ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

AMYLASES OF POTENTIAL INDUSTRIAL APPLICATION FROM MICROBIAL SOURCES

BY

MULUYE TEKA

JULY 2006

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 Applied Microbiology

Acknowledgments

I am greatly indebted to my instructor and research advisor Dr Amare Gessesse for his immeasurable helpful guidance, sincere criticism and helpful suggestions which he made during the course of this Thesis project with no reservations. I would like to pay tributes also to the South Nations Nationalities and People educational Bureau for financial assistance. I would like also to thank Addis Ababa university department of biology for providing conducive laboratory conditions. I would like to Thank Ato Abreham Tesfaye for providing fungal isolates used in the study. I take this opportunity to thank my family and all people who directly or indirectly contribute assistance during the course of this study.

Table of contents

List of Tables

List of Figure

Page

List of Abbreviations

^{O}C – Degree Celsius

Fig – Figure

g - gram

L - Litter

min - minute

mM - mili Molar

OD - Optical Density

rpm - Revolution per miute

Abstract

Glucoamylase and alpha amylase producing fungal and bacterial strains were studied. The fungal isolate previously isolated by Abreham Tesfaye belongs to the genus *Aspergillus* and was designated as Aspergillus No 43 (Asp 43). The bacterial isolate (designated as Bacillus sp CRC) which was isolated from Hot spring around lake Chitu was Gram positive, rode ,motile catalase positive and central endospor former. Asp 43 glucoamylase was optimally active at pH 4 and temperature of 65 ^{O}C. where as Bacillus CRC alpha amylase showed maximum activity at a pH range of 5-6 and temperature of 80 ^{O}C. Addition of 5 mM Ca^{2+} did not affect the temperature profile of both types of amylases. However the bacterial amylase was Ca dependent for its themostability, and this enzyme retained about 53 % of its original activity after 3 hr incubation at 80 ^{O}C. In addition Bacillus CRC was stable over a broad pH range retaining more than 80 % of its original activity in the pH range of 4.5 – 8.0 . Asp.43 grown in SSF medium at 25 ^{O}C produced maximum enzyme when the moisture content of the wheat bran used as a sole carbon source was 66.7 %. The optimum pH and temperature for maximum enzyme production by Bacillus CRC in liquid medium was 6.0 and 55 ^{O}C respectively. Bacillus CRC produced maximum enzyme when the sole carbon and nitrogen sources were starch and trypton (at 0.5 and 0.2 % respectively). Enzyme secretion by the fungal and bacterial isolates reached maximum after 96 and 36 hr of incubation respectively.

1 INTRODUCTION

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. In particular, a greater awareness of conservation issues has forced industries to consider alternative, cleaner methods (Rao *et al* 1998) With this regard, the use of enzymes as industrial catalyst is becoming the best option, and enzymes are gradually replacing chemical catalysts in many areas of industry (Smith 1996). Microbial enzymes are becoming increasingly important for their technical and economical advantages (Cherry *et al,* 2004). With annual growth rate of about 3.3 %, the global market for enzymes reached about $2 billion in 2004 (Sivaramakrishnan *et al,* 2006).

The rational to use enzymes as industrial catalysts stems from the fact that enzymes offer unique advantages over inorganic catalysts: enzymes are highly specific and efficient in catalysis; they are biodegradable, and carryout catalytic reactions under mild conditions to saving energy and resources (Kirk et al 2002).

The major classes of enzyme offering immediate application are the hydrolytic enzymes (Kirk et al 2002), which account approximately 75% of the industrial enzymes produced (Lowe, 2002). Through the use of hydrolytic enzymes many different natural and agricultural polymers can be processed and up graded for eventual human or animal consumption, or for further bioconversion in to value added products (Fogarty and Kelly 1990. The ability to commercially produce sugars from starch using amylolytic enzymes is an example of such processes.

Due to the increasing demand for enzymes in various industries, there is enormous interest in searching for enzymes suitable for application, and their cost effective production techniques (Burhan *et al* 2003).

Most industrial processes are designed to operate at elevated temperatures. Therefore the presence of enzymes with significant stability at such temperatures is crucial (Haki and Rakshit 2003). Protein engineering is one option to find thermosatble enzymes (Reddy et al 2003), however in the present day knowledge and technology this is impractical to apply for every of type enzyme. The most frequently used sources of more stable enzymes are thermophilic microorganisms that can be isolated from different environments (Sonnleitner and Fiechter, 1983; Lee *et al*, 1999; Adams. and Kelly 1998; Fitter and Heberle 2000; Kirk *et al* ,2002)

2 STARCH

Starch is the chief storage form of carbohydrate in higher plants. It occurs mainly in seeds, tubers and roots as a long-term reserve. Next to cellulose starch is the most abundant carbohydrate polymer in nature. In addition to its importance as a major source of carbon and energy for man and most other organisms, starch has many industrial applications: it is used in the manufacture of paper, textiles, pharmaceuticals and biodegradable polymers, and its hydrolysates have a wide range of importance (Van der Maarel *et al* , 2002).

Starch is present in microscopic granules in storage structures, which are quite resistant to penetration by water or hydrolytic enzymes. The highly ordered structure of starch granules can be disrupted in the presence of heat and moisture-a process known as gelatinization. When water–starch slurry is heated, the granules swell by absorbing water until a point is reached at which the swelling is irreversible. During this process, amylose leaches out of the granule and causes an increase in the viscosity of the slurry. Further increase in temperature then leads to maximum swelling of the granules and increased viscosity. Gelatinization increases the digestibility of starch and is an important process from application viewpoint. Gelatinization temperature ranges usually from 70-90 ^{O}C depending on several factors including the botanical source, amylose-amylopectin proportion and amount of

moisture available for hydration. During refrigerated storage gelatinized starch tends to gain its ordered structure. This process is called retrogradation (Alavi 2003).

Chemically starch is a polymer of glucose units linked through alpha-1,4 and the alpha-1,6,bonds.The presence of two different linkage possibilities for the monomer glucose units resulted in the existence of two types of starch molecules. As a result starch granule is a heterogeneous structure consisting of two high molecular weight polymers, amylose and amylopectin that are joined with intra and inter hydrogen bonding in the granules. The relative proportion of these polymers plays a critical role in the physical properties of native starch and its derivatives (Fogarty 1983; Van der Maarel *et al,* 2002).

Amylose is a linear polymer of alpha -1,4 linked D-glucose residues with degree of polymerization (DP) up to 6000 glucose units. The number of glucose residues varies with the origin of the starch. The average amylose content in starches can vary between almost 0 and 75%, but a typical value is 20–25 %. Amylopectin is formed when the linear polymer formed by alpha 1,4 glucose residue is branched by alpha -1,6 linkage. The degree of branching is approximately one per twenty-five glucose units in the un branched segments. Amylopectin may account for 75 to 85% of most starches. The complete amylopectin molecule contains on average about 2 000 000 glucose (Alavi 2003).

2.1. Industrial applications of starch

As a raw material starch can be used directly in textiles, paper industries, in the manufacturing of biodegradable plastic film, pharmaceuticals, adhesives etc. In addition starch can be chemically or enzymatic ally processed into a variety of starch derivatives such as, glucose, maltose and syrups of various dextrose levels that are used as sweetener or as chemical feed for further bioconversion in to different products. Starch is produced commercially from cereal crops such as corn, wheat, sorghum and rice; from the tubers and roots of plants such as cassava, potato, etc (Ramachandran *et al,* 2004

2.2. Organisms utilizing Starch

The ability to use starch as a carbon and energy source is widely distributed among different organisms. Since this polymer is water insoluble and too large to pass across the cell membrane, biodegradation occurs extracellularly. Different kinds of enzymes are required for the conversion of the starch polymer into mono and disaccharides. Animals, plants and large variety of bacteria, filamentous fungi and yeast possess starch-degrading enzymes to convert it in to usable forms. The properties of starch degrading enzymes however, vary with the source organism, and different organisms produce one kind or a mixture of these amylolytic enzymes (Castro *et al*, 1992).

2.3. Starch degrading /Amylolytic enzymes

Amylolytic enzymes are those enzymes, which catalyze the hydrolysis of alpha 1,4 and/ or alpha 1, 6 linkage in starch and related compounds. They act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved. Initially the term amylase was used to designate enzymes capable of hydrolyzing alpha-1,4-glucosidic bonds of amylose, amylopectin, glycogen and their degradation products. Today the existence of a variety of amylolytic enzymes from various sources is understood (Windish *et al*., 1965).

2.3.1. Classes Amylolytic enzymes

Based on their mode of actions enzymes that hydrolyze starch can be divided into endoamylases, exoamylases and debranching amylases (Gomes et al 2005).

2.3.1.1. **Endoamylases**

Endoamylases randomly cleave alpha -1,4 glycosidic bonds in amylose, amylopectin and related polysaccharides in the entire molecule, and produce oligosaccharides of

varied chain lengths with alpha-configuration on the C_1 of the reducing glucose unit produced (Guzman and Paredes 1995; Van der Maarel *et al,* 2002; Reddy et al 2003). Alpha -amylases (EC 3.2.1.1) are the well-known endoamylase. They are the most widely distributed enzymes in nature (Sivaramakrishnan *et al,* 2006). These enzymes are classified into two groups – liquefying and sacharifying alpha amylases. A liquefying alpha amylase hydrolyses 30- 40 %, and a sacharifying one hydrolyses 50- 60 % of the glycosidic linkage of starch (Vihienen and Mantsala, 1990). While the majority of alpha amylases are extracellular a few others were found to be intracellular. Such intracellular alpha-amylases enable producer organisms either to utilize maltodextrin or storage polysaccharides during the exponential growth phase (Ballschmiter *et al* , 2006). The extra cellular bacterial α-amylases are the most thermostable proteins known; they also show optimum pH activities from acidic to alkaline pH. On the other hand, most alpha -amylases known to be localized in the cytoplasm are reported to have a pH optimum for activity at neutral pH or below (Ballschmiter *et al* 2006)

2.3.1.2. Exoamylases

The exoamylases act preferentially on alpha -1, 4 linkages from the non-reducing end successively, resulting in low molecular weight products. Exoamylases of microbial origin are various types with respect to bond and substrate preference as well as products formed. These enzymes either exclusively cleave alpha -1,4 glycosidic bonds as beta -amylase (EC 3.2.1.2) or cleave both alpha- 1,4 and alpha-1,6 glycosidic bonds like glucoamylase (EC 3.2.1.3) and alpha -glucosidase (EC 3.2.1.20) (Sivaramakrishnan *et al,* 2006). The starch hydrolysates are also different: glucoamylase and alpha -glucosidase produce only glucose, where as beta -amylase results in maltose and beta-limit dextrin. Beta -amylase and glucoamylase also convert the anomeric configuration of the liberated product from alpha to beta (Pandey *et al*, 2000).

Glucoamylase and alpha -glucosidase differ in their substrate preference: for example alpha -glucosidase acts best on short maltooligosaccharides and liberates glucose

with an alpha -configuration while glucoamylase hydrolyzes long-chain polysaccharides best (Fogaty and Kelly 1990)

Fungal glucoamulase is the most important industrial enzyme; which has wide application in the starch processing industry. Fungal glucoamylases are known for their activity usually at acidic pH, and has low thermostability. Glucoamylases from different species differ in their properties such as resistance to proteolysis and urea denaturation. All the glucoamylase so far studied are glycoproteins the carbohydrate groups being necessary for maintaining the structural stability of the enzyme conformation (Shenoy *et al*, 1985).

