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Isolation and screening of amylase producing thermophilic spore forming *Bacilli* from starch rich soil and characterization of their amylase activities using submerged fermentation

Berhanu Andualem

Department of Biotechnology, College of Natural and Computational Science, University of Gondar, Ethiopia

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Abstract

Use of thermostable amylases from bacilli for industrial application is significant. The search for such thermostable amylases from bacilli in starch rich soil was the objective of this study. Amylase producing bacilli were isolated and their enzymes were also characterized. Effect of temperature, pH, substrate and salt concentration on amylases activity were determined. All amylases produced by different isolates were hydrolyzed greater than 90% of starch after 60 h of fermentation. There was no significant ($P \geq 0.05$) variation in enzyme productivity along with fermentation time. Amylase producing isolates were designated as A1, A2 and A3. Aamylases activities of all isolates were reached their optimum at 60°C. Amylases from isolate A1, A2 and A3 were shown hydrolysis capacity of 96.59%, 97.39% and 97.78% of starch, respectively. The optimum enzyme activity of amylase from A2 isolate was extended from pH 7 to 8 with starch hydrolysis efficiency of 99.25% but other isolates enzyme activity reaches 100% at pH 8. Thermal and pH stability of all amylases were retained above 91%. Amylases of this finding with thermophilic, alkalophilic and halophlic characterstics have wide range of huge potnetial for industrial applications.

Introduction

Amylases are one of the most significant enzymes in biotechnology that had a share of approximately 25% of world enzymes market (Rajagopalan and Krishnan, 2008). They have wide range of applications, such as in baking and bread industry, paper industry, textile desizing, detergent industry, starch liquefaction and saccharification, food and pharmaceutical industries and lastly analyses in medical and clinical chemistry (Gupta *et al.*, 2003). One of the best characteristics of such enzymes is stability at high temperature. Therefore, thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have a number of commercial and industrial applications because of their stability. Amylases working at high temperature are important for industrial application. This high temperature help to decreased viscosity of the medium, increase substrate solubility and reduce risk of microbial contamination (Kuchner and Arnold, 1997).

Enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C) by the help of thermostable amylolytic enzymes. To get such potential thermostable amylolytic enzymes, currently investigation of improved microbial strains from different ecological niches contaminated with starchy substance is significant for industrial processes. Amylases produced from such potential microorganism are significant for starch degradation to produce valuable products like crystalline dextrose, glucose, maltose, dextrose syrup and maltodextrins (Bentley and Williams, 1996; Asgher *et al*., 2007).

Although many microorganisms produce thermostable amylases, the most commonly used for their industrial application are *Bacillus licheniformis*, *Bacillus amyloliquifaciens*, *Bacillus stearothermophilus*, *Bacillus subtilis* and *Aspergillus niger* (Riaz *et al*., 2003). They are known to be good producers of thermostable-amylase and have been widely used for commercial production of the enzyme for various applications (Prakash and Jaiswal, 2009). They have wide range of useful applications in the food, brewing, textile, detergent and pharmaceutical industries. In detergents production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry (Chengyi *et al.*, 1999).

Thermostable-amylases have been reported from several bacterial strains and have been produced through the use of submerged fermentation as well as solid state fermentation (Teodoro and Martins, 2000). Submerged fermentation utilizes free flowing liquid substrates or broths efficiently to produce desired bioactive compounds. Normally, such bioactive compounds are secreted into the fermentation

*Corresponding author.
Email: *tberhanu2007@gmail.com, anberhanu2007@yahoo.com*
Tel: +2519 1870 0027; Fax: 251 5814 1931

broth. This fermentation technique is generally best appropriate for bacteria that normally require high moisture content. An additional advantage of this technique is that recovery of products is relatively simple (Subramaniyam and Vimala, 2012). Another best advantage of submerged culture is the technique for sterilization and process control is easier to engineer in these systems (Vidyalakshmi *et al.*, 2009).

