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PRODUCTION OF BACTERIAL AMYLASE AND EVALUATION FOR STARCH HYDROLYSIS

By

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Acronyms

DNS	Dinitrosalicylic acid
µl	Micro liter
U	Unit
g	Gram
°C	Degrees Celsius
g/l	Gram per liter
h	Hour
min	Minute
ml	Milliliter
mM	Millimolar
nm	Nanometer
l	liter
pH	Hydrogen ion potential
rpm	Revolutions per minute
TLC	Thin layer chromatography
V/w	Volume/weight
W/v	Weight/volume
V/v	Volume/volume
SSF	Solid state fermentation

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Abstract

In starch Degrading industry, the first step to be preformed is gelatinization of starch granules at elevated temperature. This process tends to form a viscose suspension, thus making mixing and pumping a huge challenge. Therefore, to lower viscosity of starch paste thermo stable enzymes are added for breaking down the polysaccharides into oligosaccharides by a process known as liquefaction. Therefore, availability of these enzymes can be considered a prerequisite to start a successful starch liquefaction and saccharification industry. The main objective of this study was, therefore, to produce efficient microbial amylolytic enzymes for starch hydrolysis. One bacterial isolate designated as AAU 2107 was isolated from locally collected samples. The enzyme has an optimum activity at pH of 5.0 and 70°C. Addition of calcium in the reaction mixture was shown to stabilize the enzyme. The isolate AAU 2107 was able to grow under solid state fermentation (SSF) using wheat bran as a solid substrate. Optimum enzyme production was recorded at 32°C, and at 120 h of incubation and at a moisture content of 75%. Addition of peptone to the SSF medium as an organic nitrogen supplement enhanced enzyme production. The enzyme was used to hydrolyze a 25 % (w/v) of starch at 70°C and pH 5.0, and the resulting hydrolysate was analyzed on TLC plates. After 4 h hydrolysis, the TLC chromatogram showed release of glucose, maltose, maltotriose, and other oligosaccharides, indicating that the enzyme is endo acting alpha amylase. The results of this study show that amylase from AAU 2107 has a good potential to be used in starch liquefaction and saccharification processes. The ability of the isolate to grow and produce the enzyme under SSF using cheap agro industrial wastes could help to greatly reduce the production cost of the enzyme.

Key words/phrases: Alpha-amylase, liquefying, Ca^{2+}-dependent**,** Starch hydrolysis,

1. INTRODUCTION

Starch is a polysaccharide made of glucose molecules linked together with an alpha-D-(1-4) linkage and alpha-D-(1-6) linkages at branching points (Sajilata *et al*., 2006). Apart from its food use, starch can be used in different applications such as the paper and textile industries, pharmaceutical, adhesives, beverages, plastics and such derivatives as dextrin and nitro starch (Van der Maarel *et al.*, 2002). Commercially, starch is isolated from such agricultural crop as tubers (potato), cereals (e.g maize, sorghum, millet and rice), and root (cassava, sweet potato, arrow root) (Muazu *et al.,* 2012a). In Ethiopia agriculture is considered the backbone of the national economy. Among the common crops in Ethiopia that can serve as commercial source of starch include enset (Gebre-Mariam and Schmidt, 1996a), Ethiopian yam (Gebre-Mariam and Schmidt, 1998), godare (Adane *et al*., 2006), anchote (Nigussie *et al*., 2006), cassava (Paulos *et al*., 2009) and kottee harree (Mohammed *et al*., 2007).

Starch can be hydrolyzed both enzymatically and chemically. Chemically, starch is converted into ordinary glucose and glucose syrup by mixing wetted starch with a weak solution of hydrochloric acid and heating it under pressure (Wuttisela *et al*., 2008). Enzymatically, amylases are used to break down the glycosidic bonds of the starch (Windish *et al*., 1965). Up on hydrolysis, starch is converted to a variety of derivatives such as glucose, maltose and syrups of various dextrose levels (Aiyer, 2005).

From a commercial and environmental point of view, many of the chemical processes used in starch processing industries have inherent drawbacks (Muluye, 2006). As a result, the use of enzymes as industrial catalysts offers a better option and is gradually replacing chemical catalysts (Cherry *et al.,* 2004). At the moment, the presence of a large number of microbial amylases are almost completely replacing the chemical hydrolysis of starch in starch processing industry. On the other hand, recent advancement in biotechnology enabled the use of amylase enzymes to expand in many fields of application, including the food, fermentation, and pharmaceutical industries (de Souza and Magalhães, 2010).

Amylolytic enzymes can be obtained from microorganisms, plants, and animals (Naidu, 2013). However, at present a larger part of the commercial amylases are obtained from microorganisms, mainly from the genus *Bacillus* and actinomycetes genera like *Thermonspra* and *Thermonactinomyces*), fungi (mainly from the genera *Aspergillus* and *Penicillium*) (Naidu, 2013). The different amylases used for industrial application are alpha-amylase, beta-amylase and glucoamylase (Rodriguez *et al.,* 2006). These enzymes are used in as detergent additives, as desizing agents in the paper and textile industries, beverages, fuel ethanol production, in food fermentation, and as analytical reagents (Kirk *et al*., 2002).

In the starch processing industry, enzymes are used in the conversion of starch into high fructose corn syrup**.** Thus, for complete conversion of a starch into high glucose syrup the starch must passes through a three-step processes, namely gelatinization, liquefaction, and saccharification. In the liquefaction process the starch undergoes into soluble oligomaltodextrins. The starch slurry (30–35%) of pH 6 is mixed with α-amylase and passed through a jet cooker and kept in a temperature range (95–105 °C) for 90 min to assure the removal of lipid–starch complexes. In this process the dextrose equivalent value of starch hydrolysate syrup depends on the time of incubation and the amount of enzyme added. In this process, the drawback of using a primitive α-amylases is that they are not active at a pH below 5.9 and at the high temperatures level. Therefore, the pH of the starch slurry has to be adjusted from the natural pH 4.5 to pH 6 and calcium must be added to stabilize the enzyme (Tonkova, 2006). Therefore, there is a need to develop α-amylases having enhanced thermostability, tolerant to acidic pH values, and Ca^{2+}-independent (Kirk *et al*., 2002). The advantages of using this thermostable α-amylases in industrial processes include decrement in the risk of contamination, increased diffusion rate of starch slurry and also decrement in the cost of external cooling.

The stability of enzyme is also another limiting factor in the selection of enzymes for industrial applications due to the elevated temperature or extreme pH of many biotechnological processes. In regard to this, there is a continuing demand to improve the stability of the enzymes and thus meet the requirements set by specific applications, which become a huge interest for searching a new enzyme that are cost effective and suitable for applications. Protein engineering is also the other option in finding thermosatble enzymes (Reddy *et al*., 2003). However, our present day knowledge

and available technology in relation to protein structure and function is incomplete to use the tools of protein engineering to develop novel enzymes. Another option is, therefore, to look for better enzymes from nature. The aim of this study was, therefore, to isolate new thermostable amylase producing microbial isolates for production of the enzyme using cheap agro industrial wastes.

2. OBJECTIVES

2.1. General objective

The main objective of this study was to isolate new amylase producing microbial isolates, optimize cultivation conditions, and optimize reaction conditions for efficient starch hydrolysis.

2.2. Specific objectives

The specific objectives of the study were:

i. Isolate and select the best amylase producing microbial isolates from nature that can grow on solid state media using cheap agro industrial wastes.

ii. Optimization of cultivation conditions for growth and enzyme production under solid state fermentation

iii. Characterize the enzyme and evaluate the potential application of the selected enzyme for starch hydrolysis.

3. LITERATURE REVIEW

3.1. Starch

Starch is the main storage product of many economically important crops such as wheat, rice, maize, tapioca, cassava, sweet potato and potato (Van Der Maarel *et al*., 2002). It is found in many different plant organs mainly seeds, fruits, tubers and roots, it is used as a source of carbon and energy during periods of dormancy and re-growth of a plant (Jobling, 2004). The ability of a starch to interact with other components, particularly with water and lipids, is an important issue in food industry. Among carbohydrate polymers, starch is currently attracting attention due to its usefulness in different food products and also its use in human nutrition (Copleland *et al*., 2009). Starch has also another importance in many different industrial aspects such as paper processing and textiles manufacturing. It is also used as thickener, colloidal stabilizer, gelling agent, bulking agent and water retention agent, pharmaceuticals and biodegradable polymers (Alcázar-Alay *et al*., 2015).