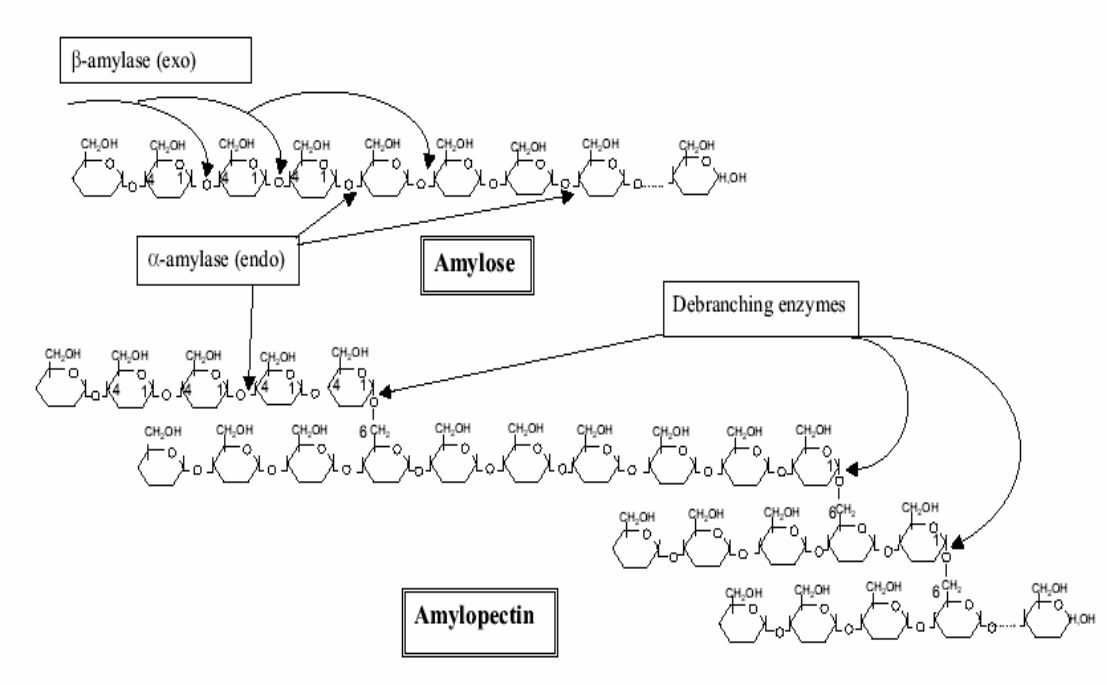

Fig 1 Action pattern of some amylases on amylose and amylopectin

2.3.1.3. Debranching Amylases

Debranching amylases include pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). Pullulanase acts specifically on alpha-1, 6 linkages in pullulan, starch, amylopectin and related oligosaccharides, whereas isoamylase (EC 3.2.1.68) hydrolyses alpha-1, 6 linkages in amylopectin. Debranching enzymes exclusively

degrade amylopectin, thus result in long linear polysaccharides (Israilides *et al*., 1999; Sivaramakrishnan *et al* 2006).).

APPLICATION OF AMYLASES

Amylases together with cellulases are important enzymes for global carbon cycle on Earth. They are capable of degrading starch and related polymers. However due to bond specificity of these enzymes, efficient and complete degradation of starch usually requires the action of many different enzymes.Alpha amylase and glucoamylase are two major commercial enzymes with wide range of applications.

In spite of the wide distribution of amylases, microbial sources, particularly fungal and bacterial strains, are used for the industrial production of the enzymes due to advantages such as cost effectiveness, less time and space requirement and ease of process modification and optimization (Burhan *et al,* 2003). Moreover amylases derived from microorganisms have been shown to display different properties towards temperature, pH and other physical factors appropriate for different industrial processes (Sogaard et al 1993).

The *Bacillus spp.: B. stearothermophilus, B. licheniformis* and *B. amyloliquefaciens* have been widely used for commercial production of alpha amylase for various applications. Today alpha amylases acco**u**nt for about 30 % of the global enzyme production (Van der Maarel *et al,* 2002) with annual sale of about $11 million (Kilara and Desai 2002). At commercial level fungal glucoamylases have been produced from *Aspergillus niger* and related *spp* (Sivaramakrishnan *et al*, 2006).

These enzymes have found applications in starch processing, desizing of textiles, paper sizing, as detergent additive, and bread improvement, ethanol and other fermentation processes (Haki and Rakshit 2003; Lowe, 2002; Gomes *et al,* 2005).

2.3.2.1. Starch processing

Conversion of starch into sugar syrups (glucose, maltose, maltotriose, dextrins sugar, or fructose syrups etc) forms the major part of the starch processing industry. The

hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products (Reddy *et al,* 2003).
The hydrolysis of starch may be carried out using either acid or enzyme as catalyst. Acid conversion has, however, many limitations: it is non-specific, lacks ways of controlling saccharide composition, require high refining coasts and is less environmentally friendly. The application of enzymes for this process has avoided these limitations (Crabb and Shetty, 1999).

In industrial starch processing the synergistic action of a bacterial α -amylase and fungal glucoamylase is employed. Thus this process occurs in two stages: liquefaction and saccharification. In the first stage the starch is cleaved into various sugar units using alpha amylase at high temperature. Therefore the presence of a thermostable alpha amylase is crucial (Haki and Rakshit 2003). The starch-sugar mixture is then degraded to form simple sugars by applying fungal glucoamylase. The resulting glucose syrup can be used to partly or fully replaced conventional sugar in many sweet and bakery products (Arasaratnam and Balasubramaniam 1993).

High fructose containing syrups (HFCS, 42% fructose) is prepared by enzymatic isomerization of glucose with glucose isomerase. The starch is first converted to glucose by enzymatic liquefaction and saccharification, and then the glucose is converted into fructose by use of glucose isomerase Thus production process involves liquefying thermostable alpha amylase, saccharifying glucoamylase and glucose isomerase sequentially. Today the production of high fructose corn syrup is a major industry, which converts large quantities of corn and other botanical starches to this and other useful sweeteners. These sweeteners are used in soft drinks, candies, baking, jams and jellies and many other foods (Ramachandran *et al,* 2004).

The application of enzymes in the production of sweeteners from starch has the potential to make food processing less dependent on sugar cane in areas, which are unsuitable for sugar production, or where starch is more cheaply accessible than cane sugar. Furthermore, starch-sweetener bioconversions offer alternative uses for starchy materials, which are highly perishable and lack proper preservation techniques particularly in the tropics (Hyun and Zeirkus 1985).

2.3.2.2.Textiles desizing

Prior to weaving of yarn into fabric, the warp yarns are coated with a removable sizing agent to lubricate and protect the yarn from abrasion during weaving. Historically, the main sizing agent used for cotton fabrics has been starch because of its excellent film forming capacity. Before the fabric can be dyed, the applied sizing agent must be removed. Before the discovery of amylase enzymes, the only alternative to remove the starch-based sizing was extended treatment with caustic soda at high temperature. However such chemical treatment was not totally effective in removing the starch, Appropriate desizing of starch is now possible by application of alpha - amylase (Kumar,*et al,1995*; Tsurikova *et al* ,2002)

2.3.2.3. Paper desizing

The use of alpha amylase in pulp and paper industry is in the modification of starches for coated paper. As for textiles, sizing of paper with starch is performed to protect the paper against mechanical damage during processing .It also improves the quality of the finished product, enhances stiffness, and elasticity of paper. Because starch is added to paper at a temperature range of 45- 60 ^{0}C, and the viscosity of the natural starch is too high for paper sizing partial degradation of this polymer is essential. Alpha amylase is employed for this purpose (Gupta *et al*, 2003).

2.3.2.4. Detergent Additive

The demand for alpha amylase for use in laundry and automatic dishwashing is very high. Alkaliphilic *Bacillus* strains often produce enzymes active at alkaline pH, including alkaline α amylase (Horikoshi, 1996). When alkaline alpha amylase is used as a component of detergents, the chelating agents usually contained in detergents

easily remove calcium, which is essential for its stability. Thus there is a search for Ca free alpha amylase (Nonaka et *al*, 2003).

2.3.2.5. Beverage alcohol and Fuel Ethanol production

In beer industries microbial amylases are used to aid cereal amylase in the production of fermentable sugar. 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. Enzymes such as alpha-amylase, glucoamylase and cellulases are important to produce fermentable sugars to produce ethanol (Kirk *et al*, 2002).

2.3.2.6. Baking

Food fermentation processes are reliant on both endogenous and microbial enzymatic activities for the degradation of starches and other polymers. In baking industries alpha amylase and glucoamylase are employed to supplement the natural enzyme coming from the grains in producing fermentable glucose by yeast. The microbial amylases are also important to improve the shelf life of the bread (Sahlstrom. and Brathen, 1997).

2.3..2.7. Other applications

The spectrum of amylase application has widened in many other fields, such as clinical, medical, and analytical chemistries (Pandey *et al.,* 2000; Cherry *et al,* 2004). To some extent amylases are also used to improve digestibility of some of the animal feed ingredients (Kumar *et al,*1995)

3. Objectives

Ethiopia is a tropical country with agriculture-based economy, and most of its population engaged in arable farming. The ability to up grade the agricultural products could have considerable economic significance. The development of starch-based industry is one option. This, however, requires among other things the presence of starch degrading enzymes of potential industrial applications. Despite this fact there was only a limited work in searching for microbial starch degrading amylases. The objective of this work was therefore:

1 To isolate amylase producing bacterial strain/s from the environment, and to select fungal strain/s positive for glucoamylase among previously isolated in our laboratory.

2 To characterize the fungal and bacterial amylases and to determine their potential application

3 To optimize cultivation conditions for enzyme production by the isolates

4 MATERIALS AND METHODS

4.1. Isolation for Amylolytic Organisms

A total of 94 fungal isolates used in this study were kindly provided by Abraham Tesfaye. (The isolate in the storage slants were designated as 1,2,3. in front of the genus name for the sake of convenience). To screen amylolytic fungal strains all the 94 isolates (most of which were grouped as *Aspergillus black* and *Aspergillus ochraceus*) were grown at 25 ^{0}C on solid agar medium supplemented with starch. After 90 hr incubation fungal colonies were flooded with 1 % Iodine solution, to select isolates which show wide clear zone around their colonies.

To isolate thermophilic amylolytic bacteria, hot spring water sample was collected from around lake Chitu. Then 0.5ml of the water sample was diluted in sterile distilled water, and spread on to petriplate agar media supplemented with starch. After 24 hr incubation at 60 ^{O}C the different colonies emerged with wider clear zone were collected and further purified.

4.1.1. Media and Culture Conditions

Fungal isolates were stored in Czapex dox agar medium at 4 ^{O}C and were periodically transferred to a fresh media every month and whenever spores for inoculation was required

The media composition used for the growth of fungal isolates (as used by Omemu et al 2005 with some modification) was g/l:
NH_4NO_3, 2; KCl 2; $FeSO_4.7H_2O$ 0.35; soluble starch 5; $MgS0_4.7H_2O$ 0.1; K_2HP0_4 0.6; KH_2P0_4 0.4; $CaCl_2$ 0.1. For solid media agar was added. After the pH was adjusted to 5.6, the media was sterilized at 121^{0}c and 15 lb for 15 min in the autoclave. Each isolates was inoculated on to agar medium by first suspending a loop full of spore on to 50 ml of saline solution (0.85 g/l), and a drop of the spore

suspension was placed on the medium. Liquid growth medium was prepared by adding 50 ml of the broth into 250 ml capacity Erlenmeyer's flasks. After sterilization, one ml fungal spore suspension prepared the same way mentioned above was added as inoculum.

