



Original Article

Thermophilic amylase from *Thermus* sp. isolation and its potential application for bioethanol production

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Abstract

Limited reserves of fossil energy stimulate researchers to explore for a new alternative energy, such as bioethanol. A thermophilic amylase producing bacterium was isolated from local hot-springs and its characteristic and potential application for bioethanol production was determined. The obtained amylase was studied to determine its optimum temperature, pH, enzymatic reaction time, and substrate concentration. Tapioca waste was used as the substrate to find the potential of the amylase for degrading starch into glucose, and then the process was continued by fermentation to produce bioethanol. The amylase producer bacterium was proposed as genus *Thermus* sp. The crude amylase that was obtained has the optimum temperature of 60°C and optimum pH of 8.0, optimum substrate concentration at 10% (w/w) and optimum enzymatic reaction time of 45 minutes. These enzymes convert the starches of waste tapioca at optimum conditions, with the result of 2.9% ethanol produced from raw materials.

Keywords: thermophilic amylase, bioethanol, *Thermus* sp., extracellular amylase

1. Introduction

Limited reserves of fossil energy have stimulated researchers to seek a new renewable energy source. One alternative energy source that is widely studied is bioethanol, a renewable energy which is more environmentally friendly and reliable. Generally, bioethanol is produced from carbohydrate materials, either from simple or complex carbohydrates. Starchy material is the most widely used raw material for bioethanol. In the bioethanol production process, starch in carbohydrates is firstly converted into glucose, and then the glucose is fermented to form bioethanol. Conversion of starch into glucose can be done chemically or enzymatically using amylase enzyme group.

In industrial process, heat may be applied such as for sterilization, improving chemical reaction or evaporation.

Therefore, enzymes for industrial application must have high temperature activity and stability. Improvements in stability, particularly thermo stability, are commonly sought by protein engineers. In industrial processes, high temperatures give benefits such as increased substrate solubility, decreased viscosity of the medium and lowered risk of microbial contamination or higher rates of concurrent non-enzymatic reactions (Kuchner and Arnold, 1997). Thermostable enzymes that are isolated mainly from thermophilic organisms have found a number of commercial applications because of their overall inherent stability (Demirjian *et al.*, 2001). Enzymes from thermophilic microorganisms are referred to as thermozymes, and display unique characteristics such as temperature, chemical and pH stability. They can be used in several industrial processes, in which they replace chemicals or mesophilic enzymes (Bruins *et al.*, 2001). Amylase is supposed to be active and stable at high temperatures such as in the processed gelatinization (100-110°C) and liquefaction for the efficiency of the process, therefore more studies are needed to find new thermophilic and thermostable amylases (Burhan

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et al., 2003). This study reports the isolation and biochemical properties of an extracellular amylase from hot spring bacteria.

2. Materials and Methods

2.1 Materials and apparatus

Hot-spring water sample was collected from local hot spring located at Purwokerto Distric, Central Java Province, Indonesia. Nutrient broth (NB) medium, nutrient Agar (NA) medium, I / KI solution (0.1% I₂ and 1% KI), Cu-tartrate, arseno-molybdate, phosphate buffer, tris HCl buffer, sodium acetate buffer, borax-NaOH buffer, sodium chloride and sodium tungstate were all bought from Merck, USA. All chemicals used were analytical grade unless otherwise noted. Main items of apparatus used were spectrophotometer UV-Vis (Shimadzu, Japan), shaker incubator (Mettler, Germany), centrifuge (Heraeus sepatech, Germany), gas chromatograph (Agilent, US), pH meter (Hanna, USA) and autoclave (All American, USA).

2.2 Methods

2.2.1 Sampling

A random sampling technique was carried out. Hot-spring water samples were taken from three random locations on the surface of hot water and were put in a sterile flask. The temperature and pH of the hot spring water for sample collection were measured.

2.2.2 Bacteria isolation

Hot-spring water sample of 20 ml was mixed with 5 ml concentrated (five times regular concentration) NB medium in a 250 ml Erlenmeyer flask. The mixture was then incubated in a shaker bath at the sample's original habitat temperature and pH for 48 hours. About 0.1 ml of the culture medium was poured on NA medium, and was then incubated under the same conditions as above. Grown colonies were isolated and purified by repeated scraping the quadrant on the same agar medium to obtain a single colony.

2.2.3 Amylase-producing bacteria screening

Amylase producer bacteria were screened based on their ability to degrade starch in the medium (Shaw *et al.*, 1995). Every single bacterial isolate was grown on starch containing NA medium at the origin habitat temperature for 48 hours, and then examined by dropping an iodine solution around the bacterial colony. A positive amylase-producing bacterium was shown by the formation of a clear zone around the bacterial colony.