Starch is accumulated in granules of an endosperm of a plant, and also deposited in layers with various amylose and amylopectin content**.** The starch granule consists of alternating semi-crystalline and amorphous layers. Starch granules have different size range (from 1 to 100 mm diameter) and shape (polygonal, spherical, lenticular), and also vary greatly with regard to content, structure and organization of the amylose and amylopectin molecules (Copleland *et al*., 2009). This diversity in size and shape of the starch granules influences starch functionality. In addition, environmental factors, such as the temperature during grain filling could have effect on both granule size and starch properties. In coldwater, the starch granule itself is insoluble which makes it relatively easy to extract starch granules from their plant source (Van Der Maarel *et al*., 2002). On the other hand, when starch is heated in the presence of excess water, it undergoes a transition phase known as gelatinization. This process occurs when water diffuses into the granule, which then swells substantially due to hydration of the amorphous phase causing loss of crystallinity and molecular order and also the leaching of amylose. This transform the starch from a semi-crystalline form to an amorphous form that is easily digestible. Similar to water, there are other solvents used to promote gelatinization (i.e., liquid ammonia, formic acid, chloroacetic acid and dimethyl

sulfoxide)**.** Gelatinization process is useful for particular processes, e.g., textile and starch liquefaction and saccharification industries.

This process also affects the rheological properties and viscosity of the starch, making the granule more accessible to enzymatic action. Subsequent cooling of concentrated colloidal starch dispersion results in the formation of an elastic gel which is called retrogradation. During this process, amylose molecules associate with other glucose units to form a double helix, while amylopectin molecules re-crystallize through association of its small chains (Alcázar-Alay *et al*., 2015).

Structurally, starch is composed of a number of monosaccharides molecules linked together with α-D-(1-4) and/or α-D-(1-6) linkages. The glycosidic bond between carbon and oxygen is stable at high pH but hydrolyzes at low pH. At the end of the polymeric chain, a latent aldehyde group is present which is known as the reducing end. The two types of glucose polymers present in starch are: amylose and amylopectin which are different in structures and properties. Amylose is a linear polymer consisting of up to 6000 glucose units and makes up ~ 30% of starch with α-1,4 glycosidic bonds and has a molecular mass of 105 to 106 g/mol. Amylopectin consists of short α-1,4 linked to linear chains of 10–60 glucose units and α-1,6 linked to side chains with 15–45 glucose units and has a molecular mass of 107 to 109 g/mol. Amylopectin is a highly branched glucan chains that makes up ~ 70% of starch. In an average it has 2 million of glucose unit, making it one of the largest molecules in nature. In general, amylose and amylopectin can be arranged in a semi-crystalline structure forming a matrix of starch granules with alternating amorphous (amylose) and crystalline (amylopectin) material (Alcázar-Alay *et al*., 2015).

3.2. Industrial applications of starch

Starch can be converted into different types of products such as, glucose syrups, fructose, maltodextrin derivatives, or cyclodextrins and starch hydrolysates (Jobling, 2004). It is also hydrolyzed into smaller oligossaccharides by alpha-amylase, which is one of the most important commercial enzyme processes in all the industrial processes such as in food, detergents, textiles and in paper industry, for the hydrolysis of starch (Gupta *et al.,* 2003)**.**

3.3. Starch hydrolyzing enzymes

Starch is enzymatically hydrolyzed by a group of enzymes collectively known as amylases and break into a diverse range of product that included dextrins, oligosaccharides, the disaccharide maltose, and the monosaccharide glucose (Dhanya *et al*., 2009). Both alpha-1, 4-glycosidic and alpha-1, 6- glycosidic linkages of starch are hydrolyzed by amylolytic enzymes. Amylases are one of the most important industrial enzymes that can be used for conversion of a starch ranging from smaller dextrin to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry. Amylase enzymes also account for about 30 % of the world's enzyme production (Naidu, 2013).

3.4. Classification of amylases

Based on the complex structure of starch, different enzymes are required to depolymerize the starch to oligosaccharides and smaller sugars, such as glucose and maltose. These enzymes are simply classified into four groups: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases

(i) Endoamylases

Endoamylases catalyze the hydrolysis in a random manner in the interior of the starch molecule. They tend to cleave α,1-4 glycosidic bonds present in the inner part of the amylose or amylopectin chain, which causes the formation of linear and branched oligosaccharides of various chain lengths. The well known endo-acting enzyme is α-amylase (EC 3.2.1.1). This enzyme is found in a wide variety of microorganisms, belonging to the Archaea as well as the Bacteria (Pandey *et al.*, 2000). Based on the enzymatic action, endo-acting enzymes can be divided into two: liquefying (hydrolyses 30- 40 % of the glycosidic linkage), and saccharifying (hydrolyses 50- 60 % of the glycosidic linkage). α-amylases are also divided into two classes which are extracellular and intracellular enzymes. The majority of the enzymes are extracellular which are known for their thermostability and also show optimum pH activities from acidic to alkaline pH while the others are intracellular enzymes that are able to utilize maltodextrin (Ballschmiter *et al.*, 2006**).**

(ii) Exoamylases

Exoamylases hydrolyse α,1-4 glycosidic bonds from the non-reducing end, resulting in small and well-defined oligosaccharides. The type of exoamylases that exclusively cleave α,1-4 glycosidic bonds are β-amylase (EC 3.2.1.2) or cleave both α,1-4 and α,1-6 glycosidic bonds which are amyloglucosidase or glucoamylase (EC 3.2.1.3) and α -glucosidase (EC 3.2.1.20). In exoamylases the hydrolysates of starch are also different: some act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α -glucosidase), or maltose and β -limit dextrin (β -amylase). β-amylase and glucoamylase also tend to convert the anomeric configuration of the liberated maltose from α to β. β -amylases usually occurs in higher plants, such as barley, wheat, sweet potatoes and soybeans. These enzymes are also not stable at temperatures above 60°C (Pandey *et al*., 2000).

Glucoamylases on the other hand have the ability to carry out almost complete degradation of starch into glucose (saccharification). At concentrations of glucose in reaction media exceeding 30–35% the glucoamylases can catalyze the reverse reactions forming maltose, isomaltose and other byproducts thereby decreasing the final yield of the process. Glucoamylases are also unstable at temperatures above 60°C and active at pH 4.5 to 5.0. Gulcoamylases are widely distributed enzymes among plants, animals and mesophilic micro-organisms. At the moment glucoamylases are used for the production of high-glucose syrup from polysaccharides originated from starch. The syrup produced by this method is often used by the food and related industries, in addition to the production of crystalline glucose. The last two exo-acting amylolytic are α-glucosidase which acts on short maltooligosaccharides and liberates glucose with an α-configuration. The other one are cyclodextringlycosyltransferase (EC 2.4.1.19), an enzyme that additionally has a transglycosylation activity (Van Der Maarel *et al*., 2002).

(iii) Debranching enzymes

Debranching enzymes are enzyme that exclusively hydrolyze α,1-6 glycosidic bonds which are isoamylase (EC 3.2.1.68) and pullanase type I (EC 3.2.1.41). The pullulanases enzyme are able to hydrolyze the α,1-6 glycosidic bond in pullulan and amylopectin, where asisoamylase can only hydrolyze the α,1-6 glycosidic bond in amylopectin (Bender *et al.,*1959; Israilides *et al.,* 1999).

The pullulanases are obtained from plants, *e.g.* rice, barley, oat and bean, as well as produced by mesophilic micro-organisms and are also heat-sensitive enzymes. There are different types of pullulanase enzymes that hydrolyze both α,1-4 and α,1-6 glycosidic bonds which belong to the II pullulanase group and are referred to as α -amylase–pullulanase or amylopullulanase which are thermostable enzymes, giving a products of maltotetraose, maltotrise, and maltobiose. A special enzyme belonging to this group of pullulanases is neopullulanase, which can also perform transglycosylation with the formation of a new α,1-4 or α,1-6 glycosidic bond (Takata *et al*., 1992**)**.

(iv) Transferases

Transferases are enzymes which separate the α,1-4 glycosidic bond of the donor molecule and transfer part of the donor linkeage to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC 2.4.1.19) are type of transferases enzyme which form a new α,1-4 glycosidic bond while branching enzyme (EC 2.4.1.18) forms a new α,1-6 glycosidic bond. Both amylomaltase and cyclodextrin glycosyltransferase are very similar enzymes with their aspect to enzymatic reaction. The major difference between amylomaltase and cyclodextrin glycosyltransferase is that amylomaltase performs a transglycosylation reaction resulting in a linear product while cyclodextrin glycosyltransferase gives a cyclic oligosaccharides with 6, 7, or 8 glucose residues and highly branched high molecular weight dextrins (Van Der Maarel *et al*., 2002).

3.5. Application of amylases

Currently, the majority of enzymes used in the industry are hydrolytic enzymes in action, which are used for the degradation of various natural substances. Amylases are among the most important hydrolytic enzymes for all starch based industries. These enzymes have received great deal of attention because of their technological significance and economic benefits. Amylases are used in biotechnological applications ranging from food, fermentation, textile, paper, pharmaceutical to sugar industries. Amylases can be derived from several sources, including plants, animals and microorganisms (Naidu, 2013).

