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Production of Alkaline Amylase from a Facultatively Anaerobic Alkaliphilic bacteria.

By

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Abberivations

AA= Alpha-amylase

Da=Dalton

DNS= Dinitrosalicylic acid

EA= Enzyme activity

EB=Enzyme blank

EC= Enzyme Commission

Fig= Figure

GA= Glucoamylase

HFCS= High fructose corn syrups/sweetener

LCBC245= Lake Chitu Bottom-mud Colony 245.

µl= Microliter

mM= Milli molar

M= molar

ND=Not detectable

OD= Optical Density

Psi= pounds per square inch

RA=relative activity

RRA=relative residual activity

rpm= Rounds per minute

RT=Room temperature

S1=Sample-1

S2=Sample-2

TDS=Total dissolved salts

TIM= Triosephosphate isomerase

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Abstract

Bacillus sp. LCBC245, which grew at a temperature of 30 ^{0}C, pH of 10.3 and under anaerobic condition, produced an alkaline amylase. The growth of the organism and amylase production reached a maximum of 0.46 and 74.09U/ml, respectively, after 36 h of incubation. The organism secreted a maximum of 69.12 and 76.96 (U/ml) of amylase when grown in liquid media containing (w/v) 1% maize and 0.5% peptone as the sole carbon and nitrogen source, respectively. On the other hand, the level of enzyme production reached a peak value of 59.52 U/ml at 0.1M NaCl and 34.23U/ml at 1% of raw starch and thereafter progressively declined in both cases. The enzyme performed optimally in the range 55-65 ^{0}C, with a peak value of 100%, and 50-60^{0}C, with a peak value of 77.59%, in the presence and absence of 5mM $CaCl_2$, respectively. LCBC245 amylase lost more than 70 % of its activity for NaCl concentrations exceeding 1M at incubation temperature of 50 ^{0}C for 15min and in the presence of 5mM $CaCl_2$. After the enzyme was incubated at 55 ^{0}C for 1 h it retained 66.91% and 61.42% of its original activity corresponding to the presence and absence of 5mM $CaCl_2$ in the reaction mixture. The optimum pH of the enzyme was 10.5 and it was stable in broad pH ranges (8-11). It had a relative residual activity of 86.33 after it was incubated for 1 h at pH 11 in 50 mM Glycine-NaOH buffer. As far as its reducing power is concerned LCBC245 amylase was able to liberate 504.25µmol/ml of reducing sugar equivalents by hydrolyzing 36.31% of a previously gelatinized starch [25 % (w/v)] after 10 h of incubation at 60 ^{0}C and pH of 10. Thus, the amylase from LCBC245 is a liquefying type. Therefore, the amylase from *Bacillus* sp., LCBC245 was stable at high alkaline pH ranges and required Ca^{+2} for enhancing its thermoactivity and thermostability. The organism produced amylase maximally when grown in media containing undefined carbon sources, 0M NaCl and organic nitrogen sources, and incubated under anaerobic condition.

Key words/phrases: Amylase, alkaline, liquefying, Ca-dependent.

1. Introduction

Many chemical transformation processes used in various industries have inherent draw backs from a commercial and environmental point of view. Such drawbacks are mainly attributed to the inorganic catalysts incorporated in the process of production. The increasing awareness of the technical and economical advantages of enzymes coupled with the need to environmentally safe technology makes the use of enzymes preferable over chemical catalysts in many areas of industry (Cherry *et al.,* 2004). Thus, with a compound annual growth rate of about 6%, the global market for industrial enzymes reached at US$ 3.3 billion in 2010 (Beyer, 2011). The merits of enzymes as industrial catalysts shoulders on their unique properties: enzymes are highly specific and efficient in catalysis; they are biodegradable and carryout catalytic reactions under mild conditions thereby saving energy and resources.

The major classes of enzyme offering immediate application are the hydrolytic enzymes (Kirk *et al*., 2002). They account approximately 75% of the industrial enzymes produced of which 30% is covered by amylases (Joshi, 2011). Amylases are of great significance in present day biotechnology with applications ranging from food, fermentation, textile, pharmaceuticals to paper industries. Although amylases can be derived from several sources, including plants, animals and fungi, microbial enzymes generally meet industrial demands (Gupta *et al.,* 2003; Saxena *et al*., 2007). With this regard alkaline amylases are of special interest in starch processing and detergent industries as they could provide advantages of reducing the risks of contamination and resisting physicochemical denaturants.

Enzyme based starch processing industries have considerable economic significance through upgrading agricultural products to qualities of demand. This would specially be more worthwhile if applied in tropical countries like Ethiopia where the economy is agrarian and most of the population is engaged in arable farming. In addition to other inputs alkaline amylases are very important ingredients in such industries. Despite this fact, only a limited work was carried out in the production and characterization of alkaline amylases from microbial sources in the country to date (Gashaw Mamo and Amare Gessesse, 1997 and 1999a, b, c). In order to fill the existing gaps of research, therefore, this study has been designed to produce amylase from alkaliphilic microorganisms, such as microbial genera.

2. Starch

2.1. Biochemical nature

Starch is the major carbohydrate of higher plants. It is deposited as a reserve material in the form of granules. The starch granule is nature's chief way to store energy in green plants over long times. The granule is well suited to this role, being insoluble in cold water and compactly packed, but still accessible to the plant's metabolic enzymes. The compact (semicrystalline) structure of the granules is resulted from the alignment of the short-chain oligosaccharide branches of amylopectin whereas the insolubility is associated with the nature of amylose component of native starch. Starch is a heterogeneous biopolymer composed of two types of macro molecules, amylose (15-25%) and amylopectin (75-85%) both containing α--D-glucose as the sole monomer. These components are separated by the addition of a polar solvent, *e.g*. n-butanol, to a dispersion of starch. The insoluble amylose complex can then be separated from soluble amylopectin fraction (Souza and Magalhães, 2010).

Amylose is essentially a linear water-insoluble polymer (with a molecular weight of ~1×10^5 to 1×10^6 Da) of glucose subunits joined together by α(1→4) bonds (~99%) and α(1→6) linkages (~1%). Amylose forms an apparent double helical crystalline structure as the linear polymers, held together by Van der Waals forces (60%) and hydrogen bonding (40%), interact in such a way that it acquires hydrophobic and hydrophilic nature in its internal and external surface, respectively. When mixed in cold water the intermolecular attractive forces become disrupted, the linear chains of amylose align themselves by hydrogen bonding and thus aggregates form spontaneously─ a process called retrogradation. Consequently, the amylose becomes viscous and insoluble (Aiyer, 2004). This condition can be reversed if the intermolecular forces are disrupted up on treating the aqueous solution of starch with high temperature or dehydrating agents like alkalis (NaOH/KOH) and salts (Imberty *et al.,* 1991). Amylose forms complex with iodine to form intense blue color and this is the basis of quantitative determination of amylase.

Amylopectin, on the contrary, is a branched, water soluble polysaccharide (molecular weight ~1×10^7 to 1×10^9 Da) comprising of short α(1→4) linked (~95%) linear chains and α(1,6) linked (~5%) side chains of glucose units. It forms the bulk (70-80%) of most starch molecules

(Dona *et al.,* 2010). The branching nature of amylopectin reduces its iodine binding power, increases its solubility in aqueous solutions, and makes it non-retrogradable. It forms double helices readily which are stabilized by hydrogen bonds and Van der Waals forces.

In general, the type of species from which starch originates determines the relative composition and degree of polymerization (chain length) of amylose and amylopectin. It also limits the differences in the size, shape and structure of the polysaccharide granules, their swelling power, gelatinization temperature, extent of esterification with phosphoric acid. Morover, the varaititon in the source of starch determines the amount of lipids and other compounds which are retained inside the hydrophobic inner surface of the amylose helices (Synowiecki, 2007).

2.2. Industrial applications

Among carbohydrate polymers starch is currently enjoying increased attention due to its usefulness in different applications. Starch contributes greatly to the textural properties of many foods and is widely used in food industries for its functionality as a thickener, colloidal stabilizer, and gelling agent, bulking agent, water binder and fat substituent. Furthermore, starch is also a potential substrate for the production of gaseous or liquid fuels, feed proteins, bakery products, sweeteners, and chemicals by microbial processes (Apar *et al.,* 2006; Robertson *et al.,* 2006). However, some properties of starch such as low shear and thermal resistance and high tendency towards retrogradation have limited its use in some industrial food applications (Nigam and Singh, 1995).

2.3. Starch-degrading (amylolytic) anaerobic alkaliphilic bacteria

Anaerobic alkaliphilic bacteria are widely distributed in extremely alkaline and saline environments (Horikoshi, 1999) such as the Rift Valley lakes of East Africa. They are adapted to high PH (9-12) and salinity up to 33%. Their extracellular enzymes are intensively studied and applied to industry on account of the organisms': (1) ubiquitous distribution, (2) non-pathogenicity, (3) high production capacity of extracellular enzymes, (4) relatively faster growth rate and (5) ease of isolation (Fujinamia and Fujisawa, 2010).

3. Alkaline and amylolytic enzymes

3.1. Mode of production

Even though they represent a rich source of energy for a wide spectrum of bacteria, starch molecules are much too large to get into the cell. To overcome this and take advantage of this potential energy source, the amylolytic bacteria secrete matured amylase and release it into the extracellular environment. The amylase released breaks the starch into sugar molecules that can be easily transported into the cell for further metabolism. Maturation of amylase is achieved after the glycosylation of immature amylase with oligosaccharides. The oligosaccharides increase enzyme synthesis, rate of secretion and stability as well as protection against protease-catalyzed degradation, stresses caused by heat, pH and pressure. It has been indicated that the oligosaccharides play such roles by enabling the protein to fold properly thereby increasing their free energy without decreasing entropy (Riederer and Hinnen, 1991). Because amylase is an extra-cellular protein, we can grow starch degraders in broth culture, remove the cell mass by centrifugation and then simply use the resulting supernatant broth containing the extra-cellular enzyme to characterize our amylase without the need for further purification.

It is known that various physicochemical factors affect the productivity and growth of amylolytic bacteria. However, the factors required for the optimum growth of the microorganism being employed for production may differ from those required for the maximum production of the enzyme. These parameters include nutrient supplementation, period of incubation, concentration of surfactants and metal ions, pH and temperature of the medium, osmotic relationships, degree of aeration, agitation and the control of contamination during incubation. Interactions of these parameters are also reported to have a significant influence on the production of the enzymes (Pandey *et al.,* 2000; Regulapati *et al*., 2007).

3.2. Thermal and alkaline stability

The adaptation of alkaliphiles to high pH draws great attention not only because they are potential sources of industrially valuable enzymes but also because of the adaptive mechanisms of their enzymes to external environmental parameters. Some anaerobic alkaliphiles produce

enzymes that are able to maintain their stability and activity under a double challenge of extremely high temperature and pH.

(i) Thermal stability

In general, the function and structure of thermozymes is stabilized at high temperature by extrinsic (non-structural) factors (such as substrates, salts, compatible solutes, pressure, *etc*) and/or intrinsic (structural) factors (such as the modulation of intra- and inter molecular attractive forces, good general conformational structure and amino acid composition). There is no a single determinant factor for thermal stability; rather a plethora of mechanisms are involved. These extensive repertoires of mechanisms are revealed from structural and non-structural studies of thermozymes and their mesophilic homologues (Vieille and Zeikus, 2001; Fitter, 2005).

The intrinsic factors include higher core hydrophobicity, greater numbers of ionic interactions, increased packing density, additional networks of hydrogen bonds, decreased lengths of surface loops, stabilization by heat stable chaperones, an increase in disulfide bond formation, and a general shortening of length. Changes in amino acid composition that account for some of these mechanisms can be detected in proteome-wide or sequential surveys. These include elevated levels of lysine, valine and glutamic acid, arginine-to-lysine replacement and a decrease in glutamine and histidine in thermophiles that can neither be explained by genomic G+C compositional basis nor by universal trends of amino acid gain and loss in protein evolution (Machius *et al.,* 2003; Li *et al*.,2005; Lieph *et al.,* 2006).

Extrinsic factors that play vital roles in thermal stability include salts (*e.g*. $CaCl_2$), substrates and pressure. Inorganic salts stabilize enzymes in two ways: (i) through a specific effect, where a metal ion interacts with the protein in a conformational manner (metal binding), and (ii) through a general salt effect (salting-out), which mainly affects the water activity (Breitung *et al*., 1992; Klein *et al*., 1993). Substrate molecules have been known to stabilize thermozymes specifically by stabilizing their active site and/or cofactor (Andreotti *et al*., 1995). The reason behind stabilization by pressure is that pressure favors the structure with the smallest volume. Enzymes stabilized mainly by hydrophobic interactions are, therefore, expected to be stabilized at high

pressure, whereas proteins stabilized by ionic interactions should be destabilized (Miller *et al*., 1989; Hei and Clark 1994).

(ii) Alkaline pH stability

A number of studies involving whole genome sequence, genetic engineering and protein structure analyses have been carried on enzymes from thermophiles, psychrophiles, and halophiles. These studies provided some insights into the mechanisms and principles of protein/enzyme adaptation to extreme temperatures and salinity. However, little is known about adaptation mechanisms of enzymes to pH extremes to date. The case gets worse when it comes to amylolytic enzymes (Fujinamia and Fujisawa, 2010).

However, comparative studies of extremely alkaline protease with its mesophilic homologue, by atomic resolution methods, have revealed that the former possesses a set of distinctive structural features. These include: (i) increase in the total number of hydrogen bonds and hydrophobic interactions at the dimer interface; (ii) enhancement in the hydrophobic interactions between the two domains in its monomers; (iii) increase in the number of negatively charged amino acid residues on the solvent-accessible molecular surface; (iv) decrease in the number of hydrophobic residues exposed to the solvent; (v) significant reduction in the total amount of ion pairs and ion networks; (vi) a preference for surface residues with long side chains; (vii) substitution of residues (*e.g*. Gly →Ala and Arg →Ser); and (vi) an increase in neutral and hydrophilic residues (*e.g*. His, Asp and Gln) (Horikoshi,2004; Dubnovitsky *et al*., 2005).

For example, in extremely alkaliphilic enzymes the numbers of arginine (Arg) and histidine (His) residues have been found to increase, whereas aspartic acid (Asp), glutamic acid (Glu), and lysine (Lys) residues decrease. The extra arginine residues contribute to an increase of hydrogen bonds or ion pairs in the enzyme. Therefore, the negative charge of the Arg residue is more stable than the Lys residue and is easier to form ion pairs with other acidic amino acid residues under alkaline pH conditions Thus, it is thought that formation of Arg-Asp ion pairs in high-alkaline enzymes is critical for stability under alkaline pH conditions.

3.3. Starch hydrolyzing processes

The functionality of starch can be achieved either through enzymatic and chemical hydrolytic methods. Sugar production by acid hydrolysis of starch was acceptable till nineteenth century. However, several drawbacks of this method have been identified gradually including high temperature (140-150 ^{0}C) and low pH (2) requirements, increase in the viscosity of the reaction mixture, low glucose yields, formation of unwanted browning and bitter tasting compounds, and the need for corrosion resistant vessels.

Currently the major starch hydrolysis is operated under enzyme based catalysis due to the advancement of knowledge on the benefits of enzymes suitable for the process. Specificity of the reaction, stability of the generated products, milder operating conditions, lower energy requirements and elimination of neutralization steps can be exemplified as the advantages of enzymatic hydrolysis (Sivaramakrishnan *et al.,* 2006). The specificity and milder operating conditions (pH 6-10 and T 60-100 ^{0}C) of enzymes allow the reduction of unwanted byproducts such as ash, browning color and bitter taste. It also reduces the refining and recovery costs in the downstream processing; and increases the yield of desired products with well defined physical and chemical properties.

