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Amylase Production by two Microbial Isolates: Isolation, Enzyme characterization, and optimization of Cultivation condition

BY

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Abbreviation

°C=Degree Celsius

μl= Microliter

DNS= Dinitrosalicylic acid

EA= Enzyme activity

EB=Enzyme blank

EC= Enzyme Commission

GH=glycosyl hydrolases

Min =Minute

mM=milli molar

OD =optical density

P^H= Power of hydrogen

RA=relative activity

Rpm= revolution per minute

RRA=relative residual activity

RT = room temperature

SMF= submerged fermentation

SSF= solid state fermentation

Abstract

Amylases are commercially important enzymes commonly used for the hydrolysis of starch to generate hydrolysates for different food and nonfood applications. Although Ethiopia has a huge potential for the production of starch, to date the starch industry is nonexistent and the country import large quantities of starch hydrolysates from abroad. The aim of this study was production of industrially important microbial amylases through solid state fermentation (SSF), optimize the cultivation condition, and characterize the enzymes. Screening using starch containing media one bacterial isolate producing α-amylase, designated as BI_{151}, and one fungal isolate producing glucoamylase, designated as FI_{97}, were isolated from soil. Both isolates grew well on SSF with wheat bran as a solid substrate. BI_{151} α-amylase produced 4,636 U/g of amylase after 96 h of incubation at 50% moisture. Isolate FI97 produced a maximum activity of 4,674 U/g after 72 h of incubation under SSF at a moisture content of 67%. For both isolates supplementation with organic or inorganic nitrogen source did not have any impact on enzyme production. This suggests that wheat bran supplemented with mineral salts and water has enough nutrients to support growth of the organisms and make the production process a lot cheaper. Amylase $BI_{151}$was optimally active in the temperature range of 65-75°C with a peak at 70°C and in the P^H range of 4.5 -5.5. On the other hand amylase of FI97 was optimally active at 65°C and at pH 6. These properties showed that both enzymes have properties that make them potentially suitable for starch hydrolysis, especially in the brewery industries. Under lab scale evaluation, both enzymes were able to efficiently hydrolyze high concentration of starch adjuncts (60% of the malt adjunct mixture) indicating their potential for application as malt enzyme supplements.

Key word: α-amylase, Glucoamylase, starch hydrolysate, relative activity, residual activity

1 INTRODUCTION

As Ethiopia being an agricultural country, farmers produce different cereal and root crops. The root crops that are widely grown in the country include enset, cassava, potato, and taro. Although both cereal and root crops can serve as sources of starch and starch derivatives, to date all starchy crops are used only as food sources. On the other hand the country currently imports different starch derivatives from abroad with the expenditure of foreign currency (Molla, 2016). Some of the starch derivatives imported include glucose hydrolysates for confectionery and other food application; glucose for medical and food application; and other starch derivatives. Most of these products are currently produced elsewhere using enzymatic hydrolysis.

Starch hydrolysates can also be used as adjuncts in breweries (Molla, 2016). At present the brewing industry in Ethiopia is expanding rapidly. Barley malt and water are the major and most important ingredients in the brewing industry. Today Ethiopia has only one malting factory (Assela Malt factory) that produce less than half of the country's malt requirement. Thus, more than half of the malt required by the different breweries is imported from abroad with the expenditure of foreign currency (Grujic, 1999).

To reduce production cost breweries in many countries use other starchy adjuncts replacing up to a quarter of the malt (O'Rourke, 1999). Depending on the geographic location, the starchy adjunct used could be maize, sorghum, potato, cassava, enset, or rice. Under Ethiopian condition root crops like cassava and enset are considered to have huge potential as brewery adjuncts (AMF, 2014). When malt is used in the brewing process, the enzymes that are induced during the malting operation are used to break down the barely starch to maltose and other fermentable sugars. This is done so because yeast cells that are involved in the fermentation process are unable to utilize starch for fermentation.

One major challenge breweries face when they use starch adjuncts is that the available enzyme of the malt may not be enough to hydrolyze all the starch. In fact even without addition of adjuncts many of the breweries in Ethiopia currently use additional enzymes to supplement the endogenous malt enzymes to bring about complete hydrolysis of the malt starch (Mulugeta, 2015). With the use of adjuncts the requirement for supplemental enzymes in breweries could be

much higher. The enzymes that are currently used as malt enzyme supplements are microbial enzymes having similar biochemical properties with the malt enzymes.

To date the brewery supplemental enzymes are imported from abroad. If these enzymes are produced locally, it could be available with cheaper price. This could in turn encourage breweries to replace up to a quarter of their malt consumption by locally produced starch adjuncts. This, in addition to saving the country substantial foreign currency, could help to create a huge market for local agricultural products.

Different amylolytic enzymes (amylases) are required to bring about complete hydrolysis of starch. These include alpha amylase, glucoamylase, beta amylase, and pullulanase. At present the best source of beta amylase is malt. But for the other three amylolytic enzymes the best sources are microorganisms (bacteria and fungi).

Ethiopia is known to have a huge microbial diversity inhabiting terrestrial and aquatic habitats. Isolation of fungal and bacterial strains from these diverse habitats is expected to give novel organism that can produce amylolytic enzyme with potential application for starch hydrolysis.

2. Objectives

2.1. General objective

The main objective of this study was to produce bacterial and fungal amylases that can be used as malt supplements in breweries and other starch processing industries.

2.2. Specific objectives

- Isolate amylase producing bacterial and fungal strains, characterize the enzymes and evaluate their potential applications
- Develop and optimize a cheap and easy enzyme production processes through solid-state fermentation
- Evaluate the potential usefulness of the different enzymes as malt enzyme supplements to hydrolyze starch adjuncts in breweries

3. Literature review

3.1. Starch

Starch is a polymer of glucose linked to one another through glycosidic bonds. Next to cellulose starch is the second most abundant heterogeneous polysaccharide synthesized by plants through a process of photosynthesis (Perez and Bertoft, 2010). It is synthesized as a long-term storage polysaccharide in amyloplasts of tuber, seeds, and roots in the form of granule with semi-crystalline structure. The size and shape of starch granules is characteristic feature of the plant species (Jane *et al.,* 1994 and Li *et al.,* 2007).

Starch containing crops commonly used as sources of food for human consumption include leguminous plants (example lentils, peas), cereal crops (such as wheat, maize, sorghum, rice, tef,and barley), root and tubers (such as cassava, enset, sweet potato and potato),and undeveloped fruit (Ramachandran *et al.,* 2004). Starches from thus varied sources differ in their morphology, retro gradation, composition, thermal and rheological properties.

Starch granules from any botanical source are usually composed of two components, amylose and amylopectin, which are distinguished from one another on the presence or absence of branching (Li *et al.,* 2007).

3.1.1. Amylose

Amylose consists of a linear chain of glucose units joined together by an α-1, 4 glycosidic linkage and can have a molecular weight ranging from 8×10^4 to 10^6. During the process of gelatinization, amylose leaches out from the granule and increases the viscosity of the starch slurry. It is the amylose component of starch granules that also forms a complex with iodine and give the characteristic blue-black color and used for qualitative and quantitative analysis of starch (Dona *et al.,* 2010).

3.1.2. Amylopectin

Amylopectin is a branched chain of glucose units linked with an α-1, 4 glycosidic bond with an α-(1, 6) glycosidic linkage at branching points (Karim *et al.,* 2000). With an average glucose unit of approximately 2,000,000, amylopectin is one of the largest molecules known in nature (Myers *et al*., 2000 and Alavi, 2003). Because of its highly branched organization, when dispersed in

water, it produces soft gels and weak films preventing the starch from becoming plastic-like (Perez and Bertoft, 2010).The branched nature of amylopectin also reduce its iodine binding power, increases its solubility in aqueous solutions, and makes it non-retrogradable (Dona *et al.,* 2010).

