Danson, 1999). However, there is an

inherent organism's basal limit of the temperature to which this is possible. In addition to the alteration of the growth of the organism at a high temperature, media components breakdown to unwanted materials, which can no more, be utilized. A number of reports have indicated that there is a decrease in both growth and no further increase in thermostability at temperatures above the optimum growth temperature (Zeikus *et al.*, 1996). The organisms (isolates ALS and MS) in this study had optimum growth temperature of 65 ^{o}C. The enzymes from ALS (2.5214 U/ml) and MS (2.5507 U/ml) isolates showed maximum enzymatic activity at this temperature (Fig.11 a-f).

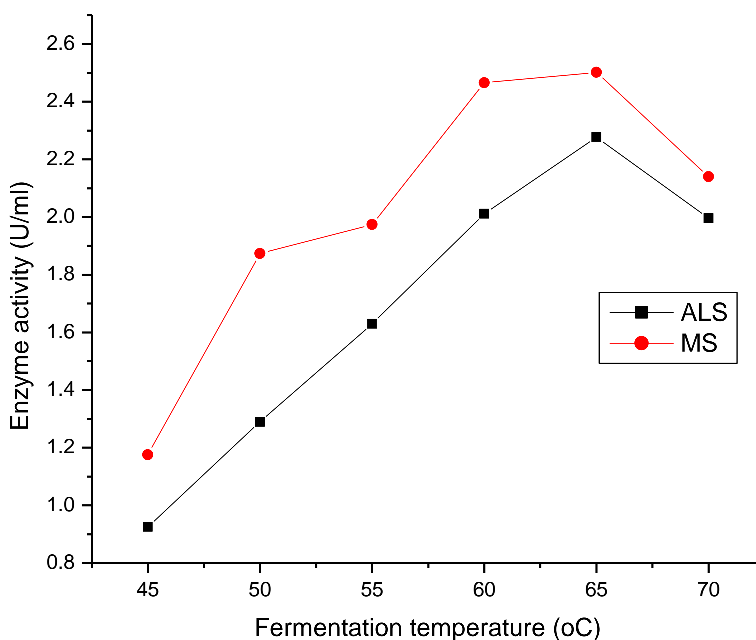

Figure 11.a Effect of incubation temperature on enzyme activity at pH 7 and 12 hr of incubation

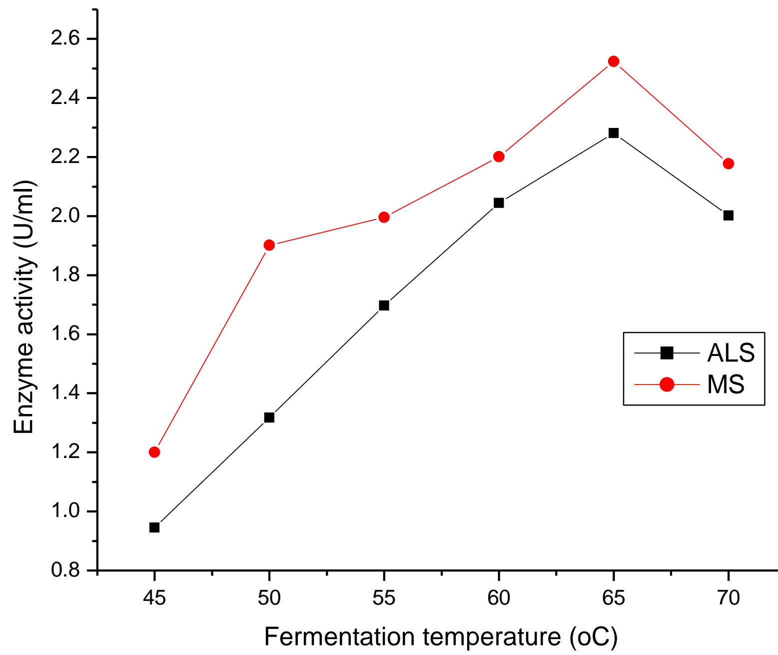

Figure 11.b Effect of incubation temperature on enzyme activity at pH 7 and 24 hr of incubation

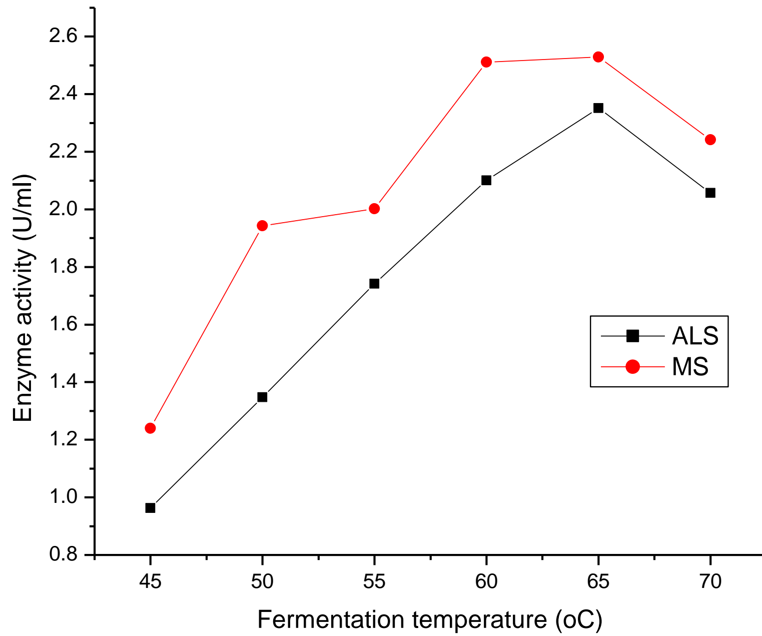

Figure 11.c Effect of incubation temperature on enzyme activity at pH 7 and 36 hr of incubation

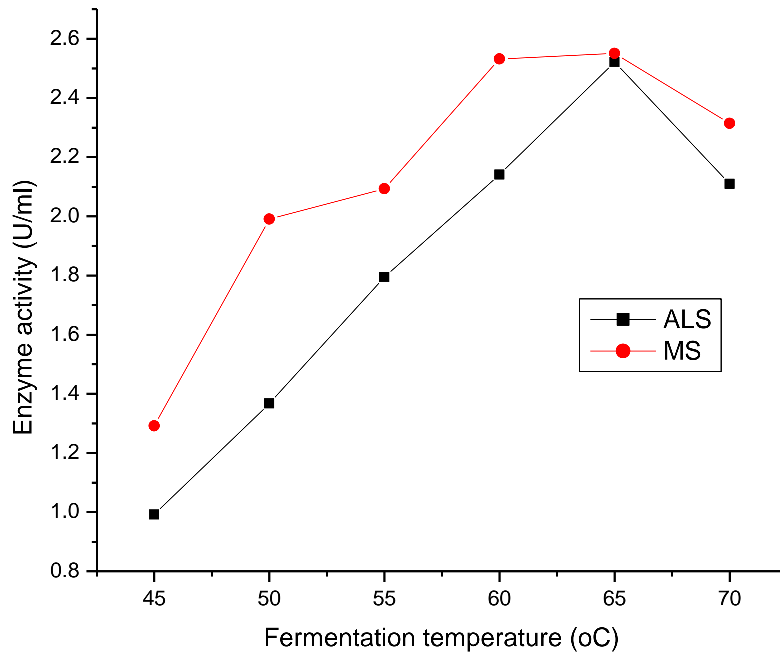

Figure 11.d Effect of incubation temperature on enzyme activity at pH 7 and 48 hr of incubation