The complex structure of starch necessitates the use of a suitable recipe containing different amylolytic enzymes for ensuring complete hydrolysis or to yield the desired hydrolysates. Additionally, a number of factors such as the botanical origin of starch, the source and operating conditions of enzymes significantly affect starch digestion and the saccharide composition of the hydrolysates (Srichuwong *et al.,* 2005).Taking these facts in to account starch is industrially hydrolyzed by a cocktail of processes such as gelatinization, liquefaction, saccharification and isomerization to obtain desired products.

(a) Gelatinization

Gelatinization of concentrated slurry of starch granules (30–40 %, w/v) is achieved by treating aqueous solution of native starch (pH 3.5-4.2) with high temperature (140-150 ^{0}C) and alkalis (NaOH/KOH) for 90 min or alkalis alone at ambient temperatures. The alkalis help adjust the pH to 6.5. The use of alkalis for gelatinizing starch at relatively lower temperatures saves energy and

thus reduces cost in commercial production. Mechanistically, both the elevated heat energy and the dehydrating effect of the alkalis and salts act by disrupting the hydrogen bonds and Van der Waals forces holding the granules together and causing the release of the water molecules that had been trapped within the granules (Imberty *et al.*, 1991). Consequently, the amylose in the granules becomes anhydrous and solublizes. This triggers the linear chains of the amylose to align quickly, with the help of hydrogen bonding, and crystallizes irreversibly. Thus, the viscosity of the solution increases dramatically and the starch retrogrades up on subsequent cooling. Therefore, the gelatinized starch becomes more susceptible to degradation than nongelatinized starch (Sivaramakrishnan *et al.,* 2006). The increased viscosity of starch, however, poses serious problems with mixing and pumping (Kunamneni and Singh,2005). To overcome these viscosity associated problems, gelatinization is coupled with liquefaction which involves partial hydrolysis and loss in viscosity.

(b) Liquefaction

The object of liquefaction is to provide a partially hydrolyzed starch suspension of relatively low viscosity, free from unwanted byproducts (*e.g*. lipid-amylose complexes), stable to retrogradation, good filtration, low turbidity and suitable for subsequent processing, *i.e.,* saccharification. In traditional liquefaction the gelatinized starch slurry is cooked at high temperature (140-150 ^{0}C) jet cooker and neutral pH for short period (5-10 min). Following that HCl or oxalic acid is added to the solution. The jet cooker and the acid serve as a mechanical and chemical thinning agent, respectively. Concomitantly the pH drops to 2. The high temperature and low pH treatments inevitably bring about many draw backs that are discussed in section 3.3. Therefore, the replacement of the acid with thermostable α-amylase from microbial sources, at relatively milder conditions of temperature (60-90 ^{0}C) and pH (6.5-6.8), not only corrects the problems associated with acid liquefaction but also maximizes yield (Crabb and Shety, 1999; Satayanarayana, 2004).

(c) Saccharification

Saccharification involves the hydrolysis of liquefact, the soluble oligosaccharides resulting from the liquefaction step, in to glucose or maltose in reactions catalysed by a thermostable

glucoamylase (GA) or β-amylase, respectively. For saccharification to be carried out optimally the pH of the liquefact must be adjusted back down to the natural pH of starch (4.2-4.5), the temperature has to be reduced to 55-60 ^{0}C, and the dry solids must be diluted to 30-32% (w/v) (Crabb and Mitchinson, 1997).

The pH adjustment serves two functions: first, it stops further action of the α-amylase so that the liquefact maintains an average chain length optimal for attachment of saccharifying enzymes; second, it moves the pH closer to the optimum for GA. These adjustments increase the chemical costs and require additional ion-exchange refining of the final product for salt removal. A promising solution to the problem could be the use of native amylases from thermoalkaliphiles or engineered amylases that are designed to fit higher pH, lower calcium levels or higher temperatures.

The high concentration (30-32% w/v) of dry-solids results in high concentration of glucose (~95%) and makes the process economical. Under these product-enriched conditions, however, GA tends to form α(1→4) and α(1→6) reversion products. This limitation can be resolved by : (a) balancing the dosage of the enzyme, the temperature of the reaction and the reaction time so that, at latter stages, thermal inactivation of the GA results in little active enzyme remaining and hence less reversion; (b) improving thermostability of GA to allow reactors to be run at higher temperatures and dry-solids levels for shorter time periods; and (c) engineering a GA that has a decreased ability to hydrolyze and, therefore, revert α(1→6) linkages.

Another drawback associated with saccharification is linked to the heterogeneous nature of starch substrate. Raw starch is a mixture of both amylose [α(1→ 4) linkages predominantly] and amylopectin [both α(1→4) and α(1→6) linkages]. GA is efficient at cleaving the α(1→ 4) links, but when it reaches α(1→6) branch point, the enzyme is slower to hydrolyze the bond and the result is a build-up of isomaltose [an α(1→6) liked disaccharide]. Pullulanase, which has the ability to degrade the α(1→6) linkages, can be blended with GA to alleviate the problem.

(d) Isomerization

The process of isomerizaion involves the production of HFCS (high fructose corn syrups/sweeteners) from dextrose mixtures resulted from saccharification step. Before

isomerization proceeds the dextrose mixture is refined, carbon-filtered and concentrated to 40% dry solids while the pH and temperature is adjusted to 7-8 and 55-65 ^{0}C, respectively. These conditions favor the isomerization of D-glucose (aldehyde form) to D-fructose (ketose form) by thermostable Glucose (xylose) isomerase (GI). The process is made continuous by immobilizing GI-producing cells or GI-containing columns or crystals.

3.4. Classification of Amylases

Classification of enzymes is based on the similarities and differences in their structure and function. Homology in amino acid sequence and global (3D) orientation of starch degrading enzymes is taken as a basis to categorize them under glycoside hydrolase (GH) family 13 (Henrissat, 1991). Members of this family: (i) act on α-glucosidic linkages and hydrolyze them to produce α-anomeric mono- or oligosaccharides or form α(1→4) or α(1→6) glucosidic linkages by transglycosylations or combination of both activities; (ii) have four highly conserved regions in their primary structures that either form the catalytic site or involve in stabilizing the TIM barrel topology; (iii) have Asp, Glu, and Asp residues as catalytic sites corresponding to the Asp231, Glu261 and Asp328 *Bacillus licheniformis* α-amylase; and (iv) possess a $(\beta/\alpha)_8$ or TIM barrel catalytic core (domain A) located in the interface between B and C domains (Sivaramakrishnan *et al.,* 2006). It has been known that all enzymes of GH family 13 are considered to catalyze the same basic reaction, a nucleophilic double-displacement mechanism with a transient covalent intermediate: two acidic amino acid residues (Asp or Glu) of protein are essentially involved in catalysis, *i.e*., one has an ionized carboxylic acid group and the other has an un-ionized carboxylic acid group (Kim *et al.,* 1995).

As a matter of fact functional differences arise from structural differences. Thus, amylolytic enzymes functionally differ in their substrate specificities, the length of the oligosaccharide fragments released after hydrolysis and the carbohydrate profile of the final product. The differences in specificities are explained in terms of differences in the numbers of subsites at each active site, in the nature of the amino-acid residues making up each subsite, in the affinity of each subsite to glucose residue, in the variability of the domains that are attached to the catalytic site or to extra sugar binding subsites around the catalytic site, and in the length and folding style of 1-8 loops for constructing subsites (MacGregor,1993; Nielsen and

Borchert,2000; Souza and Magalhães,2010). The variation in the length and the carbohydrate profile of the product is attributed to the differences in the mode of action of the enzymes. For this reason the Enzyme Commision (EC) categorized amylolytic enzymes in to endoamylases, exoamylases, debranching enzymes, and transferases (Van der Maarel *et al*., 2002).

3.4.1. Endoamylases

Endoamylases are enzymes that are able to cleave α(1→4) glycosidic bonds present in the inner (endo-) part of the amylose or amylopectin chain. This leads to a rapid decrease in the viscosity of the substrate and release of α-anomeric products including glucose, maltose, maltotriose, and α-limit dextrins (oligosaccharides of varying chain lengths with an α-configuration). α-Amylases are the major representatives of endoamylases (Nigam and Singh, 1995).

α-Amylases (AA; EC.3.2.1.1; endo-1,4-α-D-glucanglucohydrolase) are endo-acting, extracellular enzymes which randomly cleave α(1→4) linkages, but bypass α(1→6) linkages, between adjacent glucose units in starch polymers leading to the formation of linear as well as branched oligosaccharides of varying chain lengths with an α-configuration (Regulapati *et al.,* 2007). α-Amylases are sorted out in to saccharifying (free sugar producing) and liquefying (degrades starch without releasing free sugars) as they preferentially degrade substrates containing less than four and more than fifteen glucose units, respectively (Pandey *et al.,* 2000). It is assumed that α-amylase catalysis is limited at low pH by protonation of the nucleophile (Asp 231) and at high pH by deprotonation of the hydrogen donor (Glu261) thus the pH-activity profile is determined by the pKa values of these two active site groups (Dubnovitsky *et al*., 2005).

Most α-amylases are metalloenzymes, which require divalent ions in order of Ca^{2+} > Mg^{2+} >Mn^{2+} >Zn^{2+} >Fe^{2}, *etc* (Reddy *et al*., 2003). These ions play a significant role in stabilizing the structural integrity of the catalytic and/or substrate binding sites against thermal and surfactant denaturation, enhance their catalytic activity, and serve as allosteric activator in α-amylases (Sivaramakrishnan *et al.*, 2006). Nevertheless, excessive amounts of these ions induce inhibitory effects and decrease the reaction yield. On the other perspective Cl^{-} ion binding around the active site of α-amylases induces conformational change and thus enhances the affinity for the

conserved Ca^{+2}. Besides that Cl^{-} enhances the catalytic efficiency of the enzyme by elevating the pKa of the hydrogen-donating residue in the active site.

3.4.2. Exoamylases

Exoamylases are enzymes that exclusively cleave α(1→4) or both α(1→4) and α(1→6) glycosidic bonds of α-glucans (amylose, amylopectin and glycogen) from the non-reducing (external or exo) end successively to produce low molecular weight carbohydrates in the β-anomeric form. Enzymes belonging to this category include glucoamylase, α-glucosidase and β-amylase (Nigam and Singh, 1995).

(i) Glucoamylase

Glucoamylase (GA; EC.3.2.1.3; exo-1,4-α-D-glucanglucohydrolase; glucogenic enzyme; starch glucogenase; γ-amylase) is an exo-glycosyl hydrolase that catalyzes the sequential cleavage of α(1→4) glycosidic bonds from the non-reducing ends of starch and related oligo-and poly-saccharides, while slowly hydrolyzing α(1→6) linkages, to yield α-D-glucose (Kumar and Satyanarayana, 2009). In the presence of the high levels of glucose (>95% w/v) and high dissolved starch (>32% w/v), however, GA can also catalyze the reverse condensation reaction forming isomaltose [an α(1→6) linked disaccharide] and other byproducts from α-D-glucose. The α(1→6) linked oligosaccharide byproducts, commonly referred to as reversion products, decrease overall glucose yield. This is one of the draw backs of the industrial application of GA for the production of high fructose corn syrup/ sweetener (HFCS) (Crabb and Shety, 1999; Sauer *et al.,* 2000).

(ii) α-Glucosidase

α-Glucosidase (EC.3.2.1.20) hydrolyses both α(1→4) and α(1→6) glycosidic bonds of essentially short chain oilgosaccharides and thus produces only α-D-glucose units via α-retaining double displacement mechanism (Aiyer,2005).

(iii) β-Amylase

β-Amylase (EC.3.2.1.2) exclusively breaks every alternate α(1→4) glycosidic linkages, and bypasses α(1→6) glycosidic bonds, thereby librating β-maltose and β-limt dextrins. β-Amylase converts the anomeric configuration of maltose from α to β form by the same mechanism pertaining to glucoamylase. β-Amylase is characterized by a $(\beta/\alpha)_8$ or TIM barrel catalytic domain which is identical with that of α-amylase but far related to the $(\beta/\alpha)_6$ barrel of glucoamylase (Pandey *et al*.,2000). β-Amylase is commonly produced by plants (*e.g.* soybean, barely, wheat) and bacteria (*e.g*. *Bacillus* species). Bacterial β-amylases have greater industrial potential over those of plant origin as the former are thermostable and are excellent in digesting raw starch.

3.4.3. Debranching amylases

As their name signifies debranching amylases are endo-acting enzymes that hydrolyze α(1→6) glycosidic bonds existing at the branch points of amylose, glycogen, pullulan and related oligosaccharides. There are two main groups of debranching enzymes. The first group consists of pullulanase type I and isoamylase that exclusively attack α(1→6) linkages and hence liberate linear oligosaccharides of glucose residues linked by α(1→4) bonds. Pullulanase type I hydrolyzes the α(1→6) glycosidic bonds in both pullulan [a polymer of α(1→6) linked maltotriose monomer] and amylopectin where as isoamylase only hydrolyzes the α(1→6) bonds in amylopectin (Israilides *et al*., 1999; Van der Maarel *et al.,* 2002). The second group includes neopullulanases and amylopullulanases (α-amylase-pullulanases; pullulanase type II). They are active towards both α(1→6) and α(1→4) linkages and produce mainly maltose and maltotriose. Neopullulanases can also perform transglycosylation with the formation of new α(1→4) and α(1→6) glycosidic bonds.

3.4.4. Transferases

Transferases are exo-acting, starch-converting enzymes that hydrolyze an α(1→4) glycosidic bond and concomitantly links the reducing to the non-reducing end by a new glycosidic bond of α(1→4) kind as in amylomaltase (EC.2.4.1.25) and cyclodextrin glycosyltransferase (CGTase)

(EC.2.4.1.19) or of α(1→6) kind as in branching enzyme (EC.2.4.1.18). Intermolecular transglycosylatic action of amylomaltase results linear oligosaccharides. CGTase, on the other hand, produces cyclic oligosaccharides (α-, β- or γ-cyclodextrins) and highly branched dextrins (CGTase limit dextrins) via intra- and inter-molecular transglycosylation reactions, respectively (Synowiecki, 2007).

3.5. Applications of Amylases

Although amylases are universally distributed throughout the bacterial, archaeal and eukarial domains, enzymes from bacterial sources are dominating industrial applications. This is attributed to the cost effective and consistent bulk production capacity as well as the ease of process modification and optimization for the bacteria. Besides that bacteria are easy to manipulate to obtain amylases of desired characteristics. Moreover, the plasticity, vast availability and efficient catalyzing ability of bacterial enzymes at extreme conditions of pH, temperature, salinity, *etc,* while maintaining their structural stability, makes them preferable over any other sources (Schafer *et al*., 2000; Mishra and Behera, 2008).

The commercial application of amylolytic enzymes began when "Taka diastase", was produced from a wheat bran koji culture of *Aspergillus oryzae* to be used as a pharmaceutical aid for the treatment of digestive disorders. Currently amylases have been used extensively in starch processing, textile, pulp and paper, biofuel, detergent, baking and brewing industries (Gupta *et al.*, 2003). With the advent of new frontiers in biotechnology, the spectrum of amylase application has widened to clinical, medicinal and analytical areas (Van der Maarel *et al*., 2002).

3.5.1. Food processing

The discovery and application of amylolytic enzymes exhibiting different activities and substrate specificities has resulted in the development of many starch products of diverse carbohydrate profiles and functional properties. Some of the mono-, di- and oligosaccharide products include glucose, fructose, maltose, trehalose, maltodextrins, cyclodextrins and oligosaccharide mixes. Such hydrolytic products are involved in the manufacture of sweeteners, syrups, moisture conditioners, cryoprotectants, flavor and taste enhancers as well as texture stabilizers in starch-containing foods (Couto and Sanromán, 2006). Modern starch processing industries are unique in

that they are entirely dependent upon the use of enzymes for large scale production. These enzymes include α -, β- and γ-amylases, glucose isomerases and glycosyl transferases.