3.2. Physicochemical properties of starch

3.2.1. Starch gelatinization

Gelatinization is the distortion of molecular orders within the starch granule as a result of exposure to higher temperature resulting in irreversible changes in its properties (Lawal, 2004 and Vaclarik and Christian, 2003).The temperature required to gelatinize starch vary among starches from different plant species that arise as a result of differences in their degree of crystallinity. Highly crystalline starches provide structural stability and require high temperature for gelatinization. In general gelatinization temperature and pasting properties of a given starch source is closely related to its amylopectin content (Tran, *et al.,* 2001 and Kaur *et al.*, 2002).

3.2.2. Starch retrogradation

As a solution of gelatinized starch cools, the colloidal starch dispersion results in the formation of an elastic gel. This is due to the availability of less energy to keep the molecules of amylose apart in a solution or an attraction force in the hydroxyl groups of amylose that coil the molecules together (Ashogbon and Akintayo, 2014) that result in the formation of a tough gel and strong film. This process is known as retrogradation and this property of starches is more closely related to the ratio of amylose to amylopectin content of the granules (Tran *et al*., 2001).

3.2.3. Pasting and viscosity properties

Pasting properties of starch reflect its structure and it occurs after amylose leach out of the granules during gelatinization. The properties are largely governed by granular composition of amylose and continuous phase viscosity. Higher amylose content leads to the higher pasting properties of gelatinized starch. Starch exhibits unique viscosity behaviour with change of temperature, concentration and shear rate.Starches that are capable of swelling to a high degree are also less resistant to breakdown on heating and exhibit viscosity decrease (Kaur *et al*., 2004 and Morikawa and Nishinari, 2002).

3.3. Industrial applications of starch

Starch has four major physicochemical properties that make it very attractive for application in industry. These include its use as a thickening agent, gel formation, its ability to form paste, and its ability to produce strong adhesive films. In addition, the fact that starch is an easily available raw material in most regions of the world makes it an ideal raw material for different industrial applications.

In the food industry starch is used as a modifier of texture and viscosity, as moisture retention agent, as a gelling agent, and a bulking agent. Although highly viscous, starch solutions are used for mechanical manipulation, such as paper and corrugated production, and as sizing agent in the textile industry (Haki and Rakshit, 2003 and Lowe, 2002)

3.4. Amylase enzyme

Amylases are hydrolytic enzymes that catalyze the hydrolysis of glycosidic linkage in starch to generate a variety of products. At present the starch industry uses a battery of amylolytic enzymes to generate such products as dextrins, maltose, glucose, glucose syrups, and other products used for various food and non-food applications (Cordeiro *et al*., 2002 and Reddy *et al*., 2003). Although amylases can be obtained from several sources, such as microorganisms, animals and plants, the great majority of amylolytic enzymes currently used in the starch processing industry are obtained from microorganisms (Kandra, 2003; Aiyer, 2004; Kathiresan and Manivannan, 2006).

Though many microorganisms produce amylases, the commercially important enzymes are currently obtained from a limited number of bacterial and fungal genera. These include the bacterial genus *Bacillus* (Konsoula and Liakopoulou-Kyriakides, 2007) and the fungal genera *Aspergillus* and *Penicillium* (Kathiresan and Manivannan, 2006).

3.4.1 Classification of amylase

Starch converting enzymes are classified based on their substrate specificity during hydrolysis of the α-glycosidic bonds. Therefore, depending up on their mode of action amylolytic enzymes are

classified as endoamylases, exoamylases, debranching enzymes, and transferases (Van der Maarel *et al*., 2002).

a). Endoamylase: These are enzymes that randomly degrade the α, 1-4 glycosidic bonds of starch bypassing the β-1,6 linkages at branching points.Because it is an endoacting enzyme, hydrolysis of starch releases varying length oligosaccharides with the terminal glucose having α-configuration. As a result the endo-acting enzymes are commonly called α-amylase.

Based on the extent of action on degradation of starch polymers, α-amylases are grouped in to two as liquefying and saccharifying enzymes. Liquefying α-amylases degrade starch and bring about reduction in the viscosity of the starch slurry without releasing free sugars. On the other hand saccharifying α-amylases hydrolyze starch resulting in production of reducing sugars of varying glucose units (Pandey *et al* ., 2000).In order for α-amylase to hydrolyze starch, it is important that the granules disrupt upon heating, a process known as gelatinization. But gelatinization leads to an increase in viscosity. Thus to reduce viscosity, the starch industry uses thermostable α-amylases to bring about liquefaction. Therefore, almost all α-amylases currently used in industry are thermostable enzymes derived from bacteria (Dubnovitsky *et al*., 2005).

b) E xoamylase: Exoamylases are enzymes that exclusively cleave the α-1, 4 glycosidicbond from the non-reducing end of the starch polymer. The two exo-acting enzymes that fall in this group are glucoamylase (also called amyloglucosidase) (EC 3.2.1.3) and β-amylase (EC 3.2.1.2). While glucoamylase degrade the glycosidic bond of starch and release glucose units, β-amylase degrades two glucose units releasing maltose. In addition β-amylase is unable to bypass the α-1,6glycosidic bond and thus result in the formation of is called beta limit dextrin's (Sivaramakrishnan *et al.,* 2006).

c). ***Debranching amylase:*** These groups of starch converting enzymes hydrolyze only the α-1, 6 linkages at branching points of amylopectin. These enzymes, also known as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) find important application in the starch industry. While pullulanase acts on amylopectin and pullulan, isoamylase acts only on alpha-1, 6 linkages in amylopectin (Sivaramakrishnan *et al.,* 2006).

3.4.2. Amylase production through solid-state fermentation

Large-scale enzyme production of microbial amylases is carried out either through submerged state fermentation (SMF) or through solid-state fermentation (SSF). SSF is cultivation of microorganisms in the absence of free flowing water using cheap agricultural or industrial wastes as a substrate. The substrates commonly used for SSF include wheat bran, rice bran, tea waste, potato peels, maize bran, cassava waste, and sorghum pulp. The selection of solid substrate depends on the cost and the availability of the substrate (Renge *et al*., 2012).

Growth and enzyme production through SSF is influenced by a number of factors. These include moisture content, fermentation period, temperature, nitrogen sources, and particle size of solid substrates. Moisture affects enzyme production by adversely affecting microbial growth and physicochemical properties of the substrate porosity for gas diffusion (Suganthi *et al*., 2011). Therefore; to maximize enzyme production through SSF it is important to optimize the above parameters.

3.5. Industrial application of amylases

Amylases find important applications in a number of industrial processes where starch is involved as a raw material. These include food, brewing, alcohol, detergent, textile, and paper industries (Hosseinpour *et al*., 2011).

3.5.1. Amylases in the food industry

Amylases are extensively used for the production of sweeteners, maltose, and maltodextrines, products widely used in the food industry. For example maltose syrup is produced through the action of β-amylase and is used in the food industry because of its low tendency to crystallize and its non-hygroscopic nature. Similarly glucose syrups or high fructose corn syrups are produced from starch through enzymatic conversion involving different amylolytic enzymes followed by isomerization using the enzyme glucose isomerase. Glucose syrups are extensively used as sweeteners in soft drinks, confectionery, and in a number of other food products (Van Der Maarel, 2002).

Other starch-based products produced from starch through the action of amylolytic enzymes include malto-oligomers that are used as substitutes for sucrose and other saccharides or to prevent crystallization of sucrose in foods. It also serves as a moisture regulator of food with which it is mixed. These useful oligosaccharides mixture is obtained by the combined action of α-amylase, β-amylase and pullulanase on starch.