The bacterial isolates were stored in media mentioned below and were transferred in to a fresh medium every two weeks

The media used for the growth of bacterial isolates was composition of (g/l): peptone 2, yeast extract 0.1; $FeS0_4.7H_20$ 0.35; soluble starch, 5; $MgS0_4.7H_2O$ 0.1; NaCl 0.1; K_2HPO_4 0.6; KH_2P0_4 0.4; $CaCl_2$ 0.1, . KCl 0.5. For solid media agar was added. The final pH was adjusted to 6.5 before autoclaving.

Liquid media was used for enzyme production from the selected strain. After adjusting the pH and sterilization each broth medium was inoculated with 2 ml over night-cultured inoculums of bacterial isolate grown at 55 ^{0}C on a shaker

4..1,2. Solid-state fermentation (SSF) medium for fungal amylase production

SSF medium was used for enzyme production from selected isolates using wheat bran as a sole carbon source supplemented with the following nutrients g/100ml:(%): glucose , 0.05; NH_4SO_4 0.02 ;trisodium citrate, 0.027; MgSO4, 0.008; KH_2PO_4. 0.19. Five g wheat bran moistened with above nutrient solution to a level of (v/w) 66.7% in 250 ml size. Erlenmeyer flasks were used for cultivation of the fungal isolates. (The moisture level of the wheat bran (9.6%) was determined after over night oven drying at 105 ^{O}C).

The crude enzyme from SSF medium was harvested by centrifugation (5000 rpm, 10 minu). First the moldy bran was soaked in 10 mM acetate buffer pH 4,5 in (w/v) 1:10 ratio (Omemu *et al,* 2005),and shaked for 1 hr at 120 rpm on the shaker, then it was centrifuged repeatedly at 5,000 rpm (as described by Soni *et al*, 2003).

4.1.3. Screening for Fungal and Bacterial Amylases

To select the best amylase producing strains all fungal and bacterial isolates with amylolytic activity were grown in liquid medium (50 ml broth in 250 ml capacity Erlenmeyer flasks) on a shaker, 120 rpm. The fungal isolates were grown at room temperature for 4 days while the bacterial isolates were grown at 60 ^{O}C for 30 hrs. The crude enzyme from the fungal cultures was harvested by filtration through Watman's No. 1 filter paper whereas the bacterial crude enzyme was harvested by centrifugation at 10,000 rpm for 5 min in the centrifuge. The culture filtrate served as enzyme source.

4.1.4.. Screening for thermostable bacterial amylase

Crude enzyme from two selected bacterial isolates (designated as Chitu Red Colony,CRC and Chitu Lighter Colony CLC) mixed with acetate buffers, pH 5.5 (final concentration 50 mM) was incubated at 70 ^{0}C in water bath without and with $CaCl_2$ solution (final concentration 5 mM)..After an incubation period of 1hr the enzyme was assayed and residual activity was determined. This step enabled to select one bacterial isolate (designated as CRC) which produce a relatively better thermostable amylase.

4.2. Enzyme assay.

Enzyme assay was carried out based on. the dinitro salicylic acid (DNS) method .One gm soluble starch was dissolved in 100 ml 50 mM acetate buffer PH 4.8, (for fungal enzyme assay), 50 mM phosphate buffer pH 6.0 (for bacterial enzyme assay) and gelatinized on a heater.

4.2.1. Reagent

DNSA solution standard composition was prepared: g/l phenol 2; sodium sulfite 0.5; sodium potassium tartarate 200; Sodium Hydroxide (NaOH) 10; and Dinitro salicylic acid (DNSA) 10.

4.2.2. Reaction mixture

To a 0,9 ml of the substrate in a pair of test tube, 0.1 ml of appropriately diluted enzyme source was added and incubated for 10 min at 50 ^{0}C(fungal enzyme), or 80 ^{O}C (bacterial enzyme) in water bath. At the end of the incubation period the reaction was stopped by adding 2 ml DNS solution. The control used was prepared in such a way that enzyme source was added after the reaction was stopped by the DNS solution. The reaction mixture was then boiled for exactly 5 min in boiling water. Finally the test tubes were cooled in running water for about 5 min and the optical density of the resulting colored solution was measured at 540 nm against a blank.

4.3. Identification of the type of Amylase by sugar analysis on Thin layer Chromatographic (TLC)

To identify the type of amylase from the bacterial and fungal isolates based on the starch hydrolysates TLC system described by Kimura and Horikosh 1989; cited in Gashaw Mamo and Amare Gessesse 1999a) was followed. First 0.9 ml 2% soluble starch mixed with 0.3 ml crude enzyme from the respective bacterial and fungal sources was incubated for 30 minutes at 80 and 65 ^{O}C respectively in the water bath. Then each hydrlysate was spotted on TLC plate along with standard known sugar (glucose and maltose) solutions. A one dimensional ascend was done using a solvent system (v/v) of buthano : ethanol: water (5:3:2). After a total of 4 ascends air-dry TLC plates were sprayed with 50% (v/v) Methanol- H_2S0_4 mixture and heated for 10 min. at about 100 ^{O}C . The dark brown sugar spots appeared were identified by comparing with the standards.

4.4. ENZYME CHARACTERIZATION

4.4.1. Effect of pH on the activity of fungal and bacterial amylase

To determine the pH profile of the fungal and bacterial amylases the crude enzyme was assayed using 1% soluble starch prepared in 3 buffer systems pH 3.5 to 9.0 (final concentration 50 mM). These buffers were acetate buffer (pH 3.5, 4.0, 4.5, 5.0, 5.5), phosphate buffer (pH 6.0, 6.5, 7.0 and 7.5) and Tris buffer (pH 8.0, 8.5 and 9.0).

To determine the effect of pH on enzyme stability of amylase from the bacterial isolate, the crud enzyme was concentrated 10 times (10x)-using acetone (v/v enzyme: acetone 1:2). The concentrated enzyme was mixed with different buffer systems mentioned above to a final concentration of 50 mM, before the enzyme was incubated for one hr at 40 ^{O}C in the water bath.. This step lowered the final concentration of the enzyme by half. Then pre incubated enzyme was assayed in such a way that the final concentration of the incubating buffer is reduced 10 x. This was to minimize the effect of the incubating buffer by reducing its concentration from 50 mM to 5 mM.

.

4.4.2. Effect of temperature on the activity and stability fungal and bacterial amylase

To determine the effect of temperature on the activity of the amylases from the two groups of isolates (Asp 43 and bacterial isolate, CRC), the crude enzyme from these isolates was assayed in temperature ranges of 35-80 ^{O}C and 40 ^{0}C – 90 ^{0}C respectively. The assay methods were as mentioned in section 3.4.3 for the fungal amylase, however the substrate was prepared in 50 mM acetate buffer pH 4.0.

To determine the temperature stability of fungal amylases, the crude enzyme, mixed with acetate buffer pH 4 to a final concentration 50 mM, was first incubated in the temperature range (at 5 ^{O}C interval) of 45 – 80 ^{O}C for 30 min. Then the residual

activity of the enzyme was determined by assaying the incubated crude enzyme as mentioned above.

The thermostability of alpha amylase from CRC was determined after the incubation of the enzyme at a temperature range of 50-90 ^{O}C for 3 hrs in the water bath. First each labeled ependorph filled with enzyme- acetate buffer (pH 5.5 final concentration of 50 mM) mixture was placed in water the bath and one ependorph was taken out and stored in freege (1 ^{O}C) at 30 min interval. After incubation, the enzyme was assay and residual activity was determined.

4.4.3. Effect of Ca^{2+} on thermostability of the bacterial amylase

To determine Ca^{2+} requirement for thermal stability of amylase from the bacterial isolate, the enzyme was incubated at a temperature range of 50-90^{0}c for 3hr in water bath without and with 5 mM $CaCl_{2+}$ solution. After incubation, the enzyme was assay and residual activity was determined.

4.4.4 Effect of pH on the activity and stability of the bacterial amylase

The pH profile of the bacterial amylase was determined by assaying this enzyme using substrate dissolved in 3 buffer systems of pH 3.5 to 9.0, final concentration 50 mM. The buffers used were: acetate buffer (PH 3.5 4.0 4.5 5.0 and 5.5), phosphate buffer (pH 6.0, 6.5, 7.0 and 7.5) and Tris buffer (pH 8.0, 8.5 and 9.0) to a final concentration of 50 mM in the substrate.

4.5. CULTURE CONDITIONS FOR ENZYME PRODUCTION BY THE FUNGAL ISOLATE, Asp 43

4.5.1. Time course of enzyme production

To determine the maximum enzyme production period the fungal isolate, Asp 43 was grown in SSF medium (section 3.3.1) at 25 ^{0}C. The relative enzyme activity of the culture was determined after 24, 72,96, 120 and 144 hrs by harvesting the enzyme from a pair of cultivating flasks at a time.

4.5.2. Effect of moisture level on glucoamylase production by Asp 43

The optimum moisture level for enzyme production by Asp 43 was determined by growing this isolate at 25 ^{O}C in the SSF media at a moisture level (v/w) of 50%, 60%, 66.7 %, 71 %, and 75 % 78 % and 80 %. The enzyme was harvested after 96 hr and the enzyme activity was determined. One unit of glucoamylase activity was defined as the number micromoles of reducing sugar produced per min in the reaction condition.

4.6. CULTURE CONDITIONS FOR ALPHA AMYLASE PRODUCTION BY BACILLUS SP CRC

4.6.1. Effect of Cultivation Temperature

To determine the optimum temperature for enzyme production of the bacterial isolate CRC it was cultivated in a liquid medium in the temperature of 40 to 65 ^{O}C, 5^{O}C interval on incubating shaker (120 rpm). The relative enzyme activity was determined by assaying the culture filtrate obtained after 30 hr incubation.

4.6.2. Effect of Initial PH

The effect of initial media pH on enzyme production by the bacterial isolate CRC was determined by growing the test organisms at 55 ^{O}C in liquid media with an initial pH of 5, 5.5, 6.0, 6.5, 7.0 and 7.5. The crude enzyme harvested from a 30 hr incubated culture broth was assayed to determine the relative enzyme activity.

4.6.3. Time course of Enzyme Production

To determine the period of maximum enzyme secretion, in relation to growth in cell biomass, by the bacterial isolate the test organisms was first grown in liquid medium pH adjusted to 6.0 at 60 ^{O}C on an incubating shaker. The change in the relative enzyme activity, and cell biomass was evaluated by taking aseptically 2 ml of the culture broth at every 3 hr interval. The cell biomass was measured at 600 nm (as described by Soni *et al,* 2003) against a blank, and the relative enzyme activity of the culture filtrate was determined based on the standard assay method.

4.6.4. Effect of carbon source on Enzyme production

The effect of carbon source on enzyme production by Bacillus CRC was determined by growing the test organism in liquid medium (pH adjusted to 6.0) in which 0.5% of either of the following carbon sources: glucose, sucrose, lactose, fructose, galactose maltose dextrin and starch was used as a sole carbon source. After 36 hr incubation at 55 ^{O}C on a shaker, 120 rpm the crude enzyme was harvested and assayed to determine enzyme activity. One unit of enzyme activity was defined as the number of micromoles of reducing sugar produced per minute in the reaction condition.

4.6.5. Effect of Nitrogen source on enzyme production

To determine the appropriate nitrogen source for amylase production by the bacterial isolate, this test organism was grown in liquid medium (the same initial pH of media, cultivation temperature and agitation as in section 3.7.3 above) in which 0.2 % (w/v) of either of the following nitrogen sources: NH_4Cl, NH_4N0_3, $(NH_4)_2$ $S0_4$, peptone, tryptone, yeast extract, urea and casein was used assole nitrogen source. At the end of 36 hr incubation the crude enzyme harvested was assayed to determine the enzyme activity.