(i) Manufacture of oligosaccharide mixes

Oligosaccharide mixture (maltooligomer mix) is obtained by digestion of corn starch with α -amylase, β-amylase and pullulanase. Maltooligomer mix is highly hygroscopic thus it serves as a moisture regulator of food with which it is mixed. It also prevents the crystallization of sucrose in foods and maintains a certain level of hardness to the texture during storage by preventing the retrogradation of the starch gradient. Its solution shows lower viscosity and sweetness than corn syrup because of its low content of glucose and sucrose, respectively. For this reason maltooligomer mix is used as a mild substitute for sucrose. Besides it has low water activity which is convenient in controlling microbial contamination (Parasanna, 2005).

(ii).Manufacture of sweeteners and syrups

The industrial conversion of starch in to sugar sweeteners and a broad range of syrups has undergone an impressive growth following new technological breakthroughs in relation to the discovery, isolation and application of various amylolytic enzymes including thermostable α-amylase, glucoamylase and glucose (xylose) isomerase (GI) (Nigam and Singh, 1995). Sugar sweetener production involves the liquefaction of corn starch by thermostable α-amylase to liberate oligosaccharides followed by the saccharification of the oligosaccharides in to glucose by glucoamylse. Subsequently, the glucose is isomerized in to an equilibrium mixture of glucose (45%) and fructose (55%), which is referred to as HFCS, by glucose isomerase (GI). The HFCS completes the circle back to wild honey— the oldest sweetener known (Crabb and Shety, 1999). Because it participates and intensifies Maillard reactions, HFCS is used as a sweetener and enhancer of citrus flavor, crystallization inhibitor of stored food and an agent of triggering desirable browning to fried or baked foods.

(ii)Manufacture of maltose

When β-amylase is applied on starch it cleaves the α(1→4) linkages of the polymer; reduces the intermolecular association of the straight chain portion of amylose; and thus results in the

production of maltose and the inhibition of retrogradation. The resulting maltose has a low level of coloring, a mild-type sweetness and nonhygroscopic nature. As a result of which maltose is used as a sweetener in confectionery and candy. Its high moisture retention capacity and resistance to crystallization, coupled with the anti-retrograding effect of β-amylase, makes maltose suitable to improve the softness and the shelf-life of starch-derived foods. In this application, however, contamination of the enzyme preparation even with a very small quantity of α-amylase causes stickiness of the product.

The industrial manufacture of maltose demands the use of microbial β-amylases than those of plant origin. The reason is that unlike β-amylases of plant origin, bacterial β-amylases: 1) can act on raw starch there by liberating maltose with a great potential for wide range of applications; 2) hydrolyze starch at higher temperatures because of their thermostability; 3) do not require allergen-related labeling and can be utilized for gluten-free food applications; and 4) can be produced in surplus amounts with a reduced cost of production.

(iii)Manufacture of Cyclodextrins

In the manufacture of cyclodextrins α-amylases and pullulanases are used in bulk to degrade starch and produce unbranched and helical oligosaccharides of 6-8 glucose units. The helical nature of the oligosaccharides enables the reducing and non-reducing ends of each molecule to come in close proximity to each other. Eventually, cyclodextrins of α, β or γ kind will be formed following the transglycolytic activity of CGTases between the ends. The OH groups of cyclodextrins are located on the surface of the rings thereby making the molecules hydrophilic externally and hydrophobic internally. The hydrophobic interior can easily form inclusion complexes with hydrophobic compounds of adequate size and structure. This property makes cyclodextrins suitable for many applications in the food; cosmetics and pharmaceutical industries where they can capture undesirable taste or odor stabilize volatile compounds and increase the solubility of hydrophobic substances in water. Therefore, cyclodextrins are used for debittering of citrus juices, protecting lipids against oxidation and removing cholesterol from eggs (Fujinamia and Fujisawa; 2010).

3.5.2. Textile desizing

In textile industry, starch is becoming a very attractive sizing agent as it is relatively cheaper, highly abundant and easily removable or desizable from fabric. In textile weaving, gelatinized starch is applied to the yarn before fabric production to: (i) strengthen the warp thread and prevent it from breaking during weaving process; (ii) prevent the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of the string; and (iii) ensure fast production. After weaving the cloth the starch is removed and the cloth goes to scouring and dyeing. Starch is desized by the application of α-amylases without attacking the fibers of the cloth (Souza and Magalhães, 2010).

3.5.3. Paper sizing

Starch is used for coating paper in paper industry. The coating treatment serves to improve the writing quality and erasibility of the paper as the starch sufficiently smoothen its surface as well as enhances its stiffness and strength. The viscosity of natural starch, however, is too high for paper sizing. Interestingly, α-amylases are employed to resolve this bottle neck as they are able to partially degrade (liquefy) the starch polymer and produce starch of reduced viscosity. The resulting liquefact is used for sizing the paper (Guptal *et al.,* 2003).

3.5.4. Detergent additive

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. Amylases are the second most important type of enzymes, next to proteases, used in the formulation of enzymatic detergents. About 90% of liquid detergents contain amylases (Hmidet *et al.,* 2009). Several factors pertaining to amylases could be accounted for this high demand. These include: (i) their ability to maintain stability and activity by resisting extreme detergent conditions such as lower temperatures (at which raw starch is hardly soluble in water), alkaline pH, and oxidative conditions; (ii) their ability to withstand surfactants, metal ion chelating agents and proteolytic degradation from proteases; and (iii) their catalytic products are environmentally friendly (Nielsen and Borchert, 2000). The role of amylases in laundary and automatic dishwashing is to degrade the residues of starchy foods to dextrins and other smaller

oligosaccharides. In addition to that particulate matter that would be attracted and adhered to starch, and thus to the cloth or porcelain, can be avoided. This ensures whiteness benefit.

3.5.5. Bioethanol production

Since the past three decades the ever escalating price of crude oil has become the burning agenda worldwide. This has triggered a paradigm shift from crude oil-energy dependence to bioethanol production from renewable resources such as starch from non-crop substrates and agricultural byproducts (Robertson *et al.,* 2006). The starch obtained can be processed by a single-step or two-step method to produce ethanol. In the two-step method starch is sequentially hydrolyzed under high temperature gelatination, liquefaction, and sacchsarification processes to generate fermentable sugar. Subsequently, the sugar is fermented by *Saccharomyces cerevisiae* to produce ethanol (Kirk *et al.*, 2002). Because it requires high temperature operating conditions this method remains energy deficit. The single-step method, however, involves the direct utilization of starch by wild type or genetically engineered microorganism(s) to produce ethanol under relatively economical and simpler conditions. The use of extremophiles or their GMO equivalents with a number of desired genes encoding different amylases including α-amylase, β-amylase, GA and pullulanase may potentially fill the gap in question (Pandy *et al.,* 2000).

3.5.6. Baking

Thermostable amylases, particularly α-, β- and γ-amylases, play vital roles in the baking industry. α-Amylases are natural components of flour. However, their content in flour depends on the climatic conditions during ripening and harvesting. When the weather is very humid the grain starts to germinate and the content of amylolytic enzymes is too high for the preparation of good quality bakery goods. In contrast, the flour obtained from cereals cultivated in a hot and dry climate often has very low α-amylase content and its deficit needs to be supplemented with microbial sources. The α-, β- and γ-amylases have different but complementary functions during the bread making process provided that they are mixed in appropriate proportions (Van der Maarel *et al.,* 2002).

The α-amylases break down polymers of starch into dextrins which contribute to the browning of the crust, improvement of the flavor of bread, retardation of staling, enhancement of moisture

retention capacity and the shelf life of baked foods. Staling, the stiffness or reduced water retention capacity and crumb elasticity of bread, is caused by starch retrogradation (Parasanna, 2005). Over dosing of α-amylases, however, results in excessive levels of dextrin formation that often causes collapse of the bread after baking. This leads to a final product with an unacceptable gummy and sticky structure.

β – and γ- Amylases are added in to chilled or frozen dough to assist the conversion of starch and dextrins in to fermentable sugars. This provides yeast with sufficient sugars to act up on and grow fast thereby enhancing the rate of fermentation and reducing the viscosity of the dough. Subsequently, the dough rises and the crumb volume of the bread increases due to enough amount of carbon dioxide released during fermentation. Besides that the sweetness of the bread increases and the toasting qualities improved due to the sugars generated (Souza and Magalhães, 2010).

3.5.7. Medicinal and clinical values

There are several processes in the medicinal and clinical areas that involve the application of amylases. For example, amylases are being used as a basic component of stable, liquid reagents for clinical use, and as a biosensor for the biochemical analysis of cyclodextrins (Becks, *et al.,* 1995; Menzel *et al*., 1998), sugar syrups and higher oligosaccharides (Giri *et al*., 1990; Pandey *et al*.,2000; Cherry *et al.,* 2004).

3.5.8. Miscellaneous applications

On top of the ones discussed above amylases also play vital role in many other sectors. For example, treatment of starchy wastes with amylases not only produces valuable and renewable starchy substrate for biomass production but also reduces the disposable solid content of sludge. The starchy substrate could be used directly or need to be hydrolyzed to simple sugars by amylases alone or in combination with acids before they can be assimilated. Moreover, amylases are employed to supplement the diastatic activity of flour and to improve digestibility of some of the animal feed ingredients. Beyond that amylases have been used in the commercial manufacture of lactic acid from hydrolytic products of starch and clarification of haze formed in beer or fruit juices (Kokab *et al*., 2003; Coutoa and Sanroman, 2006; Ribeiro *et al.,* 2010).

4. Objectives

4.1. General objective

- ❖ To produce an alkaline amylase from alkaliphiles and characterization of the prospective amylase using different physicochemical parameters thereby evaluating its industrial potentials.

4.2. Specific objectives

- ➢ To isolate amylase producing alkaliphilic microorganisms from Lake Chitu and screen them for high productivity of amylase as well as high thermal activity of the enzyme.
- ➢ To optimize cultivation conditions for growth and amylase production.
- ➢ To determine whether different physicochemical factors such as temperature, pH, concentration of NaCl and presence and absence of $CaCl_2$ affect the activity and stability of amylase from the potent isolate.
- ➢ To evaluate industrial potentials of the enzyme under consideration.

5. Materials and methods

5.1. Isolation of amylolytic organisms

Microbial samples were obtained from the Bottom-mud of Lake Chitu where the depth, temperature, pH, TDS and oxygen concentration was 17 m, 22 ^{0}C, 10.3, 5.8% and 0 M, respectively. A 10 g of this sample was diluted using 20 ml of sterile distilled water. The mixture was then inoculated aseptically in to a 100 ml Erlenmeyer flask containing 80 ml of sterile liquid medium. The flask was sealed with parafilm and incubated at a temperature of 30 ^{0}C and pH of 10.3 for 48 h. Following that 1ml culture of OD value two at 600nm was serially diluted (10^{-1}-10^{-6}) with saline solution (0.85 g/l) to a final volume of 10 ml. Thereafter, 100 µl of the diluted seed culture was inoculated and spread on agar plates aseptically. The plates were placed in anaerobic jar and incubated at 30 ^{0}C for 3 days. Following that distinct colonies were picked and streaked on freshly prepared agar plates and incubated for 48 h. After successive rounds of purification pure isolates were obtained and labeled with code numbers beginning with a prefix LCBC (for designating Lake Chitu Bottom-mud Colony).

5.1.1. Media and culture conditions

Media preparation for enrichment and enzyme production was modified from Gashaw Mamo and Amare Gessessse (1999a). The percentage composition of the components of the modified media was (g/ml): peptone, 0.5; NaCl, 2.6; Na_2CO_3, 3; $NaHCO_3$, 0.3; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.02; $CaCl_2.2H_2O$, 0.02; wheat starch, 1; and trace mineral solution (v/v), 1. However, for isolating starch degrading microorganisms peptone was replaced by 0.5% $(NH_4O)_2SO_4$, and wheat starch (0.5%) was used as the sole carbon source. The trace mineral solution was composed of (g/l): H_3BO_3, 30; $ZnSO_4.7H_2O$, 10; $NaMO_4.2H_2O$, 3; $MnCl_2.4H_2O$, 3; $NiCl_2.6H_2O$, 2; $CoCl_2.6H_20$, 10; and $CuCl_2.2H_2O$, 1.0. It is prepared by filter sterilization using Whatman filter paper with a pore diameter of 0.2µm. For solid media 2% (w/v) of agar was added. Except for trace mineral solution the other components of the medium were sterilized by autoclaving at a temperature of 120 ^{0}C and pressure of 15 psi for 15 min. Components like NaCl, Na_2CO_3 and $NaHCO_3$ were sterilized by autoclaving separately and mixed aseptically to the basal medium which was cooled to 45-50 ^{0}C to avoid blackening of the final medium. The pH of

the final medium was adjusted to 10.30 after autoclaving using a sterile solution of 0.5M Na_2CO_3.

Agar medium was used for isolation, purification and preservation of isolates. A loop full of each isolate was streaked on agar plates and left to grow for 48 h. After testing the pure isolates for amylase production those which were positive for the test were further screened for maximum production of the enzyme. Then the selected isolate was preserved on agar slants and transferred to fresh medium every 3 month.

On the other hand, liquid medium was used for seed culture preparation and enzyme production. Seed culture was obtained by inoculating a loop full of isolates from a fresh agar slant in to 90ml broth in a 100ml Erlenmeyer flask and incubating it for 24 h. Enzyme production was carried out using 500ml Erlenmeyer flasks filled with 480ml broth with 2 % (v/v) seed culture of OD 2 at 600nm. Some cultivation conditions, in both the liquid and solid media were stimulated to those of the natural environmental conditions to which the organisms adapted to. In view of this the temperature of incubation and the pH of the medium was adjusted to 30 ^{0}C and 10.3, respectively. Whereas the anaerobic condition was simulated by the depth of the liquid medium and placement of agar plates in anaerobic jar together with the sealing of the containers with parafilm.

5.1.2. Screening of potent organisms for amylase production

Four hundred twenty five (425) bacterial colonies were screened for amylase production by flooding the plates with Lugol's Iodine solution (1 g iodine and 2 g of potassium iodide in 100ml of distilled H_2O) (Gashaw Mamo and Amare Gessesse, 1999a; Muluye Teka, 2006). The formation of a transparent halo around the colonies indicates the production of amylase. Isolates which were positive for amylase production were further screened for maximum productivity on the basis of the diameter of the halo around the colonies. To select the best isolate that produces amylase of high thermal activity all the isolates with a halo of relatively wider diameter were grown in liquid medium. After 48 h of incubation the culture was centrifuged at 10,000rpm for 5min under ambient temperature and atmospheric pressure. The cell free supernatant was harvested and stored at -20 ^{0}C for later use as crude enzyme. The temperature profiles (40-70 ^{0}C)

of the crude enzymes from three prospective isolates (LCBC16A,LCBC16B and LCBC 245) were compared in the presence and absence of 5mM of $CaCl_2$ following the standard enzyme assay procedure described in section 5.3 below. Further screening of potent isolates (LCBC16B and LCBC245) was made based on the temperature profile of their respective amylases for a temperature range of 30-80 ^{0}C.

5.2. Biochemical and morphological characterization of *Bacillus* sp. LCBC245.

The isolate, which was chosen as the best for producing thermostable amylase, was characterized on the basis of different biochemical and morphological parameters to tentatively identify it to the genus level. The parameters used were test for Gram, Catalase, Oxidase and Nitrate reductase reactions. Microscopic examination and colony feature characterization were also employed.