One of the factors used to produce amylase is optimization of culture condition. A number of investigators have been conducted researches to optimize culture condition for amylase production (Saxena *et al*., 2007). The chemical and physical parameters like pH, temperature, salt concentration, incubation time, etc. of microbial fermentation process play great role in enzyme production.

Isolation and identification of soil microorganisms with best amylase activity could contribute a lot for the discovery of novel potential amylases appropriate for industrial and biotechnological applications (Mohapatra *et al*., 2003). The bacilli isolated from soil are considered as an ideal source for the production of bulk extracellular amylase for industrial application (Riaz *et al*., 2003). Therefore, isolation and screening of thermophilic bacteria from soil samples are significant to discover novel new industrial enzymes. There is a need to isolate and screen amylase producing bacilli bacteria from starch rich soil associated with high temperature environment. Most of the time, the temperature in environment around cereal grinding machine is high and the soil is normally rich in content of starch of cereal flour. The soil in such area may be rich in bacilli species of bacteria with huge potential to produce thermostable amylase. Therefore, the objective of this study was to produce amylases from bacilli species and characterize their amylase activities using submerged fermentation process.

Materials and Methods

Isolation and screening of amylase producing Bacillus strains

This study was conducted from March to April, 2012. Samples were collected from Maraki (Gondar town) grain mill house soils rich in content of starchy flours. The sample site was located in the Northwest part of Ethiopia. To isolate an aerobic, rod shaped, gram-positive, thermophilic and spore-forming *Bacillus* sp., from each soil samples 1 g was introduced into 9 ml of distilled sterilized water and heated at 80°C for 10 min and then 1 ml was first enriched on starch broth containing 1% soluble starch (w/v), 0.5% peptone (w/v) and 0.50% yeast extract (w/v) at pH 7 and incubated at 45°C for 24 h with constant shaking at 150 rpm. Two percent of the enriched liquid medium was then spread on starch agar (1% soluble starch, 0.5% peptone, 1.5% yeast extract, 1.5% agar (Andualem and Gessesse, 2013). The plates were incubated at 45°C for 48-72 h until bacteria typical colonies obtained. The colonies were further sub-cultured on starch agar plates to get pure colonies. Iodine solution (1% iodine in 2% potassium iodide w/v) was folded over the surface of the plate in order to select amylase producing isolates. Those colonies having clear zone (more than 10 mm diameter) were selected for further investigation. Morphological characteristics of isolates were identified using gram staining techniques and colony morphology. The cultures were maintained on nutrient agar slants at 4°C.

Enzyme production

Amylase activity was assayed using starch as substrate 14, 27-28. The selected bacterial isolates (designated as A, A1, A2) were separately cultured at 45°C for 120 h, in 100 ml of starch broth (1% starch, 0.5% peptone and 1.5% yeast extract) in 250 ml flask and constantly mixed using rotary shaker at 150 rpm. The pH of the medium was adjusted at 7. The broth from each culture was centrifuged at 6000 rpm for 20 min and the supernatant was collected as crude enzyme extract. The crude extract was used for characterization of the enzyme activity and stability in different conditions (Bajpai and Bajpai, 1998).

Enzyme assay and characterization

The assay was carried out based on the reduction in blue colour intensity due to enzyme hydrolysis of starch (Bajpai and Bajpai, 1981; Oboh, 2005; Andualem and Gessesse, 2013). In this assay, 1 ml enzyme (cell free supernatant) and 10 ml of 1% starch solution were mixed and incubated at 45°C for 10 min. The reaction in the test tube was stopped by adding 10 ml of 0.1N HCl. One more dilution was made by mixing 1 ml of this acidified solution with additional 10 ml of 0.1 N HCl. From this, 1 ml was added to 10 ml iodine solution (0.05% iodine in 0.5% KI). Optical density (OD) of the solution was measured by spectrophotometer at 660 nm. A standard curve was prepared using starch (0 to 2.0 mg/ml) and a linear regression analysis was used to determine the total reducing sugar present as % starch equivalents. The same procedure was done using 1 ml-distilled water instead of 1 ml enzyme sample (Yang and Liu, 2004). One unit of activity was defined as the amount of enzyme that reduces the intensity of blue colour of

starch-iodine solution by 1% at the assay conditions. Stock solution and solution for enzyme assay was prepared from1% or 1000 mg/100 ml soluble starch.