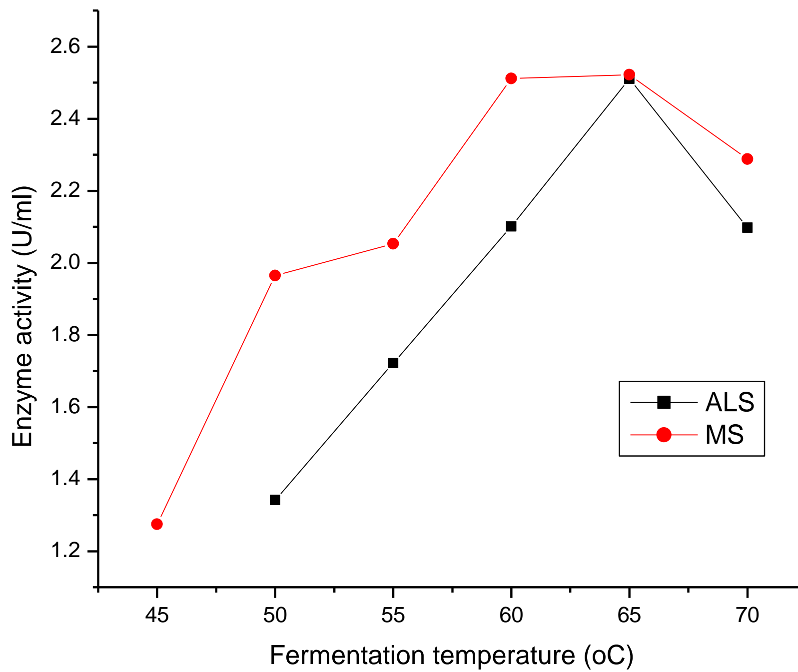

Figure 11.e Effect of incubation temperature on enzyme activity at pH 7 and 60 hr of incubation

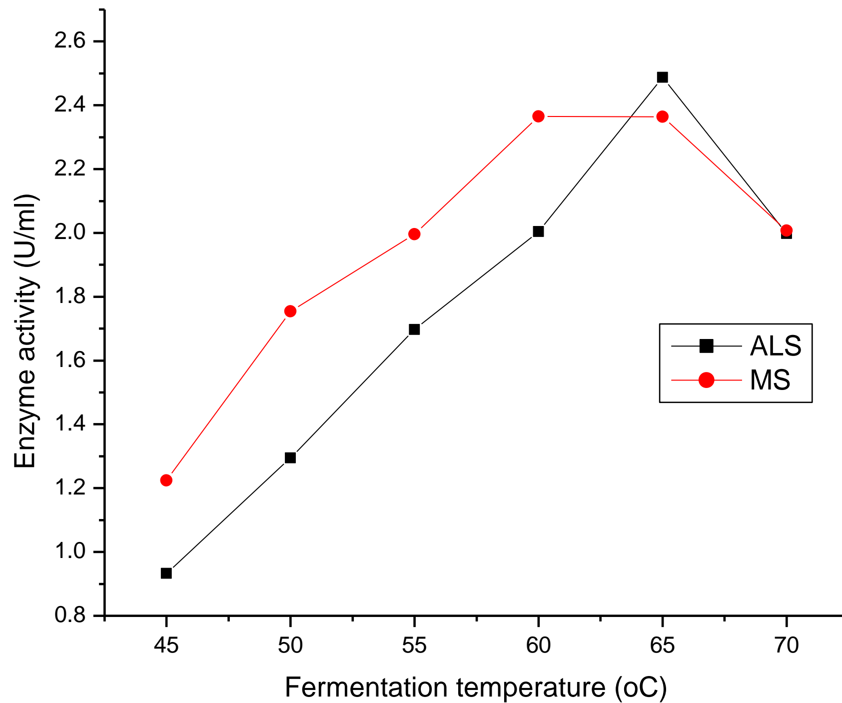

Figure 11.f Effect of incubation temperature on enzyme activity at pH 7 and 72 hr of incubation

4.8 Effect of Initial pH on Enzyme Production

After achieving an optimization strategy, major problem in industrial bioprocess operations still remains if there is a failure to use the optimum pH, since strong acids and alkali exert a pronounced bactericidal effect. Soccol and Vandenberghe (2003) reported that a thermostable α-amylase production from thermophilic and aerobic *Bacillus* sp. JF strain was enhanced between pH 7.2-7.4. Enzyme activity of *Bacillus caldolyticus* reached 13.5 U/ml when pH of the medium was adjusted to 7 (Zayed and Mostafa, 1992).

In this study, initial pH of the enzyme production medium was adjusted to 4-11. In these shake flask cultures activity of the amylase was then monitored at optimum fermentation temperature (65 ^oC) and optimum fermentation period (48 hours). Results presented in (Fig. 12) shows that enzyme production from ALS isolate was enhanced at neutrality to basic (pH 7-9). In the contrary amylase from MS isolate was active at pH values from neutrality to acidity (pH 5-7).

Optimum pH for the amylase production by ALS was found to be 8. The isolates from ALS were found to favor ph range from neutrality to basic. These isolates did not grow in production medium below pH 5. MS isolates were found to favor optimum pH of 6 and favorably grow at acidic environments up to pH value of 4.These isolates did not grow in environments with pH values of 10 and above. The enzyme activities at the optimum pH and temperature were 2.3110 U/ml and 2.5791 U/ml for ALS and MS respectively.