(i). Starch hydrolysis test

To determine whether Bacillus sp. LCBC245 was able to hydrolyze starch Lugol's iodine reagent was flooded on the starch-agar plates containing a three day grown. And it was left to stand for 10 min.

(ii). Gram reaction

To test the organism for Gram reaction 3% KOH was added drop by drop on a 3 day culture placed on a slide and smeared with thoothpick for 1min.The after the viscous cell content was dragged upward using the tip of the toothpick.

(iii). Nitrate reduction

As far as testing its nitrate reducing ability was concerned 10 drops of each of reagent A (8 g sulfalinic acid dissolved in 1litre of 5N acetic acid) and reagent B (6 g N,N-Dimethyl-1-naphthylamine dissolved in 1 litre of 5N acetic acid) was added in to 15 ml of culture grown in broth containing starch and $NaNO_3$ as the sole carbon and nitrogen source, respectively.

(iv). Catalase test

In view of catalase reaction there was a drop by drop addition of 3% H_2O_2 on a culture grown for three days and mounted on a slide.

(v). Oxidase test

A toothpick *c*ontaining a fresh culture at its tip was striked across Whatman filter paper which was previously soaked in an oxidase solution. The oxidase solution was perpared by dissolving 0.03 g of L(+)-Ascorbic acid and 0.03 g of N,N,N',N'-tetramethyl-p-ohenylenediamine dihydrochloide ($C_6H_{16}N_2.2HCl$) in 10ml of distilled water.

(vi). Oxygen requirement

On the perspective of its oxygen requirement the method described in section 5.4.1 below was followed.

(*vii). Microscopic examination and colony features*

Liquid culture that was grown for 48 h was examined microscopically at a magnification of 1000x in order to observe its motility, cell shape and presence or absence of spores. The color and texture of the colony were observed from a 48 h culture grown on the agar medium containing 1% starch.

5.3. Enzyme assay

Amylase activity was assayed based on the amount of reducing sugar released following the modified dinitrosalicylic acid (DNS) method (Gashaw Mamo and Amare Gessesse, 1997).The DNS reagent was composed of (g/l): phenol, 2; sodium sulfite, 0.5; sodium potassium tartarate (Rochelle salt), 200; sodium hydroxide (NaOH), 10; and dinitrosalicylic acid (DNS), 10.

The substrate was produced in such a way that a 2% soluble starch was gelatinized by heating at 90-100 ^{0}C and diluted with equal volume of 100mM glycine-NaOH buffer (pH 10) to make the final concentration of starch 1% (w/v) and that of the buffer 50mM. To this mixture a 100mM $CaCl_2$ was added with a final concentration of 5mM in the reaction mixture.

The reaction mixture, which was composed of 0.9ml of the substrate and 0.1ml of appropriately diluted crude enzyme, was filled in 20ml test tubes. After 15 min of incubation at 50 ^{0}C under water bath the reaction was terminated by adding 2ml of DNS reagent. The control used was prepared in such a way that the crude enzyme was added after the reaction was stopped by the DNS solution. The reaction mixture was then boiled for exactly 5 min in boiling water (92 ^{0}C). Finally, the test tubes were cooled to ambient temperature using a cold running water bath for

about 5 min and the optical density of the resulting colored solution was measured at 540 nm against a reagent blank for calibration. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to glucose per min per ml of enzyme used under the assay condition. It was thus calculated by the formula: *EU= [(13.08*OD*df+0.34)*10/15] U/ml/min*. The amount of reducing sugar was calculated based on glucose standard curve whose equation was *y=[(13.08*OD*DF+0.34)*1000/180] µmol/ml* with a correlation coefficient (r^2) of value 0.996, where y is the amount of glucose equivalents; OD is optical density (absorbance); and DF is the dilution factor of the enzyme. The value of DF was 10 in the standard assay method*, i.e.,* excluding enzyme dilutions made ahead of the enzyme-substrate mixing.

5.4. Optimization of culture conditions for amylase production by *Bacillus* sp. LCBC245.

Different factors affecting enzyme productivity of the isolate were optimized so as to obtain maximum enzyme production. These factors include period of incubation, carbon and nitrogen sources, and concentration of starch and NaCl. The cultivation pH, temperature and oxygen relationships were simulated to those of the natural environment of the test organism. Ones an optimum condition was determined it was used in all the succeeding experiments.

5.4.1. Effect of aerobic /anaerobic conditions

To investigate whether the test organism requires aerobic or anaerobic conditions for maximum production of amylase the organism was allowed to grow in either aerobic or anaerobic conditions while keeping all the other culture conditions at their optimum values obtained previously. The aerobic culture condition was achieved by closing the mouth of the conical flask with sterile gauze while maintaining the volume of the culture to be $2/3^{rd}$ of the volume of the flask in which the culture was growing. Whereas the anaerobic-microaerobic condition was maintained by keeping the volume of the culture to be $3/4^{th}$ of the volume of the flask used for culturing and then closing the mouth of the flask with a gauze enrolled with aluminum sheet under sterile condition. After inoculation the flask was then sealed with parafilm. In both groups of the experiment the incubation temperature and initial pH was adjusted to 30 ^{0}C and 10.3,

respectively. From 48 up to 68 h of incubation crude enzyme was harvested for every 6 h interval and assayed under standard assay conditions to obtain enzyme activity.

5.4.2. Time course of enzyme production

In order to determine the time for maximum enzyme production, in relation to cell biomass, *Bacillus* sp. LCBC245 was allowed to grow in liquid media containing 1% raw starch at a temperature of 30 ^{0}C and under stationary and anaerobic conditions for 96 h. The initial pH of the media was adjusted to 10.3 at ambient temperature. A 2ml of culture sample was drawn aseptically every 6 h beginning from 0 h and continued up to 96 h. Subsequently, the growth of the culture was measured spectrophotometrically at an absorbance of 600nm against a blank (Soni *et al.,* 2003). The final pH was also measured simultaneously. Crude enzyme was harvested and its activity was determined based on the standard assay procedure described in section 5.3 above.

5.4.3. Effect of carbon sources

The effect of carbon source on enzyme productivity was determined by growing the test organism in liquid medium (pH 10.3) in which 1% of a single carbon source was used either from chemically define or undefined carbon sources (Gashaw Mamo and Amare Gessesse, 1999c). The chemically defined carbon sources consisted of glucose, sucrose, maltose and wheat starch whereas chemically undefined carbon sources included Teff flour, barely flour, maize flour, and wheat flour. After incubating the organism at 30 ^{0}C for 48 h under anaerobic condition the crude enzyme was harvested and assayed under standard conditions to determine enzyme activity.

5.4.4. Effect of nitrogen sources

To determine the appropriate nitrogen source for maximum enzyme production LCBC245 was grown in liquid medium in which 0.5% (w/v) of a single nitrogen source was used from either of the inorganic or organic nitrogen sources. The inorganic nitrogen sources included $NaNO_3$, KNO_3, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$ and NH_4HPO_4 while the organic nitrogen sources were peptone, yeast extract, beef extract, casein, gelatin and tryptone (Aiyer, 2004). The incubation

temperature, initial pH of the media and anaerobic treatment were same as in section 5.4.2 above. At the end of 48 h incubation the crude enzyme was harvested and assayed to determine the enzyme activity.

5.4.5. Effect of concentration of NaCl

LCBC245 was allowed to grow in liquid media in which the concentration of NaCl was varying from 0-3.5M with an interval of 0.5M between successive sets of treatments in duplicate (Ashwini *et al*., 2011). After 48 h of incubation at a temperature of 30 ^{0}C the culture filtrate was harvested by centrifugation of the culture at 10,000 rpm for 5min. Subsequently, the enzyme activity was determined following the standard assay method. Growth was also measured in terms of biomass at an absorbance of 600 nm.

5.4.6. Effect of concentration of starch

To determine the effect of starch concentration on growth and amylase production the test organism was grown in liquid media, of which the initial pH was 10.3, containing either of different concentrations of wheat starch (w/v): 0, 0.5, 1, 1.5, 2, and 2.5 (Mishra and Behera, 2008). After 48 h of incubation at a temperature of 30 ^{0}C and anaerobic condition crude enzyme was harvested and enzyme activity was determined following the standard assay method. The final pH and biomass of the culture were also measured concomitantly.

5.5. Enzyme characterization

For characterizing the amylase from LCBC245 the organism was allowed to grow under optimum conditions. After 48 h of incubation crude enzyme was harvested by centrifuging the culture filtrate at 10,000rpm for 5min. Following that enzyme activity was measured based on the standard assay method described in section 5.3 above.

5.5.1. Thermostability of the amylase in the absence of Ca^{+2}

The crude enzyme was diluted 3x with 50mM glycine-NaOH buffer at pH 10 and incubated at a range of temperatures between 35-70 ^{0}C (with an interval of 5 ^{0}C) for 30 min without $CaCl_2$. A simultaneously prepared enzyme-buffer mix was stored at 4 ^{0}C for 30 min to be used as a

control. All the enzyme preparations were assayed simultaneously following the standard assay procedure. Thus, the residual activity and hence the thermostability of the enzyme was determined as follows:

$$\textit{Residual activity} = \frac{\textit{Enzyme activity obtained after incubation at a specific T treatment}}{\textit{The original enzyme activity at 4 } ^0C} * 100\%.$$

5.5.2. Effect of temperature and Ca^{+2} on the activity & stability of amylase

To determine the effect of temperature and Ca^{+2} on the activity of amylase the crude enzyme from LCBC245 was diluted 5x with 100mM glycine-NaOH buffer (pH 10 and final concentration of 50mM) and prepared with and without 5mM $CaCl_2$. The resulting enzyme preparations were assayed in the temperature range of 35-70 ^{0}C (with an interval of 5 ^{0}C) and relative activities were computed for each pair of treatments following the formula:

$$\textit{Relative activity (RA)} = \frac{\textit{Individual enzyme activity}}{\textit{The highest enzyme activity in the block}} * 100\%$$

The effect of temperature and Ca^{+2} on the stability of amylase was evaluated as follows. The crude enzyme from the test organism was diluted 3x with 100mM glycine-NaOH buffer (pH 10 and final concentration of 50mM) and treated with and without 5mM $CaCl_2$. Such enzyme preparations were incubated at a temperature of 50, 55 and 60 ^{0}C for 60min. Following that 100µl of the enzyme solution was taken in every 10 min interval from each treatment pairs and was immediately mixed with a freshly prepared substrate. Enzyme preparations from with and without $CaCl_2$ treatment groups were filled in eppendroffs and stored at 4 ^{0}C to serve as controls. Finally the residual activity was calculated following the standard assay method.

5.5.3. Effect of pH on the activity and stability of amylase

So as to evaluate the pH profile of the amylase from LCBC245 the crude enzyme was diluted 5x with distilled water and assayed in three buffer systems of pH 6-11 and final concentration 50mM. The buffers with their corresponding pH range were: Na-Phospate buffer, 6-8; Tris-HCl buffer, 7.5-9; and Glycine-NaOH buffer, 8.5-11 with an interval of 0.5 in all pH ranges (Gashaw

Mamo and Amare Gessesse, 1999b). The relative activity of the enzyme was determined based on standard assay procedure.

The effect of pH on the stability of the amylase was determined after the crude enzyme was diluted 3x in three buffer systems (each with a final concentration of 50mM): Na-Phospate buffer (pH 6-8), Tris-HCl buffer (pH 7.5-9) and Glycine-NaOH buffer (pH 8.5-11) with an interval of 0.5 in all pH ranges. Following the incubation of the enzyme at 40 ^{0}C for 1 h under water bath assaying was carried out under standard conditions and thus relative residual activity was calculated to determine the stability of amylase as below:

$$\textit{Relative residual activity (RRA)} = \frac{\textit{Individual residual activity}*100\%}{\textit{The highest residual activity}}$$

5.5.4. Effect of NaCl on the activity of amylase

The effect of NaCl on amylase from LCBC245 was evaluated after the crude enzyme was mixed with NaCl in a range of concentrations (0-2M) with an interval of 0.5 and assayed under standard conditions (Aygan *et al.,* 2008; Carvalho *et al.,* 2008). After measuring activity spectrometrically at an absorbance of 540nm the effect of ionic strength on the enzyme was determined.

5.5.5. Time course of starch hydrolysis

The amount of reducing sugar released from the hydrolysis of wheat starch by amylase was quantified as a function of time following the method used by Gashaw Mamo and Amare Gessesse (1999b) with some modifications. A 10 g of wheat starch was dissolved in 10ml distilled H_2O and 4.5ml 1M NaOH and mixed thoroughly using a glass rod. After 30 min of gelatinization of the starch solution 4.5ml 1M HCl, 0.1g Na_2CO_3 and 20ml of crude enzyme were added sequentially to it. Next to that the reaction mixture, with an initial pH of 10.3, was incubated at 60 ^{0}C for 10 h with shaking every 30min interval. A1ml of sample was taken every 1h starting from zero time and was centrifuged at 10,000rmp for 5min. The cell free supernatant was harvested and served as a source of reducing sugar released from enzymatic hydrolysis of the starch. The supernatant was diluted to 25x with distilled H_2O to a final volume of 1ml and assayed under standard conditions to provide activity. Finally, the amount of reducing sugar

released and the percentage of starch hydrolysis were calculated based on glucose standard curve and the following formulas.

1) *Y=[(13.08*OD*DF)+0.34] mg/ml*
2) *Y=[(13.08*OD*DF+0.34)*1000/180] µmol/ml*
3) *% Starch hydrolyzed=*$\frac{\text{Mass of reducing sugar equivalents*total vol. of reaction mixture*100\%}}{\text{The original mass of starch substrate (g)}}$

Where, Y=the amount of glucose equivalents liberated from starch hydrolysis

OD=Optical density measured at 540nm

DF=25x, the dilution factor of the released glucose equivalent.

6. Results

6.1. Isolation and screening of organisms.

A total of 425 pure colonies were isolated and labeled with code numbers all beginning with a prefix LCBC (Lake Chitu Bottom–mud Colony). All of these isolates were tested for amylase production by flooding Lugol's iodine reagent on starch-agar medium on which the isolates grew for three days. The result showed that 21 isolates (4.94%) were found to be positive. Further screening of the 21 isolates for having a relatively wider clear zone around the colony resulted in the selection of three prospective isolates, namely, LCBC16A, LCBC16B and LCBC245. It is based on the fact that the wider the diameter of the clear zone is the higher the amylase is produced by the organism provided that the thickness of the media kept about 0.75 cm. The clear zone formed around LCBC245 bacterial colonies is depicted in figure-1 below.

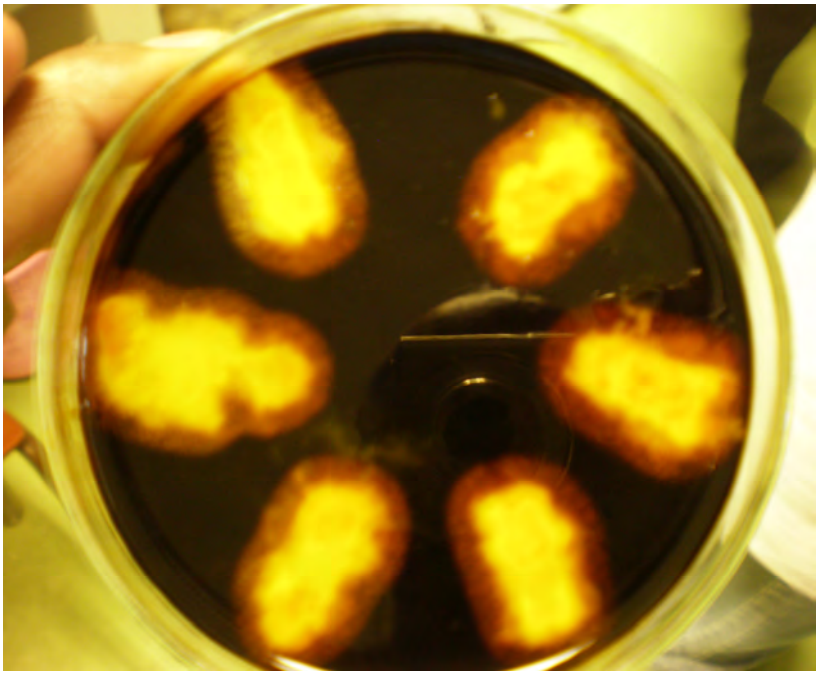

Fig.1.Halo formed around *Bacillus* sp. LCBC245 bacterial colonies after flooding them with Lugol's iodine reagent.