Effect of temperature on amylase activity

The effect of temperature was determined at different temperatures (40, 45, 50, 55, 60, 65, 70, 75, 80^{o}C) at pH 7. One ml of crude culture extract enzyme was mixed with 10 ml of 1% soluble starch in sodium phosphate buffer (pH 7) and incubated in a water bath at different temperature for 10 min. The reaction was stopped by using 10 ml of 0.1 N HCl. One more dilution was made by mixing 1 ml of this acidified solution with additional 10 ml of 0.1 N HCl. Then 1 ml from this solution was added to 10 ml iodine reagent containing 0.05% iodine and 0.5% KI. The OD-value was measured at 660 nm (Andualem and Gessesse, 2013).

Effect of pH on amylase activity

The effect of pH on amylase activity was determined on starch solutions (1%) at different acetate buffer and sodium phosphate buffer pH range (4.0-9.0) at 60^{o}C for 10 min. The amylase activity was similarly determined from reduction in blue colour intensity (Andualem and Gessesse, 2013).

Effect of substrate concentrations on amylase activity

Amylase activity of various crude amylase preparations was assayed at various substrate concentrations of 0.5, 1.0%, 1.5%, 2.0%, 3.0% and 4.0 % starch solutions in sodium phosphate buffer at pH 8 and temperature of 60^{o}C for 10 min of incubation (Oboh, 2005; Andualem and Gessesse, 2013).

Effect of NaCl concentration on the stability of amylase

The enzymes were incubated in 1% starch solution containing 0, 1, 2, 3, 4, 5 M NaCl solution for 10 min at 60^{o}C and pH 8 (Andualem and Gessesse, 2013).

The time course of starch hydrolysis by crude amylase extract from thermophilic soil bacillus bacteria

Determination of incubation time was carried out up on reaction mixture to estimate the time required for maximum amylase activity (2, 5, 10, 15, 20, 30 min).

Growth rate and enzyme production dynamics

Growth rate and enzyme activity was determined by taking samples aseptically from submerged fermentation at the interval of 20 hrs (0, 20, 40, 60, 80, 100).

Stability of the enzyme toward pH and temperature

The enzyme stability was done by measuring the residual activity of the enzyme after being incubated for specific period at specific pH and temperature based on the method applied by Yang *et al*. (2004). The thermal activity of each enzyme was evaluated by incubating each enzyme at 65^{o}C for 60 min. At specific interval time (10 min) the activity of enzyme was determined. (0, 10, 20, 30, 40, 50, 60) (Andualem and Gessesse, 2013).

Data analysis

The data in this study were analyzed using SPSS version 16.0. Means and standard deviations were calculated using analysis of variance (ANOVA) to analyze the significance differences between the means using Duncan's Multiple range test ($p < 0.05$) when the F-test demonstrated significance. The triplicates mean values of tests were analyzed. Significant difference was defined as $p < 0.05$.

Result and Discussion

Several species of *Bacillus* are widely known to produce various kinds of extracellular enzymes having wide range of industrial application. Of those intercellular enzymes, amylases are the most significant for industrial production. The amylase activity and characteristics of all bacterial isolates were determined after 120 h of fermentation (Figure 1). The production of amylase and rate of bacterial isolates growth (Figure 2) were analyzed in the medium containing 1% starch as source of carbon and 0.5% peptone as a source of nitrogen. This medium was supplemented with 1.5% yeast extract. The measurement of cell growth rate and enzyme activity of bacteria isolates were carried out at every 20 h time intervals as shown on Figures 1 and 2. All isolates which are designated as A1, A2 and A3 were gram positive bacilli and they were screened based on size of clear zone diameter (> 15 mm) formed on starch agar. In all bacterial isolates, amylase production was increased together with cell mass increment after 40 h of fermentation. Moreover, those isolates showing high concentration of product (fermentable sugars) i.e., above 90% after 120 h fermentation were selected for further investigation. With regard to enzyme production kinetic or dynamics of amylase producing spore former bacilli, all isolates were able to hydrolyze > 90% of starch after 60 h of submerged fermentation. High biomass (OD) of amylase producing thermopile *Bacillus* bacteria was seen after 120 h of incubation period. Isolate A2 was shown the highest (3.65 OD) biomass in comparison with other