Nowadays, a large number of enzymes are produced from microbial source because of their different properties towards temperature, pH and other physical factors which are appropriate for different industrial processes **(**Kirk *et al.,* 2002**)** and making them commercially available has completely replaced the chemical hydrolysis of starch in starch processing industry, which makes the starch degradation process cleaner and environmental friendly. Amylases enzymes such as :α-amylases, β-amylases and glucoamylases are one of the most commercially important enzyme used in the field of biotechnology. These enzymes are produced from several bacteria, yeasts and fungi, however, enzymes from fungal and bacterial sources have dominated the applications sectors of the industry. The α-amylase produced by *Bacillus* species have a large variety of extracellular enzymes of which are significantly important for starch degrading industries. A mesophile *Bacillus* species also offer highly thermostable and alkaline α-amylases (Naidu, 2013). On the other hand, glucoamylases produced from *Aspergillus niger* and *Rhizopus* species have a great deal of use in the production of glucose syrup from liquefied starch (Nigam and Singh, 1995).

3.5.1. Starch processing

The enzymatic conversion of starch into sugar syrups such as glucose, maltose, maltotriose, dextrins sugar, or fructose syrups forms the major products of the starch processing industry. These type of 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) and also used as carbon sources in fermentation process of beverages industries (Reddy *et al.*, 2003).

The conversion of starch by enzymatic process is done through liquefaction and saccharification process. The first step in starch processing industry is gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension. After this liquefaction process takes place with the involvement of thermostable alpha amylase that partially hydrolyze the starch granules and loss in viscosity. Lastly, saccharification process takes place with the help of glucoamylase and glucose isomerase sequentially to further hydrolyze the granules into glucose and maltose.

3.5.1.1. Manufacturing of maltose

Maltose is a naturally occurring disaccharide. It is the main component of maltose sugar syrup**.** Maltose is produced when β-amylase is applied on starch granules to hydrolyze the α(1-4) linkages of starch polymer; which result in production of maltose and the inhibition of retrogradation. Maltose is type of sugar widely used as sweetener in candy and confectionery. Agricultural products like corn, potato, sweet potato and cassava starches are the suitable plant for maltose manufacture**.** The concentration of starch slurry is adjusted to be 10-20% for production of medical grade maltose and 20-40% for food grade (Aiyer, 2005).

3.5.1.2. Manufacture of oligosaccharides mixture

Oligosaccharides mixture (Maltooligomer mixture) is type of sugar prepared from the digestion of corn starch with alpha-amylase, beta-amylase and pullulanase. Maltooligomer mix is a new commercial product with the composition of glucose, 2.2%; maltose, 37.5%; maltotriose, 46.4%; and maltotetrose and larger malto oligosaccharides, 14%. Even though, Maltooligomer mix is type of sugar with lesser sweetness tastes. It is used as main product for substituting sucrose and other saccharides. It is also used in the prevention of the sucrose crystallization in foods and keeping a certain level of hardness of the texture during storage. It is also a type of sugar with lower viscosity than corn syrup (Aiyer, 2005). Maltooligomer mix powder obtained by spray drying is highly hygroscopic. Therefore, it serves as a moisture regulator of the food with which it is mixed.

3.5.1.3. Manufacture of maltotetraose syrup

Maltotetraose syrup (G4 syrup) is type of syrup with sweetness as low as 20% of sucrose .The syrup is produced by permitting the starch to the action of maltotetraose forming amylase (G4 amylase). Maltotetraose syrup are used as replacement for sucrose to reduce the sweetness of foods without affecting their taste and flavor. It has a capacity to retain substantial amount moisture which could serves as a prevention mechanism for retrogradation of starch ingredient and also used as an improvement mechanism in food texture. G4 syrup is also used as a controller of freezing points of frozen foods. G4 syrup also have a property of glossing which can be used in a paper sizer industries (Aiyer, 2005).

3.5.1.4. Manufacture of high fructose containing syrup

High fructose containing syrups (HFCS) 42 F (Fructose content equal to 42%) is produced by enzymic isomerization of glucose with glucose isomerase. First, the starch is converted to glucose by enzyme (liquefaction) and then converted to glucose and maltose (saccharification) **(**Aiyer, 2005**).**

3.5.1.5. Manufacture of high molecular weigh branched dextrins

Branched dextrins of high molecular weight are produced from hydrolysis of corn starch with α-amylase. The extent of starch degradation depends on the type of the starch and physical properties desired (Dhanya *et al*., 2009). They are obtained as powder after chromatography and spray drying, which can be used in a powdery food as extender and a glossing agent for production rice cakes **.**

3.5.2. Textile desizing

In textile industry, the first step in creating a fabric is warping which is done by applying a starch paste on to threads of the fabric. Being a cheap and easily available product in most regions of the world, starch is used as the main sizing agent for weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of the string due to laid down wrap and quite easy to be removed. After this process the cloth under goes scouring and dyeing. Before this processes takes place, the sizing agent (starch) have be removed from the cloth, which is done by applying an alpha amylase on starch coated fabric (Aiyer, 2005).

3.5.3. Application in the paper industry

In paper manufacturing industry starch is applied as coating agent of the paper. Which serves as an improving agent of the writing quality and erasibility of the paper as the starch sufficiently smoothen its surface as well as enhances its stiffness and strength. In this application, the viscosity nature of the starch is too high for paper sizing and this can be altered by partially degrading the polymer with α-amylases in a batch or continuous processes. The use of α-amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch (Souza, 2010).

3.5.4. Detergent industry

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. In earlier times the automatic dishwashing detergents were very harsh, caused injury when ingested and were not compatible with delicate china and wooden dishware. This forced the detergent industries to search for milder and more efficient solutions. The use of amylase in detergents formulations gives a great advantage in a removal of tough stains and making a detergent that is environmentally safe. Amylase enzyme is used in 90% of all liquid detergents. Amylases are also used in a laundry detergents and automatic dishwashing to remove starch residues of starchy foods (Mukherjee *et al*., 2009). Amylases have activity at lower temperatures and alkaline pH, maintaining the necessary stability under detergent conditions. Amylase also have an oxidative stability and a good whitening agent for starchy caused stains (Kirk *et al*., 2002; Chi *et al*., 2009).

3.5.5. Beverage alcohol and fuel ethanol production

The price increment of crude oil has become a burning issue worldwide. This has triggered the world to form a systematic change in the production of fuel from crude oil production to bio ethanol production from renewable resources such as starch from non-crop substrates and agricultural byproducts (Robertson *et al.,* 2006). For the ethanol production, starch is the highly used substrate due to its low price and easily available raw material in most regions of the world (Chi *et al*., 2009). In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars. The two steps involved in bioconversion of starch into ethanol are liquefaction and saccharification process, where starch is converted into sugar using α-amylase, followed by fermentation, in which the sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* (Moraes *et al.,* 1999; Öner, 2006**)**.

3.5.6. Baking

Baking industry has made use of enzymes for hundreds of years to manufacture a wide variety of high quality products. For decades, enzymes such as malt and microbial α-amylases have been widely used in the baking industry. These enzymes are used in bread and rolls to give these products a higher volume, better color, improve flavor, a softer crumb and as an anti-staling agent.

Currently, microbial enzyme such as α-amylases have been used in baking industry to enhances the rate of fermentation and reduces the viscosity of the dough. There is also an improvement in the volume and texture of the product, which also generates additional sugar in the dough, subsequently improving the taste, crust color and toasting qualities of the bread (Van Dam and Hille, 1992**).** The other enzyme used in the bread industry are proteases, lipases, xylanases, pullulanases, pentosanases, cellulases, glucose oxidase, and lipoxygenases. Such enzymes are used in baking industries to enhance the quality of bread when wheat flour has been substituted with polished flour. Polished flour contains large amounts of dietary fiber, vitamins, minerals and antioxidant compounds, where as wheat flour mainly consists of the endosperm. (Kim *et al*., 2006)**.**

3.5.7. Other applications

Amylases are one of the most widely used enzymes in different sectors of application such as clinical, medical, and analytical chemistries (Pandey *et al.,* 2000; Cherry *et al.,* 2004). To some extent amylases are also used to improve digestibility of some of the animal feed ingredients and also used for treatment of starch based wastes (Kumar *et al.,* 1990).