3.5.2. Application of amylases in beer production

Beer, which is made from the starch of cereals, is the most widely consumed alcoholic beverage in the world (Nelson, 2005). In the process of beer production the first step involves enzymatic saccharification of the starch from the cereal (usually barely) followed by fermentation of the resulting reducing sugar. The saccharifying enzymes are normally derived from the malt (Aastrup *et al*., 2004). But due to failure in the malting or drying process the amount of enzyme in the malt could occasionally be less than the required amount to bring about complete saccharification of the available starch. If the enzyme activity present in the malt is low, it could greatly affect the final beer quality and make the fermentation process slow. To overcome such challenges breweries normally add exogenous enzymes as supplements to the malt enzymes (Gupta *et al*., 2008).

Because malt is very expensive in many countries up to a quarter of the malt required for beer production is replaced by starch from cereal or root crops and is known as adjuncts. Although the use of adjuncts helps to substantially lower the production of beer, because the added starch lack its own enzyme (for example root starches), the endogenous enzymes of malt may not at times be enough to bring about compete saccharification of all the available starch. Therefore, to ensure complete saccharification commercial amylolytic enzymes are often used added as supplements of the malt enzymes (Taylor, and Dewar, 2001).

3.5.3. Amylases in the bakery industry

The bakery industry is a large consumer of amylases which is used to generate readily available sugars for yeast fermentation. In addition amylolytic enzymes are used to improve shelf life of backed foods preventing stalling or increasing softness, an action commonly known as anti-stalling effect (Gupta *et al*., 2008). Glucoamylase delay the baked bread staling, acting on the external side chains of glucose polymer to awkward amylopectin towards retrogradation.

3.5.4. Application in the textile industry

The weaving process in modern textile production exerts huge force leading to breakage of the warp thread. Unless tied up immediately by a technician breakage of even a single thread leads to stoppage of the machine and these results in significant losses due to prolonged idle time. Therefore, to increase efficiency textile factories need to strengthen the warp by applying appropriate coating agents, a process known as sizing. Because it is easily available, cheap, and can be easily removed from the fabric, thus starch is the most commonly used as sizing agent used by the textile industry.

After the fabric is woven, the starch must be removed before it is dyed, a process called desizing. If the starch coat is not removed, the dying operation fails to be uniform and also pose other challenges in subsequent operations. Because the starch coat is very difficult to wash away with water, α-amylase is commonly used. The enzyme acts on the starch, leaving the fibers unaffected and ready for scouring and dyeing (Aiyer, 2005 and Souza and Magalhães, 2010).

3.5.5. Paper industry

Like the textile industry the paper industry also use large quantities of starch sizing to produce a smooth surface on paper products. For this application α-amylase are widely used in pulp and paper industry to lower the viscosity starch size through partial hydrolysis. This helps to reduce the high viscosity of natural starch solution making it suitable for paper coating (Gupta *et al*., 2003).

3.5.6. Detergent industry

Today the detergent industry is the largest consumer of enzymes in both volume and value. Although the most widely used enzymes for detergent application are proteases, amylases also finding important application as detergent additives, especially for dishwashing detergents (Hmidet *et al.,* 2009). Because starch can attract soil particle on to the clothes its removal by amylases is considered important to maintain the whiteness of clothes. The use of enzymes making the detergent process environmentally safe, enhances the detergents ability to remove tough stains (Haq *et al.*, 2010) and also allow for lowering of washing temperatures (Gupta, *et al*., 2003).

3.5.7. Biofuel production

At present, in many countries, ethanol is the most utilized liquid biofuel replacing a portion of crude oil derived gasoline. In some countries, such as USA ethanol is produced through fermentation from grain starch (mainly maize). Since yeast cells cannot metabolize starch hydrolysis using amylases is a prerequisite for ethanol production through fermentation. Production of ethanol from starch has two basic enzymatic steps, liquefaction (catalyzed by α-amylase and scarification (saccarifying amylase), followed by fermentation (using fermentative microorganism) (Juge *et al*., 2006).

4. Materials and methodology

4.1. Sample collection and isolation

Soil samples were collected from wheat agricultural land and transported to Addis Ababa university Biotechnology laboratory in ice box and stored at 4°C until used.

For isolation of amylolytic bacteria, one gram of soil sample was mixed with 9 ml of sterile water and shaken well for five minutes in 20 ml test tubes. Then 1ml of the suspension was serially diluted from 10^{-1} to10^{-6} with sterile water to a final volume of 10 ml. From the 10^{-4} to10^{-6}dilutions 0.1 ml sample was spread on a freshly prepared starch agar plates followed by incubation at 37°C for 48 h. Distinct colonies showing different morphological characteristics were picked and transferred into freshly prepared starch agar plates and incubated at 37 °C for 48 hours. After repeated streaking and sub culturing, pure isolates were obtained and stored at 4°C until required for further analysis and studies.

4.2. Media used for growth and screening

The medium used for amylase production was composed of (g/l) of yeast extract, 3; peptone, 2; NaCl, 5; K_2HPO_4, 0.6; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1 and soluble starch, 5. For solid media 15 g/l of agar was added. For sterility the media was autoclaved at 120°C and pressure of 15 psi for 20 min. For enzyme production under submerged fermentation and preparation of inoculums for solid state fermentation, 50 ml of starch containing liquid media was used.

The media used for enzyme production under solid-state fermentation 10 g of wheat bran with(mg/g) of: NaCl, 0.5; $CaCl_2.2H_20$, 0.1; K_2HPO_4, 1 and $MgSO_4.7H_2O$, 0.2 was transferred to 250 ml Erlenmeyer, the moisture content adjusted to 67%, and autoclaved at 121°C and a pressure of 15 psi for 20 min.

4.3. Screening of amylase producing microbes

To screen microbes that release extracellular amylase, the isolated microbes were incubated at 37°C for 48 h in duplicates on starch agar plates. One set of the plates was used to test amylase production by flooding with Lugol's Iodine solution (1% iodine with 2% KI) (Thippeswamy, 2006). Amylase positive strains gave a clear hallo zone around the colony against a dark blue

background formed due to formation of starch iodine complex. The positive isolate that were produced maximum clear zone were kept on agar slants for second round of screening on liquid media.

4.4. Characterization of amylolytic strains

The selected isolates, which were chosen as thermostable amylase producer, were further characterized by their morphology and biochemical properties. The parameters used were colony feature, Gram reaction, catalase activity, indole test, casine and gelatine hydrolysis.

4.5. Enzyme production

Under submerged fermentation, 50 ml medium containing 1% soluble starch in 250 ml Erlenmeyer flask were inoculated with the bacterial and fungal strains and incubated in a shaker incubator at 37°C at 120 rpm for 48 h for the bacteria strain (or 72 hours for fungal strain). The culture was centrifuged at 1000 rpm for 10 min and the cell free culture supernatant was used as a crude enzyme source.

For enzyme production under SSF the 10 g wheat bran containing and all the required additives in 250 ml Erlenmeyer flask was inoculated with 2 ml of an overnight culture of the selected bacterial or fungal isolate. After incubation at 37°C for three days, amylase was extracted by adding 100 ml distilled water per h flask and placed in a shaker at RT for one hour. The mixture was filtered and the cell free filtrate was used as crude enzyme source for amylase assay.

4.6. Amylase activity assay

Amylase activity was determined following the DNS method as modified by Gashaw Mamo and Amare Gessesse, (1999). To 900 µl of 1% soluble starch in 50 mM phosphate buffer at pH 6.8,100 µl of enzyme was added and incubated for at 60°C in a heating water bath. After 15 min incubation the reaction was stopped by adding 2ml DNS and heated in a boiling water for exactly five minutes. After cooling in running water, absorbance was measured at 540 nm against a reagent blank. DNS reagent was composed of (%): 3,5-Dinitrosalicylic acid, 1; sodium hydroxide, 1; sodium potassium tartarate, 2; Sodium bisulphate, 0.05 and phenol, 0.2.

Each assay was done in triplicate and the results are averages of the three determinations. One unit (U) of enzyme activity was defined as the amount of amylase that releases 1 µmol of reducing sugar equivalent to glucose per minute under the assay condition.