5.RESULTS

5.1 Isolation and screening

5.1.1. Screening for Glucoamylase Producing Fungal strains

. Based on amylolytic activity which was reflected by wide clear zone formation around the colony on the solid starch containing agar media, out of a total 94 isolates, 31 isolates (21 *Aspergillu*s black, 8 Aspergillus *coraceus* and 2 P*enicillium* groups) were selected for further screening.

To select the best glucoamylase producing strain all the 31 amylolytic isolates were grown in liquid medium. Based on this step three isolates – two *Aspergillus black* groups, No 7(*As*p 7), & No 43 (Asp 43), and one Aspergillus *coracles group,* No 36 (*Ochra* 36) were selected and the properties of their enzymes towards pH and temperature was determined (Table 1 and Table 2)

Table 1 Effect of temperature on the activity of amylase from three selected fungal isolates

Assay temperature (^{O}C)		30	35	40	45	50	55	60	65	70	75
Relative Enzyme activity (%)	Asp.43	11	30	38	69	78	83	90	100	74	32
	Asp 7	34.7	65	81.9	91	100	75	58	30	19	8
	Ochra 36	27.5	42.7	79	100	73	43	20	14	5	1.2

Table 2. Effect of pH on enzyme activity from the three fungal isolates

Assay pH	Relative enzyme activity(%)		
	Asp 43	Asp7	Ochra 36
3.5	98	59	49
4.0	100	71	81.7
4.5	90.2	82	96
5.0	36	100	100
5.5	8	70	77

Because of maximal activity at relatively high temperature of its enzyme (table 1), isolate Aspergillus black No 43 (Asp 43) was selected for further study.

5.1.2. Isolation and screening alpha amylase producing bacteria isolate

A total of 17 different colonies positive for amylolytic activity were screened form starch agar plates incubated at 60 ^{O}C. These colonies were purified, and were stored in freege (1 ^{O}C) for further screening.

To select the best alpha amylase producing strain the relative activity at 80 ^{O}C of the crude enzyme from the 17 isolates grown in liquid media was compared. Based on this method two isolates designated Chitu water red colony (CRC) and Chitu water white colon (CLC) were selected. When thermostability of the enzyme from the two isolates is compared enzyme from CRC was found more heat stable and thus CRC was used for further study

5.2. Morphological Characterization of the isolates

The fungal isolate Asp 43 was previously identified to belong to the genus *Aspergillus* (Abreham Tesfaye 2006)

The bacterial isolate (Isolate CRC) used in this study was a Gram-positive rod, motile, catalase positive, central endospore former. Based on these morphological features this isolate was tentatively grouped under the genus *Bacillus*.

5.3. Identification of the type amylases from fungal and bacterial isolates by sugar analysis on Thin Layer chromatographic (TLC) method

The starch hydrolysates produced by enzymes from selected fungal isolate Asp 43 and from two bacterial isolates (Bacillus CRC and Bacillus CLC) were run on TLC plate. The major spot developed by the fungal amylase was glucose (Fig 2) indicating that it is glucoamylase, and those by the bacterial isolate were various dextrins and maltose, indicating that it was alpha amylase type (iFig 3)

Fig 2 Spots developed from starch hydrolysates on TLC by the action of Asp 43 amylase

Fig 3 Spots developed from starch hydrolysates on TLC by the action of Bacillus CRC and Bacillus CLC alpha amylase

5.4. Enzyme Characterization

5.4.1. Effect of pH on the activity of Asp 43 Glucoamylase

To determine the pH profile of the glucoamylase this enzyme was assayed using a substrate prepared in a pH range of 3.5 – 9.0.The highest activity was observed in the pH range of 3.5 to 4 and the maximum activity was observed at pH 4.0. The activity of this enzyme sharply decreased above pH 5.5 values (Fig 4)

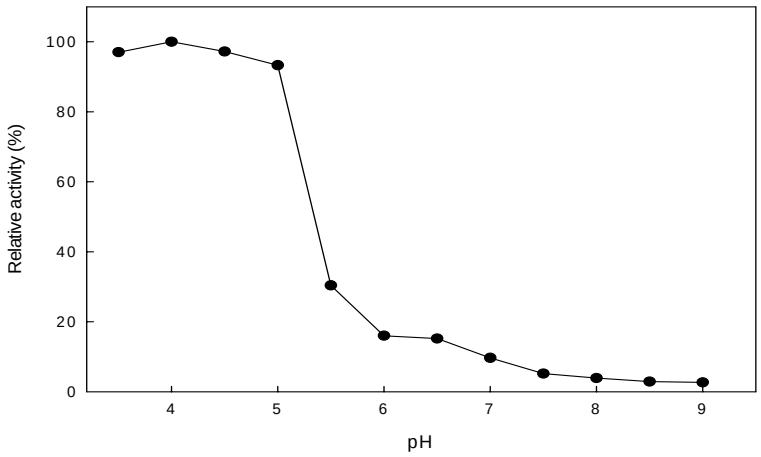

Fig 4 pH profile of Asp Glucoamylase

5. 4..2. Effect of Temperature on the activity and stability of the Glucoamylase

The least glucoamylase activity was observed at 35 ^{0}c, but with increase in assay temperature glucoamylase activity also increased. However above its optimum temperature (65^{0}c) the activity sharply decreased. (Fig 5.)

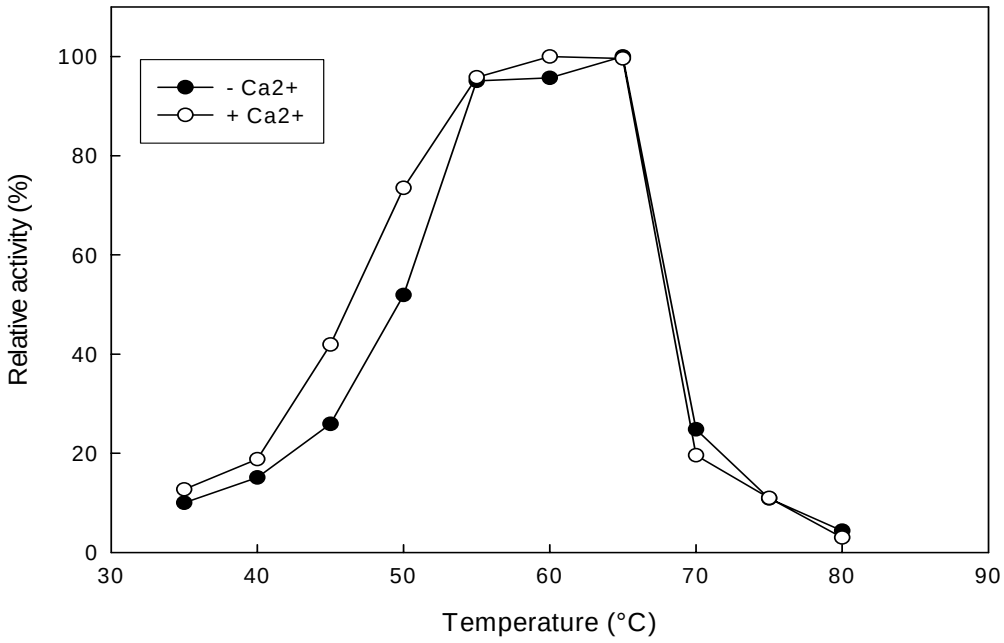

Fig 5 Temperature profile of Asp 43 in the presence and absence of 5 mM $CaCl_2$ solution

The temperature stability of Asp 43 glucoamylase was determined by assaying the preincubated enzyme. The relative activity of this enzyme decreased with increase in incubation temperature .It maintained about 54% of its original activity at its maximum temperature of activity (65 ^{O}C).after 30 min (Fig 6).

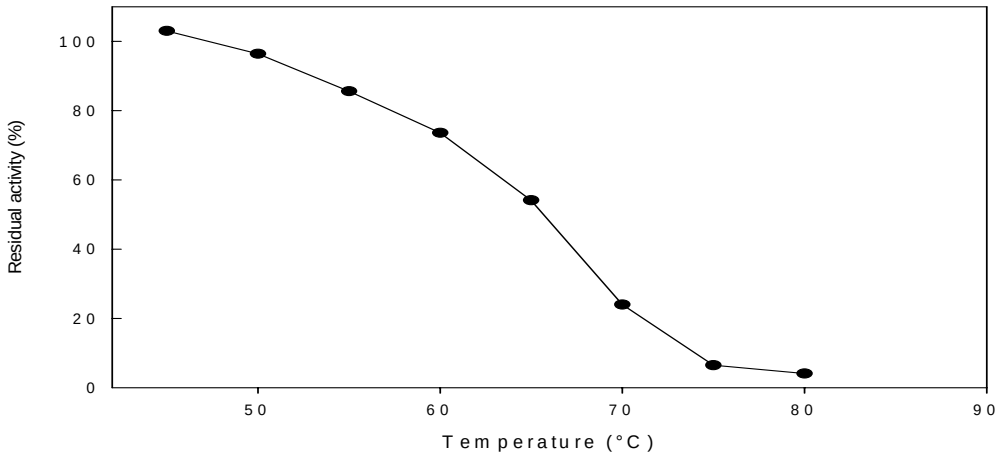

Fig 6 . Effect of Temperature on the stability of Asp 43 glucoamylase

5.4.3. Effect of ca^{2+} on the activity of Glucoamylase

When it was assayed with out and with 5 mM ca^{2+} in the temperature range of 35 ^{0}C – 80 ^{0}C (at 5 ^{O}C interval), the temperature profile of Asp 43 glucoamylase was more or less similar. The optimum activity of this enzyme was observed at pH 6.5 in both cases. Thus the addition of 5 mM Ca^{2+} did not affect the temperature profile of this enzyme (Fig 5).

5.4.4 Effect of pH on the activity of Bacillus CRC alpha amylase

The optimum pH of *Bacillus* sp CRC alpha amylase was observed in the pH range of 5.0 to 6.0. This enzyme maintained about 85 % of its activity at pH 4.5. The lowest activity was observed at pH 3.5 (Fig 7).

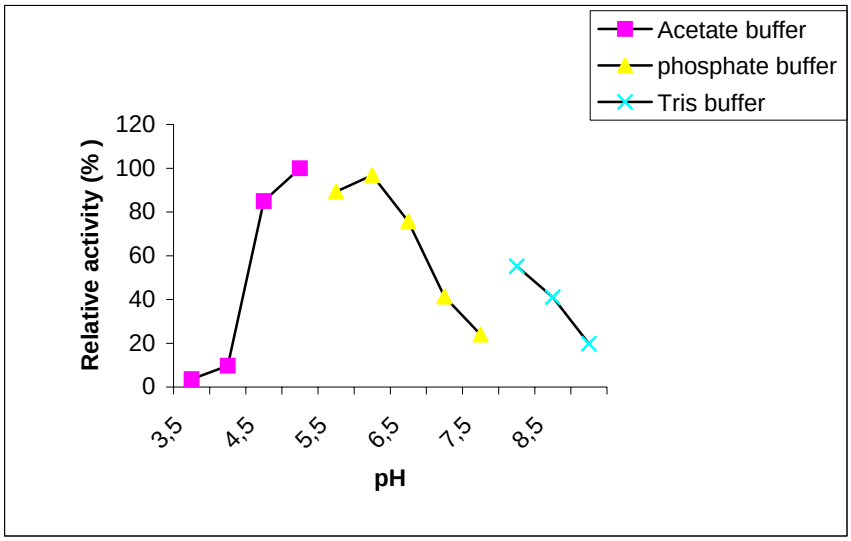

Fig 7. pH profile of *Bacillus sp CRC* alpha amylase

5.4.5. Effects of temperature and Ca^{2+} on the activity of *Bacillus sp* CRC alpha amylase

The activity of Bacillus CRC alpha amylase increased with assay temperature from 50-80 ^{O}C. The optimum temperature for maximum enzyme activity was observed at 80 ^{O}C. The addition of 5 mM ca^{2+} in the assay mixture did not affect on the temperature profile (Fig 8).