4. MATERIALS AND METHODS

4.1. Isolation for amylolytic organisms

Bacterial isolates were obtained from water sample collected from hot spring water at Afdera, Afar region and soil samples collected from Addis Ababa University Arat Kilo campus. The water sample was diluted using 20 ml of sterile distilled water and 5 ml of the water sample was aseptically transferred into a 250 ml Erlenmeyer flask containing 50 ml sterile enrichment liquid medium. The flask was then incubated on shaker at 45°C-55°C. After 24 h incubation, 1ml of culture was taken and serially diluted (10^{-1}-10^{-7}) with sterile distilled water. Thereafter, 100 µl of the diluted culture was inoculated and spread on agar plates aseptically, with the same composition of enrichment media mentioned in (section 4.2) with the addition of agar. The plates were placed in an incubator and incubated at 45°C for 48 h. Isolation from soil sample was carried out as follows. First, 1g of soil was mixed with 9 ml of sterile water and then serially diluted (10^{-1}-10^{-7}) with sterile distilled water. One hundred µl of the diluted culture was spread on agar plates and incubated at 32°C. After 48 h incubation, distinct colonies were picked and streaked on freshly prepared agar plates and incubated for 48h. For fungal isolation, soil sample and plant sample were collected from different environment. First, the plant samples were cleaned by 75% ethanol for 1 min then washed by 3% hypochlorite for 3 min after this, cleaned by 96% ethanol for 30 min. At the end, cleaned three times by sterile distilled water and cut the edge of the plant and putted on solid media agar. The root of the plants were firstly cleaned by 75% ethanol for 3 min then cleaned by 3% hypochlorite for 10 min. After this, it was cleaned by 96% of ethanol for 2 min lastly cleaned three times by sterile distilled water and cut down the edges of the root of the plant and putted on solid media agar with the media composition mentioned in (section 4.2). The plates were placed in an incubator and incubated at 32 °C for 168 h. The soil sample was grounded and 1g of soil sample was serially diluted (10^{-1}-10^{-7}) with sterile distilled water. Then after, 100 µl of the diluted culture was inoculated and spread on agar plates aseptically and placed in an incubator and incubated at 30°C for 168h. After successive rounds of purification pure isolates were obtained (Sajedi *et al*., 2005; Vaseekaran *et al*., 2010).

4.2. Media and culture conditions

The media used for the growth of bacterial isolates was composed of (g/l): peptone 1.5, yeast extract 2.5; soluble starch 2.5; $MgSO_4.7H_2O$ 0.1; NaCl 2.5; K_2HPO_4 0.3; KH_2PO_4 0.2; $CaCl_2.2H_2O$ 0.05. For solid media 7.5 (g/l) agar was added. The enrichment media used for bacterial growth was composed of (g/l): soluble starch 1.25; peptone 0.5; yeast extract 0.5; $CaCl_2.2H_2O$ 0.1; $MgSO_4.7H_2O$ 0.1; K_2HPO_4 0.5; NaCl 2.5; 50 ml of soil extract. For solid media 7.5(g/l) of agar was added (Shibuya *et al*., 1986; Mamo and Gessesse, 1997). Fungal isolates were stored in a media composition same as bacterial media composition. Then media was sterilized at 121°C and 15 lb for 15 min in the autoclave. After sterilization, 0.05 (g/l) of chloramphenicol was added for Fungal media composition (Hankin and Anagnostakis, 1975). The solid media was used for isolation and purification of isolates. For bacterial isolates loop full of each isolate was streaked on agar plates and left to grow for 48 h. After screening 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. For fungal isolates the colonies were cut out and put on agar plates with the same media composition for fungal isolates and left to grow for 168 h. After screening the pure isolates form 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. Liquid growth medium was used for seed culture preparation and enzyme production from the selected strain by adding 50 ml of the broth into 250 ml capacity Erlenmeyer's flasks.

4.3. Screening for amylase productions

To select amylase producing strains all fungal and bacterial isolates were screened for amylase production by flooding the agar plates with Lugol's Iodine solution (1 g iodine and 2 g of potassium iodide in 100ml of distilled H_2O) (Peltier and Beckord, 1945). The appearance of clear zone around the colonies indicated production of amyloltic enzyme. The positive isolates for screening was grown in liquid media (50 ml broth in 250 ml capacity Erlenmeyer flasks) on a shaker incubator, 120 rpm. The bacterial isolates were grown at 32°C in shaker incubator for 48 h while the fungal isolates were grown at 30°C for 168 h. The crude enzyme of both bacterial and fungal cultures was harvested by centrifugation at 10,000 rpm for 10 min in the centrifuge. The supernatant was harvested, which was used as crude enzyme and stored under refrigerator (-22°C). The crude

enzyme from the selected bacterial isolates and fungal isolates were assayed with standard enzyme assay method described in (section 4.6) with phosphate buffers, pH 7.00 (final concentration of 50 mM) in the absence of 5mM $CaCl_2$ solution and incubated in a water bath with a temperature range of (50-85) °C with slight modification of (McTigue *et al*., 1995)

4.4. Biochemical and morphological characterization of AAU 2107

4.4.1. Gram reaction, microscopic examination and morphology of colony

To test the organism for gram reaction the organism was fixated on slide. Consequently, the slide was immersed in crystal violate solution for 1 min and it was instantly washed for 5 min by running water. Next, the slide was put in iodine containing solution for 1 min then washed by running water for 5 min. Subsequently the slide was deepen in 96% of ethanol for 30 sec and then washed away by running water. After this, the slide was immersed in saffranine for 1 min and washed by running water for 5 min. Finally, the slide was observed under microscope (Sneath, 1986).

4.4.2. Catalase test

The catalase reaction was determined by adding 3% H_2O_2 drop by drop on a liquid culture media grown for 48h and mounted on a slide and the presence or absence of bubbles or foam formation was observed.

4.5. Solid-state fermentation (SSF) medium for bacterial amylase production

SSF media was used for enzyme production for the selected isolates using wheat bran as a sole carbon source with the addition of the following nutrients g in 13ml of distilled water (%): NaCl 0.5; $CaCl_2.2H_2O$ 0.01; $MgSO_4.7H_2O$ 0.02; KH_2PO_4. 1; 10g wheat bran was moistened with above nutrient solution to a moisture level of 60% (v/w ratio) in 250 ml Erlenmeyer flasks for cultivation of the bacterial isolates. After sterilization, each SSF media was inoculated with 2 ml of overnight-cultured inoculums of bacterial isolates. They were incubated in an incubator at 32°C for 120 h. The crude enzyme from SSF media was harvested by adding 100 ml of distilled water and shaken for 1 h at 120 rpm on the shaker, then filtered using cotton sheet (Babu and Satyanarayana, 1995).

4.6. Enzyme assay

Enzyme assay was carried out using the dinitro salicylic acid (DNS) method (Bertrand *et al*., 2004). The assay mixture was composed of 1% starch dissolved in 50 mM acetate buffer, pH 5.0 (or phosphate buffer, pH 7.0). To 0.9 ml of the assay substrate, 0.1 ml of appropriately diluted enzyme was added and incubated at 70°C for 10 min in a water bath. The reaction was stopped by adding 2 ml of DNS solution followed by boiling for exactly 5 min. After cooling in a running water for about 5 min, the optical density of the resulting colored solution was measured at 540 nm against a reagent blank.

4.7. Enzyme characterization

4.7.1. Effect of pH on the activity of amylase

To determine the pH profile of the bacterial amylase enzyme, the enzyme was assayed using 2% soluble starch in 3 buffer systems pH 3.5 to 9.0 (final concentration 50mM). The buffers are acetate buffer (pH 3.5, 4.0, 4.5, 5.0, 5.5), phosphate buffer (pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and Tris buffer (pH 7.5, 8.0, 8.5 and 9.0). To determine the effect of pH on enzyme stability, the crude enzyme was diluted by acetate buffer pH 5.00 (v/v enzyme: buffer 1:10). The diluted enzyme was mixed with different buffer systems with a final concentration of 50 mM, then enzyme was assayed in the same procedure mentioned in (section 4.6) but, the enzyme was incubated at 60°C in a water bath.

4.7.2. Effect of temperature on the activity amylase enzyme with or without Ca^{2+}

To determine the effect of temperature on the activity of amylases, the crude enzyme from bacterial isolate was diluted by acetate buffer pH 5.00 (v/v enzyme: buffer 1:10) and assayed in temperature ranges of 45-85°C (within 5°C interval). The assay methods used for the bacterial enzyme was mentioned in the (section 4.6), but the substrate solution was prepared in 50 mM acetate buffer pH 5.00. Then the residual activity of the enzyme was determined by assaying the incubated enzyme as mentioned in the above. The Ca^{2+} requirement of a bacterial enzyme on the effect of temperature was determined by incubating the enzyme at a temperature range of 45-85°C (within 5°C interval) with 5 mM $CaCl_2$ solution. After incubation, the residual activity was determined (Lo *et al*., 2001).

4.7.3. The thermostability of the amylase enzyme with or without Ca^{2+}

The thermostability of bacterial amylase enzyme was determined after incubating the enzyme at temperature values of 70 and75°C for 1 h in a water bath. First, each labeled eppendorf filled with enzyme were placed in the water bath and the other eppendorf was taken out and stored in refrigerator (+4°C) at 10 min interval. After incubation, the enzyme was diluted (1:10 (v/v) ratio) with acetate buffer pH 5.00 (final concentration of 50 mM) then assayed and residual activity was determined. To determine Ca^{2+} requirement for thermal stability of the bacterial enzyme, the crude enzyme was incubated at a temperature 75°C for 1 h in water bath with 5mM $CaCl_2$

solution. After incubation, the enzyme was assayed and residual activity was determined (Egas *et al*., 1998).