4.7. Enzyme characterization

4.7.1 Effect of temperature on enzyme activity and stability

The optimum temperature for the activity of amylase was measured by incubating the appropriately diluted enzyme and starch as substrate within 100mM sodium phosphate buffer with final concentration of 50 mM at pH 7 in temperature ranges from 50-80°C in a 5°C unit interval. The assay was done in a standard assay procedure that wrote in section 4.6 and the relative activity of amylase was calculated in the following equation.

$$\text{Relative activity (RA)} = \frac{\text{Individual enzyme activity at each T°}}{\textit{The highest enzyme activity in the block}} * 100\% \ldots\ldots\ldots\ldots \text{eq. 1}$$

In order to determine the temperature stability, amylases of both isolates were diluted 10x and added in to a different eppendrof tube. For each of the isolates 7 eppendrofs containing amylase with uniform dilution were prepared and incubated at 60 and 65 °C. One eppendrof from both bacterial and fungal amylase was picked out from a water bath in 0, 10, 20, 30, 40, 50, 60, 90, and 120 mins of incubation and then each of the incubated enzymes was assayed in the standard assay procedure at 65 and 70°C respectively for $\boldsymbol{FI_{97}}$ and $\boldsymbol{BI_{151}}$. The residual activity of amylase was calculated as follow.

$$\text{Relative residual activity (RRA)} = \frac{\textit{Individual residual activity}}{\textit{The highest residual activity}} * 100\% \ldots\ldots\ldots\ldots\ldots.\text{eq. 2}$$

4.7.2 Effect of calcium on thermal activity and stability of amylase

The effect of calcium on the activity of amylase of $\boldsymbol{BI_{151}}$ and $\boldsymbol{FI_{97}}$ was determined by measuring the activities of the enzyme within and without the presence of 0.05M $CaCl_2$ solution. The reaction mixture prepared in both conditions was assayed by the standard assay procedure. Relative activity of amylase was calculated with the equations used in section 4.7.1 of eq.1.

To determine whether amylase require or not Ca^{2+} to increase stability was determined by incubating the crude amylase enzyme with and without 0.05M Ca^{2+}in a 6 different eppendrof

tubes for each isolates in each test. And that one eppendrof per each test for each isolate was picked out from boiling water bath in 10 min interval for 1 h. The assay was done for each of the sample taken and the residual activity was calculated in eq. 2 of section 4.7.1.

4.7.3 Optimum pH for the activity and stability of amylase

To determine optimum pH condition for highest amylase activity the assay was done in a different buffer system. About 0.1 M of acetate buffer (pH 3.0-5.5) and phosphate buffer (pH 5.5- 8.0) was prepared in a 0.5 unit interval with 0.05 M final concentration in a reaction mixture in the assay. The relative activity of amylase was calculated in the formula written in section 4.7.1 of e.q.1.

To determine the stability of amylase in a different pH values, the crude enzymes of fungal and bacterial isolates was diluted in 100 mM of each of the buffer ranges from (4.5 - 8) and incubated at 60°C and 65°C respectively for 1 h. The assay was performed for each test the same as the above assay procedure at a temperature of 60°C for fungus and 65°C for bacterial amylases. The stability was determined by calculating the relative residual activity's by the equation as described in section 4.7.2 of eq.2.

4.8. Optimization of culture condition for amylase production

4.8.1 Optimization of moisture level

The optimum moisture level for production of amylase was determined by incubating the culture media containing 10 g of wheat bran and mineral salts within a 33.3, 50, 60, 66.6, 71.4 and 75% (w/v) of moisture level at a temperature of 37°C for 72 h followed by enzyme extraction following the standard procedure. To optimize the extraction process the residue was re-extracted up to three times and each extract tested for enzyme activity separately. Those extracts having significant activity were then pooled together and the activity measured from the pool. Enzyme production under SSF was expressed as U/g.

4.8.2 Requirement of Nitrogen Sources for Amylase Production

The requirement and selection of the best nitrogen source were tested by supplementing of each organic and in organic nitrogen sources to a fermentation media containing 10g of wheat bran with a moisture level of 66.6% and 50% respectively for fungal and bacterial isolates. The organic nitrogen source added were yeast extract and peptone whereas the in organic sources

were added in the form of $(NH_4)_2SO_4$ and $NaNO_3$ with a percentage composition of 0.5 %(w/v). The inoculum size and mineral contents were the same as used in section 4.8.1.

4.8.3 Effect of temperature condition on amylase production

The optimum temperature for higher productivity of amylase for both fungal and bacterial isolates was determined by incubating 10g of wheat bran containing media with moisture level of 66.6% and 50% respectively at a temperature of 25°C, 28°C, 30°C and 37°C. All other supplements of conditions were the same as used in the above section and the enzyme was harvested using the same technique used as in section 4.8.2 after three days of incubation. The assay was done for each of the enzymes produced in each temperature and the best temperature were determined by calculating the amount of reducing sugar produced from the dry solid substrate that was added.

4.8.4 Time course of enzyme production

The time course of amylase production was determined by incubating the medium containing 10g of wheat bran with mineral salts having a moisture content of 50% (for the bacterial isolate) or 66.6% (for the fungal isolate) at a temperature of 30˚C or 28°C, respectively. Enzyme extraction and assay was carried out following the standard procedure.

4.9. Starch hydrolysis and testing of the starch hydrolysate

Potato and sweet potato starch was isolated in the laboratory through manual grating, settling, and drying. The dry starch sample was used for hydrolysis studies using bacterial and fungal *amylases.*

4.10. Optimization of enzyme supplementations of malt

Malt from Assela Malt Factory (AMF) was grounded to a powder and sifted. Gelatinized raw starch was prepared and mixed with malt with the starch adjunct accounting for 60% of the total mass. Different amounts of laboratory produced bacterial and fungal amylases (measured in Units) were added to the malt -starch adjuncts and incubated at 60˚C for 30 min. To determine the level of starch hydrolysis, a sample of the starch-malt mixture was mixed with an iodine solution and absorbance was measured at 660nm.The level of hydrolysis was expressed a percentage hydrolysis compared with the treatment with the malt enzyme alone (no exogenous amylase added).

5. Result

5.1. Isolation and screening of amylolytic microbes

A total of 922 isolates (731 bacteria and 191 fungal isolates) were isolated from soil based on formation of clear halo zone on starch agar plates (Fig. 1). Of these about 4% (26 of bacterial and 11 of fungal isolates) were positive for amylase production and were selected for further screening in liquid culture.

Figure 1: Amylase positive isolates on starch agar plates after flooding with iodine solution.

Based on their activity at elevated temperature and the amount of amylase produced two fungal and four bacterial isolates were selected. The activity of these six isolates was tested in temperature range of 50°C-75°C in the presence and absence of Ca^{2+}. One bacterial isolate designated as BI_{155} with good activity at 70°C and one from fungal isolates designated as FI_{97} with optimum activity at 65°C were selected for further study. Ability to grow in solid-state culture was also considered as part of the criteria to select the isolates. And both isolates were grown well and produced appreciable level of amylase activity under solid substrate cultivation.

5.2. Characterization of amylolytic isolate

Isolate BI151 was gram negative; rod shaped forming a chain, and motile (Table1). On starch agar medium it forms white and dry colonies with irregular margins. It was catalase positive and negative for indole production. In addition to starch hydrolysis this isolate was capable of hydrolyzing casein and gelatin.