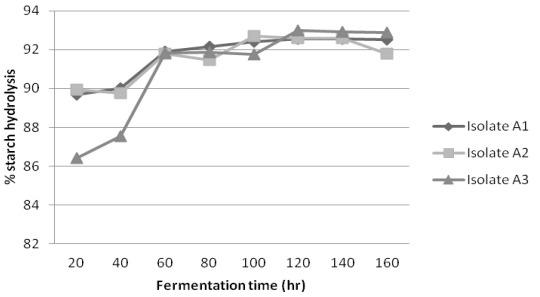

Figure 1. Enzyme production kinetic or dynamics of amylase producing spore former bacilli against time (h) of fermentation. Amylase producing spore former thermopile bacteria were designated as isolate A1, isolate A2 and isolate A3.

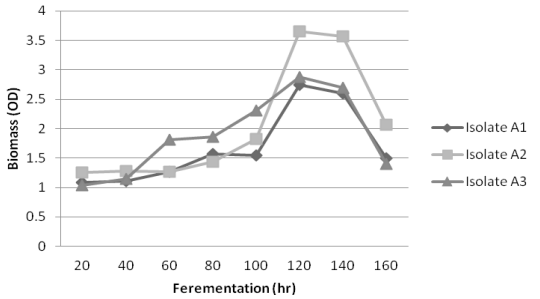

Figure 2. Biomass of amylase producing thermophile bacillus bacteria at different incubation period (h)

two isolates (range from 2.72 to 2.87 OD) at stationary phase after 120 h incubation. Among studied isolates, there was no significant ($P \geq 0.05$) variation in enzyme productivity but there was significant ($P \leq 0.05$) difference in biomass. When the isolated bacteria were cultured in submerged fermenter, gradual increment of biomass up to 120 h of fermentation but the amylases activity were continued to remain up to 160 hour of fermentation. In brief enzyme synthesis started after 40 h of fermentation and extended up to 120 h of fermentation process. Determination of period of bacterial growth and amylase productivity are significant to optimize time of product recovery.

Determination of effect of temperature and pH for amylases produced from spore former bacilli are significant to optimize fermentation process during enzyme production. The optimum temperature of amylase reaction was analyzed by the incubation of crude amylase extract at temperature range of 40-80°C (Figure 3). Crude amylase activity of all isolates was relatively increased from 40°C up to 55°C and sharply reaches their optimum reaction activity at 60°C. After optimum temperature, as temperature increases, amylase activities of all isolates were reduced. This could be due to the breakage of secondary, tertiary and quaternary bonds that maintain the three dimensional structure of enzymes at high temperature and thereby would lead to conformational changes of the enzyme active site. It is known that enzyme activity increases with increasing temperature up to the optimum