4.8. Thin layer chromatographic (TLC) analysis of reaction products

To identify the type of amylase enzyme produced by bacterial isolate**.** The reaction mixture was made of 25% (w/v) raw starch mixed with 22.8 U concentrated crude enzyme from the bacterial isolate and 10.85 U of malt enzyme extracted using (section 4.10) method which was incubated at 70 and 60°C for 4 h in the water bath but the malt enzyme was included in the reaction after 3 h of incubation period. One ml of the hydrolysate was collected in a eppendorf with in 1 h interval and stored in refrigerator (4°C). Then the hydrolysate was spotted on TLC plate along with standard known sugar (glucose and maltose) solutions **(**as used by (Gashaw Mamo and Amare Gessesse, 1999a) with some modification). A one dimensional ascend was done using a solvent system (v/v) of butanol : ethanol: water (5:5:2). After a total of 4 ascends air-dry TLC plates were sprayed with 50% (v/v) methanol-H_2SO_4 mixture and heated for 5 min at about 160°C. The dark brown sugar spots appeared was identified by comparing with the standards.

4.9. Optimization of culture conditions for amylase production of AAU 2107

4.9.1. Time course of enzyme production

The maximum period for the production of bacterial enzyme was determined by growing the bacterial isolate on SSF media (section 4.5) at 32°C. The relative activity of the enzyme was measured after 24, 48, 72, 96, 120 and 144 hs, respectively. By harvesting the enzyme from cultivating flasks at a time (Babu and Satyanarayana, 1995).

4.9.2. Effect of moisture level on enzyme production

The optimum moisture level for amylase enzyme production of bacterial isolate was determined by growing bacterial isolate in SSF media at 32°C at a moisture level (v/w) of 33.3%, 50%, 60%, 66.7% and 75%. Then the enzyme was harvested after 120 h of incubation period and assayed to determine the enzyme activity of the bacterial isolate (Ramesh and Lonsane, 1990).

4.9.3. Effect of nitrogen source on enzyme production

The appropriate nitrogen source for amylase enzyme production of bacterial isolate was produced by growing the bacterial isolate in SSF media within 75% (v/w) with the following nitrogen sources: inorganic nitrogen source ($NH_4(So_4)_2$ and $NaNO_3$) and organic nitrogen source (peptone and yeast extract) and also without nitrogen source. After 120 h of incubation the crude enzyme was harvested and assayed to determine the enzyme activity (Meer and Saramma, 2004; Akcan *et al*., 2012).

4.10. Beta-amylase extraction

Beta amylase was extracted from a barely malt flour following the method of Buttimer *et al.,* 1998 with slight modification. To 0.5 g malt powder, 5 ml of acetate buffer (pH 5.0) was added and intermittently vortex mixed for 1 h. The mixture was then incubated in a refrigerator (+4°C) for 5 min followed by centrifugation at 12,000 rpm for 5 min. The supernatant was collected and kept at (+4°C) until used.

4.11. Enzyme hydrolysis of raw starch

A 2 g of raw potato starch was mixed with 6 ml acetate buffer, pH 5.0 containing 10 mM $CaCl_2$. Then 22.8 Units of concentrated crude bacterial enzyme preparation and incubated at 70°C. After 3h the reaction mixture was taken out, 10.85 U of malt enzyme extract was added, and incubated at 60°C for a further 4 h. At 1 h interval 1 ml of the hydrolysate was collected in a eppendorf and stored (+4°C) and the amount of reducing sugar was measured following the DNS method after 1:200 dilution with distilled water (Legin *et al*., 1998; Mamo and Gessesse, 1999).

4.12. Drop in starch viscosity

In order to measure the viscosity of the starch hydrolysates produced by alpha amylase, the following method was used. 3 ml of water was taken in a funnel sealing it at the bottom. It was then released to flow downward and the speed was measured in time. The water completed its flow in 9 second. Using this method the flow of the starch hydrolysates in 9 second was measured in volume.

5. RESULTS

5.1. Isolation and screening of organisms.

A total of 289 (226 bacterial and 63 fungal) isolates were screened for amylolytic activity on starch-agar plates. A total of 180 isolates (130 bacterial isolate and 50 fungal isolates) were positive for extracellular amylase production. The activity of fungal enzymes were very low compared to the bacterial enzymes. Based on this, bacterial isolates were further evaluated for the level of amylase production based on the diameter of the halo zone formed (Fig 1).

Fig. 1. Clear zone formed around bacterial colonies after flooding them with Lugol's iodine regent

To select the best amylase producing isolate(s) all the 180 positive isolates on starch agar were grown in liquid media and the relative activity of the crude enzyme was assayed at 50°C - 85°C. Four of the amylase enzymes from the bacterial isolates were found to be active at 70°C. When thermostability of the four enzymes were tested and compared, only one bacterial isolate designated as AAU 2107 produced an enzyme that was more heat stable and selected for further study.

5.2. Biochemical and morphological characterization of isolate AAU 2107.

For gram reaction the organism was found to be rod shape and a purple in color when seen under microscope. Thus, indicates that the organism was gram positive bacteria. In catalase reaction, the organism was able to produce a gas (oxygen) bubble indicating the organism was able to produce catalase enzyme. On the other hand, the colonies of the organism were yellow, creamy and circular edged.

Table1. Summary of the biochemical and morphological features of isolate AAU 2107

Parameter	Observation	Result
1.Gram reaction	Purple in color	Gram positive
2.Catalase test	Produce a gas bubble	Catalase positive
3. Microscopic exam.	Rod shape	
4.Colony features	Yellow, Creamy, Circular edged	

5.3. Properties of the Enzyme

5.3.1. Effect of pH on activity of amylase

For determination of pH profile the bacterial amylase enzyme was assayed in 3 buffer systems with a pH range of 3.5 – 9.0. Based on this, the optimum pH of the enzyme was observed at pH 5.0. The least activity of the enzyme was observed at pH 3.5 (Fig.2).

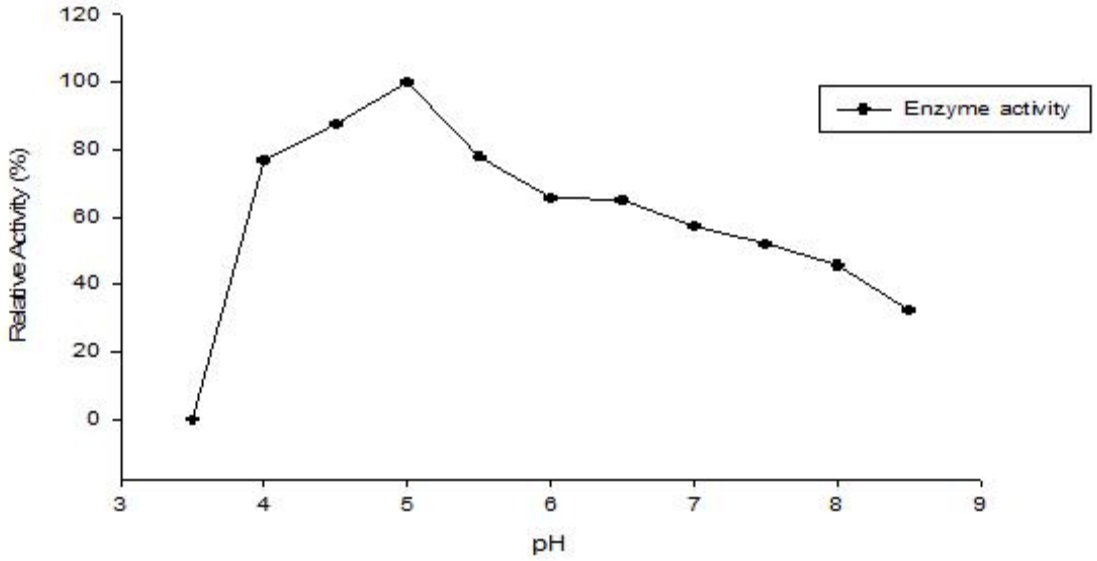


Fig. 2. Effect of pH on the activity of AAU 2107 amylase

5.3.2. Effect of temperature on activity and stability of amylase

To test the thermal activity and calcium requirement of AAU 2107 amylase, the crude enzyme was assayed in temperature ranges of 45-85°C with and without 5 mM Ca^{+2}. The optimum relative activities of the enzyme was measured in the absence of 5 mM Ca^{+2} in temperature range of 55-65°C. Maximum activity was recorded at 70°C and after 75°C the relative activity of the enzyme started to decline as shown in (Fig.3). In the presence of 5 mM Ca^{+2}, the maximum activity of the enzyme was recorded at 65°C (100%). The relative activity measured at 70°C and 75°C was 92.81% and 70.76%, respectively. This result showed that the enzyme activity in the presence of 5 mM Ca^{+2} after 70°C tend decline slowly, showing that the enzyme was more stable in the presences of 5 mM Ca^{+2} (Fig.3).