Table 1 Summery of biochemical and morphological characterstics of BI151

Test	Observation	Result
Gram stain	Pink, rod shaped with chain	Gram negative
Microscopic feature	Motile, white with irregular margin	
Color and Texture	White and dry	
Motility	The organism grew thinning out towards the side of the test tubes	motile
Catalase test	Air bubble formation	Catalase positive
Indole test	Any red color was not observed The same result observed as the control	The organism was not utilize the amino acid tryptophan to produce indole
Starch hydrolysis	A clear halo zone against a dark blue back ground	Amylolytic microbe was positive for amylase production
Casein hydrolysis test	A clear halo observed around the colony	Microbes was positive for protease production
Gelatin hydrolysis test	Liquefaction of the gel was observed	Amylolytic microbe was positive for gelatinase production

5.3. Characterization of the amylases

5.3.1 Effect of temperature on enzyme activity and stability

The optimum temperature for BI151 amylase was at 70°C and it retained 75% of its maximum activity at 75°C (Fig. 2a). For amylase FI97 optimum activity was recorded at 65°C and showed 79% of its maximum activity at 70°C (Fig. 2b). The presence of calcium ion did not change the optimum temperature of both enzymes.

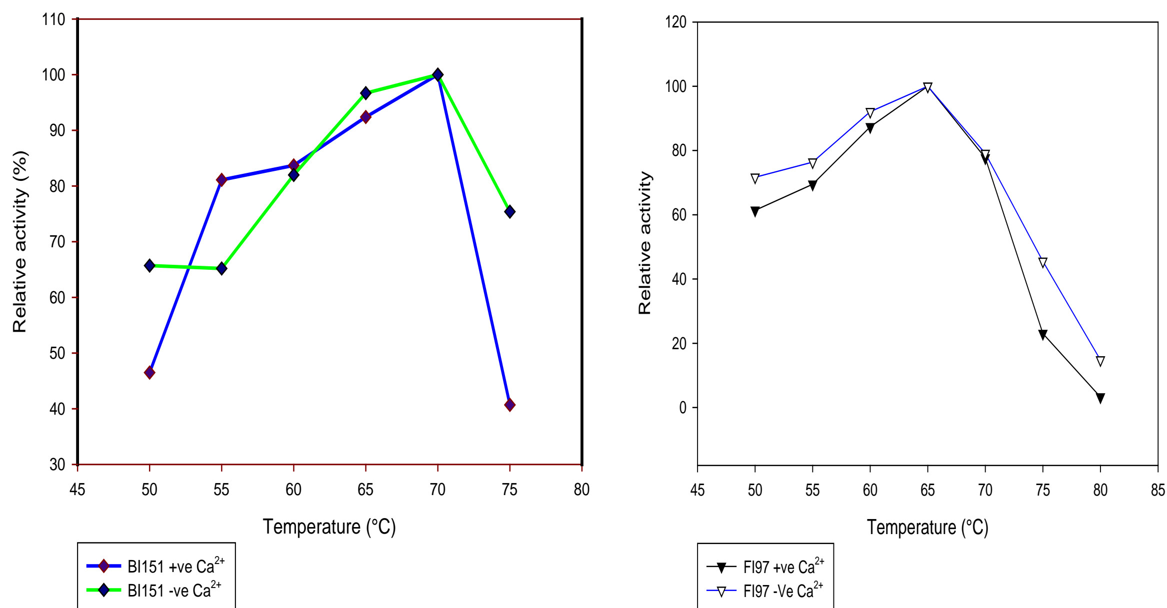


Figure 2(A). Temperature profile of BI151 amylase and (B). FI97 amylase, Enzyme activity was measured at different temperature in 50 mM phosphate buffer, pH 6.8 and in the prescence and absence of 5mM $CaCl_2$ solution.

Measured at 60˚C and 65˚C both enzymes showed low thermal stability (Fig. 3). At 60˚C amylase BI151 retained only 38.5% of its original activity after 20 min of incubation at a temperature of 60 and 53% of its original activity was retained at 65°C, respectively. As incubation time of about 50 min it retained 13.18% and 4.7% residual activity (Fig.3A). FI97 also retained 54% and 49.4% of its original activity after 20 min of incubation at 60 and 65°C, respectively (Fig.3B). In both of the incubation temperature the amylase of both isolate was high stability towards incubation without Ca^{2+}.

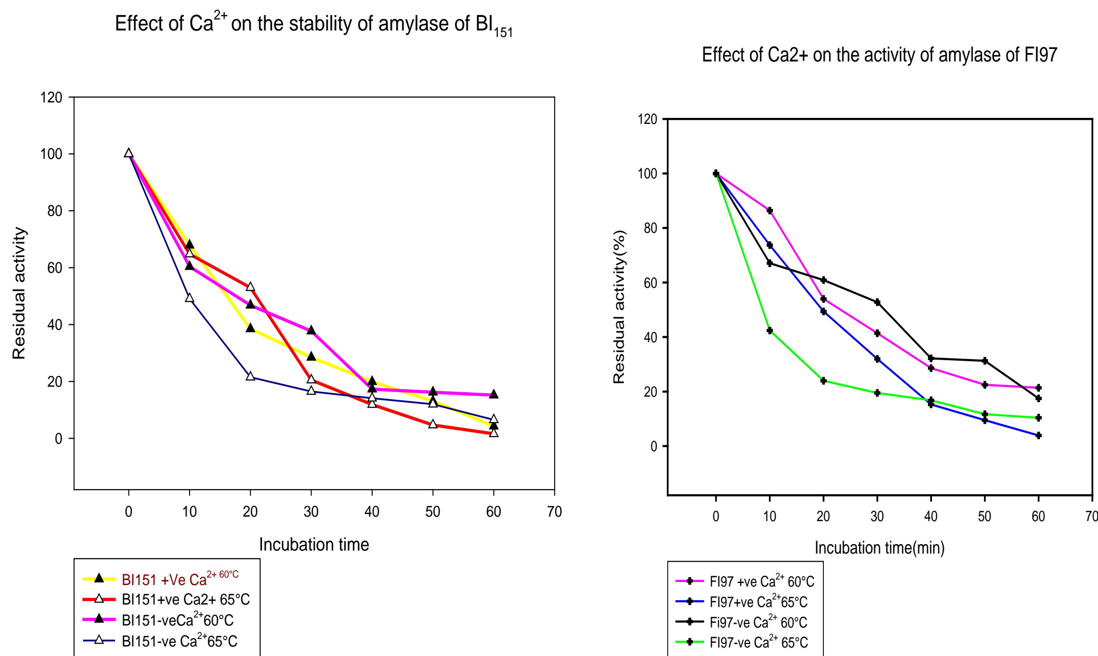
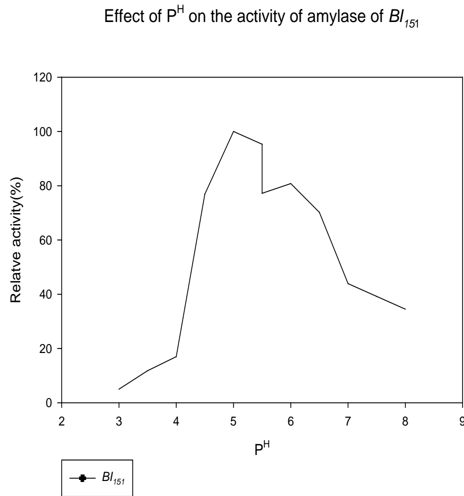


Figure 3: Thermal stability ofBI151 amylase (a) and FI97 amylase at 60°C and 65°C in the presence and absence of Ca^{2+}.

5.3.2 Effect of pH on the activity and stability

The optimum pH for the activity of BI151 was in the range of 4.5-6.5 with a peak activity at pH 5. The optimum pH of amylase of FI97 was in the range of 4.5 and 7.0 with a maximum activity at pH 6.5. Amylase of FI97 showed higher activity to a slightly neutral pH values.