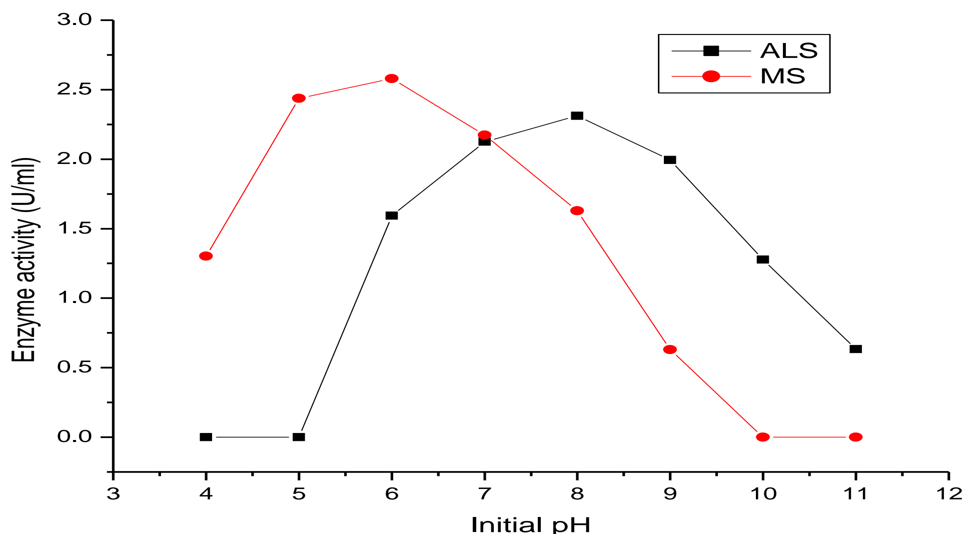

Figure 12. Effect of initial pH on enzyme activity at optimum fermentation temperature and incubation time

4.9 Effect of Substrate Concentration on Enzyme Production

Starch is a generally accepted nutritional component for induction of amylolytic enzymes. Lucca and Tepper (1994) reported that of the carbon sources tested, soluble starch was the best inducer for amylase production while glucose was a poor inducer at approximately 50%. Also, in a study reported by Pandey *et al*. (1999), higher cell density and higher specific growth rate were obtained from glucose but higher enzyme activity and higher specific enzyme activity were obtained from starch.

When the starch concentration in the basal media was increased from 0.2 - 2%, the activity of the enzyme showed increment up to 1% starch concentration (Fig. 13) at optimum pH and fermentation temperature. The amylase activity for ALS increased rapidly as the concentration

of the substrate (soluble starch) increased from 0.25% to 1% and sharply declined as the concentration further increased to 2%. The enzyme had maximum amylase activity at 1% concentration of the substrate.

There was a minimal increment of enzyme activity for MS as we increase the starch concentration from 0.25% to 0.5% and started to increase steadily up to 1% starch concentration which gave the maximum amylase activity. The maximum activity was relatively maintained up to 1.25 % concentration and started to decline as the concentration was increased up to 2%. Pandey *et al*. (1999) reported that starch at concentration of 1 % (w/v) supported optimal enzyme production, followed by a decline at higher concentrations. This can be attributed to the high viscosity of culture broth at such concentrations, which interferes with O_2 transfer leading to limitation of dissolved O_2 for the growth of bacteria.

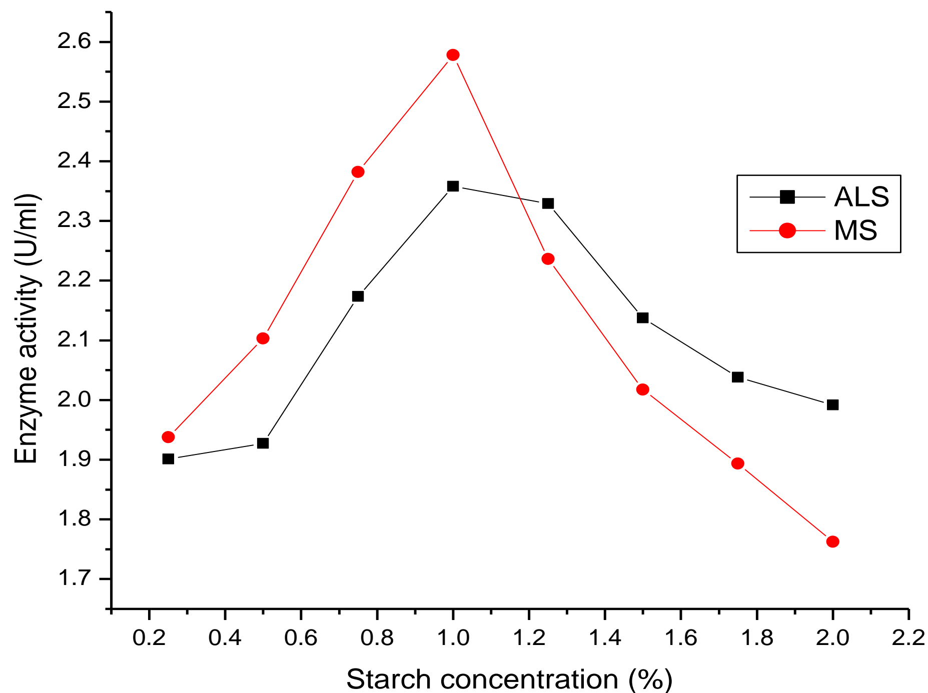

Figure 13. Effect of starch concentration on enzyme activity at optimum fermentation temperature, time, and pH

4.10 Enzyme Production at Optimum Enzyme Production Conditions

Over production of primary and secondary metabolites is complex (Amartey *et al*., 1991), and requires mastery of the fermentation process, media optimization and the fine-tuning of process conditions.

The identification of essential growth requirements has traditionally been done in shake flasks by adding or omitting certain nutrients and incubating statically or shaken (Amartey *et al*., 1991). Among the basic requirements of organisms, water, carbon, nitrogen, salts, trace metals and growth factors are essential. The general objectives of fermentation conditions optimization are to maximize productivity, minimize by-products and costs and ensure steady product quality.
On the basis of the results obtained with all the optimum parameters, viz., pH (pH 6 for MS, and pH 8 for ALS), incubation temperature (65 ^{o}C) and incubation period (48 Hrs), the enzyme was produced by submerged fermentation and assayed.

Table 6. Enzyme activity at optimum enzyme production conditions

Sample code	Enzyme activity (U/ml)
ALS	2.4657 ± 0.07
MS	2.5471 ± 0.1

4.11 Temperature Optima for the Enzyme Activity

It would be preferable to use enzymes at high temperatures in order to make use of increased rate of reaction and the protection it affords against microbial contamination. Enzymes, however, are proteins and undergo essentially irreversible denaturation entailing a loss of biological activity high temperatures. The actual loss of activity is the product of the duration of incubation and this may be due to covalent changes such as the deamination of asparagin residues or non-covalent changes such as the rearrangement of the protein chain. Inactivation by heat denaturation has a profound effect on the enzyme's productivity. When considering the effects of temperature on the activity and stability of an enzyme, it must be remembered that they are time-dependent. Increasing the temperature will result in an increase in activity and temperature-induced

denaturation. In general, thermostability of a particular enzyme is a reflection of its structure (Huimin *et al*., 2002).