The test for the thermal activity and calcium requirement of amylases from these isolates revealed a number of differential features of the enzymes (Fig.2). It has been observed that the amylases from LCBC16A and LCBC16B were found to have higher relative activities in a range of temperatures (40-70 ^{0}C) in the presence of 5mM Ca^{+2} than that in the absence of 5mM Ca^{+2}. However, the trend was different for the amylase from LCBC245 as it showed higher relative activities at lower temperature ranges (40-50 ^{0}C) and lower relative activities at higher temperature ranges (60-70 ^{0}C) in the presence and absence of Ca^{+2}, respectively. The amylase from LCBC16A showed a maximum relative activity of 65.39% at 50 ^{0}C and 43.04% at 60 ^{0}C in the presence and absence of Ca^{+2}, respectively. At 60 ^{0}C LCBC16B showed a maximum relative activity of 83.24% and 71.37% in the presence and absence of Ca^{+2}, respectively. The relative activity of LCBC245 amylase reached a maximum of 100% and 95.09% at 60 ^{0}C in the presence (+) and absence (-) of 5mM Ca^{+2}, respectively.

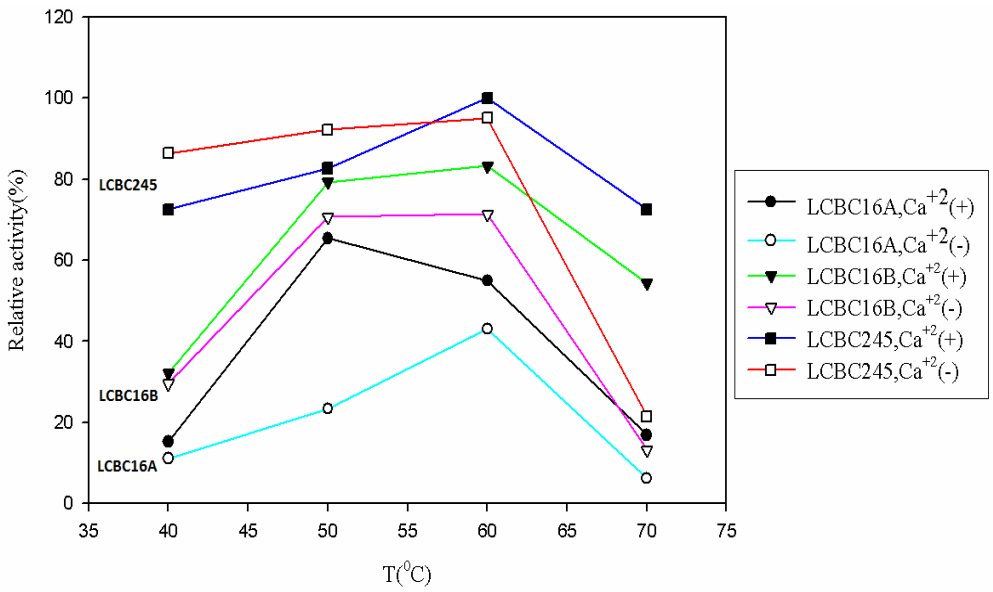

Fig.2. Effect of Ca^{+2} on the temperature profile of amylases from LCBC16A, LCBC16B and LCBC245.

To avoid any doubt about the seemingly overlapping relative activities of amylases from LCBC16B and LCBC245 a final screening was carried out based on high thermal activity in the presence of 5mM Ca^{+2}. The result vividly indicated that the amylase from LCBC245 had higher relative activities for a wide range of operating temperatures (40-65 ^{0}C) (Fig.3). Its peak value (100%) occurred at 50 ^{0}C. In contrast to that the amylase from LCBC16B was found to have lower relative activities, all falling within a narrow range of temperatures (45-65 ^{0}C), with a peak value of 91.07% at 50 ^{0}C. With all its desirable characteristics, as discussed above, the amylase from LCBC245 was chosen as the best candidate for further studies.

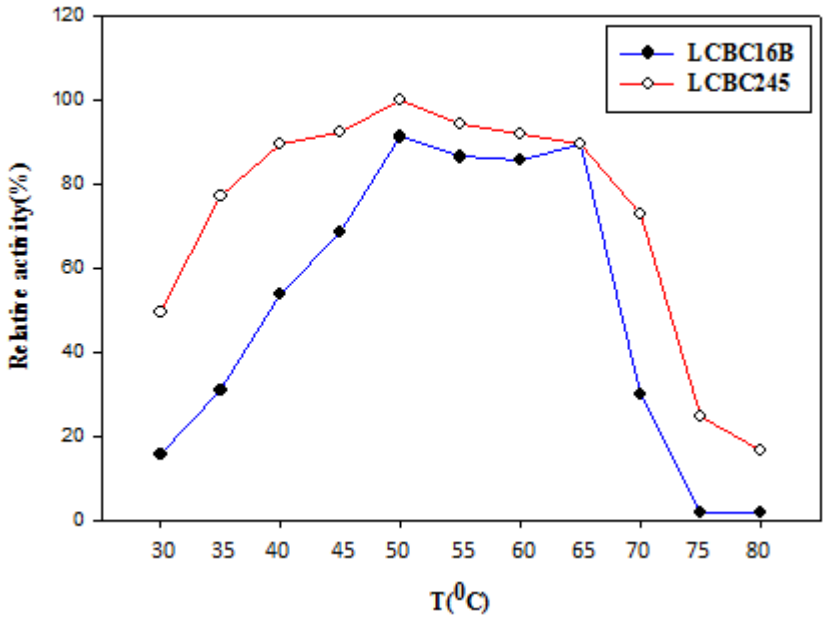

Fig.3.Temperature profile of amylases from LCBC16B and LCBC245 in the presence of 5mM Ca^{+2}.

6.2. Biochemical and morphological characterization of *Bacillus* sp. LCBC245

(i). Starch hydrolysis test

A clear zone (halo) was formed around *Bacillus* sp. LCBC245 colonies against a blue background located further from each colony. This indicates that the organism released

exracellular amylase and thus hydrolyzed the surrounding starch substrate to generate simple sugar molecules out of it and use them as carbon and energy source (Table-1).

(ii). Gram reaction

There was no strip formation when the smeared culture debris was daragged with the tip of the thoothpick. This implies that the alcohol didn't hydrolyze the cell wall completely to release DNA strands and other cell contents to be stripped out and the organism was Gram positive.

(iii). Nitrate reduction

After 15min shaking red pigmentation was observed implying that the nitrate in the medium was reduced in to nitrite as well as used as oxygen source and attainment of anoxic condition. It was, therefore, clear that the red precipitate (prontisol) was formed due to the reaction of the nitrite first with salinilic acid followed by alpaha-naphthyle amine. Hence, *Bacillus* sp. LCBC245 was found to be positve for producing nitrate reductase and thus for nitrate reduction reaction.

(iv). Catalase test

There was no evolution of gas (oxygen) bubble. Thus, the organism was unable to produce catalase that catalyzes the breakdown of the poisnous H_2O_2 molecule in to the non-poisonous oxygen and water molecules.

(v). Oxidase test

There was no development of blue colour along the striked path. It indicated that there was no phenyle-amine derivatives to be oxidized by cytochrome C to produce a bluish endophenol.

(vi). Oxygen requirement

The organism grew facultatively with a preferrential to anaerobic condition.

(*vii). Microscopic examination and morphology of colony*

The organism was found to have rod shape and with an apical spore. The colonies of the organism, on the other hand, were creamy white, submerged and circular edged.

On the basis of the above results the test organism (LCBC245) was, therefore, catagorized tentatively under the genus *Bacillus*.

Table 1. Summary of the biochemical and morphological features of *Bacillus* sp. LCBC 245

Parameters	Observation	Result
1.Starch hydrolysis test	Halo formed around the colony	Positive for amylase production
2.Gram reaction	No strip formation	Gram positive
3.Nitrate reductase test	Red precipitate (prontosil) formed	Positive for nitrate reduction
4.Oxidase test	No blue color formed	Oxidase negative
5.Catalase test	No evolution of gas bubble	Catalase negative
6.Oxygen relationships	The organism grew faster under anaerobic condition	Facultative anaerobic
7. Microscopic exam.	Motile, rod shaped, apical spore	*Bacillus* sp.
8.Colony features	submergid, creamy white in color and with circular edge.	*Bacillus* sp.

6.3. Optimization of culture conditions for amylase production by *Bacillus* sp. LCBC245.

6.3.1. Effect of aerobic /anaerobic conditions.

LCBC245 was able to generate a relatively higher amylase activity when cultivated under anaerobic condition than that of aerobic condition (Fig.4). From this result it has been observed that after 48 h of incubation the organism produced 57.95 and 39.12 (U/ml) amylase activities under anaerobic and aerobic condition, respectively. After 68 h of incubation the production of amylase activity reached a maximum of 89.17 and 85.94 (U/ml) for anaerobic and aerobic cultivation condition, respectively. Interestingly the variation in the production of amylase was insignificant around 62 h of incubation under both aerobic and anaerobic conditions. Numerically it was 83.5 (U/ml) for the aerobic and 83.24 (U/ml) for the anaerobic cultivation condition.

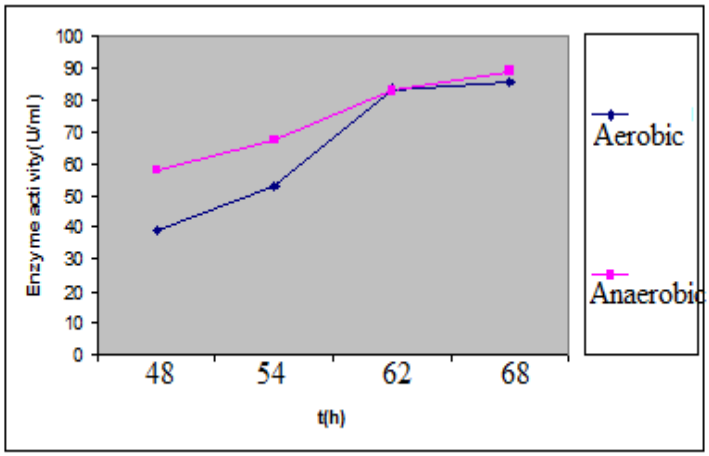

Fig.4. Patterns of enzyme production by *Bacillus* sp. LCBC245 under aerobic and anaerobic cultivation conditions.

6.3.2. Time course of enzyme production

Growth and amylase production progressively increased from 6 to 42 h of incubation, remained more or less stationary until 66 h and started to decline afterwards (Fig.5). The rate of amylase production was lagging behind when compared with that of cell division until 36 h of incubation. Between 36-48 h of incubation the rate of enzyme production was overtaking that of growth. The maximum amylase production and growth occurred after 36 h of incubation. Beyond 66 h of incubation the decrease in biomass was faster than that of amylase production. The minimum value of cell biomass (0.01) and amylase production (3.72 U/ml) occurred around 6 and 96 h of incubation, correspondingly.

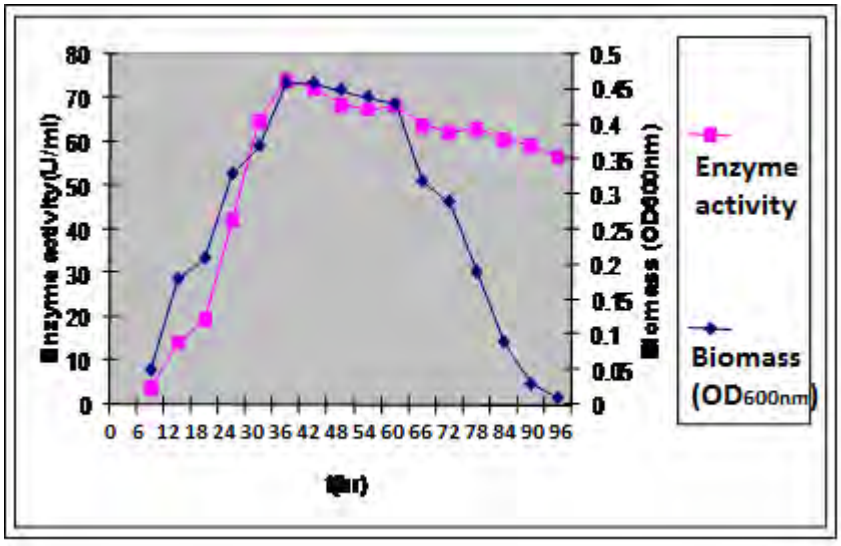

Figure 5.Growth dynamics and profile of amylase production against incubation period.

6.3.3. Effect of carbon sources

Amylase productivity by LCBC245 was affected by different carbon sources (Fig. 6). Chemically undefined carbon sources favorably influenced amylase production while chemically defined carbon sources effected in from high stimulation to inhibition. The use of maize (corn) flour yielded the greatest enzyme activity (69.12U/ml) compared to all other carbon sources used in the experiment. Barely, teff and wheat flour followed with amylase activities 63.88, 58.65 and 55.16 U/ml, respectively. Among chemically defined carbon sources starch supported the highest

amylase activity (60.39 U/ml) followed by that of maltose (29.87 U/ml). The minimum of all amylase activities (1.09 U/ml) was the one corresponding to glucose.

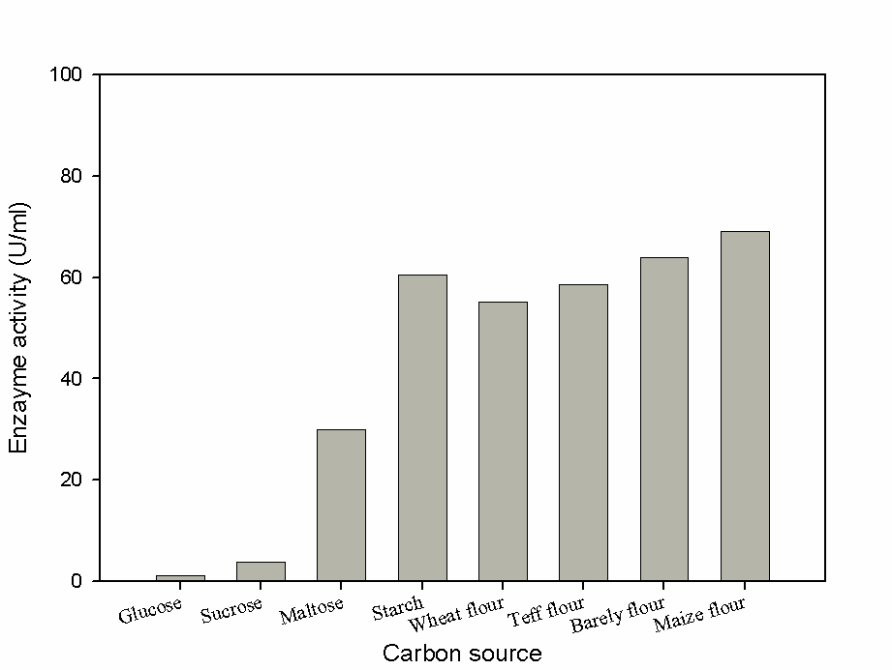

Fig.6.Effect of different carbon sources on amylase production by *Bacillus* sp. LCBC245.