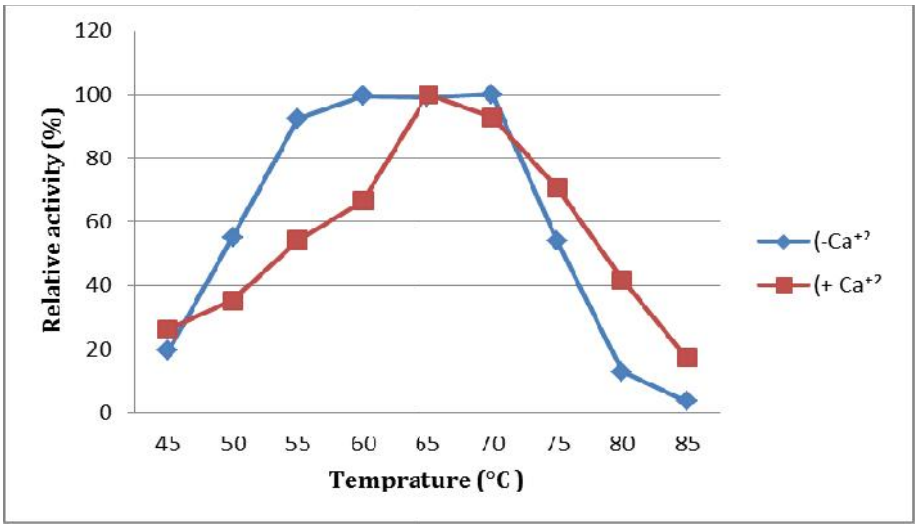


Fig. 3. Effect of temperature on the activity of AAU 2107 enzyme in the presence and absence of Ca^{+2}

The thermal stability of bacterial amylase enzyme was determined by incubating the enzyme for 60 min in the presence and absence of 5mM Ca^{+2} in the temperature values of 70°C and 75°C. In the absence of Ca^{+2} and 70°C, the enzyme retained about 24.51% of its original the enzyme lost more than 90% of its original activity within 20 min of incubation (Fig. 4).

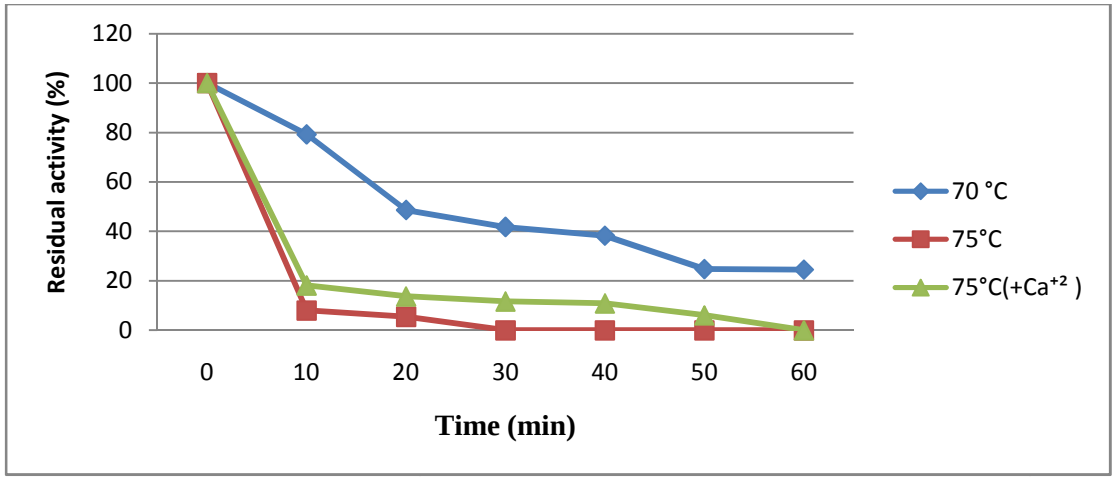
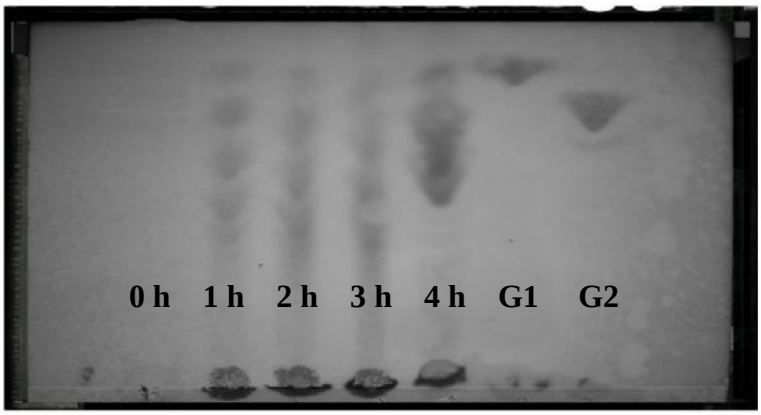


Fig. 4. The thermostability of the bacterial enzyme with or without Ca^{2+}

5.4. Thin layer chromatographic (TLC) analysis of reaction products

TLC analysis of the starch hydrolysate produced by AAU 2107 amylase showed production of glucose, maltose, maltotriose, and different types of oligosaccharides indicating that it is an endo-acting alpha-amylase (Fig. 5).



- ✓ **G1-Glucose**
- ✓ **G2-Maltose**

Fig. 5. Spots developed from starch hydrolysatese on TLC by the action of bacterial amylase

5.5. Optimization of culture conditions for amylase production

5.5.1. Time course of enzyme production

Amylase production by AAU 2107 through SSF reached to maximum production at 120 h of incubation (Fig. 6) after which enzyme production declined.

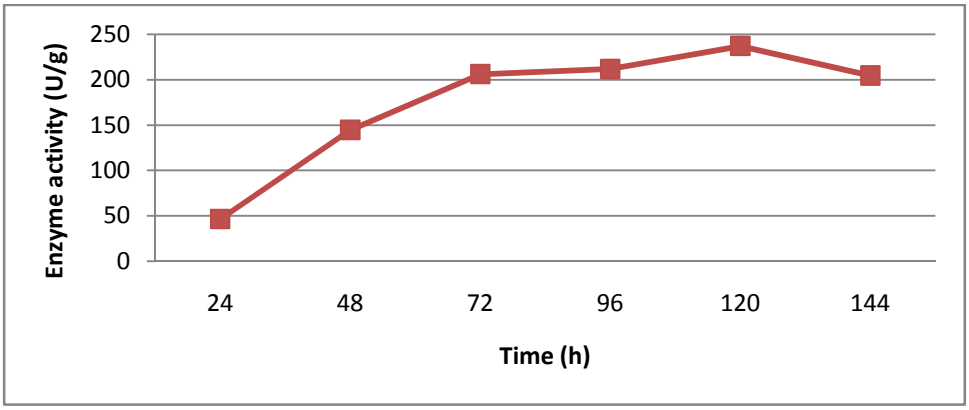


Fig. 6.Time course of enzyme production by AAU 2107

5.5.2. Effect of moisture level on enzyme production

The optimum moisture level for alpha-amylase production by AAU 2107 was observed at a moisture level of 75% (Fig.7). At lower moisture level enzyme production was low.

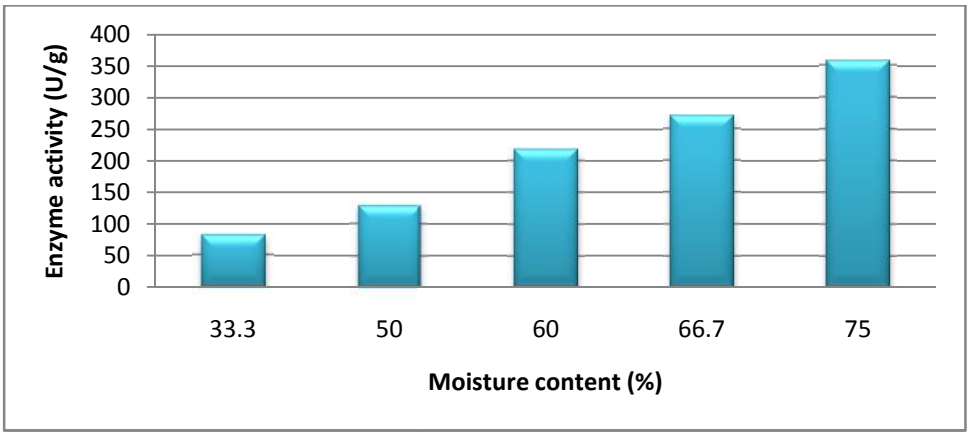


Fig. 7. The moisture level of SSF for enzyme production of AAU 2107

5.5.3. Effect of nitrogen source on enzyme production

Amylase production by isolate AAU 2107 was slightly higher in the presence of an organic nitrogen supplement peptone (Fig. 8). However, enzyme production in the presence of other nitrogen supplements or in the absence of any nitrogen supplement showed no significant difference.