The activity of the α-amylase obtained from ALS and MS was assayed at different temperatures ranging from 45 - 115℃ at optimum pH (pH 8.0 for ALS and pH 6 for MS). The optimum temperature for activity of α- amylase was 90°C for ALS and 85 ^{o}C for MS (Fig.14). The activities of the enzymes sharply dropped as the temperature increased. The enzymes were inactivated at temperature of 105 and 110 ^{o}C and above for MS and ALS respectively.

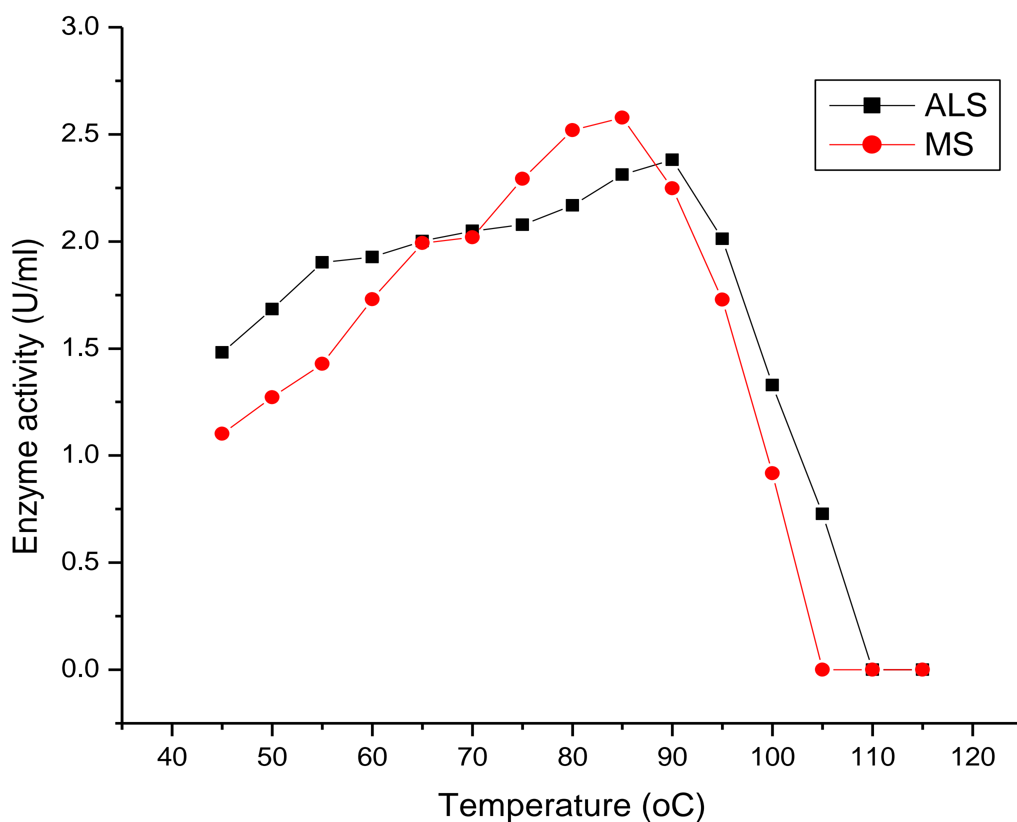

Figure 14. Effect of temperature on enzyme activity at optimum pH

4.12 pH Optima for the Enzyme Activity

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. Changes in charges with pH affect the activity, structural stability and solubility of the enzyme (Tobin *et al*., 2000).

In the pH range between 4 -11, the activity of α-amylase produced by the isolates (ALS and MS) was studied at the optimum temperatures for enzyme activity for each isolate (90 and 85 ^{o}C for ALS and MS respectively) and the optimum was 8 for ALS and 6 for MS (Fig.15). The enzymes from ALS were inactivated as the pH value of 5 and below. And it was active up to pH value of 11. The amylase from MS was functional even at lower pH value of 4 yet it was inactivated at pH value of 10 and above.

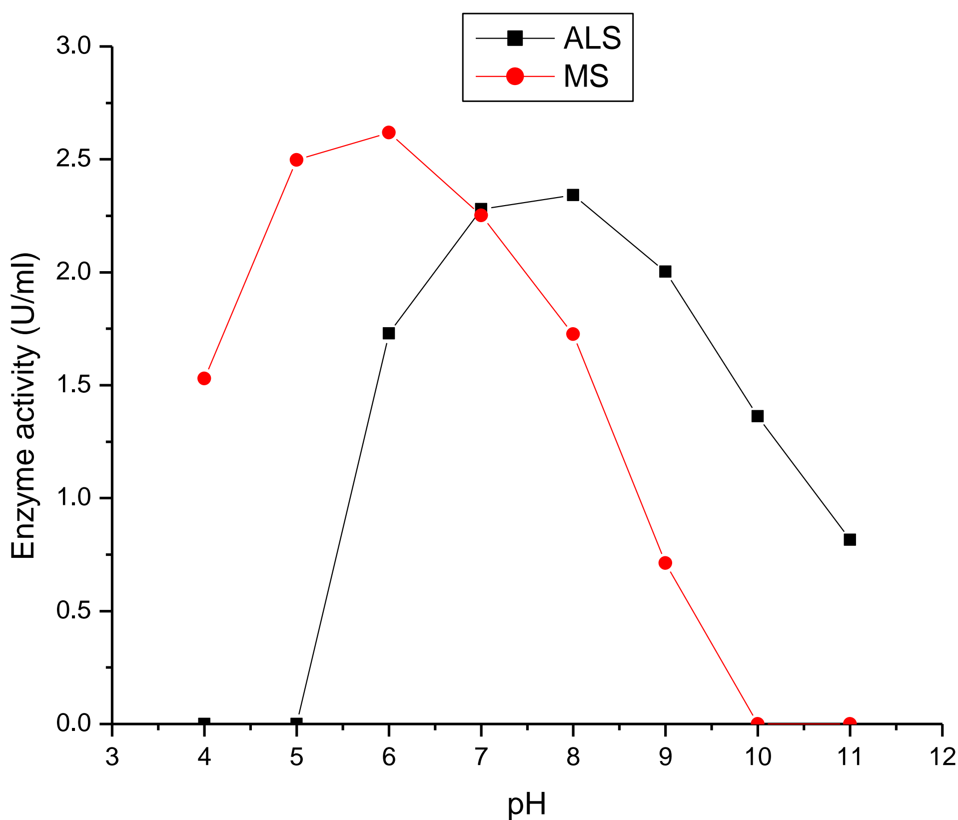

Figure 15. Effect of pH on enzyme activity at optimum temperature

4.13 Thermostability of the enzyme

Thermostability of α-amylase was investigated by incubating the enzyme solution at various temperatures from 10 to 60 minutes and residual activity was calculated (Figures 16 and 17). The enzyme from ALS showed high thermostability in which it approximately maintained 62% of its original activity (2.3817 ± 0.11 U/ml) for 40 minutes of incubation at 90 ^{o}C. However, the stability was only 6% of its activity at 100 ^{o}C for 40 minutes. The enzyme was deactivated after

30 minutes (retaining only 5% at 105 °C of its original activity). The activity was lost completely at incubation temperature of 110 °C in 10 minutes yet retained 5% of its activity after 10 minutes at 105 °C. This enzyme is more stable than amylases produced from *B. amyloliquefeciens* (Milner *et al*., 1997) but less stable than *B. lecheniformis* (Jaenicke, 1991).