6.3.4. Effect of nitrogen sources

The amount of amylase produced by *Bacillus* sp.LCBC245 was affected by both inorganic and organic nitrogen sources but with variable extents (Fig.7). Generally, higher amylase activities were obtained when the organism was provided with organic nitrogen sources than those of inorganic sources with the concentration of each nitrogen source fixed to 0.5 % (w/v). The highest of all amylase activities, 76.96 (U/ml), was obtained when peptone was used as the sole nitrogen source. It was followed by activities 65.63, 58.65, 56.91, 42.95 and 28.13 U/ml corresponding to that of tryptone, yeast extract, beef extract, casein and gelatin. On the other perspective the greatest amylase activity obtained from that of inorganic nitrogen sources, 15.92 U/ml, was almost half of the least value from among the organic sources, 28.13 U/ml. From among the inorganic sources ammonium monohydrogen phosphate [$(NH_4)_2HPO_4$] and ammonium sulfate [$(NH_4)_2SO_4$] produced a relatively higher enzyme activity of 15.92 and 14.18

U/ml, respectively. A minimum amylase activity of 10.69 (U/ml) was obtained in medium containing ammonium chloride (NH_4Cl).

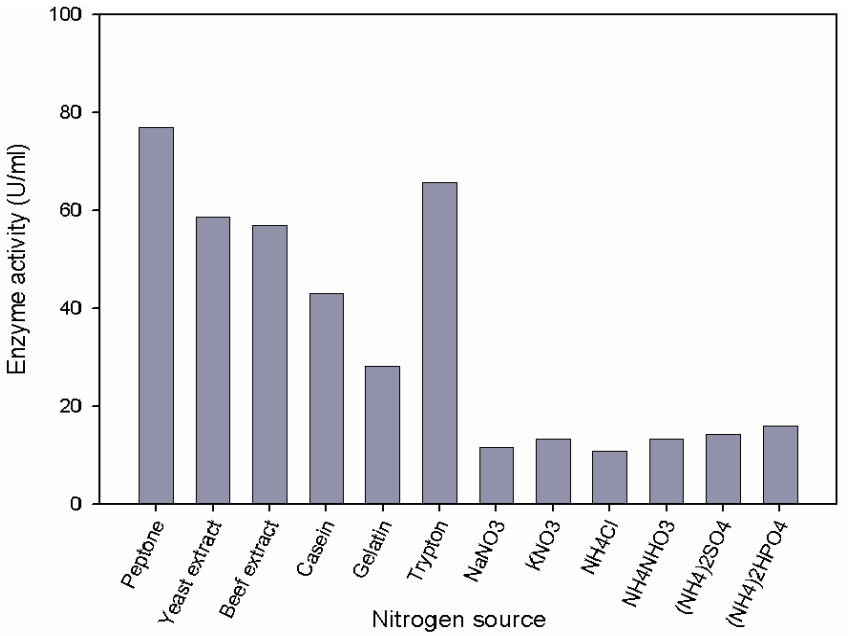

Fig.7.Effect of different nitrogen sources on amylase production by *Bacillus* sp. LCBC245.

6.3.5. Effect of concentration of NaCl

Amylase productivity by *Bacillus* sp.LCBC245 was affected by NaCl in such a way that lower concentrations of the salt enhance enzyme production. The maximum amylase activity (59.52 U/ml) and growth (0.44 at OD_{600nm}) was obtained corresponding to 0.1M NaCl. Growth and amylase productivity was successively declined corresponding to salt concentrations exceeding 1M (Fig.8). For example, enzyme productivity reached a minimum value of 1.97 U/ml at 3.5M NaCl.

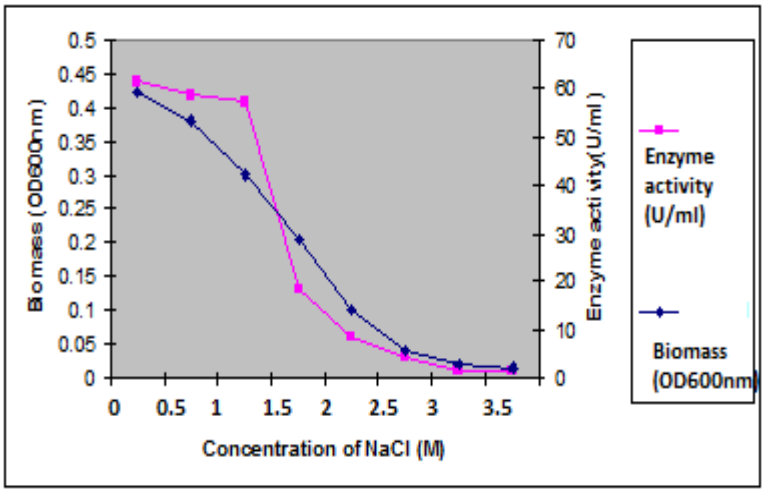

Fig.8. Effect of the concentration of NaCl on the growth and amylase productivity of LCBC245.

6.3.6. Effect of concentration of starch

Different concentrations of starch were found to affect amylase production variably. Amylase activity and biomass were successively increasing corresponding to the increase in the concentration of starch from 0-1% (w/v) and sharply declined afterwards (Table-2). When the organism was provided with a 1% starch it was able to release a pretty high amount of amylase activity (34.23 U/ml). This is almost four fold of that obtained in the absence of starch (8.94 U/ml). Talking of the final pH there was a sharp decline, from the initial pH of 10.3 down to 9.34, along with the increase in the concentration of starch from 0-1%. However, it was maintained around 8 for starch concentrations greater than 1.5%.

Table 2. Effect of the concentration of starch on growth and amylase production

Starch(%)	final PH	Biomass (OD_{600nm})	EA (U/ml)
0.0	10.28	0.16	8.94
0.5	9.94	0.39	22.89
1.0	9.34	0.43	34.23
1.5	8.61	0.28	22.89
2.0	8.04	0.18	15.92
2.5	8.02	0.07	13.31

6.4. Enzyme characterization

6.4.1. Thermostability of the amylase in the absence of Ca^{+2}

In the absence of 5mM Ca^{+2} the amylase from LCBC245 retained a residual activity of 97.43% and 92.2% corresponding to incubation temperature of 35 and 40 ^{0}C for 30 min, respectively (Fig.9). It also maintained more than 71.46% of its activity after it was incubated at temperatures below 55 ^{0}C for 30min. However, above 60 ^{0}C the enzyme lost more than 72.35% of its activity for 30 min of incubation. The loss in activity was so pronounced at 65 and 70 ^{0}C, which was more than 94.21%, for the same incubation period as lower temperatures.

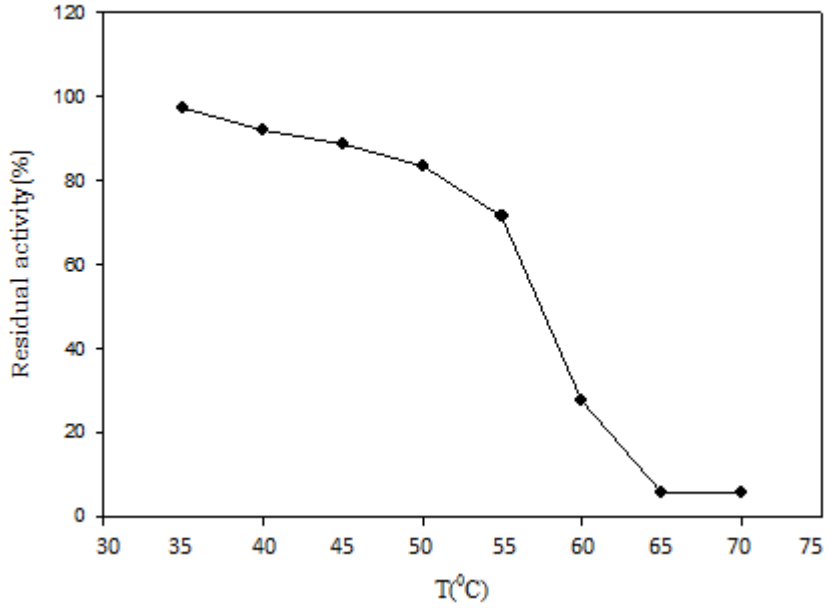

Fig.9. Effect of temperature on the stability of amylase in the absence of 5mM Ca^{+2}.

6.4.2. Effect of temperature and Ca^{+2} on the activity & stability of amylase

The temperature profile of LCBC245 amylase was found to vary greatly when incubated in a range of temperatures (35-70 ^{0}C) with and without 5mM Ca^{+2} (Fig.10a). The activities of the enzyme corresponding to the presence and absence of 5mM Ca^{+2} was found to be concurrent at incubation temperature of 45 ^{0}C below and above which the profile varied. For incubating temperatures lower than 45 ^{0}C amylase activities were relatively higher in the absence of 5mM

Ca^{+2} than that in the presence of 5mM Ca^{+2}. Conversely, for incubation temperatures greater than 45 ^{0}C amylase activities were relatively higher in the presence of 5mM Ca^{+2} than that in the absence of 5mM Ca^{+2}. A fairly high relative activities of the enzyme occurred in the range of 55-65 ^{0}C and 50 - 60 ^{0}C in the presence and absence of 5mM Ca^{+2}, respectively. The maximum relative activity of the enzyme was 100% at 55 ^{0}C and 77.59% at 60 ^{0}C in the presence and absence of 5mM Ca^{+2}, correspondingly.

The thermal stability of the amylase in relation to calcium requirement was also investigated by incubating the enzyme at 50, 55 and 60 ^{0}C for 1h with and without 5mM Ca^{+2} (Fig.10b). When the enzyme was incubated at 50 ^{0}C for 60min it retained more than 80.29% and 71.88% of its original activity in the presence and absence of 5mM Ca^{+2}, respectively. After 60min of incubation at 55 ^{0}C the amylase had a residual activity of 66.91% and 61.42% corresponding to the presence and absence of Ca^{+2} treatments. However, incubation of the enzyme at 60 ^{0}C for only 30min resulted in a rapid loss of activity and was able to maintain a reduced residual activity of 48.58% and 32.39% with and without calcium treatments, respectively.

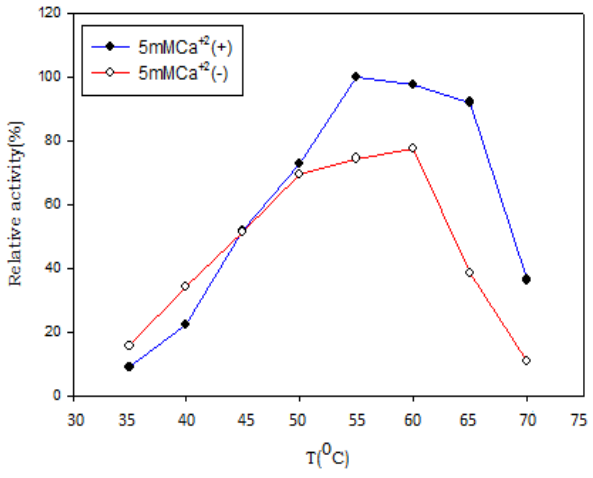

Fig.10a. Effect of temperature and Ca^{+2} on the activity of amylase

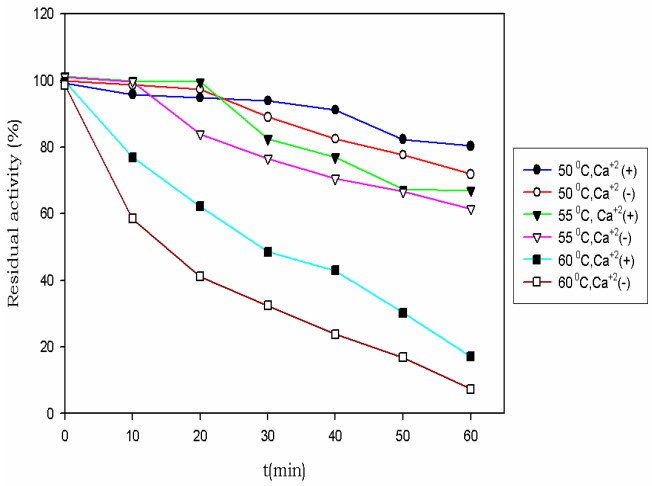

Fig.10b. Effect of temperature and Ca^{+2} on the stability of amylase

6.4.3. Effect of pH on the activity and stability of amylase

The activity of LCBC245 amylase increased along with the increase in pH and across a range of three buffers with overlapping pH values. Its relative activity was greater than 52.42% above pH 9.5 and reached a maximum of 100% at pH 10.5 in Glycine-NaOH buffer (Fig.11a). The activity of the amylase in Glycine-NaOH at pH 10.5 was two and three fold of that of Tris-HCl at pH 9 and Na-phosphate at pH 8, correspondingly. Beyond pH 10.5 the relative activity of the enzyme sharply declined.

The pH stability of the enzyme was favored by the buffer systems in increasing order of Na-phosphate, Tris-HCl and Glycine-NaOH along with the increase in their corresponding pH values (Fig.11b). After the amylase was incubated at 40 ^{0}C for 1h in Na-phosphate buffer at pH 8, Tris-HCl buffer at pH 9 and Glycine-NaOH buffer at pH 10.5 it was able to retain a relative residual activity of 79.18%, 96.16% and 100%, respectively.

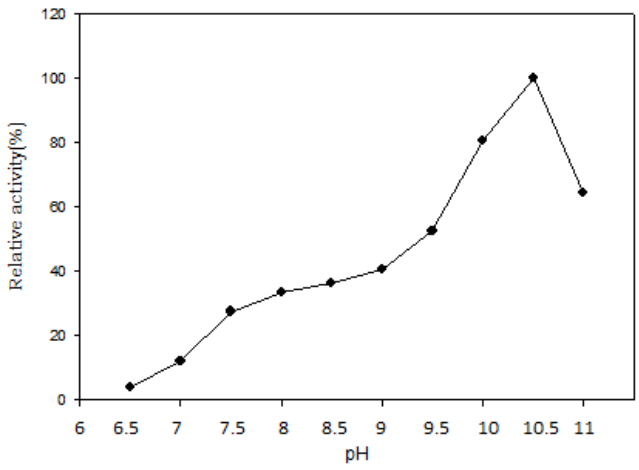

Fig.11a. Effect of pH on the activity of amylase

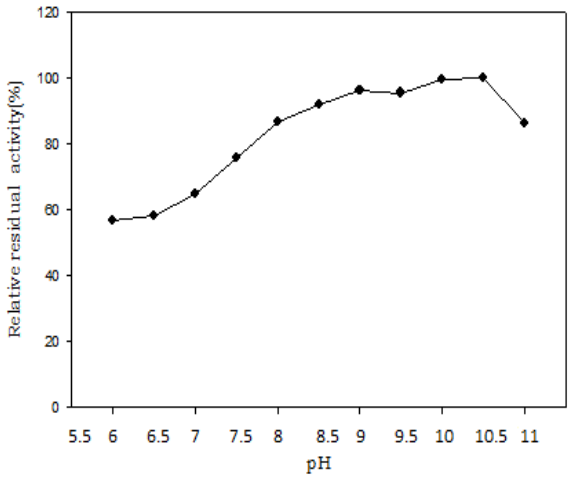

Fig.11b.Effect of pH on the stability of amylase

6.4.4. Effect of NaCl on the activity of amylase

The activity of the amylase from the test organism reached a maximum of 100% when NaCl was not added in the reaction mixture in the standard assay process. Beyond a concentration of 1M NaCl the activity of the enzyme was reduced by half of the peak value. All in all the activity of

the amylase descended almost in a linear fashion against the increase in the molar concentration of NaCl (Fig.12).