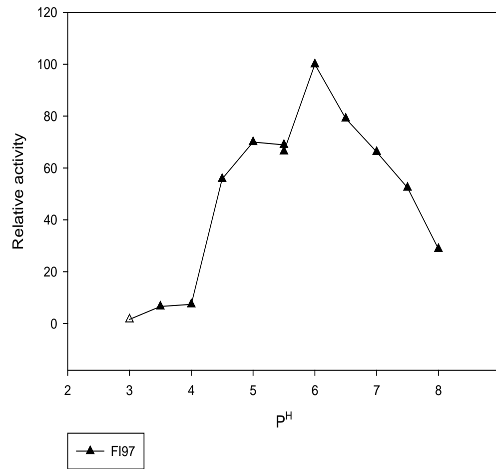
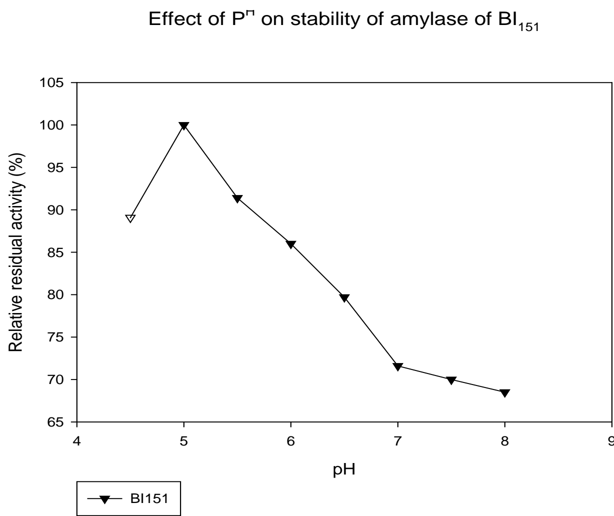


Figure 4 PH profile of amylase of BI151 (A) amylase of FI97 (B) assayed at 70°C and 65 °C respectively

Amylase BI151 was retained 91.4% of its original activity at a pH 5 (Fig.5a). On the other hand amylase of FI97 also retained 75% of the peak activity in the pH range of 4.5 to 8 (Fig. 5b).



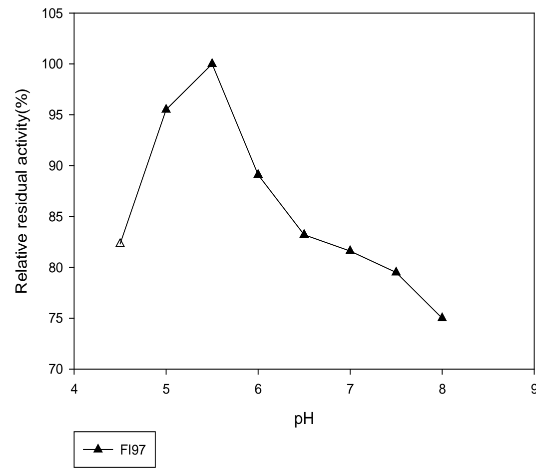


Figure 5 PH s tability of am ylase of B I151 (a) a mylase o f F I97 (b) as sayed a t 70 and 75° C respectively

5.4. Enzyme production through solid state fermentation

5.4.1 Optimum moisture level for amylase production

Bacterial isolate BI151 produced the maximum amount of amylase activity (4,663 U/g) at a moisture level of 50% after a growth period of 72h. Increasing the moisture level above 50% resulted in a decrease in the level of enzyme production (Fig. 6A). For the fungal isolate FI97 highest amylase production (4,013 U/g) was recorded at a moisture level of 67% above which the level of enzyme production progressively decreased (Fig.6B).

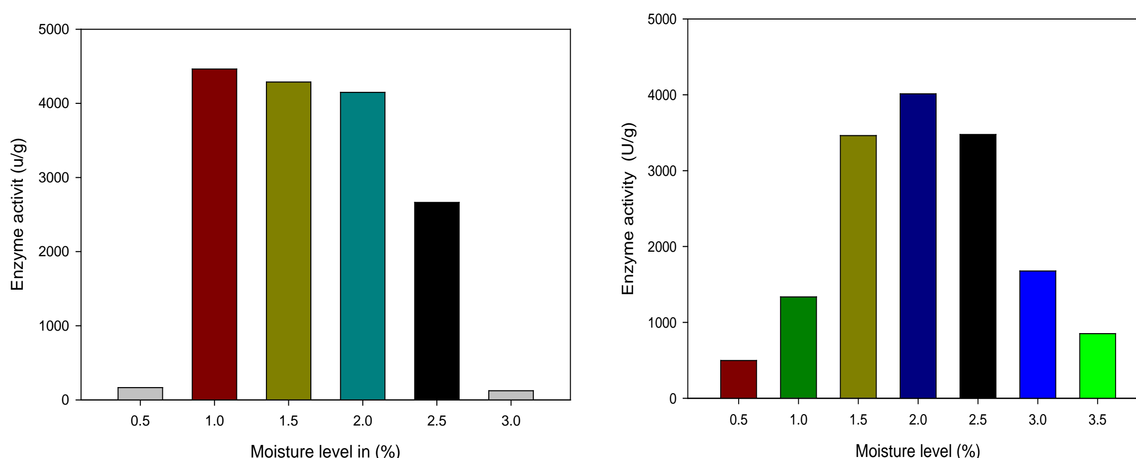


Figure 6(A) Effect of Moisture level on the level of amylase production by BI151 (B) and FI97, grown under SSF

5.4.2 Effect of nitrogen supplementation on amylase production under SSF

Under solid-state culture bacterial isolate BI151 produced the highest level of amylase production (7307 U/g) when cells were grown in wheat bran medium without any nitrogen supplement. Of the different nitrogen supplements tested enzyme production was highest in the presence of ammonium sulphate. Addition of ammonium nitrate led to a significant reduction in the level of amylase production (Fig. 7). The level of enzyme production was also very low when organic nitrogen sources (peptone and yeast extract) were added in the solid-state culture as nitrogen supplements.

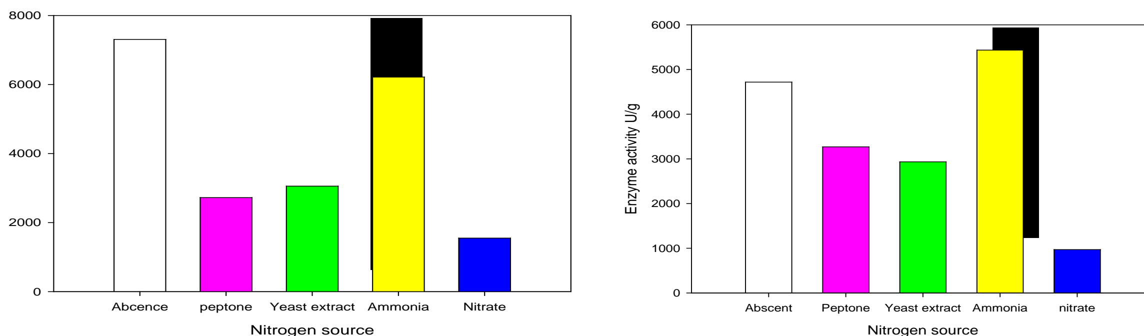


Figure 7(A). The e ffect of di fferent or ganic a nd i norganic ni trogen sources on am ylase production by isolate BI151 and (B) FI97, growen under SSF

For the fungal isolate FI97 the highest enzyme production (7,302 U/g) was recorded when ammonium sulfate was used as a nitrogen supplement. The next highest productivity was recorded when wheat bran alone was used as the nitrogen source. Low enzyme production was recorded in cultures supplemented with nitrate and organic nitrogen sources (Fig. 7B).