The enzyme from MS has shown thermostability and maintained 52% its original activity (2.5782 ± 0.56 at 85 °C) after 50 minutes of incubation at 85 °C. However, it had only 4% of its activity at 100 °C when incubated for 40 minutes. In addition, the enzyme was completely deactivated at temperatures above 100 °C in 10 minutes of incubation. This enzyme is more stable than amylases produced from *Thermomyces langinosus* (Hamer, 1995) and less stable than amylases from *Myceliophthora thermophilia* (Vieille and Zeikus, 2001).

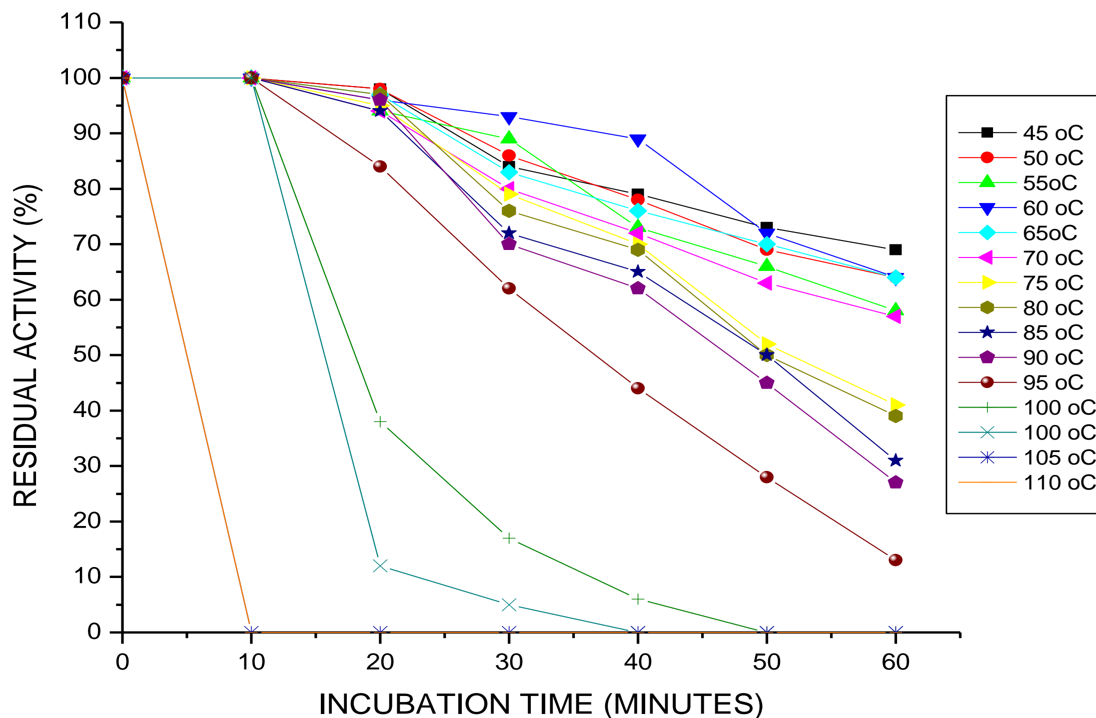

Figure16. Effect of temperature on stability of the enzyme from ALS

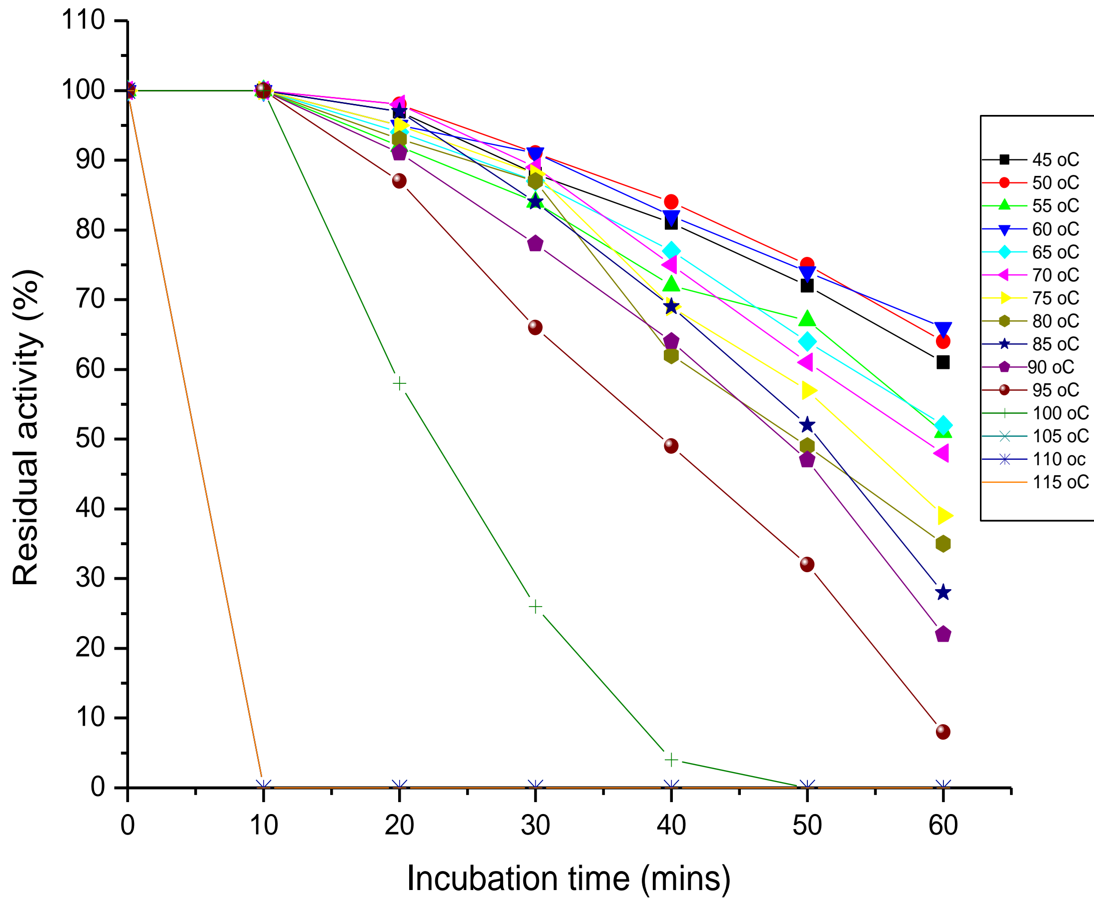

Figure 17. Effect of temperature on stability of the enzyme from MS

4.14 pH stability

Figure 18 shows that the enzyme from ALS is fairly stable for 30 minutes maintaining more than 50 % of its activity at pH values of 6-9, with no activity at pH values less than 6. It had 68 % of its activity at pH value of 8 after 40 minutes of reaction time and maintained 18 % of its original activity at pH value of 11 after 20 minutes of reaction time.