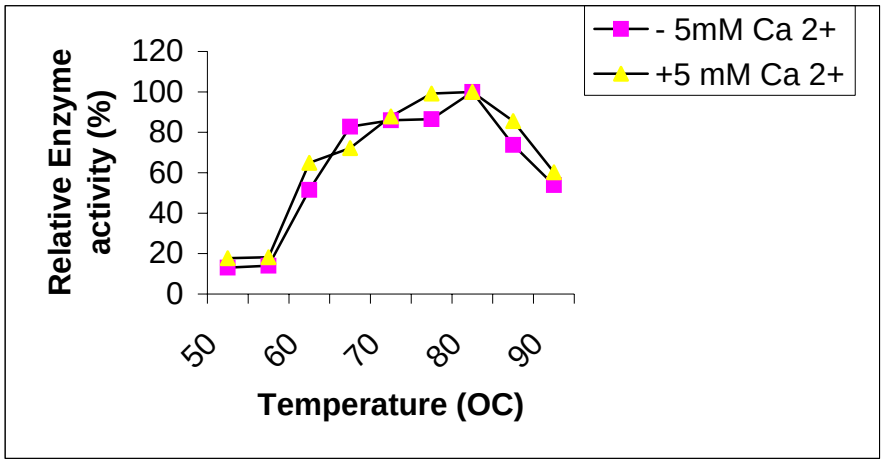

Fig 8. Temperature profile of Bacillus CRC alpha amylase with and without 5 mM Ca^{2+}

5.4.6.. Temperature stability and Ca^{2+} requirement of Bacillus CRC alpha amylase

The temperature stability of Bacillus CRC alpha amylase determined after incubating the enzyme with and without 5 mM Ca^{2+} About 70 % of its original activity was retained after 90 min and 30 min incubation at 75 and 80 ^{O}C respectively. In the presence of 5 mM Ca^{2+} this enzyme retained high portion of its original activity. The enzyme maintained more than 53 % of its original activity after 3 hr incubation at 80 ^{O}C (Fig 9).

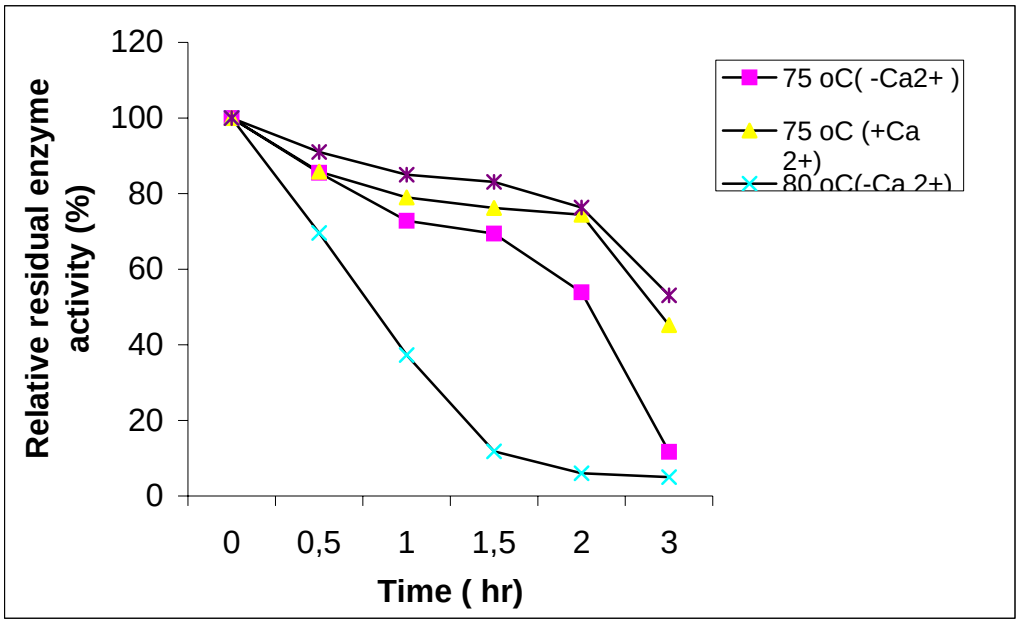

Fig 9 Effect of Temperature and ca^{2+} on the activity of CRC amylase

.5.4.7. PH Stability of Bacillus CRC alpha amylase

The pH stability of Bacillus alpha amylase was determined by incubating the enzyme in the pH range of 3.5- 9.0 and assaying the residual activity. The enzyme was stable in wide pH range retaining more than 80 % of its original activity in the pH range of 4.5 – 8.0. The least stability was observed at pH 9.00 (Fig. 10).

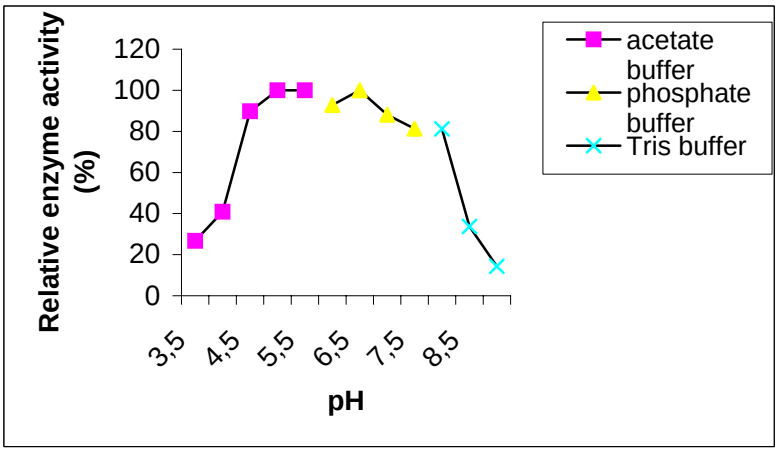

Fig 10 Effect of Temperature and ca^{2+} on the activity of CRC amylase

.5.4.8.Cultivation conditions for enzyme production

Solid-state fermentation has been used in the production of industrial enzymes like amylase by fungal strains and it has great potentials in the developing countries due to its simplicity of operations, low capital cost and high volume productivity (Akpan *et al 1*999; Ramachandran *et al*, 2004). On the other hand most bacteria are unable to grow in SSF medium since they require high water activity for growth. As a result submerged state fermentation method is mostly used in enzyme production.

In this study amylases with optimal activity at elevated temperature had been identified from a fungal and a bacterial isolates. For scale production the genes that encode these enzymes must be cloned on to appropriate host organism. However gene cloning was beyond the scope of this study. Because of this regardless of the amount of enzyme produced, some cultivation conditions for enzyme production by the wild Asp 43 and Bacillus sp CRC isolates was evaluated.

5.4.9. Time course of enzyme Production by Asp. 43

Solid-state fermentation system was used for enzyme production by Asp. 43. Maximum enzyme production was observed after 96 hr incubation at 25 ^{O}C. Enzyme production increased as time of incubation increased from 0 hr – 96 hr (Fig.11). There was however a gradual reduction in the relative enzyme activity after 96 hr. of incubation.

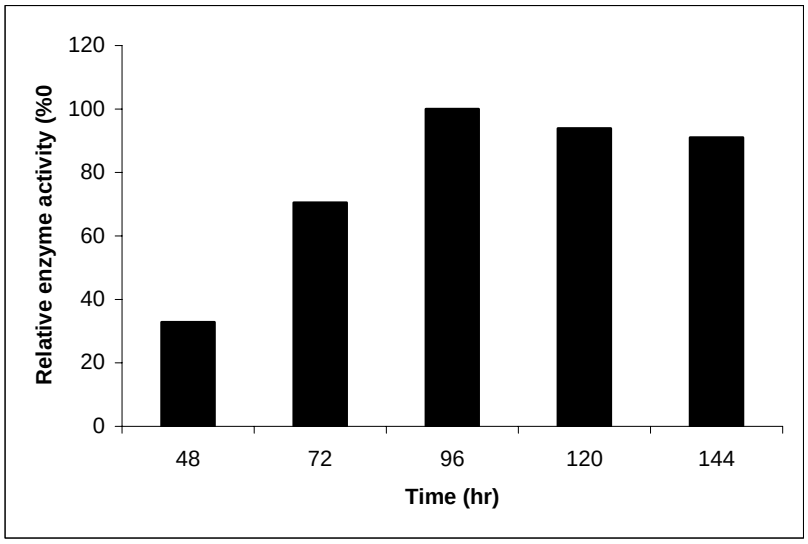

Fig 11 Time course of enzyme production by Asp 43

5.5.0.. Optimum moisture level for Enzyme production by Asp. 43 on SSF medium

To determine the optimum moisture level for enzyme production Asp 43 was grown on wheat bran containing different moisture levels. Maximum enzyme production was observed at moisture level of 66.7%. With increasing moisture level the level of enzyme production decreased. The least activity was observed at 80% moisture level (Fig 12).

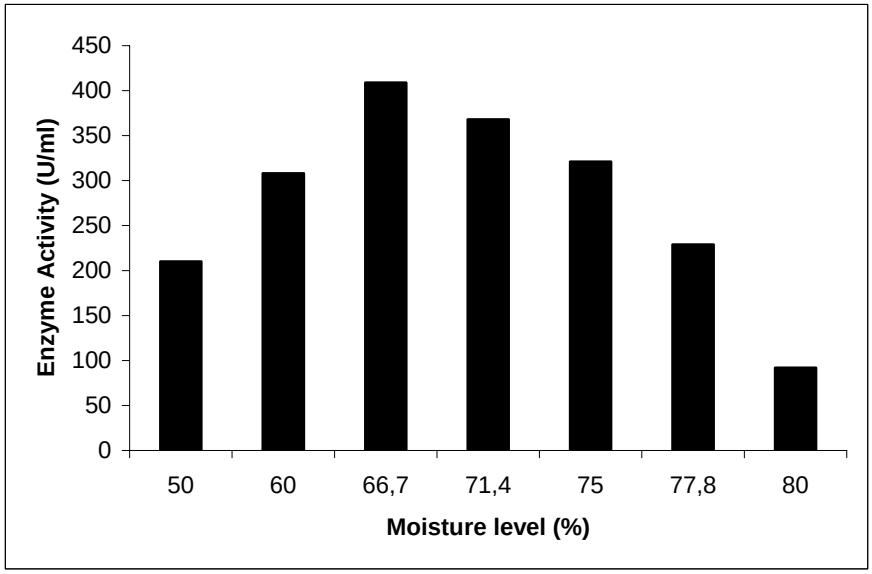

Fig 12 Moisture level of SSF medium and enzyme production by Asp 43

5.5.1. Effect of Growth temperature on Enzyme production by Bacillus CRC.

Bacillus spp.CRC was grown in the temperature range of 40-65 ^{O}C. Maximum enzyme production was observed at 55 ^{0}C. The least activity was observed at 37 ^{O}C (Fig 13)

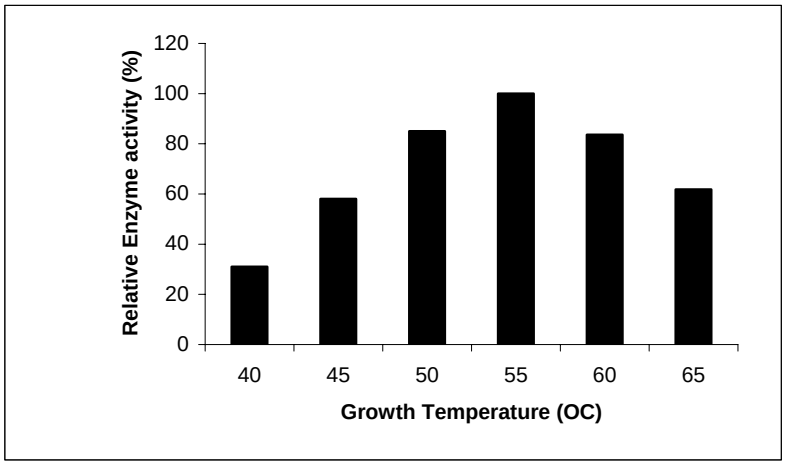

Fig 13 Effect of Growth Temperature on Enzyme production By Bacillus CRC

.