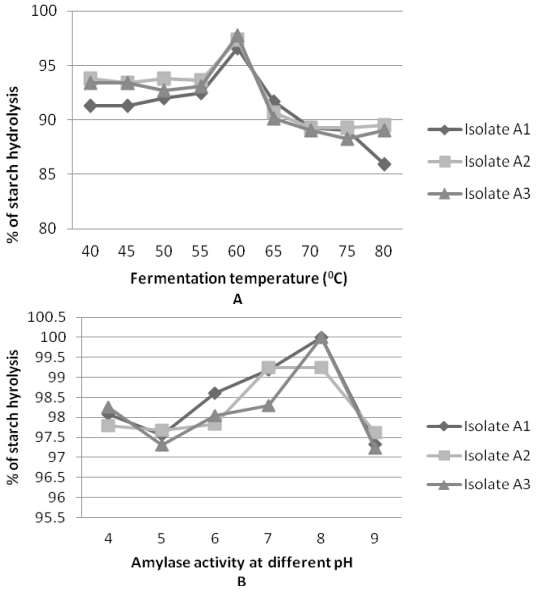

Figure 3. Effect of temperature (A) and pH (B) on the activity of amylase produced from thermophilic bacillus

temperature as the result of increasing kinetic energy, which can favor rate of collisions between substrate and enzyme during hydrolysis process. The amylase activity of isolate A1 was statistically ($P \leq 0.05$) reduced after 75°C. In this study, the optimum temperature for amylases produced from all bacteria isolates was 60°C. At this optimum temperature, amylase from isolate A1, A2 and A3 have shown starch hydrolysis capacity of 96.59%, 97.39% and 97.78%, respectively. High optimum temperature activity of the amylase offers some advantages for industrial process such as reduce the cooling cost, lower viscosity of the substrate, reducing the risk of microbial contamination and provides better solubility at high temperature (Burhan *et al*., 2003). The amylase activities of all enzymes produced from three bacterial isolates in this study were in line with some scientific reports (Burhan *et al.*, 2003; Afiukwa *et al.*, 2009). However, the optimum temperature of amylases reported in this study was lower in comparison with amylases produced from the spices of *Thermus* (70°C) (Shaw *et al*., 1995) and *Thermus filiformis* (95°C) (Egas *et al*., 1998). Thus, amylases with optimum activity at 60°C, like in this study, have properties considered to be significant for industrial starch liquefaction. Generally, all amylases produced from all bacterial isolates were not shown significant ($P \geq 0.05$) activity along with different temperature treatment.

The effect of pH on the enzyme activity of all isolates in this study is shown on Figure 3. The amylase activity of A2 was increased from 97.6% at pH 5 to 99.25% at pH 7. Its optimum enzyme activity was extended from pH 7 to 8 with starch hydrolysis efficiency of 99.25%. At the same time, amylase

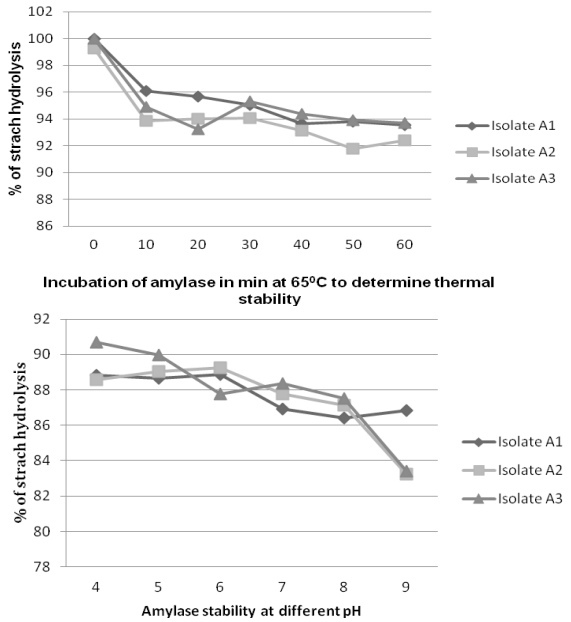

Figure 4. The effect of temperature (A) and pH (B) on the stability of amylase produced from thermophilic bacilli