Figure 19 indicates that the amylase from MS is fairly stable at pH values of 5-7 for 40 minutes, with no activity at pH values above 9. It retained 69% of its activity at pH value of 6 after 40

minutes of reaction time and maintained 16 % of its activity at pH value of 9 after 30 minutes of reaction time. Stranes (1990) confirmed that starch liquefaction enzymes active at the saccharifying pH of 4.5 are attractive in the starch industry. Considering the requirement of starch liquefaction process of an enzyme with a low pH optimum, this enzyme may offer an advantage since it is working between pH values of 4-9.

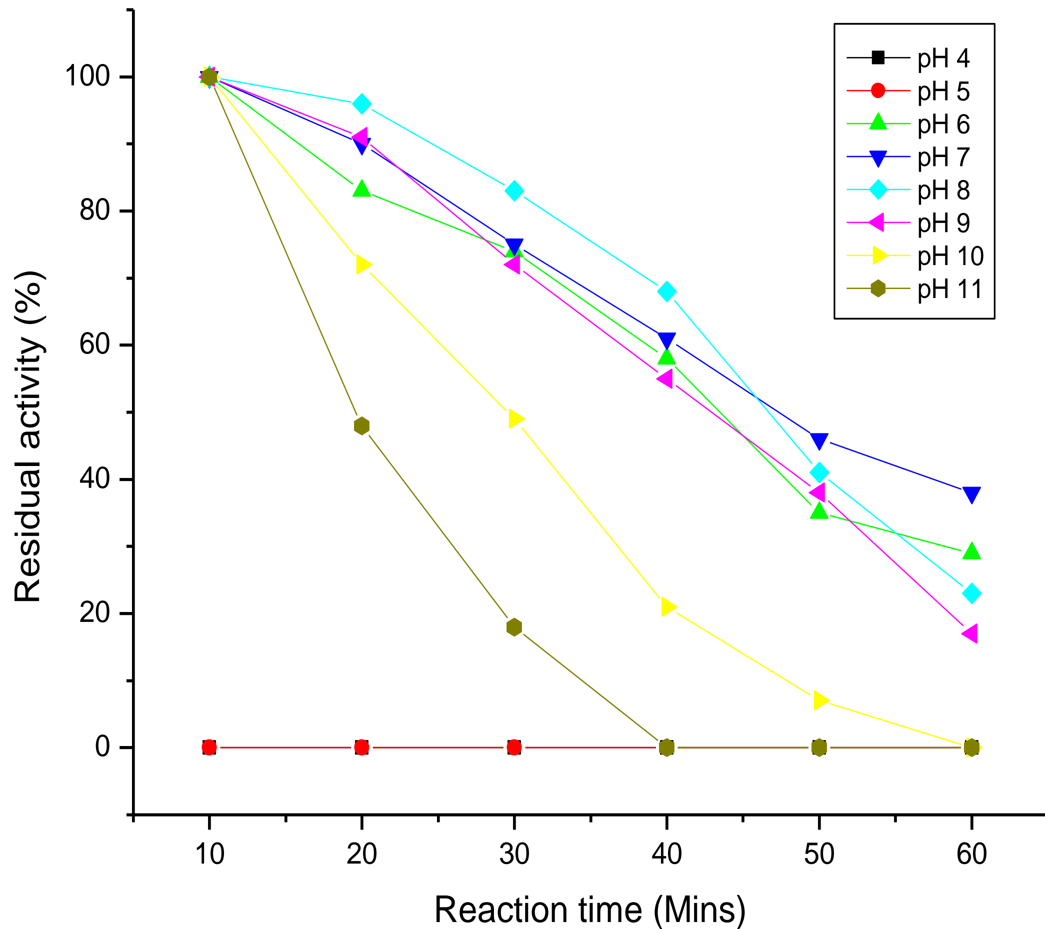

Figure 18. Effect of pH on stability of enzyme from ALS

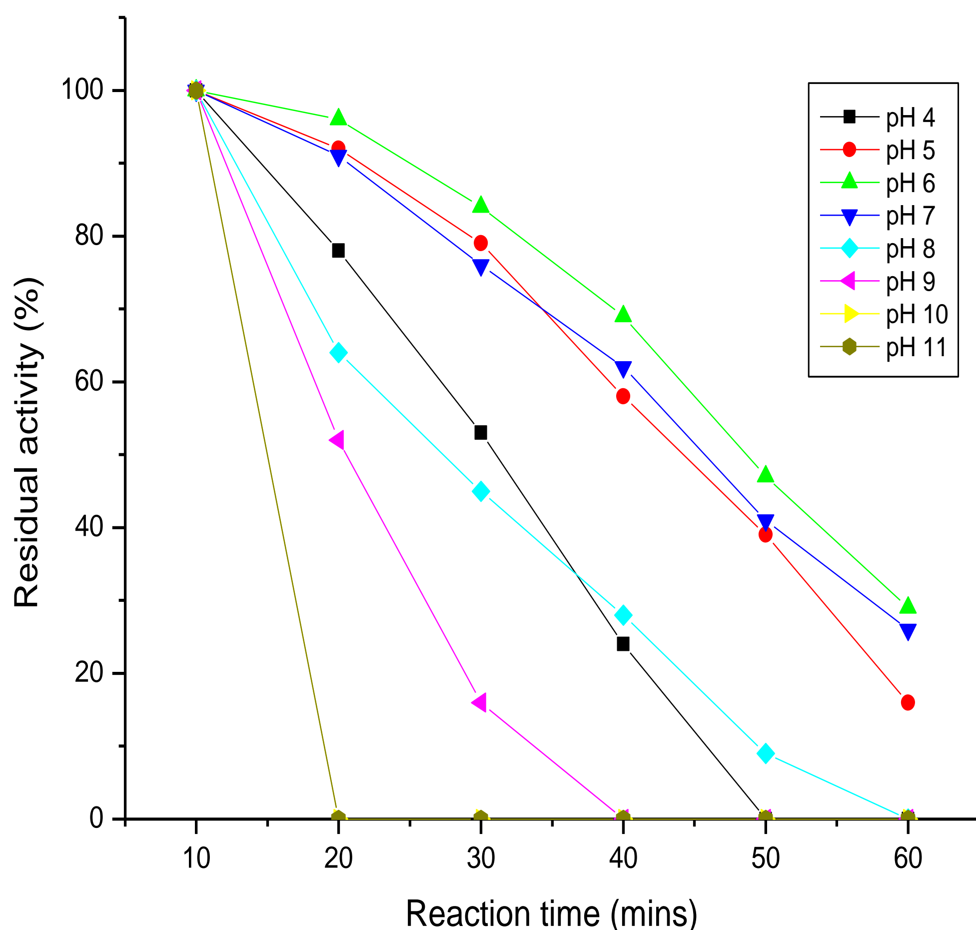

Figure 19. Effect of pH on the stability of enzyme from MS

4.15 Effect of Chemicals and Cations on Enzyme Activity

Establishing the enzyme characteristics with regard to stability and increased activity is vital for industrial application purposes. Therefore, evaluation was made to learn the effects of some metals on the enzyme activity. Activity of amylase was dependent on the chemicals and cations tested (Table 6).

The ion Zn^{2+} and higher concentrations (10 mM) of Mg^{2+} strongly inhibited enzyme activity while 5mM concentration of Ca^{2+} and Ba^{2+} induced higher catalysis. Addition of $CaCl_2$

improved at enzyme activity all concentrations tested. However, the highest improvement of amylase activity for ALS (42%) and for WS (28%) was achieved by addition of 5 mM of $CaCl_2$. Likewise, the addition of 5 Mm $BaCl_2$ gave 33 and 20 % improvement for ALS and MS enzyme activity respectively.

However, addition of all levels of concentration of $MgCl_2$ and $ZnCl_2$ showed no improvement of enzyme activity in both ALS and WS. In contrary, the activity of enzyme decreased as the concentration of both increased to from 1mM to10mM in the ALS and WS cases.

The presence of chelating substances like EDTA and Urea in small concentrations had little effect on the activity of the enzyme. Addition of 1 mM EDTA yielded an improvement of 6% and 11%, respectively for ALS and MS while urea increased 9% and 16% for ALS and MS, respectively.

Table 6. Effect of chemicals and cations on enzyme activity

Activator/ Inhibitor	Concentration (mM)	Residual activity (%)	
		ALS	MS
Control	-	100	100
CaCl2	1	118	115
	5	142	128
	10	130	122
BaCl2	1	114	109
	5	133	120
	10	126	114
MgCl2	1	92	86
	5	71	61
	10	58	54
ZnCl2	1	66	63
	5	51	48
	10	37	34
EDTA	1	106	111
Urea	1	109	116

5. FINDINGS, CONCLUSION AND RECOMMENDATION

5.1 Findings

5.1.1 Screening and selection of thermophilic amylase producing microorganisms

For the study, five water samples and five soil/mud samples were taken from Afar region where the altitudes of the study areas range from 628 m above sea level (Meteka) to 1690 m above sea level (Awash). The pH of the water samples varied from 6.43 (Meteka) to 8.70 (Awash), whereas, for the soil/mud samples it ranged from 6.5 (Meteka) to 9.31 (Blen Buri). The temperature of the water samples at the spot were in the range of 26.4 ^oC (Tendaho) to 88.2 ^oC (Alelobad), whereas, soil/mud sample temperatures were between 24.3 ^oC (Tendaho) and 65.1^oC (Meteka).