5.5.2. Effect of Initial pH on Enzyme Production by Bacillus CRC

The highest relative enzyme activity was observed in culture filtrates in which the initial pH was 6.0. There was also relatively high enzyme activity in all culture filtrates with an initial pH 5.5 - 7. When the initial pH was 7.5 enzyme production was almost negligible (Fig 14).

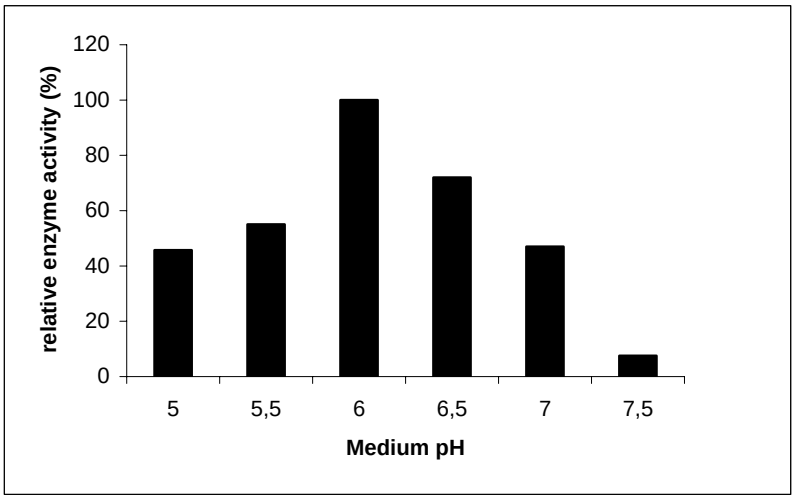

Fig 14 Effect of growth medium pH On Enzyme production by Bacillus CRC

5.5.3. Effect of Carbon source on Enzyme production by Bacillus CRC

The highest amylase production by Bacillus sp CRC (0.5 U/ml) was observed when 0.5 % starch was used as a sole carbon source. In addition, dextrin and maltose support the next higher production. All other disaccharides and monosaccharides tested supported little enzyme production (Fig 15).

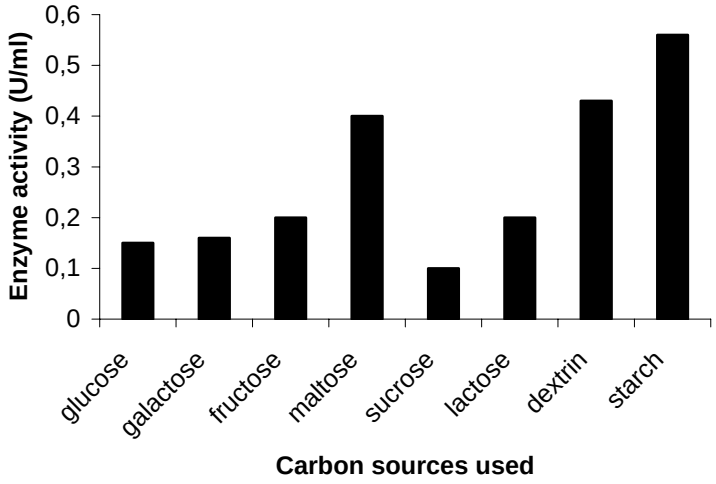

Fig 15 Effect of Carbon source on Alpha amylase production by Bacillus CRC

5.5.4. Effect of Nitrogen source on Enzyme production by Bacillus CRC

Of all the nitrogen sources tested trypton at 0.2 % supported the highest enzyme production. In addition all other organic nitrogen sources (except caesin) supported a relatively high enzyme production. On the other hand the inorganic nitrogen sources did not support much enzyme production (Fig 16)

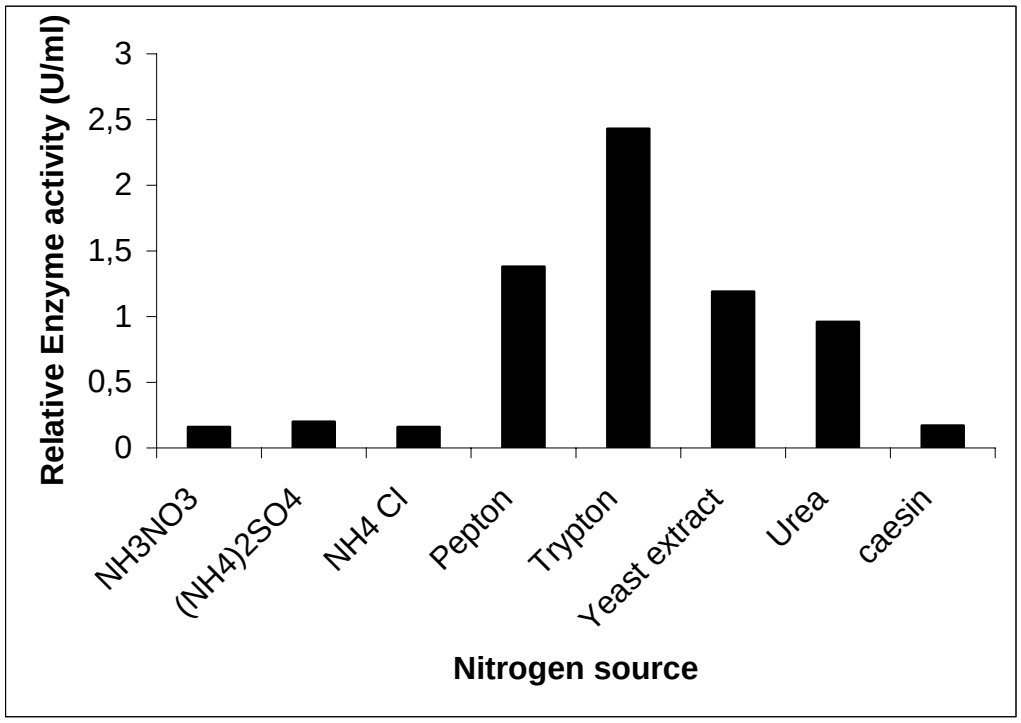

Fig 16 Effect of Nitrogen source on alpha amylase production By the isolate Bacillus CRC

5.5.5. Time course of Enzyme Production by Bacillus sp CRC

Maximum enzyme production was attained after 36 hr and the enzyme activity remained more or less constant up to 60 hr Bacillus CRC reach its stationary phase after 27 hr and the biomass gradually decreased but after 40 hr it remained constant (Fig.17).

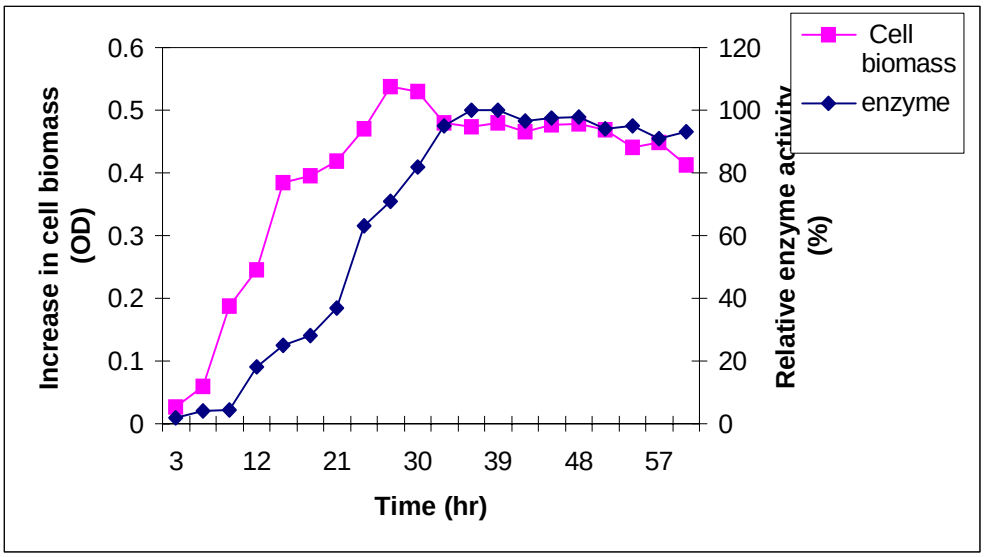

Fig 17 Cell growth – Time course of enzyme production

6. DISCUSION

In countries like Ethiopia the development of enzyme technology could enable to effectively exploitation of agricultural commodities such as starchy material. Starch degrading enzymes can be produced from microorganisms. Amylases are important in many industrial processes. A number of microbial sources exist for the efficient production of this enzyme, but only a few selected strains of fungi and bacteria meet the criteria for commercial production (Schafer et al., 2000). In this study one fungal strain that belongs to the genus Aspergillus (coded *as Aspergillus* No 43, Asp.43), and one thermophilic bacterial isolate (coded as *Bacillu*s CRC) were isolated culture conditions evaluated and their enzymes characterized

Different culture conditions greatly affect on the production of amylase (Cherry *et al,* 2004). The culture conditions for enzyme production by the fungal isolate. Maximum enzyme production by Asp 43 was evaluated by growing it in SSF medium using wheat bran with different moisture content. The highest enzyme activity was observed at 66.7% moisture level (Fig 12). As moisture content increased from the optimum, enzyme production was decreased. The least enzyme activity was observed at 80% moisture level. As moisture level increased anaerobic condition could be created as water molecules occupy the air space in the bran. As the fungus is aerobic this could result in reduced enzyme production. A second possible explanation is catabolic repression. According to Sauza and Peralta (2001), the adverse effect of catabolic repression in SSF system is related with moisture content- high moisture content leads to high catabolic repression. This is because as water activity increases rate of glucose diffusion also increases making catabolic repression to occur

The major factors involved in the biosynthesis of amylases include growth phase, type and concentration of chemicals in the growth media, as well as inducibility and repressibility of the enzyme (Ikran *et al,* 2003). In this study the effect of carbon and nitrogen sources, growth temperature, initial pH of the medium on enzyme production

as well as the growth phase at which maximum enzyme secretion occurred in relation with growth in cell biomass of Bacillus CRC was evaluated.