activity of isolate A3 was increased from 97.3% at pH 5 to 100% at pH 8. Thus, its optimum amylase activity was at pH 8. On other hand, amylase activity of isolate A1 was increased from 97.57% at pH 5 to 100% at its optimum pH 8. The optimum amylase activity (99.25%) of A2 was significantly ($P \leq 0.05$) reduced in comparison with the rest isolates enzyme activities (100%) at pH 8. All bacterial isolates amylase activities were increased with increment of pH up to optimum and reduced after their optimum pH, i.e., pH 8. The optimum pH of amylases activities found in this study was in line with that of optimum amylase activity reported by Fatoni and Zusfahair (2012). Changes in pH, like that of temperature, above or below optimum point alter the active site of three dimensional structure of the enzyme and substrates binding speed to produce maximum product will be highly reduced (Garrett and Grisham, 1999). The optimum pH of the amylase in this study was in line with the optimum pH (i.e., pH 8) activity of amylase produced by *Thermus* sp. (Fatoni and Zusfahair, 2012) and amylase from *Bacillus* KSM-K38 (Hagihara *et al*., 2001). However, this optimum pH was higher when compared with that of amylase produced by other *Thermus filiformis* of 5.5-6.0 (Egas *et al.*, 1998) and *Thermus* sp. of 5.5-6.5 (Shaw *et al*., 1995). In other studies it was shown that optimum pH could range from 5.0 to 10.5. Amylase isolated from such bacteria has high enzymatic activity at alkaline pH has huge potential for detergent industry. It is known that enzymes in detergents have potential abilities to remove tough stains without any environmental effects. Amylases are the second type of enzymes used for formulation of enzymatic detergents (Gupta *et al*., 2003; Hmidet *et al*., 2009). Currently, such type of enzyme formulations are widely used for laundry and automatic dish washing in order to remove starchy food substances derived from gravies, potatoes, chocolate, custard, and other smaller oligosaccharides (Mukherjee *et al*., 2009).

The thermal stability of each enzyme was evaluated by incubating each enzyme at 65ºC for 60 min (Figure 4). Samples were taken at 10 min interval and starch hydrolysis activity was estimated. In this study, 100% of the original activity of amylase produced by isolate A1 was reduced to 96.11%, 95.65%, 95.05%, 93.65%, 93.81% and 93.56% at 10, 20, 30, 40, 50 and 60 min of thermal treatment, respectively. With regard to amylase produced by isolate A2, 99.25% of the original enzyme activity was reduced to 93.85%, 94.02%, 94.04%, 93.13%, 91.75% and 92.42% at 10, 20, 30, 40, 50 and 60 min of thermal treatment, respectively. The original amylase activity (100%) of isolate A3 was reduced to 94.89%, 93.94%, 95.3%, 94.39%, 93.90% and 93.70% at 10, 20, 30, 40, 50 and 60 min of temperature treatment, respectively. The thermal stability of all amylases in this study was retained above 91% of the original enzyme activity. All enzymes of this investigation have high thermal stability. Thermostable amylases from three bacilli isolates can be used for starch hydrolysis up to 60ºC. This is the basic property that to be considered to be very important for industrial starch hydrolysis.

The pH stability of amylase enzymes obtained from three soil bacilli is shown on Figure 4. A wide range of pH stability might have significant advantage of preserving and handling the enzyme for commercial and industrial process. In this investigation, all three isolates exhibited nearly or over 86.95% of the original enzyme activity over a wide range of pH (4 -8) (Figure 4). Similar pH stability for amylase from three *Bacillus* isolates and *Bacillus licheniformis* (Saito, 1973) were reported.

Effect of salt on enzyme activity was presented on Figure 5. Salt tolerance of amylases in this study were evaluated in the presence of different NaCl concentration (0 to 8 M NaCl). Except isolate A2, the rest two isolates amylases activities or starch hydrolysis capacity were 100% starting from 0 to 5 M NaCl solution but isolate A2 starch hydrolysis potential was 99.25% at 0 M Nacl concentration. The amylase activity of isolate A2 and A3 were sharply decreased after 5 M Nacl concentration i.e., 99.22% at 8 NaCl concentration. This result implies that the enzymes of these isolates are halotolerate and are comparable with some halophilic amylases reported in the literature (Carvalho *et al*., 2008).