Growth temperature was taken as the primary screening criteria among the microbes from each sample. For this, the microorganisms were grown at high temperatures (45-70 ^oC) and all were able to grow at all temperatures. Among the colonies which grew on starch nutrient agar at 70 ^oC, thirty five colonies were selected from each sample and colonies which showed positive amylolytic activity were selected and their colony size and clear halo sizes were measured and the ratio of halo size to colony (HCR) size was taken as a means for the selection of best amylase producers. Based on this result, isolates from soil samples of Alelobad (ALS) and Meteka (MS) were chosen for further characterization since they have the highest HCR (5.707 for ALS and 5.638 for MS).

5.1.2 Enzyme production optimization

On the basis of the results obtained with all the optimum parameters, viz., substrate concentration (starch concentration of 1%, fermentation medium initial pH of 6 for MS and 8 for ALS, fermentation temperature of 65 ^oC for both ALS and MS, and fermentation period of 48 hours for ALS and MS, the enzyme was produced by submerged fermentation.

5.1.3 Enzyme characterization

The enzyme from ALS has shown higher activity between 80 ^{o}C and 90 ^{o}C, with maximum activity at 90 ^{o}C (2.3817±0.11). The enzyme's activity gradually increased as the incubation temperature increased from 45-90 ^{o}C and sharply declined as the temperature increased to 105 ^{o}C. It lost all its activity at temperatures 110 ^{o}C and above. This enzyme retained 62% of its original activity (2.3817 ± 0.11 U/ml at 90 ^{o}C) after 40 minutes of incubation at 90 ^{o}C however, maintained 6% of its activity at 100 ^{o}C. The enzyme was deactivated after 30 minutes (retaining only 5% at 105 ^{o}C of its original activity). The enzyme was fairly stable for 30 minutes maintaining more than 50 % of its activity at pH values of 6 - 9, with no activity at pH values less than 6. It had 68 % of its activity at pH value of 8 after 40 minutes of reaction time and maintained 18 % of its original activity at pH value of 11 after 20 minutes of reaction time. The ion Zn^{2+} and higher concentrations (10 mM) of Mg^{2+} strongly inhibited enzyme activity while 5 mM concentration of Ca^{2+} and Ba^{2+} induced higher catalysis. The addition of 5 mM of $CaCl_2$ and $BaCl_2$ improved the amylase activity of ALS by 42% and 33% respectively. However, the activity started to decrease (130% and 126 %) as the concentration was increased to 10mM. The addition of 1 mM of $ZnCl_2$ highly reduced the activity of the enzyme (by 24%). The inhibition effect of these chemicals increased as the concentration increased to 10mM (63%). There was no significant effect on the activity due to the addition of EDTA and Urea at 1mM level.