Alpha amylase is an inducible enzyme (Saito and Yamamoto 1975). Of the different carbon sources used as sole carbon sources starch at 0.5% supported maximum enzyme secretion. Similar observations were reported earlier (Welker and Campbell 1963; Gashaw Mamo and Amare Gessesse 1999a; Dharani, 2004)). The next higher enzyme production was observed when dextrin and maltose were used each as sole carbon source. According Dharani, (2004) however fructose was the second to support a better alpha amylase production by *B. licheniformis*.. On the contrary enzyme synthesis was greatly suppressed when the test organism was grown either on monosaccharides, (glucose gelatos and fructose) or the disaccharides (sucrose and lactose) (fig 15). The least enzyme activity was observed from culture media in which glucose and sources each were used as sole carbon sources. The effect of simple sugars on alpha amylase production is as a result of catabolic repression. Like most other inducible enzymes alpha amylase production is also subject for catabolic repression by glucose and other sugars (Teodoro and Martins, 2000)

The effect of nitrogen source on enzyme production by Bacillus CRC was evaluated by growing the test organism in media supplemented with different organic and inorganic nitrogen sources that serve as a sole nitrogen source. The highest enzyme activity was observed in a culture filtrate in which 0.2 % trypton was used as a sole nitrogen source. In addition, high enzyme activity was observed in all media in which organic nitrogen sources (except casein) were used. (Fig 16).. According to Haq- et al (2002), however, alpha amylase production by *Bacillus subtilis* was significantly increased with the addition of $NaNO_3$ and 4 mM $CaCl_2$ to the fermentation medium. . In this study however, low enzyme activity was observed in all media in which inorganic nitrogen sources were used as the sole nitrogen source. The reason why casein suppressed enzyme secretion in Bacillus *CRC* was not clear. It was however assumed that the organism might not proteolytic, because it grows and produce high enzyme in simplified nitrogen sources such as tryptone peptone or yeast extract

High enzyme production was observed at a temperature range of 50-60 ^{O}C the maximum being at 55 ^{O}C (Fig 13). Similar observations were reported earlier (Lin et al 1997; Gashaw Mamo and Amare Gessesse 1999 b). Bacterial amylases are produced at a much wider range of temperature .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 or thermophilic.

The pH of the fermentation medium has very critical role in the production of alpha amylase (Sindhu et al 1997; Haq *et al*, 2002). To determine the optimum pH for maximum enzyme production the bacterial isolate Bacillus CRC was grown in liquid media with an initial pH of 5 to 7.5 (at 0.5 interval). The highest enzyme production was observed when initial pH of the medium was 6.0. The least enzyme activity was observed at initial pH of 7.5. Generally, high enzyme activity was observed in all culture filtrates whose initial pH was between 5.5 and 7 (Fig 14). The effect of initial media pH in the production of alpha amylase by *Bacillus spp* CRC was compatible with the existing facts in the literature. According to Mielenz (1983) pH affects the synthesis, secretion and stability of alpha –amylase. According to Zhang *et al,* (1983) glycine added in the early growth phase prevented lowering of pH and has increased alpha amylase production by *B. amyloliquefacience* by a factor of 300.

The period at which an enzyme is harvested from the culture is an important factor in enzyme production. Most reports in the literature confirmed that enzyme secretion usually occurs maximally during the late exponential and early stationary phases. According to Gupta *et al* (2003) enzyme secretion during the late growth phases occurs maximally because of reduction in catabolic repression as the nutrient in the growth medium depletes. In this study time course of enzyme production by the fungal and bacterial isolates was examined. The highest enzyme production by Asp 43 was observed after 96 hr of incubation under optimum culture conditions. Similar observation was reported earlier by Soni *et al* (2003). As time of cultivation exceeds 96 hr there was a gradual reduction in enzyme activity (Fig 11). Based on these observations it was concluded that Asp.43 was a slow growing fungus.

Under optimum culture conditions, maximum enzyme activity by *Bacillus* CRC was observed after 36 hr incubation. In addition as tine of incubation was extended to 60 hr there was no significant reduction in enzyme activity (Fig 17). The ability of this enzyme to maintain its activity over such a long period could be due to its high resistance to wards chemical denaturants in the culture medium. According to Scandurra *et al*, (2000) enzymes from thermophillic microorganisms have high resistance to denaturation by temperature, proteolysis or chemicals.

The Maximum cell biomass growth of *Bacillus sp* CRC was observed after 27 hr of incubation under optimum cultivation conditions. Although there was a slight reduction just after 27hrs, cell biomass showed only a small reduction when the period of incubation was prolonged to 60 hr (Fig 17).. This gradual change in biomass of *Bacillus CRC* was not clear. However assumed to be due to either spore formation or dead cell accommodation or both.

How much active and stable an enzyme is during application and stronger condition is an important factor for its industrial application. Successful industrial use of amylases requires that they are sufficiently stable and active at application conditions for example at high temperatures in starch-liquefaction processes. Besides the parameters, such as temperature and pH, the influence of divalent metal ions is important (Nielsen et al 2003). In this study the effect of temperature, pH and Ca^{2+} on the activity and stability of bacterial and fungal amylases was evaluated.

The optimum temperature for maximal activity of Asp 43 glucoamylase was observed at 65 ^{O}C. There was also a significant activity at 70 ^{O}C. This result was in agreement with reports of Gashaw Mamo and Amare Gessesse (1999c) The minimum enzyme activity was observed at 35 ^{O}C (Fig 5). In addition the presence of 5 mM Cacl2 in the assay mixture did not affect the temperature profile of this enzyme.

The activity of *Bacillus sp* CRC alpha amylase increased with assay temperature from 50 ^{O}C to 80 ^{O}C The optimum temperature for this enzyme was observed at 80 ^{O}C. The lowest enzyme activity was observed at 50 ^{O}C (Fig 8.) This result was similar with earlier reports(Burhan et al ,2003; Gashaw Mamo et al 1999a). The result

obtained from the temperature profile enabled to conclude that Bacillus CRC alpha amylase was a thermostable enzyme

The ability to carry out liquefaction and saccharification reaction at elevated temperature has a great advantage in starch processing industries: it avoids excessive cooling time and offer high reaction rate with no problem of contamination (Nielsen 2003). Bacillus CRC alpha amylase and Asp 43 glucoamylase showed their optimal activity at high temperature of 80 ^{O}C and 65 ^{O}C. Thus the parent organisms could be potential candidates for industrial production of the enzymes.

Most repots in the literature confirm that the optimum pH of Aspergillus glucoamylase is between 4 and 5 The maximum activity for Asp 43 glucoamylase was observed at pH 4 (Fig 4). Similar observation were reported earlier by Omemu et al (2005).

Alpha amylase from *Bacillus CRC* on the other hand maintained its optimal activity at a pH range of 5.5 – 60 (Fig 7). Similar observation was reported earlier by Gashaw Mamo and Amare Gessesse (1999a). Enzymatic liquefaction of starch in industries is carried out at a pH range of 5 -7. The pH of liquefied starch has to be lowered to pH 4 - 5 to facilitate saccharification by fungal glucoamylase. This process involves the addition of acid (HCl), which could decrease the quality of the product and hence incurs additional cost for later purification (Van der Maarel *et al*, 2002). In this regard Bacillus CRC alpha amylase with optimum activity at pH 5 could be a potential candidate for industrial application.

Thermostability of an enzyme is affected by many factors like presence of stabilizers such as calcium and substrate (Vihinen *et al*., 1990). In the presence of 5mm Ca^{2+} *Bacillus* CRC alpha amylase showed a better stability. Although it was not clear this amylase showed unusual property in that in the presence of 5 mM Ca^{2+} the enzyme was more stable at 80 ^{O}C: about 45% and 53% of the original activity was observed after 3hr incubation at 70 and 80 ^{O}C respectively (Fig 9). Generally however it was clear that *Bacillus* CRCalpha amylase was a Calcium dependent enzyme for its temperature stability. The calcium dependency of a Bacillus sp alpaha mylase is a well documented fact. All known alpha amylases bind at least one strongly conserved

Ca^{2+} ion, which is required for structural integrity and stability (Aghajari *et al* 2002, Sivaramakrishnan *et al* 2006). At liquefying temperature commercially used amylases require Ca^{2+} as stabilizer (Kumar et al 1990) since heat energy causes the loss of chemically bound Calcium.

PH stability of *Bacillius CRC* alpha amylase was evaluated by assaying the enzyme after incubation of the enzyme in three buffers systems of pH value 3.5 to 9.0 for one hr. This enzyme was observed to maintained 100 % activity between pH values of 5.0 to 6.5 and 80 % of its original activity in a pH rage of 4.5 to 8.0..Based on these observations it was concluded that Bacillus CRC alpha amylase had a broad rage of pH stability (Fig 10).

7. CONCLUSION

To alleviate the ever-growing demand for fuel energy the production of fuel ethanol from plant material is the focus of research. To this effect the application of amylolytic enzymes in the production of the fermentable sugar from starchy crops is indispensable. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits Omemu et al (2005). On the other hand the production of amylases for different application can be achieved by growing the parent organisms on starchy material. The presence of abundantly available year round agricultural by products such as wheat bran in this country could enable their use as substrate for starch utilizing organisms in the production of amylases Starch degrading enzymes like amylase have received great deal of attention because of their technological significance and economic benefits

There is a very huge demand to improve the stability of the enzymes to meet the requirements, especially with respect to temperature and pH (Reddy et al 2003). Asp 43 and Bacillus sp CRC isolated and characterized in this study produce amylases that carryout amylolysis maximally at 65 and 80 ^{O}C, These isolates could be potential candidates for industrial production of glucoamylase and alpha amylase. It is hoped that more efficient strains with enzymes of required quality cold be isolated if the search for such organisms is carried out over the different agro- ecological zones of the country. In addition the development of gene coning techniques is important to produce the enzymes in large scale so as to met industrial demands

8. **REFERENCES**

Abreham Tesfaye (2006). A study on ochratoxin A and ochratoxigenic fungi in coffee. M.Sc Thesis

Adams, M.W.W. and Kelly, R.M. (1998). Finding and using thermophilic enzymes. *Trends Biotechnol*. **16**: 329-332.

Aghajari N., Feller G., Gerday C. and Haseri R. (2002). Structural basis of amylase activation by chloride. *Prot. Sci* **11**:1435 – 1441

Akpan,I., Bankole,M.O., Adesemowo,A. M. and Latunde- Dada, G. O. (1999).Production of amylase by *A. niger* in a cheap solid medium using rice bran and agricultural materials. *Trop. Sci*. **39**:77-79

Alavi, S. (2003). Starch Research over the years. *Food Res. Intr l***36**: 307-308.

Arasaratnam, M. and Balasubramaniam, L. (1993). Synergystic action of alpha amylase and glucoamylase on raw starch. *Microbiol. Biotechnol* **7** : 37-46

Ballschmiter, M., Fütterer, O., and Liebl, W. **(**2006) Identification and Characterization of a Novel Intracellular Alkaline alpha-Amylase from the Hyperthermophilic Bacterium *Thermotoga maritima* MSB8. *Appl. Environ. Microbiol.* **72**: 2206-2211

Burhan.A. Nisa, U., Gökhan,C., Ömer.C., Ashabil,A.,Osman,G. (2003). Enzymatic properties of a novel thermostable, thermophilic, alkalineand chelator resistant amylase from an alkaliphilic Bacillus Sp. Isolate ANT-6. Proc. Biochem*. **38**: 1397-1403*

. Castro ,P.M.L.,Hayter, P.M., Ison,A.M.,and Bull, A.T.,(1992) Application of statistical design to the optimization of culture medium for recombinant interferon – gama productionby chinese hamster ovary cells. *App. Microbiol Biotechnol* **3:** 38- 90

Cherry, H.M., Hossain, M. T., and Anwar ,M..N. (2004). Extracellular Glucoamylase from the Isolate *Aspergillus fumigatus. Pakit J Biol. Sci.* **7** (11): 1988-1992

Crabb, W.D. and Shetty, J.K., (1999). Commodity scale production of sugars from starches. *Curr. Opin. Microbiol*. **2**: 252–256 Crab, W. and Mitchinson C. (1997). Enzymes involved in the processing of starch to sugars. *Trend Biotechnol*. **15:** 349-352

Dharani, A. P. (2004) Effect of C:N ratio on alpha amylase production by B. Licheniformis SPT- 27. Afr. J. Biotechnol **3** (10): 519 – 522

Fitter, J. and Heberle, J.(2000). Structural equilibrium fluctuations in mesophilic and thermophilic alpha –amylase. *Biophys. J.* **79**: 1629-1636

Fitter, J. Hermann, R., Dencher, N..A.,Blume, A. and Hauss, T. (2001). Activity and stability of thermostable alpha amylase compared to its mesophilic

homologue: mechanism of thermal adaptation. *Biochemistry* **40**(35): 10723-10731.