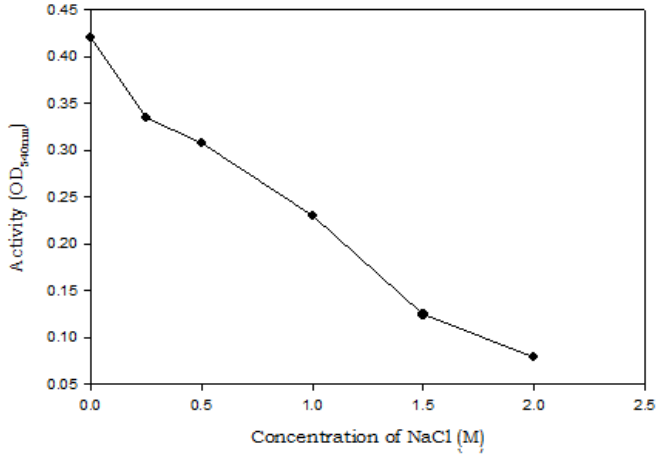

Fig.12. Effect of concentration of NaCl on amylase activity.

6.4.5. Time course of starch hydrolysis

The amylase under consideration hydrolyzed a gelatinized starch with a concentration of 25% (w/v) in a linear fashion for the first 4 h of incubation at 60 ^{0}C and pH 10 (Fig.13). Afterwards the rate of hydrolysis reached its peak and remained more or less constant thereby causing the hydrolytic profile of the starch to follow a sigmoid curve. After 10 h of incubation at 60 ^{0}C the enzyme liberated 504µmol/ml of reducing sugar equivalents corresponding to 36.31% hydrolysis of raw starch.

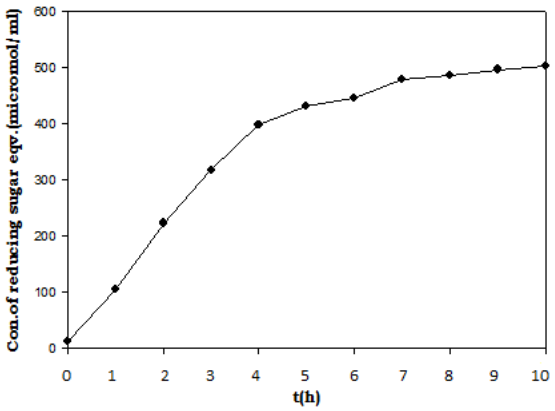

Fig.13. Profile of starch hydrolysis by LCBC 245 amylase

7. Discussion

The underlying reason for why LCBC245 was positive for amylase and nitrate reductase may be associated with the adaptation of the organism to depend on starch as carbon source and nitrate as nitrogen source, respectively. The nitrate may also serve as source of oxygen for respiration until anoxic/anaerobic condition was attained. Gram positive result indicates the failure of KOH to disrupt the thick peptidoglycan cell wall and thus absence of bare DNA strands and other contents to be dragged off. The negative result for oxidase test indicates that the organism preferably uses alternative route that surpasses Cytochrome C in the respiratory chain. Therefore, LCBC245 may able to avoid oxygen as a final electron acceptor and preferably require anaerobic condition. This is further supported by the submerged growth of its colony grown on agar plates. Hence, on the basis of the overall biochemical and cultural characterstics LCBC245 was tentatively identified as a strain of the genus *Bacillus*. Gram positive *Bacilli* are the dominant amylolytic strains in saline environments (Ghasemi, *et al.,* 2010).

Different culture conditions have been found to have a profound influence on the production of amylase (Cherry *et al.,* 2004). 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,* 2002).

After 48 h of incubation in aerobic condition the amount of amylase activity produced by LCBC245 was only 2/3rd of that of anaerobic condition (Fig.4). After 54 h of growth, however, the gap was narrowed down as the amount of dissolved oxygen depleted and the CO_2 released from the organism grown under aerobic condition accumulated and created anaerobic atmosphere thereby favoring amylase production.

It is empirically confirmed that amylase secretion usually occurs maximally during the late exponential and early stationary phases, when the cell population reached the peak, for cultures growing under stationary condition (Cordeiro *et al*., 2002; Ray *et al*., 2008). Thus, *Bacillus* sp. LCBC245 may be unusually sensitive to catabolite repression. Effective induction may not occur until the stationary phase has been reached and the readily available carbon source was depleted (Rasooli *et al.,* 2008). The decrease in the activity of the amylase harvested from 36-96 h of

incubation was insignificant (Fig. 5). The ability of this enzyme to maintain its activity over such a long period could be attributed to its high resistance towards physicochemical denaturants in the culture medium including the temperature, dynamics of pH and chemicals as well as the onset of proteolysis (Scandurra *et al*., 2000). From the perspective of industrial application the high resistance of the amylase towards denaturing agents coupled with the maximum production of the enzyme in such short period of incubation reduces the risk of contamination and cost of production (Gashaw Mamo and Amare Gessesse, 1999a).

Bacillus sp. LCBC245 released a high level of cell-free amylase in media containing 1% (w/v) starch and maltose from among refined carbon sources (Fig.6). These carbon sources are known to induce amylase production in the genus *Bacillus* (Prakash *et al*., 2009; Joshi, 2011). The organism produced a minimum amount of amylase when grown in media containing 1% (w/v) glucose and sucrose. It could be explained in such a way that the biosynthesis and secretion of the amylase may be subject to catabolic repression by readily metabolizable substrates such as glucose (Teodoro and Martins; 2000; Muluye Teka, 2006; Asgher *et al.,* 2007) and sucrose (Aiyer, 2004; Suman and Ramesh; 2010). Among undefined carbon sources maize and barely flour, both at 1% (w/v), supported maximum production of amylase by *Bacillus* sp. LCBC245 with an enzyme activity (U/ml) of 69.12 for the former and 63.88 for the latter. On the other hand, Gashaw Mamo and Amare Gessesse (1999c) reported that a higher level of amylase activity was produced corresponding to barely flour (36.6U/ml) than that of maize flour (30.0 U/ml) in their finding. From an economical point of view, it is interesting that LCBC245 produces a higher amount of amylase on cheaper and more easily available resources than on expensive and refined substrates.

Growth and amylase synthesis by several microorganisms has been correlated to the presence or absence of various amino acids and complex nitrogenous sources in the culture medium. *Bacillus* sp. LCBC245 produced the highest amount of amylase activity (76.96U/ml) when grown in liquid medium containing peptone as a sole nitrogen source. The next higher values were 65.63, 58.65, and 56, 91 (U/ml) corresponding to tryptone, yeast extract and beef extract (Fig.7). These results were in accordance with the reports of Aqeel and Umar (2010). It is believed that simple and organic nitrogen sources like peptone have a stimulatory effect on both the growth rate and

amylase synthesis and thereby shortening the lag phase of the culture (Teodoro and Martins; 2000). However, when the organism was grown in liquid media containing inorganic nitrogen sources amylase productivity was so reduced and reached a minimum value of 10.69 (U/ml) corresponding to that of ammonium chloride. This could be attributed to the assumption that ammonium salts may have an inhibitory effect on amylase production though that was not always the case (Saxena *et al*., 2007). The reason why casein and gelatin did not support a significant increment on enzyme secretion by *Bacillus* sp.LCBC245 was not clear. It is, however, assumed that the organism may not be proteolytic and able to degrade a relatively complex nitrogenous source.

Regarding the effect of the concentration of NaCl on the growth and amylase productivity of LCBC245 lower concentrations of the salt (0.1-1.5M) were found to favor maximum growth and enzyme production (Fig.8). This could be explained in terms of the pH homeostasis mechanism of alkaliphiles during growth. When grown under alkaline pH cells withstand the alkaline stress by increasing the inwardly directed sodium gradient and thus derive sodium–coupled substrate transport activities. This may enabled the organism grew faster and reached early stationary phase in short period and thus amylase production reached maximum values (42.08-59.52U/ml) so long as the lower concentrations of Na^+ were counter balanced with that of K^+ available in the medium. However, the higher concentrations (>1.5M) of Na^+ may outcompete that of K^+ as a result of which cells became more sensitive to cytoplasmic Na^+ toxicity. This may inhibit growth and hence reduction in the secretion of amylase (Slonczewski *et al.,* 2009; Sumrin *et al.,* 2011).

Starch concentration in the fermentation medium is a well known factor affecting growth and enzyme productivity in the genus *Bacillus*. In this study LCBC245 produced amylase maximally at starch concentration of 1% (w/v) below and above which growth and amylase production successively decreased (Table. 2). The underlying reason could be the generation of a repressor from the catabolism of starch substrate. Amylase production could proceed until the concentration of the catabolite repressor reached high enough to inhibit enzyme production and growth (Nusrat and Rahman, 2007). Contrary to this finding Suman and Ramesh (2010) reported earlier that a thermostable extracellular amylase from a *Bacillus* sp. was produced maximally at a starch concentration of 0.6% and the value declined thereafter.

In the absence of 5mM Ca^{+2} the amylase from *Bacillus* sp. LCBC245 had a residual activity higher than 71.46% after the crude enzyme was incubated at temperatures lower than 55 ^{0}C for 30 min (Fig. 9). This implies that the amylase is moderately thermostable.

The temperature profile (Fig.10a) and thermostability (Fig. 10b) of the amylase was reasonably different with respect to the presence and absence of 5mM Ca^{+2} in the reaction mixture. Therefore, it is clear that LCBC245 amylase was Ca^{+2}- dependent for both thermostability and thermoactivity. Divalent calcium cations are well known to increase both the thermostability and thermoactivity of most amylases from most *Bacillus* sp. (Aygan *et al.,* 2008; Al-Quadan *et al.,* 2009; Devi *et al*., 2010). It is a well documented fact that Ca^{+2} enhances the thermostability of bacterial amylase by binding on the surface of the protein so that it salts out the hydrophobic residues. Eventually, the enzyme adopts a compacted structure, reduced flexibility and increased T_m (Breitung *et al*.,1991; Saboury, 2002). Calcium also increases the thermoactivity of amylase by inducing the conformational stability of the catalytic and/or substrate binding sites as well as by serving as an allosteric activator (Sivaramakrishnan et *al.*, 2006). The decline in the activity and stability of the amylase beyond 65 ^{0}C can be explained due to the loss of the chemically bound calcium from the surface of the enzyme as heat energy increases.

The LCBC245 amylase was found to have an optimum pH of 10.5 indicating that the enzyme is alkaliphilic (Fig.11a). Such pH activity profile of the enzyme is in accordance with thermostable and alkaline amylases from *Bacillus ciculans* PN5 with a single pH peak value (Arikan, 2007; Joshi, 2011). The activity of the amylase declined more sharply in the alkaline side than in the acidic side of its optimum pH. This may be associated with the excessive deprotonation, triggered by Glycine-NaOH buffer at pH >10.5, of residues in the catalytic site (usually Glu) and/or on the surface of the enzyme (Behal *et al.,* 2006). This destabilizes its conformation and ultimately leads to the loss in activity.

As shown in fig.11b LCBC245 amylase was so stable at high alkaline conditions of broad pH range (8-11) in which the enzyme retained a relative residual activity of at least 86.33%. This may be taken as an advantage for enzymatic liquefaction of starch industrially at moderately alkaline condition (pH around 9) provided that the subsequent saccharification step is also catalyzed by an alkaliphilic glucoamylase. As a result it can avoid problems that would pose if

acidic–liquefaction was used including undesired byproduct formation, high cost of product recovery in the downstream processing, and reduced amount of yield. Beyond pH 10.5 the enzyme showed a gradual decline in stability. The underlying reason for this could be the excess hydroxyl ions released from the Glycine-NaOH buffer may cause unfavorable deprotonation of residues on the solvent–accessible surface of the enzyme. This could bring about conformational stress and destabilization of the 3D structure (Dubnovitsky *et al*., 2005).

The maximum enzyme activity 100% occurred in the absence of NaCl and thereafter gradually decreased and reached a minimum value of 18.57% as the concentration of the salt increased to 2M (Fig.12). Contrary to this Carvalho *et al.* (2008) reported earlier that an amylase from a thermophilic *Bacillus* sp. was able to retain a residual activity of 63.4% of its original activity after it was incubated at 45 ^{0}C for 2 h in the presence of 2M NaCl. The loss of activity of LCBC245 amylase against the increase in NaCl concentration may be accounted to the disruption of ion net works, owing to their interaction with Na^+ at the surface of the native structure thereby causing conformational stress (inhibition) and/or salting in of the enzyme.

As far as its reducing power is concerned LCBC245 amylase was able to liberate 504.25µmol/ml of reducing sugar equivalents by hydrolyzing 36.31% of a previously gelatinized starch [25 % (w/v)] after 10 h of incubation at 60 ^{0}C and pH of 10. Such reducing power indicates that the amylase is a liquefying type (El-Tayeb *et al*., 2007). The rate of hydrolysis reached more or less constant after 7 h of incubation (Fig.13). One reason may be associated with the inability of the enzyme to degrade the hydrolytic products further and increase the amount of reducing sugar products. Putting it in another way there could be formation of limit dexrins and reversion products with α(1→6) glycosidic linkages (Gashaw Mamo and Amare Gessesse, 1999b). Such bonds could resist cleavage by the enzyme (Regulapati *et al.,* 2007) thereby allowing these byproducts over accumulated along the course of incubation period. On the other perspective some hydrolytic products could inhibit the enzyme (Konsula *et al*., 2004). Loss of activity due to heat treatment (60 ^{0}C) for over 7 h may be another explanation. This property of the enzyme may be good enough to exploit it for rapid liquefaction of starch in short period in textile and detergent industries where saccharification step can be omitted to reduce cost of production.

8. Conclusion and Recommendation

In recent years, the potential of using microorganism as sources of industrially applicable enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity. Alkaline amylases of bacterial origin are of particularly significant importance as they fit the extreme physicochemical operating conditions in the industries. On this perspective LCBC245 amylase can be a potential candidate to be applied in starch liquefaction process owing to its moderate thermostability (45-55 ^{0}C) and thermal activitity (55-65 ^{0}C) as well as its high pH activity (10.5) and stability (8-11). Enhancing the thermostability and thermoactivity of the enzyme is possible by the addition of 5mM of divalent calcium ion. The induction and maximum production of the amylase using easily available carbon sources coupled with a rapid starch-hydrolyzing ability of the enzyme is of great economical advantage.

The nature and activities of LCBC245 amylase calls for further study whether the enzyme is α or β-amylase. Moreover, the enzyme should be characterized further, purified and tested for small scale production which leads to the large scale industrial applications.