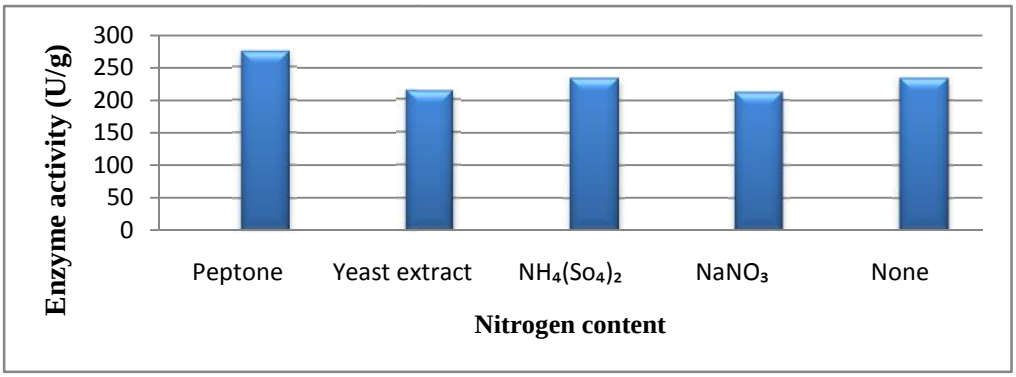


Fig. 8. Effect of nitrogen source on alpha amylase production

5.6. Enzyme hydrolysis of raw starch

A 25% (w/v) paste was hydrolyzed using 22.8 U of alpha-amylase at 70°C. After 3 h incubation 10.85 U malt enzyme (β-amylase) was added and incubated at 60°C for a further 1h. Thus after 4 h of incubation the enzyme released 413.27 µmol/ml of reducing sugar equivalents corresponding to 27.90% hydrolysis of raw starch (Fig. 9).

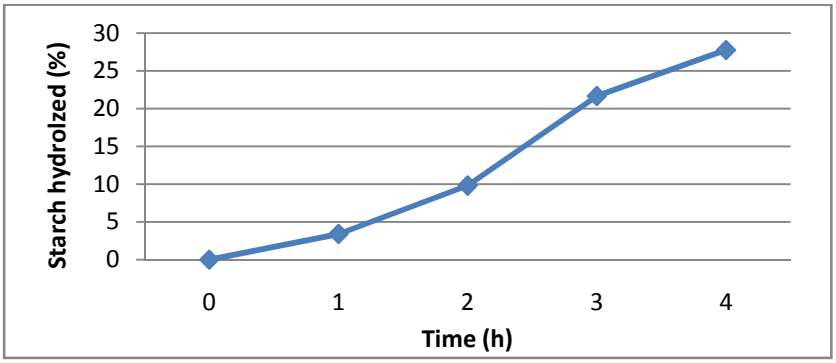


Fig. 9. A profile of starch hydrolysis by AAU 2107 enzyme against incubation period

5.7. Drop in starch viscosity

The viscosity of starch hydrolysates produced by alpha amylase enzyme was determined by comparing the volume of the starch hydrolysates for down flow speed within 9 second. Based on this, 2.9 ml of a starch hydrolysate which were incubated for 4h was able to follow in 9 second (Fig. 10).

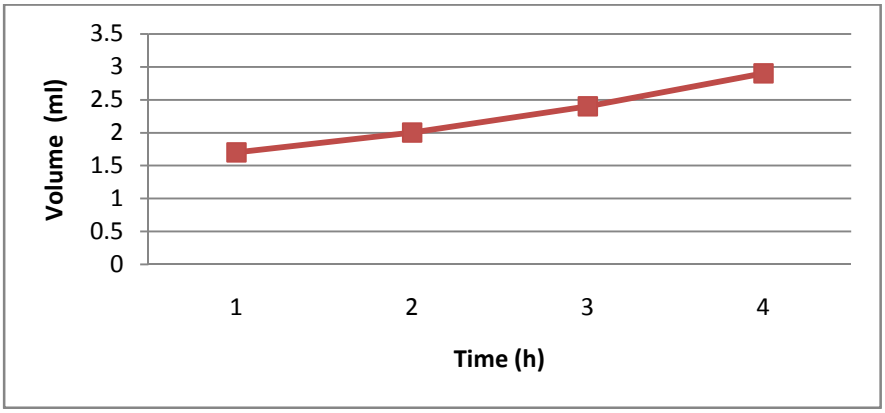


Fig. 10. A profile of viscosity starch hydrolyzed by AAU 2107 enzyme

6. DISCUSSION

Amylases are among the most important enzymes for industrial application, with application in different industrial processes (Burhan *et al*., 2003). Existence of diverse of applications indicates the need for amylases having different properties. The production of amylases can be carried out by submerged fermentation (SmF) or through solid state fermentation (SSF). Submerged fermentation has been a traditionally used method for the production of amylolytic enzymes. Whereas, solid state fermentation involves growth in the absence of a free flowing water using cheap agro industrial wastes. Thus SSF involves low capital investment for machinery and substrate making preferred technology for developing countries.

In addition to low capital cost, some of the advantages of solid-state fermentation include lower levels of catabolic repression and end product inhibition, low waste water output, better product recovery, and high quality production (Pandey, 1992). Enzyme production under SSF is affected by various physical and chemical parameters such as temperature, pH, period of incubation, carbon sources acting as inducers, surfactants, nitrogen sources, phosphate, different metal ions, moisture and agitation with regards to SSF. Amylase production by isolate AAU 2107 reached to maximum at 120 h growth. In most bacterial isolates enzyme production starts at around the logarithmic stage of the growth and reach to maximal point at the stationary growth phase (Meer *et al*., 2004). In the case of AAU 2107 enzyme, the increment of incubation period beyond 120 h led to a decrease in enzyme production could be because of depletion of essential nutrients or due to accumulation of toxic substance produce.

Moisture is one of the most important parameters affecting enzyme production through SSF (Pandey *et al.*, 2000). For isolate AAU 2107, the highest enzyme production was observed at 75% (v/w) moisture level (Fig.7). Some researchers reported that, at high moisture content substrate porosity is reduced thus or limiting the transfer of oxygen in the particle (Sivaramakrishnan *et al*., 2006). However, for isolates AAU 2107 enzyme production progressively increased with increasing moisture level**.** It has been proposed that at high moisture content solubility of nutrients of substrate particle is high causing a better facility for utilization of the substrate by microorganisms (Anto *et al*., 2006; Ibrahim *et al.*, 2012).

Another parameter that determine enzyme under SSF addition of nitrogen supplements. The requirement of specific nitrogen source differs from organism to organism. From all the nitrogen sources, AAU 2107 showed the highest enzymatic production for organic nitrogen source which is peptone (273.58 U/g)(Fig.8). Some studies showed that organic nitrogen sources are preferred for the production of α-amylase than inorganic nitrogen sources (Gupta *et al*., 2003). Organic nitrogen sources contain readily available amino acids and peptides that are easily taken by the cell directly and incorporated into protein or changed into another cellular nitrogenous constitutes (Mongi *et al.*, 2005).

Peptone is a richest nitrogen source among other organic nitrogen source. It is also rich in vitamins and other growth stimulating compounds (Wang *et al*., 2010). However, the reason why amylase production was low in the presence of yeast extract (213 U/g) is not clear. Other authors have also reported that peptone resulted in an increased enzyme production while yeast extract exhibited no effect on alpha amylase production (Tanyildizi *et al.*,2005). Aiyer (*2004*) also reported that peptone is the better nitrogen source for enzyme production by *B. licheniformis* SPT 278 than ammonium hydrogen phosphate. When the SSF was supplemented with inorganic nitrogen content ($NaNO_3$), the organism showed (212.03 U/g) in the enzymatic production (Fig.8). Inorganic nitrogen source have a less complex nitrogen content which makes the cell to spend more energy and time in synthesizing amino acid for protein synthesis.

In starch degrading industries the rate of hydrolysis of starch by amylase enzyme depends on many process conditions such as: pH, temperature, stability, nature of substrate, substrate concentration, enzyme concentration, presence of Ca^{2+} ions and other stabilizing agents (Sivaramakrishnan *et al*., 2006). On the basis of temperature profile of AAU 2107 amylase enzyme, in the absences of 5 mM Ca^{2+} the highest residual activity was observed when the enzyme was incubated at 70°C (Fig.3). This is because, when the temperature increases the rate of the enzyme activity will be high because substrates collide with active sites of the enzyme more frequently as the molecules move rapidly (Reece *et al.,* 2011). However, the enzyme activity starts to decline when it reaches at 75°C. Some evidence shows that the activity of an enzyme is dependent upon enzyme flexibility (Daniel *et al*., 1996). Enzyme flexibility could be affected due to an increment of temperature on the covalent and hydrogen bonds of the enzyme.