The starch hydrolysis activity of bacteria isolates

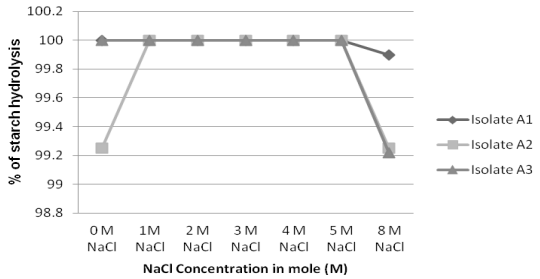

Figure 5. Effect of concentration of substrate and NaCl concentration on the activity of amylase produced from thermophilic bacillus

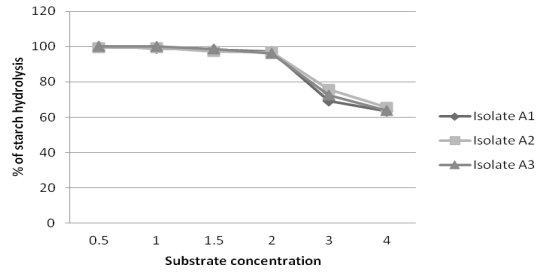

Figure 6 .The effect of concentration of substrate on amylase activity

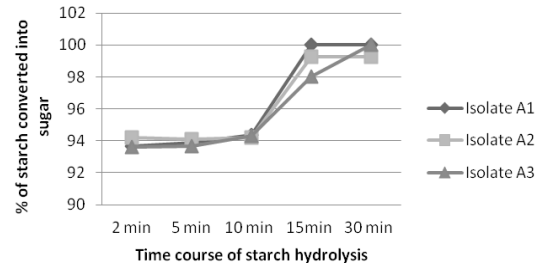

Figure 7. The time course of amylase from thermophilic bacteria required to hydrolysis starch

was measured at optimal pH and temperature (pH 8 and 60°C) (Figure 6). In all bacilli isolates, there was a high enzyme activity in the range between 0.5 to 2% of starch concentration. This result was in agreement with that of Alli *et al.* (1998) and Oboh (2005). In this result, the amylase activity of isolate A1 was only reduced from 100% to 63.23% from the range of 0.5% to 4% starch substrate, respectively. The amylase hydrolysis activity of isolate A2 was reduced from 99.25% to 65.43% within the range of 0.5 to 4% starch concentration, respectively. With regard to isolate A3, the amylase activity was reduced from 100% to 63.88% in the range from 0.5% to 4% starch concentration, respectively. According to this finding, optimal hydrolysis of starch into fermentable sugars, the concentration of starch may not be surpasses over 2%. This data is significant to optimize fermentation process within this range of substrate concentration.

The incubation time of amylases activity of bacterial isolates were determined and measured at various incubation times (Figure 7). The optimum enzyme activity of isolate A1 and A2 was 100% and 99.25%, respectively, at 15 min of incubation, while 100% starch hydrolysis was observed by isolate A3 at 30 min of incubation. Determination of time course of starch hydrolysis is significant to hydrolyze the substrate efficiently during industrial fermentation process.

Conclusion

In this study, three best amylase producing bacilli were isolated from soil rich in content of starch at grain milling house. Determination of period of bacterial growth and amylase productivity are significant to optimize time of product recovery. Amylases obtained with optimum activity at 60°C could be significant for industrial starch liquefaction. Amylase isolated from such bacteria in this study with high enzymatic activity at alkaline pH has huge potential for detergent industry. Thermostable amylases from three bacilli isolates may be used for starch hydrolysis up to 60°C, which is a basic property of an enzyme for industrial starch hydrolysis. A wide range of pH stability have significant advantage of preserving and handling the enzyme for commercial and industrial process. Generally, high temperature and pH stability of such enzymes have also potential application in industrial food biotechnology. The out come of this study implies that the enzymes of these isolates are halotolerate. Determination of time course of starch hydrolysis is significant to hydrolyze the substrate efficiently during industrial fermentation process.

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