Fogarty, W.M. (1983). Microbial amylases. In: *Microbial Enzymes and Biotechnology* (Fogarty WM, eds), pp 1–92, Elsevier Applied Science, London

Fogarty, W.M. and Kelly, C.Y. (1990). Recent advances in microbial amylases. **In** *Microbial Enzymes and Biotechnology*, 2nd edn pp. 71–132.: Elsevier Science Publishers London, UK

.

Gashaw Mamo and Amare Gessesse (1999a). Production and characterization of two raw starch digesting thermostable alpha amylases from thermophilic *Bacillus sp*. *Enz Microbial Technol* **25** : 433- 438.

Gashaw Mamo and Amare Gessesse (1999b). Effect of cultivation conditions on growth and α-amylase production by a thermophilic *Bacillus* sp *Lett Appl Microbiol* **29**(1): 61-65

*Gashaw Mamo and Amare Gessesse (1999c) Production of raw starch degrading amyloglucosidases by Aspergillus sp Gp-21 in solid state fermentation. J. Indus Microbiol. Biotechnol.. **18:**26-29*

Gomes, E., Sauza, S. R., Grandi,R. P.and Silva, R. (2005) Glucoamylases produced from *A. flavus* A1.1 and *T. Braz. J. Microbiol* **36** (1): 75-82

Gupta, R., Gigras, P., Mohapatra, H., Goswami,V.K.and Chauhan, B. (2003) Microbial alpha amylases: a biotechnological perspective. *Proc biochem.* **38** : 1599 – 1616.

Guzman, M. H.and P, L. O. (1995). Amylolytic enzymes and products derived from starch: a review. *Crr Rev. Food Sc. Nut*. **35**::373-403.

Haki, G. D. Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: a review. *Bioresour Technol*. **89**: 17-34.

. Haq I., Rani, S.. Ashraf, H. and Qadeer M. A. (2002) Biosynthesis of Alpha Amylase by Chemicall Treated Mutant of *Bacillus subtilis. On Line J. Biol Sci* **2**(2): 73-75

Horikoshi, K (1996). Alkaliphiles from an industrial point of view. *FEMS Microbiol. Rev*. **18**:259-270.

Hyum, H.H.and Zeirkus, J.G. (1985). Simultaneous and Enhanced Production of Thermostable Amylases and Ethanol from Starch by Co cultures of *Clostridium thermosulfurogenes* and *Clostridium thermohydrosulfuricum. Applied and Environmental Microbiology* **49** (5): 1174 -1181

Ikram . H , Rani S., Ashraf H. and Qadeer M. A. (2002) Biosynthesis of Alpha Amylase by Chemicall Treated Mutant of *Bacillus subtilis. On Line J. Biol Sci* **2**(2): 73-75

Israilides, C., Smith, A., Scanlon, B.and Barnett, C. (1999). Pullulan from agro-industrial wastes. *Biotechnol. Genet. Eng. Rev*. **16**: 309–324

Kilara, A. and Desai, M. (2002) Enzymes. **In**: *Food Additives* (A.L. Branen, P.M. Davidson, S. Salminen, J.H. Thorngate eds), Marcel Dekker Inc., New York, USA pp. 661–706.

Kirk,O., Borchert, T. V. and Fuglsaang, C. C. (2002). Industrial enzyme applications. *Curr opin biotechnol* **13** :345-351

Kumar, S. S., venkateswara, R. M. and Das, D. (1990).Studies on glucoamylase produced from Aspergillus awamori (NRRL-3112) and their effect on saccharification of potato starch. *Indian J Exp Biol.* **33** (12):957-61

Lee, D.W., Koh, Y.S., Kim, K.J., Kim, B.C., Choi, H.J., Kim, D.S.; Suhartono, M.T. and Pyun, Y.R. (1999). Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiol. Lett*., **179**: 393-400,

Leveue, E., Janacek, S.and Haye, B. (1989).Thermophilic archeal amylolytic enzymes. *Trend biotechnol* **7:** 49-53.

Lin, L.L., Hsu, W. H. and Chu, W. S. (1997). A gene encoding for alpha amylase from thermophilic *Bacillus sp* strain TS-23and its expression in *E. coli*. *J.. Microbiol* **82**: 325-334

Liu ,H. L. Ford C. and Reilly, P. J**. (** 1999) Mutations to alter *Aspergillus awamori* glucoamylase selectivity *Prot. Engineering*, **12**,(2) 163-172.

Lowe, D.A. (2002) Production of enzymes In : *Basic biotechnology 2nd edn* (Colin ratledge and Bjarn Kristiansen eds) pp 391 - 407 Cambridge University press, UK.

Mielenz, J. R. (1983). *Bacillus stearothermophilus* contains a plasmid-
-borne gene for a-amylase, *Proc*. *Natl*. *Acad*. *Sci*. ***80*** : 5975–5979.

Nielsen, A. D., Fuglsang, C. C. and Westh, P. (2003) Effect of calcium ion on the irreversible denaturation of a recombinant Bacillus halmapalusa- alpha amylase: a calorimetric investigation. *Biochem. J*. **373**: 337–343.

Nonaka,T.,Fujihashi,M., Kita,A., Hagihara,H., Ozaki, K., Ltso,S. and Miki, K. (2003) crystal structure of Calcium free alpha amylase from *Bacillus sp*. Strain KSM-K38 (amyK38) and its sodium ion Binding sites. *J. Biol. chemi* **278**(27): 24828 – 24824.

.

Omemu, A. M. akpan, I. Bankote, M. O. amd Tenioda O.D. (2005). Hydrolysis of raw tuberstarchesby alpha amylase of A. niger Am07 isolated from the soil. *Afr. J. Biotechnoi* **I4** (1): 19-25

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.Y.,Singh, D. and Mohan, R. (2000). Advances in microbial amylases. *Biotechnol.Appl. Biochem.* **31**: 135–152

Rao, M. B, Tanksale, A.M., Gathe, M.S., Deshpande, V.V. (1998). Molecula and biotechnological aspects of microbial proteases*. Microbiol. Mol.Biol. Rev*. **62**(3):597-635

Ramachandran,S., Patel, A., Nampoothiri, K. M. Chandran ,S.. Szakacs, G., Soccol C.R. and Pandey, A. (2004). June Alpha Amylase from a Fungal Culture Grown on Oil Cakes and Its Properties *Braz Arch Biol Technol* **47(** 2) : 309-317

Reddy, N.S., Nimmagada , A. and sambasiva Rac, R.S (2003) An over view of the Microbial alpha amylase Minireview. *Afri. J. Biotechnol* **2**(27): 645-648

Sahlstrom, S. Brathen, E. (1997). Effects of enzyme preparations for baking, mixing time and resting time on bread quality and bread staling. *Food Chem*. **58:** 75–80

Saito, M. and Yamamoto, R. (1975). Regulatory factors affecting alpha amylase production in Bacillus licheniformis. *J. Bacteriol*. **121**(3) :848 –85

Sauza, D. F. and Peralta, R. M. (2001). Production of amylases by A. tamarrii in SSF at high initial glucose concentration. *Maringal* **22** (2): 599-602

Schafer, T., Duffner, F.and Borchert, T.V. (2000). Production and properties of alpha amylase from Thermophilic Bacillus sp Brazil J. Microbiol 33: 57 - 61

Scandurra, R., Consalvi, V.,Chiaraluce, R.,Politi L., Engel, P. C. (2000).Protein stability in extremophillic Archaea. *Front Biosci* **5:**787-795

Shenoy, B C.,Katwa, L. C., Appurao A. G. and Raghavendrarao , M. R. (1985) Fungal Glucoamylase. *Biosci* **7** (3&4): 399 – 419

Sindhu, G.S. Sharma, P. Chakrabarti T, Gupta, J.K.(1997). Strain improvement for the production of a thermostable α-amylase..*Enzy Microbiol. Technol .* **24**:584-589

Smith,J.. E.(1996) .Enzyme technology In*: Biiotechnology 3rd edn pp 68 –83 Cambridge Unoversity press, UK.*

Sogaard, M., Abe, J., Martin-Eauclaire, M. F. and Svensson, B. (1993) alpha-Amylases: structure and function, *Carbohy. Polym.* **21**: 137- 146

Soni, S. K. Kaur, A. and Gupta, J. K. (2003) A solid state fermentation Based bacterial alpha amylase and fungal glucoamylase synthesis and its suitability for hydrolysis of wheat starch. Proc Biochem **39:** 185 – 192

Sonnleitner, B. and Fiechter, A. (1983) Advantages of using thermophiles in biotechnological processes: expectations and reality. *Trend Biotechnol.* **1**: 74-80.

Sivaramakrishnan,S. gangadharan,D., Nampoothiri, K. M., soccol, C. R. and pandey, A. (2006). Alpha amylase from microbial sources: an over view on recent developments. Food Technol Biotechnol 44 (2): 173- 184

Teodoro, C.E.de S. and Martins, M. L.L (2000) Culture conditions for the production of Thermostable alpha amylase by *Bacillus sp*. *Braz J. microbial* **31**: 298-302

Tsurikova, N..M..Nefedova,,L.I.,Kostileva, E.V., Zvenigodskii,V.I., Asinovskii ,V.G. Voeikova,T.A. and Sinitsyn,A. P. (2002) Preparation of an active strain of *B. licheniformis*-producer of thermostable alpha amylase .PrklBiokhim Mikrobiol **38** (5) :502-508

Van der Maarel M . J. E. C., Van der Veen, B.,Utdehaag, J. C. M., Leenhuis, H. and Dijkhuizen, L. (2002). Properties and applications of starch converting enzymes of the alpha amylase family. *J. Biotechnol* **94**: 137-155.

Vihinen, M. Mantsala, P. (1990) Microbial amylolytic enzymes. *Crit. Rev.Mol. Biol.* Biochem **24:**329- 418

Welker, N. E. and Campbell, L. L. (1963). Effect of carbon source on the formation of alpha amylase by *Bacillus stearothermophilus*. *J. Bacteriol* **86** : 681-685

Windish, W..W., Mhatre, N.S. (1965). Microbial amylases. In: Adv. Appl. Microbiol **7**:273-304.

Zhang, S. and Udeka,S. (1983).Increased production of alpha amylase by *B. amyloliquefacience* in the presence of glycine *Appl. Environ. Microbiol* **46** (1): 393- 395

Declaration

I, the under signed, declare that this thesis is my original work. It has never been submitted in any institution and that all sources of materials used for thesis have been dully acknowledged.

Name: Muluye Teka

Signature _____

Date _____

This thesis has been submitted for examination with our approval as

Advisor: Amare Gessesse, Ph.D

Signature _____

Date _____