The chemical bonds that make up the shape of the enzyme and enzymes' function is related to its shape, thus the temperature increment causes the weaker hydrogen and ionic bonds of the enzymes to be broken resulting a conformational change in the active site of the enzyme causing a decrement in the activity of the enzyme (Reece *et al.*, 2011). The activity of an enzyme can be demonstrated at temperatures below the glass transition where mobility does not occur but catalytic-centre activities are very low (More *et al*., 1995). In the presence of 5 mM Ca^{2+} the activity of the enzyme in temperature range 65°C - 75°C was relatively similar. This implies that calcium ions have the ability to stabilize the conformational structure of the catalytic and/or substrate binding site (Sivaramakrishnan *et al.*, 2006). Some of the finding shows that Ca^{2+} ion has the ability to increase the α-amylase activity of an alkaliphilic *Bacillus* sp. ANT-6 (Burhan, 2003).The other challenge in starch degrading industry is the stability of an enzyme. Scientifically, the stability of an enzyme is the result of a delicate balance between large stabilizing (nature of an enzyme) and large destabilizing forces which could be increment of temperature. Based on this, the thermo stability of alpha-amylase in the absence of 5 mM Ca^{2+} the enzyme was able to sustain 24.51% of its original activity at 70°C and at 75°C the enzyme only retain 5% of its original activity within 20 min of incubation (Fig. 4).

Some reported that the conformational stability of an enzyme is the result of compromise between two opposing factors: flexibility, for the catalytic function of the enzyme and rigidity, for conformational stability (Padayachee, 2006). In the case of AAU 2107 enzyme, the enzyme resulted in a conformational change of the active site after 20 min incubation (denaturation) at 75°C. It has been reported that the conformational stability of an enzyme depends upon a stabilizing forces arising from a large number of weak interactions between the hydrophobic residues, which are opposed by almost an equal large destabilizing force due mostly to conformational entropy (Daniel *et al*., 1996). The rigidity of the hydrophobic interaction protects them from unfolding and preserves their catalytically active structure (Padayachee, 2006). Soni *et al.* (2003), reported the α-amylase of a *Bacillus* species. have an optimum temperature 70°C and thermal stability greater than 60°C (Soni *et al*., 2003).

On the other hand, in the presence of 5 mM Ca^{2+} the enzyme retained 6.05% of its original activity at 75°C with in 50 min of incubation (Fig.5). It is well documented that, calcium ions 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 and reduced flexibility of the enzyme (Breitung *et al.*, 1991; Sabour, 2002). Some researchers reported that the thermostabilities of Bacillus licheniformis CUMC 305 was as high as 4 h at 100°C have been reported (Krishnan and Chandra, 1983). In general, the enzyme was a thermostable enzyme. In the starch industry starch slurry is heated in a jet cooker at temperature range 100 - 175°C in order to disrupt the starch granules and release the amylose and amylopectin into the water (gelatinization). Then the heated starch slurry is passed directly into a hydrolysis reactor for further enzymatic treatment (liquefaction). To make the process economical, a-amylases used for this application are preferred to be active and stable at the high temperatures of gelatinization (100–110 °C) and liquefaction (70–90 °C) (Sivaramakrishnan *et al.*, 2006). This shows that amylase AAU 2107 has a good potential to be used in the process of starch liquerification.

pH is another factor affecting the rate of hydrolysis of starch by a-amylase. In a starch degradation process, the starch slurry containing (30-40%) of dry solids is cooked in a jet cooker. The enzyme will be added after the starch has been cooked and cooled, in this process the pH may be in range 2- 5 (Aiyer, 2005). Every enzyme has its own optimum pH. In the case of alpha amylase of AAU 2107, the enzyme was able to maintain its optimum activity in the pH range of 4.0 − 8.0 and the highest activity of the enzyme was observed at pH 5.0 (Fig. 2). This indicate that, pH 5.0 is an optimum pH for amylase AAU 2107.

The optimum pH of an enzyme means that the concentration of H^+ ions in the solution is the right concentration to give the tertiary structure the best overall shape. As stated that an important part of catalysis in the active site relies on charged groups of on the R-groups of the amino acids that make up the active site. So, increasing or decreasing the pH (the concentration of H^+ ions) will alter the charges that are around the active site. This would happen because more H^+ ions would be attracted towards any negatively charged groups that are in the active site (amino acids form part of the active sites and are negatively charged. These H^+ ions are therefore attracted to these groups and cluster around them. By doing this, it interferes with the binding of the substrate to the active site and it therefore changes the rate of activity of the enzyme - the rate of an enzyme controlled reaction). Based on this, when the H^+ ions of the solution decreases the activity of amylase AAU 2107 starts to decrease. The excessive increment

and decrement of H^+ ions concentration in the solution can cause an effect on the intra molecular forces and change the enzyme's shape -- potentially to the point where it is rendered ineffective. In conclusion α-amylase of AAU 2107 is an acidic thermostable enzyme.

In starch degradation process, the gelatinization of starch requires a high-energy input forming a starch-based products with an increased production cost. The hydrolysis of raw starch below gelatinization temperatures has gained an importance point views in energy costs, effective utilization of natural resources and viscosity problems. In starch liquefying and saccharifying industry the sweetness of a starch syrup depends on the degree of hydrolysis and a complete hydrolysis of starch results in the formation of only glucose or dextrose. The amount of dextrose in syrup is given by the DE (amount of reducing equivalents expressed as glucose per unit dry weight). As far as reducing power is concerned, AAU 2107 amylase was able to hydrolyze a 25 % (w/v) of gelatinized starch in 4 h of incubation and released 413.3 µmol/ml of reducing sugar equivalents corresponding to 27.9 % hydrolysis of starch. This is implies that when the incubation period increase the enzyme would be able to catalyses the hydrolysis of internal α-1,4-glycosidic linkages of the starch in to a low molecular weight products, such as glucose, maltose and maltotriose units (Sivaramakrishnan *et al.,* 2006). This indicates that the amylase is a liquefying and saccharifying enzyme (Ayalew, 2011). A thermostable alpha-amylase of *Bacillus*sp. I-3 was also reported for the hydrolysis raw potato starch at a concentration of 12.5 % within 12 h (Soni *et al*., 2005). Therefore, enzymes that are capable of digesting raw potato starch are economically attractive for the increase range of starch sources for direct saccharification (Gashaw Mamo and Amare Gessesse, 1999a).

The other main problem in starch liquefying and saccharifying industry is viscosity. Viscosity is a result of further heating of a starch granule above its gelatinization temperature. In this research, the viscosity of the raw starch was measured by comparing the volume of starch hydrolsates produced by alpha amylase for down flow speed within 9 seconds. The result shows that in the presence of α-amylase the viscosity of the starch starts to drop as the incubation period increases as result the fluidity of a starch increases (Fig.10). Since the viscosity of starch is related mainly to the swelling of amylopectin molecules and the reduction of viscosities of hydrolyzed starches in relation to the original starches can be explained by the enzyme action on the amylose and amylopectin molecules (Bean *et al.,* 1978). Thus, by using amylase enzyme as

thinning agent when alpha amylase start to hydrolyze the gelatinized starch, the swollen granules will be converted into another molecules or degraded into its lowest form (unit). Some of the scientific reports indicates that the viscosity of a starch is essentially the principal measure of the potential application of starch in industry (Sarker *et al*., 2013; Gryszkin *et al*., 2014). Some of the researchers also indicated that the concentration, temperature, volume fraction of the swollen granules, the deformability of the granules, and the degree of molecular entanglement affect the viscosity of starch pastes during pasting (Doublier, 1990; Noosuk *et al.*, 2003).

7. CONCLUSION AND RECOMMENDATION

The recent development of industrial biotechnology has promoted a renewed interest in investigation of microorganisms capable of producing an extracellular enzyme. East Africa region has a unique microbial diversity that could serve as a source of novel enzymes for industrial application. Alpha amylase of BACC 2107 has a property of low pH activity and a thermo stability (70^{o}C - 75^{o}C) and thermal activity (60^{o}C - 75^{o}C) that can also be enhanced with the addition of 5mM Ca^{2+}. Based on this, amylase enzyme of BACC 2107 could be a good candidate for used for starch liquefaction and saccharification processes. Additionally, the presence of abundantly available year round agricultural by products such as wheat bran in country like Ethiopia could enable the production of amylase enzyme by BACC 2107.

Since East Africa is a potential region for the production of starch based products the maximal production of amylase using easily available carbon sources coupled with a rapid starch hydrolyzing ability of the enzyme is one of great economical advantages. The ability of Alpha amylase of BACC 2107 to hydrolyze different types of botanical source like potato makes it a favorable enzyme for the production of candies, jams, jellys, etc. It can also be used to supplement malt amylase in breweries and the hydrolsates produced by this enzyme can also be used as a carbon source in the fermentation process, which makes BACC 2107 strain the a potential candidate for the production alpha amylase enzyme acceptable by the commercial standard of enzyme production. Therefore, it is recommended that, the enzyme should be characterized further, purified and produced in large scale to be easily accessible for large scale industrial applications. Additionally, the gene coding for BACC 2107 strain should be cloned and modify some of the properties through protein engineering.

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