The enzyme from MS has shown higher activity between 45°C and 85°C, with maximum activity at 85 ^{o}C (2.5782±0.56). The enzyme's activity gradually increased as the incubation temperature increased from 45 – 85 ^{o}C and sharply declined as the temperature increased to 100 ^{o}C. It lost all its activity at temperatures 105 ^{o}C and above. This enzyme retained 65% of its original activity (2.5782 ± 0.56 U/ml at 85 ^{o}C) after 40 minutes of incubation at 85 ^{o}C however, maintained 4% of its activity at 100 ^{o}C. The enzyme was deactivated at 105°C. The enzyme was fairly stable for 40 minutes maintaining more than 50 % of its activity at pH values of 5 - 7, with no activity at pH values higher than 9. It had 69 % of its activity at pH value of 6 after 40 minutes of reaction time and maintained 53 % of its original activity at pH value of 4 after 30 minutes of reaction time. The ion Zn^{2+} and higher concentrations (10 mM) of Mg^{2+} strongly inhibited enzyme activity while 5 mM concentration of Ca^{2+} and Ba^{2+} induced higher catalysis. The addition of 5 mM of $CaCl_2$ and $BaCl_2$ improved the amylase activity of MS by 28% and 20% respectively.

However, the activity started to decrease (126% and 144 %) as the concentration was increased to 10 mM. The addition of 1mM of $ZnCl_2$ highly reduced the activity of the enzyme (by 27%). The inhibition effect of these chemicals increased as the concentration increased to 10mM (66%). There was no significant effect on the activity due to the addition of EDTA and Urea at 1 mM level.

5.2 Conclusion

Considering the requirement of starch liquefaction process of an enzyme with a low pH optimum, the enzyme isolated from MS may offer an advantage since it functions well in acidic pH value of up to 4.

Even though the enzyme activities of ALS and MS improved with the addition of $CaCl_2$, they had good amylolytic activities in the absence of Ca^{2+}which is a requirement in large-scale starch hydrolysis processes. Therefore, the enzymes from ALS and MS can be employed for starch hyhrolysis in large-scale processes.

Since the enzymes from ALS and MS are highly active at high temperatures (90 and 95 ^oC) they can be used in processes using high temperature.

5.3 Recommendation

Further studies will be needed to detail studies on enzyme activity, stability, specificity, alter pH optimum, and achieve free Ca $^{+2}$ requirements of enzymes from ALS and MS. These can be achieved through the following study designs:

1. Studying the interactions among different enzyme production factors and effects of enzyme production conditions,
2. Further purification of the enzymes,
3. Molecular characterization of the genes of the enzymes and biotechnological manipulation to get the desired characteristics,

4. Application of the enzymes in processes which employ starch hydrolysis processes, production of fuel ethanol, etc. In order to achieve the efficient, large-scale production, and
5. Structural and functional relationships of α-amylases have to be known in detail in order to achieve efficient and large-scale production.

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APPEDICES

Appendix A. Total microbial counts

Table A. 1 Total microbial counts

SAMPLE SITES	TOTAL AEROBIC COUNTS (CFUs/ml)	
	REFRIGERATED	SHELVED AT ROOM TEMPERATURE
MS	22.80×10^6	23.05×10^6
MW	8.20×10^6	8.35×10^6
AWS	18.00×10^6	17.60×10^6
AWW	10.60×10^6	10.73×10^6
ALS	15.70×10^6	15.24×10^6
ALW	11.00×10^6	11.05×10^6
BBS	10.40×10^6	10.77×10^6
BBW	3.00×10^6	2.97×10^6
TS	8.60×10^6	8.82×10^6
TW	4.50×10^6	4.67×10^6

Appendix B. Standard curve

Table B. 1 Standard curve for maltose

Concentration (mg/ml)	**Absorbance (540nm)**
0.00	**0.00**
0.05	**0.03**
0.10	**0.09**
0.15	**0.12**
0.20	**0.16**
0.25	**0.22**
0.30	**0.25**
0.35	**0.31**
0.40	**0.35**
0.45	**0.39**
0.50	**0.43**

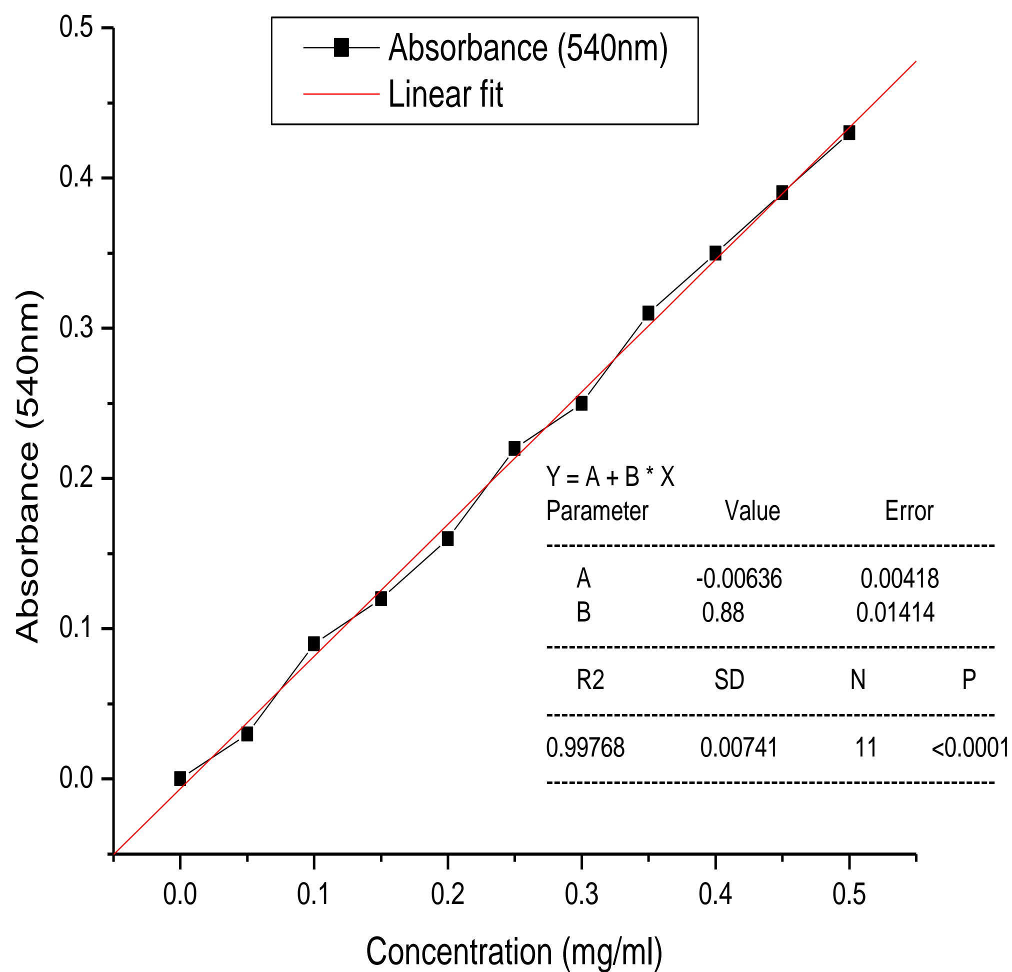

Figure B. 1 Figure showing prediction of appropriate concentration