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Appendix

A. Media formulations

i. Composition of media for growth and amylase production

Ingridients	Quantity (g/100ml)
NaCl	2.60
Raw starch	1.00
Peptone	0.50
yeast extract	0.50
K_2HPO_4	0.10
$MgSO_4.7H_2O$	0.02
$CaCl_2.2H_2O$	0.02
Na_2CO_3	3.00
$NaHCO_3$	0.30
Trace mineral solution(v/v)	1.00
Agar (for solid media only)	2.00

ii. Composition of trace mineral solution

Ingridients	Quantitiy (mg/ml)
H_3BO_3	30.00
$ZnSO_4.7H_2O$	10.00
$NaMo_4.2H_2O$	3.00
$MnCl_2.4H_2O$	3.00
$NiCl_2.6H_2O$	2.00
$CoCl_2.6H_2O$	10.00
$CuCl_2.2H_2O$	1.00

B. Effect of aerobic /anaerobic conditions on amylase production.

Aerobic					Anaerobic			
t(h)	EB	Mean	Net OD_{540nm}	EA (U/ml)	EB	Mean	Net OD_{540nm}	EA (U/ml)
48	0.29	0.74	0.45	39.12	0.29	0.95	0.66	57.95
56	0.39	0.99	0.60	52.72	0.38	1.15	0.77	67.55
62	0.16	1.11	0.95	83.5	0.16	1.11	0.95	83.24
68	0.16	1.14	0.98	85.94	0.16	1.18	1.02	89.17

C. Time course of amylase production.

	Amylase activity (OD_{540nm})					EA		Biomass
t (h)	EB	S1	S2	Mean	Net	(U/ml)	pH	(OD_{600nm})
0	….	….	…..	…..	……	…	10.33	ND
6	0.21	0.21	0.29	0.25	0.04	3.72	10.28	0.05
12	0.21	0.37	0.37	0.37	0.16	14.18	10.12	0.18
18	0.21	0.41	0.45	0.43	0.22	19.41	9.91	0.21
24	0.21	0.69	0.68	0.69	0.48	42.08	9.77	0.33
30	0.21	0.93	0.97	0.95	0.74	64.75	9.56	0.37
36	0.21	1.05	1.07	1.06	0.85	74.09	9.39	0.46
42	0.21	1.02	1.06	1.04	0.83	72.37	9.31	0.46
48	0.21	0.99	0.99	0.99	0.78	68.41	9.31	0.45
54	0.21	0.96	0.97	0.97	0.77	67.37	9.32	0.44
60	0.21	0.99	0.99	0.99	0.78	68.24	9.32	0.43
66	0.21	0.94	0.94	0.94	0.73	63.88	9.29	0.32
72	0.21	0.92	0.92	0.92	0.71	62.14	9.25	0.29
78	0.21	0.91	0.95	0.93	0.72	63.01	9.24	0.19
84	0.21	0.90	0.90	0.90	0.69	60.39	9.23	0.09
90	0.21	0.90	0.87	0.885	0.68	59.09	9.22	0.03
96	0.21	0.90	0.81	0.855	0.65	56.47	9.21	0.01

D. Effect of carbon sources on amylase production

C-sources	Final pH	Biomass OD_{600nm}	Amylase activity (OD_{540nm}) EB	S1	S2	Mean	Net	EA (U/ml)
Chemically defined								
Glucose	9.25	1.47	0.06	0.06	0.08	0.07	0.01	1.09
Sucrose	9.36	1.34	0.03	0.08	0.09	0.07	0.04	3.72
Maltose	9.44	1.51	0.04	0.37	0.39	0.38	0.34	29.87
Starch	9.32	1.65	0.03	0.74	0.71	0.72	0.69	60.39
Chemically undefined								
Wheat flour	9.49	0.09	0.04	0.66	0.68	0.67	0.63	55.16
Teff flour	9.35	0.11	0.02	0.69	0.69	0.69	0.67	58.65
Barely flour	9.33	0.19	0.03	0.76	0.76	0.76	0.73	63.88
Maize flour	9.34	0.21	0.02	0.82	0.80	0.81	0.79	69.12

E. Effect of nitrogen sources on amylase production

N-sources	Final pH	Biomass OD_{600nm}	EB	Amylase activity (OD_{540nm}) S1	S2	Mean	Net	EA(U/ml)
Organic N-sources								
Peptone	9.46	0.46	0.09	0.99	0.95	0.97	0.88	76.96
Yeast extract	9.44	0.34		0.75	0.77	0.76	0.67	58.65
Beef extract	9.58	0.45		0.75	0.73	0.74	0.65	56.91
Casein	9.84	0.16		0.59	0.57	0.58	0.49	42.95
Gelatin	9.97	0.14		0.41	0.41	0.41	0.32	28.13
Trypton	9.73	0.21		0.85	0.83	0.84	0.75	65.63
Inorganic N-sources								
Sodium nitrate	10.19	0.12		0.22	0.22	0.22	0.13	11.56
Potassium nitrate	10.14	0.21		0.23	0.25	0.24	0.15	13.31
Ammonium chloride	9.71	0.21		0.21	0.21	0.21	0.12	10.69
Ammonium nitrate	9.83	0.15		0.23	0.25	0.24	0.15	13.31
Ammonium sulfate	9.77	0.12		0.25	0.25	0.25	0.16	14.18
Ammonium monohydrogen phosphate	9.76	0.14		0.28	0.26	0.27	0.18	15.92

F. Effect of the concentration of NaCl on amylase production

[NaCl]		finalPH	Biomass	Amylase activity (OD_{540nm})					
(M)	%(w/v)	at RT	OD_{600nm}	EB	S1	S2	Mean	Net	EA(U/ml)
0.00	0.00	9.82	….	0.06	ND	ND	ND	ND	ND
0.10	2.59	9.63	0.44		0.73	0.75	0.74	0.68	59.52
0.50	2.93	9.31	0.42		0.68	0.66	0.67	0.61	53.42
1.00	5.85	9.35	0.41		0.54	0.54	0.54	0.48	42.08
1.50	8.78	9.71	0.13		0.39	0.39	0.39	0.33	29.00
2.00	11.70	9.82	0.06		0.21	0.23	0.22	0.16	14.18
2.50	14.63	9.73	0.03		0.11	0.13	0.12	0.06	5.46
3.00	17.55	9.66	0.01		0.09	0.09	0.09	0.03	2.84
3.50	20.48	9.55	0.01		0.08	0.08	0.08	0.02	1.97

G. Effect of concentration of starch on amylase production

Starch(%)	final PH	Biomass (OD_{600nm})	Amylase Activity (OD_{540nm})					
			EB	S1	S2	Mean	Net	EA(U/ml)
0.0	10.28	0.16	0.02	0.13	0.11	0.12	0.1	8.94
0.5	9.94	0.39	0.08	0.35	0.33	0.43	0.26	22.89
1.0	9.34	0.43	0.05	0.43	0.44	0.44	0.39	34.23
1.5	8.61	0.28	0.09	0.34	0.36	0.35	0.26	22.89
2.0	8.04	0.18	0.16	0.33	0.35	0.34	0.18	15.92
2.5	8.02	0.07	0.18	0.32	0.32	0.32	0.14	13.31

H. Thermostability of amylase in the absence of divalent calcium ion.

	Amylase activity (OD_{540nm})						
T (°C)	EB	S1	S2	Mean	Net	Resid.Act.	Loss in activity
4	0.01	1.17	1.33	1.25	1.24	100	0.00
35		1.16	1.28	1.22	1.21	97.43	2.57
40		1.16	1.14	1.15	1.14	92.20	7.80
45		1.09	1.09	1.09	1.08	88.81	11.19
50		1.02	1.07	1.05	1.04	83.6	16.40
55		0.87	0.92	0.89	0.88	71.46	28.54
60		0.32	0.38	0.35	0.34	27.65	72.35
65		0.07	0.08	0.08	0.07	5.79	94.21
70		0.07	0.08	0.08	0.07	5.63	94.37

I. Effect of temperature and Ca^{+2} on the activity and stability of LCBC245 amylase.

i) Effect of temperature and Ca^{+2} on the activity of LCBC245 amylase

		With 5mM CaCl2					Without 5mM CaCl2				
T (^{0}C)	EB	S1	S2	Mean	Net	RA(%)	S1	S2	Mean	Net	RA(%)
30	0.044	0.014	0.074	0.044	0.000	0.00	0.018	0.028	0.023	0.000	0.00
35		0.114	0.135	0.125	0.081	8.65	0.188	0.191	0.190	0.146	15.58
40		0.252	0.250	0.251	0.207	22.09	0.372	0.356	0.364	0.320	34.15
45		0.524	0.534	0.529	0.485	51.76	0.509	0.543	0.526	0.482	51.44
50		0.727	0.722	0.725	0.681	72.68	0.692	0.695	0.694	0.650	69.37
55		0.979	0.983	0.981	0.937	100.00	0.737	0.744	0.741	0.697	74.39
60		0.929	0.985	0.957	0.913	97.44	0.750	0.792	0.771	0.727	77.59
65		0.897	0.912	0.906	0.862	92.00	0.272	0.240	0.480	0.436	38.42
70		0.405	0.362	0.384	0.340	36.29	0.138	0.148	0.143	0.099	10.57
75		0.371	0.345	0.358	0.314	33.51	0.133	0.140	0.137	0.093	9.93

ii) Effect of temperature and Ca^{+2} on the stability of LCBC245 amylase

Temperature (^{0}C) = 4												
	Ca^{+2}(+)						Ca^{+2} (-)					
t (min)	EB	S1	S2	Mean	Net	Relat.Act.	EB	S1	S2	Mean	Net	Relat.Act.
0	0.004	1.091	1.098	**1.095**	1.091	100	0.002	1.096	1.106	1.101	1.099	100

50 (^{0}C)										
	Ca^{+2}(+)					Ca^{+2} (-)				
t (min)	S1	S2	Mean	Net	Resid.Act.	S1	S2	Mean	Net	Resid.Act.
0	1.088	1.082	1.086	1.082	99.2	1.099	1.098	1.099	1.097	99.80
10	1.011	1.086	1.049	1.045	95.78	1.00	1.172	1.086	1.084	98.64
20	1.010	1.066	1.038	1.034	94.78	1.13	1.008	1.069	1.069	97.27
30	1.022	1.034	1.028	1.024	93.86	0.939	1.016	0.978	0.978	88.98
40	0.974	1.022	0.998	0.994	91.11	0.872	0.940	0.906	0.906	82.44
50	0.861	0.942	0.902	0.898	82.30	0.854	0.860	0.857	0.853	77.62
60	0.827	0.933	0.880	0.876	80.29	0.648	0.900	0.774	0.790	71.88

55 (^{0}C)										
	Ca^{+2}(+)					Ca^{+2} (-)				
t (min)	S1	S2	Mean	Net	Resid.Act.	S1	S2	Mean	Net	Resid.Act.
0	1.080	1.122	1.109	1.105	101.30	1.127	1.093	1.110	1.110	101
10	1.082	1.106	1.094	1.090	99.86	1.099	1.094	1.097	1.095	99.64
20	1.088	1.091	1.090	1.086	99.54	0.864	0.984	0.924	0.922	83.89
30	0.823	0.983	0.903	0.899	82.40	0.746	0.940	0.843	0.841	76.52
40	0.822	0.878	0.850	0.846	76.95	0.724	0.830	0.777	0.775	70.52
50	0.759	0.717	0.738	0.734	67.28	0.703	0.763	0.733	0.732	66.60
60	0.749	0.712	0.736	0.730	66.91	0.666	0.688	0.677	0.675	61.42

	60 (^{0}C)									
	Ca^{+2}(+)					Ca^{+2} (-)				
t(min)	S1	S2	Mean	Net	Resid.Act.	S1	S2	Mean	Net	Resid.Act.
0	1.083	1.092	1.088	1.084	99.50	1.048	1.120	1.084	1.082	98.50
10	0.759	0.926	0.843	0.839	76.90	0.556	0.733	0.645	0.643	58.51
20	0.630	0.734	0.682	0.678	62.15	0.511	0.397	0.454	0.452	41.13
30	0.514	0.554	0.534	0.53	48.58	0.340	0.376	0.358	0.356	32.39
40	0.469	0.476	0.473	0.469	42.99	0.257	0.271	0.264	0.262	23.84
50	0.302	0.365	0.334	0.330	30.25	0.211	0.163	0.187	0.185	16.83
60	0.167	0.214	0.191	0.187	17.14	0.077	0.088	0.083	0.081	7.37

J. Effect of pH on the activity of amylase from LCBC245.

		Na-phosphate buffer					Tris-HCl buffer					Glycine-NaOH buffer				
PH	EB	S1	S2	Mean	Net	RA	S1	S2	Mean	Net	RA	S1	S2	Mean	Net	RA
6	0.003	0.004	0.002	0.003	0.000	0.00										
6.5		0.006	0.015	0.011	0.008	3.65										
7		0.010	0.048	0.029	0.026	11.87										
7.5		0.064	0.061	0.063	0.060	27.40	0.071	0.074	0.073	0.070	31.44					
8		0.066	0.062	0.064	0.061	28.31	0.074	0.076	0.075	0.072	33.39					
8.5							0.082	0.080	0.081	0.078	36.29	0.066	0.064	0.065	0.062	28.50
9							0.091	0.095	0.093	0.090	40.34	0.077	0.081	0.079	0.076	35.31
9.5												0.115	0.112	0.113	0.110	52.42
10												0.179	0.176	0.178	0.175	80.80
10.5												0.209	0.228	0.219	0.216	100.00
11												0.133	0.149	0.141	0.138	64.38

K. Effect of pH on the stability of amylase from LCBC245.

		Na-phosphate buffer					Tris-HCl buffer					Glycine-NaOH buffer				
PH	EB	S1	S2	Mean	Net	RRA	S1	S2	Mean	Net	RRA	S1	S2	Mean	Net	RRA
6	0.007	0.464	0.44	0.452	0.445	56.83										
6.5		0.466	0.462	0.464	0.457	58.37										
7		0.534	0.494	0.514	0.507	64.75										
7.5		0.566	0.53	0.548	0.541	69.09	0.608	0.593	0.601	0.594	75.86					
8		0.619	0.634	0.627	0.62	79.18	0.745	0.625	0.685	0.678	86.59					
8.5							0.711	0.694	0.703	0.696	88.89	0.63	0.653	0.641	0.634	80.97
9							0.722	0.719	0.721	0.714	96.16	0.65	0.653	0.652	0.652	89.47
9.5												0.7	0.697	0.696	0.696	95.62
10												0.73	0.726	0.726	0.726	99.72
10.5												0.73	0.725	0.728	0.728	100
11												0.69	0.68	0.683	0.683	86.33

L. Effect of NaCl on the activity of amylase from LCBC 245

$T_{incub.}$	[NaCl]		Amylase activity(OD_{540nm})					
(^0C)	(M)	%(w/v)	EB	S1	S2	Mean	Net	RA (%)
50	0.00	0.00	0.115	0.52	0.547	0.535	0.42	100.00
50	0.25	1.46		0.45	0.443	0.449	0.33	79.52
50	0.50	2.93		0.42	0.426	0.423	0.31	73.33
50	1.00	5.85		0.34	0.352	0.344	0.23	54.52
50	1.50	8.78		0.24	0.235	0.239	0.13	29.52
50	2.00	11.70		0.20	0.186	0.193	0.08	18.57

M. Starch hydrolysis profile of LCBC245 amylase

$t_{incubation}$ (h)	ER	Activity of Amylase (OD_{540nm}) S1	S2	Mean	Net OD	Concentration of reducing sugar eqvt. mg/ml	(µmol/ml)	Hydrolysis of starch(%)
0	0.115	0.153	0.153	0.153	0.038	2.298	12.766	0.92
1		0.438	0.439	0.4385	0.324	19.132	106.288	7.65
2		0.797	0.799	0.798	0.683	40.263	223.681	16.11
3		1.083	1.087	1.085	0.970	57.156	317.530	22.86
4		1.327	1.325	1.326	1.216	71.635	397.972	28.65
5		1.437	1.431	1.434	1.319	77.698	431.653	31.08
6		1.479	1.471	1.475	1.361	80.170	445.387	32.07
7		1.579	1.577	1.578	1.463	86.174	478.741	34.47
8		1.601	1.605	1.603	1.488	87.645	486.916	35.06
9		1.631	1.639	1.635	1.520	89.529	497.380	35.81
10		1.657	1.655	1.656	1.541	90.765	504.247	36.31
24		1.725	1.723	1.724	1.609	94.767	526.483	37.91

Declaration

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

Name: Ayalew Damte

Signature: _____

Date: May, 2011

This thesis has been submitted for examination with our approval as

Advisor: Amare Gessesse (Ph.D)

Signature _____

Date _____