5.4.3 Effect of growth temperature on amylase production

The optimum temperature for amylase production under SSF was at 30˚C for isolate BI151 and 28°C for isolate FI97. Both isolates showed the next highest enzyme production at a growth temperature of 25˚C. At 37˚C both isolates had the least amount of enzyme production (Fig. 8)

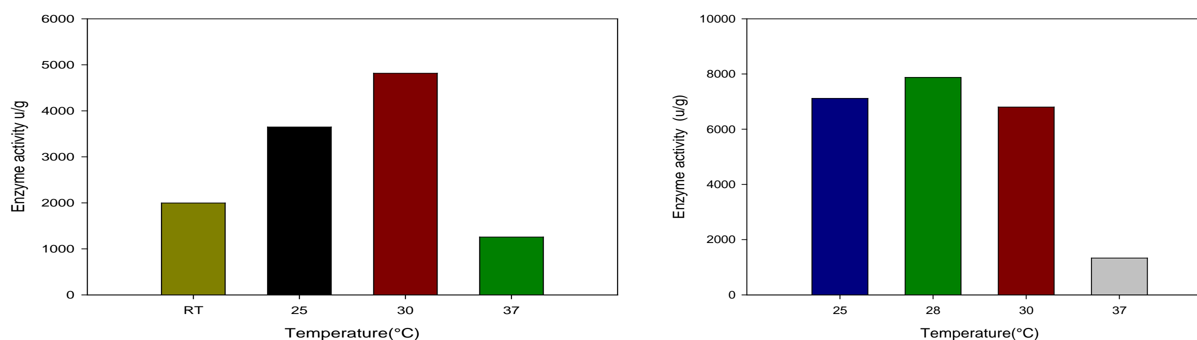


Figure 8(A) Effect of growth temperature on amylase production by BI151 (B) and FI97, grown under SSF

5.4.4 Time course of amylase production

Amylase production by BI151 reached to a maximum after 96 h of incubation, with a productivity of 4,636 U/g. Further incubation after 96 h resulted in a significant decrease in the level of amylase production (Fig. 9A). For isolate FI97 the amount of amylase produced increased with increasing incubation time and reached to a maximum (4,674 U/g) after 72 h incubation and after that productivity progressively decreased (Fig. 9B).

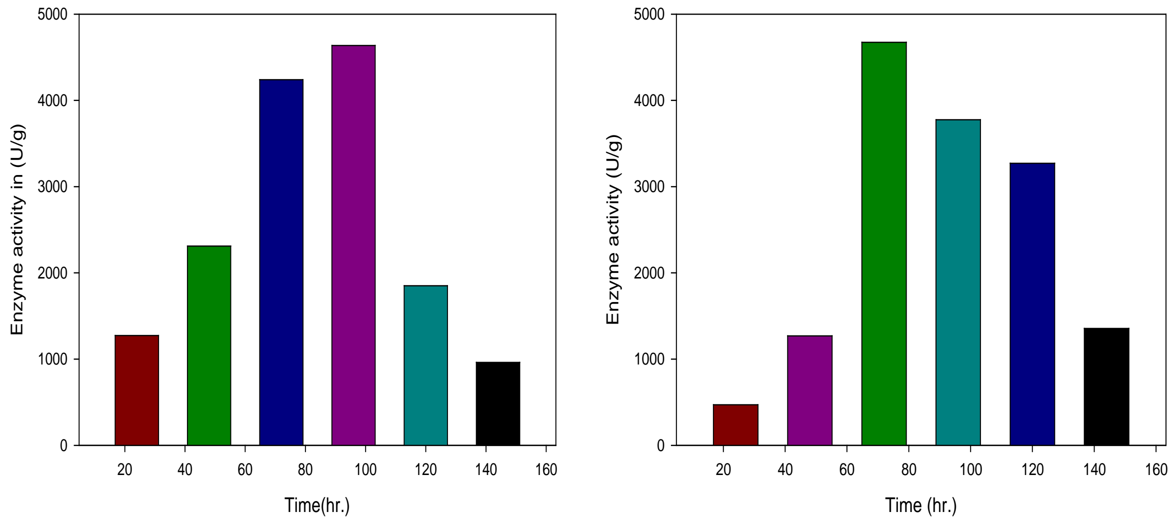


Figure 9(A) Time course of amylase production by BI151 (B) and isolate FI97, grown under SSF

5.5. Evaluation as malt enzyme supplements for the hydrolysis of starch

To test if amylase BI151and amylase FI97 could be used as malt enzyme supplements, standard malt powder was mixed with starch at a ratio of 4:6 (adjunct accounting for 60% of the mixture). The starch was separately gelatinized and different concentrations of the two enzymes (measured as Unites) were added and the degree of hydrolysis of the whole starch mixture measured by measuring the residual starch amount using the iodine test. For both enzymes the degree of starch hydrolysis progressively increased with increasing enzyme concentration. For amylase BI151 almost complete hydrolysis was reached in the presence of 25 U of the enzyme while for FI97 only 10 U was required.

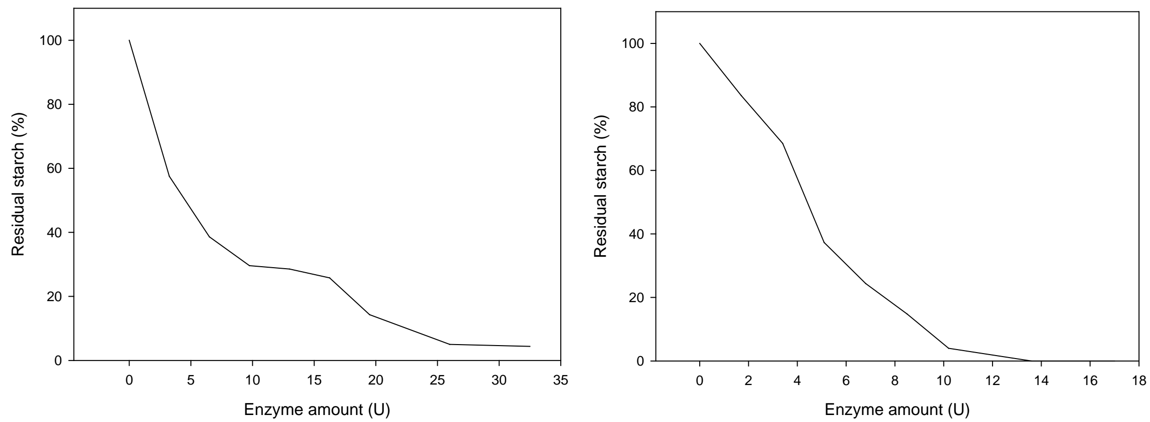


Figure.10(A): Hydrolysis profile of malt-starch adjunct mixture using amylase of BI151 (B)and amylase of FI97. After mixing malt (accounting for 40% of the total) and starch (60% and separately gelatinized), different amounts of the enzyme was added and incubated at the optimum temperature of each enzyme. After 30 min incubation residual starch was measured using quantitative iodine test.

6 Discussion

Worldwide large quantities of starch from different botanical sources is used for a number of food and none food applications. Because plants synthesize starch as a granule held together by hydrogen bonding, prior to any use it is essential to disrupt the granule by breaking the hydrogen bonding through heat treatment and resulting in gelatinization. Gelatinization of starch is associated with an in increase in viscosity. Although the high viscosity is important for some applications, for other applications it poses serious technical challenges. To reduce viscosity it is important to break the starch polymer into smaller oligosaccharides either using acid or enzyme hydrolysis. At present enzyme based hydrolysis using thermostable amylases is widely used for starch liquefaction (El-Tayeb *et al*., 2007). In addition, other amyloytic enzymes are used to generate other hydrolysis products from the liquefied starch, such as glucose, glucose syrup, maltose syrup, etc. The majority of these enzymes are obtained from microorganisms (fungi and bacteria).

Amylase producing microorganisms can be isolated from different habitats. But, soil is known to be the best source of amylase producing microorganisms and is sometimes considered as the storehouse of amylolytic microorganisms (Fossi *et al*., 2005 and Omemu *et al*., 2005). In this study over 900 bacterial and fungal isolates were isolated from soil. Only about 4% were found to have good amylase production. The initial screening procedure for amylase production was based on detection of extracellular enzyme production on agar plates. Thus the low amylase positive frequency might show low extracellular enzyme production rather than lack of enzyme activity. In some microorganisms amylases are produced but remain cell bound (Sheridan *et al.,* 2000).

Based on the characteristics of their enzymes and their suitability for starch hydrolysis, one bacterial isolate designated as BI151 and one fungal isolated designated as FI97were selected for further characterization. In the brewing process malt starch is hydrolyzed through stepwise increase of temperature from about 45˚C to 70˚C, a temperature range where malt amylases are optimally active (Bertoft, and Henriksnäs, 1983). Therefore to develop enzymes that are active in this temperature range were considered ideal. In this study optimal activity in this temperature was used as criteria for the selection of the two strains which were optimally active in the temperature range of 60˚C – 70˚C.

In the production of industrial enzymes the cost of fermentation medium accounts for up to 30% of the overall production cost (Pandey *et al*., 1999). Therefore, use of cheap substrates for enzyme production can have significant cost implication for large-scale use of enzymes. In this respect growth of amylolytic microbial strains under solid-state fermentation using cheap agricultural wastes help to significantly reduce enzyme production costs (Sodhi *et al*., 2005). In this study ability to grow under SSF was used as additional criteria for the selection of the two strains, isolates FI97 and BI151. Both isolates grew well under SSF using wheat bran as the solid substrate and produced high level of amylolytic activity. Earlier reports have also showed high level of amylase production using wheat bran as a substrate (Hema Anto *et al*., 2006; Mamo and Gessesse, 1999). Wheat bran is a cheap readily available substrate that is released as a byproduct in the process of flour milling.

Supplementation of wheat bran with different nitrogen sources, compared to non-supplemented culture, did not bring about any improvement on the level of enzyme production (and in some cases even lowered enzyme production). This shows that wheat bran has a good balance of the required nutrients for microbial growth. Earlier reports showed that wheat bran is composed of different chemical components occurring in varying proportions (Oluwatoyin *et al*., 2015). It contains 60 - 75% total carbohydrate (of which between 9 and 39% is starch), 10 - 19% proteins, and 4 - 8% ash. The ash component contains such essential minerals as Zn, Fe, Mn, Mg, and P. It also contains a range of vitamins and other phytochemicals (Oluwatoyin *et al*., 2015). This shows that wheat bran contains all the major nutrients required for the growth of microorganisms. The fact that these organisms do not require additional supplements greatly help to lower enzyme production cost.

Moisture is one of the crucial parameters affecting growth of microorganisms and influencing the level of enzyme production under solid state fermentation (Ellaiah *et al*., 2002).The optimum moisture level for enzyme production by isolates BI151and FI97 was 50% and 67%, respectively. Increasing or decreasing the moisture level above or below the optimum value resulted in a significant reduction in the level of enzyme production. This is due to the fact that at low moisture content solubility of nutrient is reduced and there could be a higher water tension thus leading to a lower enzyme yield. Increasing moisture level above the optimum, on the other hand, causes water molecules to occupy the air space in the wheat bran thus reducing gas

exchange due to less diffusion. This results in production of lower amount of amylase as a result of impaired oxygen transfer (Lévêque *et al.*, 2000).

Temperature is one of the other factors influencing growth and enzyme production under SSF. For isolates *FI97* and *BI151* the optimum temperature for maximum enzyme production was 28°C and 30°C, respectively. Enzyme production increased with increasing growth temperature until it reaches to the optimum and then progressively decreased with further increase in growth temperature. This may be due to the loss of moisture in the substrate which adversely affects the metabolic activities of the microbes leading to reduced growth and decline in enzyme production (Sivaramakrishnan *et al*., 2006).

Both isolates showed a fast growth rate on wheat bran under SSF. After 24 h of incubation both FI97 and ***BI151*** produced 1, 274 U/g and 470U/g of amylase activity, respectively. But maximum enzyme production for both isolates was observed after 72 and 96 h of incubation, respectively. Further incubation after the optimum period resulted in a sharp decrease in amylase production probably due to the accumulation of metabolic waste products, depletion of one or more nutrients, or release of proteolytic enzyme.

The optimum temperature for amylase of ***BI151*** was between a temperature of 65°C -75°C with a peak activity at 70°C. Similarly amylase FI97 showed highest activity at 65°- 70°C. Optimum activity at this temperature makes these two enzyme ideal candidates as supplementary enzymes in the brewing industry because malt enzymes are optimally active in the temperature range of 65 -70°C (Brányik *et al.,* 2005).

Commercial thermostable amylases currently used by the starch industry require $Ca^{2+}$for activity and stability (Nwagu and Okolo, 2011). Therefore, $Ca^{2+}$is normally added to the reaction mixture to stabilize the enzyme. But in subsequent steps the Ca^{2+}must be removed by ion exchange because it is inhibitory to the isomerase that is used to convert glucose into fructose (Reyed, 2007). This process adds up to the overall production cost. Therefore, amylases that do not require Ca^{2+} could offer significant advantages. Addition of $Ca^{2+}$to amylase **BI151** effect on its activity and stability at high temperature and this indicates its potential importance for starch processing.

pH is another factor that markedly affects the activity of amylases (Alva *et a l*., 2007) and has significant implication in its application. Amylase BI151 displayed 70% of its peak activity in the pH range of 4.5-6.5 with an optimum at pH 5. It was also stable in the pH range of 4.5-7.5. Most bacterial amylases are known to be optimally active and stable in the pH range of 6 to 8 (Oyeleke and Oduwole, 2009 and Daniel *et a l*., 2010). The use of such enzymes for starch processing is of great advantage because avoids any need for pH readjustment. This is especially important in the brewing industry where the commonly accepted optimal P^H range for starch conversion in the pH range of 5.2 - 5.7. But studies showed that quality of beer improves if the pH is lowered into the 5.2 - 5.4 range (Malomo *et al*., 2012). It is to be noted that the pH of the natural starch salary is 4.5. Therefore use amylase ***BI151*** as a brewing adjunct is expected to make P^H readjustment unnecessary.

Most of the fungal amylases are reported to be optimally active and stable in the acidic pH range (Hernandes *et al*., 2006). But amylase FI97 was optimally active in the pH range of 5-7 with a peak activity at PH 6and stable in the pH range of 4.5-8.0. This indicates that the pH at which the two enzymes from the bacterial and fungal isolates have an overlaps making them ideal to be used for starch processing, especially as brewery supplemental enzymes.

The two amylases (amylases BI151 and FI97) were separately tested as supplements of malt enzymes in the presence of very high concentration (60%) of starch adjunct. Both enzymes were very effective in completely saccarifying the adjunct. Currently Ethiopia imports about 50% of its malt requirement from abroad. In many countries different starch adjuncts are used as adjuncts replacing at least a quarter of the malt (Mulugeta, 2015). But as all the enzymes for starch hydrolysis s contributed by the malt component, addition of adjuncts could lower the amount of available enzyme for starch hydrolysis thus requiring the addition of exogenous enzymes. Implementation of such procedure could, therefore, allow significant savings in Ethiopia foreign currency expenditure. In addition, some reports showed that substitution of barley malt with adjuncts could result in the production of unique beer flavor/aroma (Abou-Elela, 2009). Commercial industrial enzymes in addition to their role in improving adjunct liquefaction, also proved important in the production of low-carbohydrate beer and shortening

beer maturation time (Malomo *et al* ., 2012) The two enzymes reported in this study might therefore find important application in the brewing industry.

7 Conclusion and recommendation

The two amylases did not require addition of calcium for activity and thermal and stability, and are optimally active and stable in the acidic pH range close to the optimum for malt amylases. These shows the potential importance of these two amylases for the starch industry, especially as malt enzyme supplements in the brewery industry. Both organisms grow and produce high level of enzyme activity under solid state fermentation using cheap and easily available raw material thus allowing significant reduction in the production cost of the enzymes and offer great economic advantage. In addition, the use of these enzymes as brewery supplements allow the use of locally available starch sources creating a huge market for locally produced agricultural products.

Based on the observations from this study it is recommended that the enzymes are tested under actual application conditions and the economic benefits of adding adjuncts and supplementary enzymes are critically evaluated.

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Declaration

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

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