2.2.4 Crude amylase isolation and optimum production time optimization

Amylase production curve was required to obtain the incubation period with the highest amylase activity. An inocula were prepared by transferring 100 ml of culture into 25 ml of inoculum medium and incubating the mixture in a shaker bath with agitation of 200 rpm for 24 hours at sample's origin temperature and pH. Two ml of inoculum was poured into 100 ml production medium and was incubated under the same condition. The extracellular crude amylase was reduced and secreted by bacteria on the grown medium. The amylase was separated from the biomass using centrifugation at 3000 x g for 15 minutes. Optimum enzyme production time was determined by analyzing the activity of amylase at various production times i.e. 6, 12, 18, and 24 hours. Bacterial growth was monitored by measuring its optical density at 660 nm using a spectrophotometer. Extracellular amylase was then produced using the same procedure and conditions mentioned above at the optimum production time.

2.2.5 Amylase activity

The activity of the amylase was tested using starch as the substrate. The reducing sugar released as an enzymatic reaction product was measured according to the Somogyi-Nelson method (Nelson, 1944). A sample tube containing 0.5 ml of 1% starch was incubated for 5 minutes at 37°C. After five minutes 0.5 ml of amylase and 0.5 ml of 0.85% NaCl were added and the incubation was continued for 30 minutes. The enzyme activity was stopped by adding 1 ml of 10% sodium tungstate and 1 ml of 0.7 NH₂SO₄. A control tube was also prepared using the same procedure in the absence of amylase. The sample and control solution was filtered and the reducing sugar was measured in the filtrate obtained.

The reducing sugar was measured by preparing a series of 5 ml test tube, each was filled with 0.1 ml of sample, control and standard solution of glucose at the concentrations of 100, 200, 300, 400, and 500 mg.ml⁻¹. 0.2 ml of alkaline cupric tartrate was added in each tube and was held at 100°C for 30 minutes. This mixture was then cooled and 0.2 ml arseno molybdate followed by 7.5 ml of distilled water added. The absorbance of the samples was measured at 660 nm and the reducing sugar was calculated using the following formula:

The number of reducing sugar produced each ml enzyme = reducing sugar in sample - reducing sugar control (mg.ml⁻¹).

2.2.6 Enzymatic starch hydrolysis condition optimization

1) Substrate concentration optimization

The substrate that was used in this study was tapioca

solid waste. About 100 g of starch was dried to a maximum of 16% water content. One gram of the substrate was dissolved in 50 ml phosphate buffer pH 7. Starch solution was incubated in the oven at 70°C for 15 minutes and then the solution was transferred to an incubator at the temperature of 45°C. Substrate concentration variation was performed using the same procedure as the enzyme activity test, with concentrations of 2%, 4%, 6%, 8%, 10%, 12% and 14% (w/v).

2) Optimum pH determination

Substrate at the optimum concentration was dissolved in 5 ml of various buffers (pH 4.0 to 10.0). For pH of 4.0 and 5.0, sodium-acetate buffer was used, for pH of 6.0 and 7.0 sodium phosphate buffer was used, for pH of 8.0 and 9.0 Tris-HCl was used, whereas for pH of 10 borax NaOH buffer was used. This procedure was performed under the same procedure as the amylase activity test with various pH conditions.

3) Optimum temperature determination

This procedure was performed under the same procedure as the amylase activity test, using optimum pH and substrate concentration, and various temperatures of 30, 35, 40, 45, 50, 55, 60, 65, and 70°C for 45 minutes. The temperature that resulted in the highest reducing sugar was the optimum temperature for enzymatic reaction.

4) Determination of optimum enzymatic reaction time

Using the optimum substrate concentration, pH and temperature, various enzymatic reactions were tested at 30, 45, 60, 75, and 90 minutes.

2.2.7 Hydrolysis of starch and fermentation into bioethanol

This procedure was performed under the same procedure as the amylase activity test, using optimum pH, substrate concentration, temperature, and reaction time. The glucose as the enzymatic product reaction in the last step was fermented using commercial yeast to produce bioethanol. About 10 ml of glucose solution was added with 1 ml of 0.3 g.ml⁻¹ yeast solution and was incubated in anaerobic condition for 48 hours at 37°C. Bioethanol produced in the solution was assayed using gas chromatography with n-propanol as the internal standard. One ml of bioethanol solution was put in a 10 ml volumetric flask and 0.5 of n-propanol and demineralized water to the total volume of 10 ml added. About 0.1 ml of mixture was injected into the gas chromatograph with an initial temperature of 75°C, final temperature of 175°C and rate of 2°C.min⁻¹. (Institute of Medicine, Committee on Food Chemicals, 2003)

3. Result and Discussion

3.1 Bacteria isolation and identification

The hot-spring water sample was taken in the rainy season with the observed temperature of 52°C and pH 8.0. This original habitat temperature was used for bacterial growing incubation for producing thermophilic amylase. Bacterial amylases are produced at a wide range of temperature, for example *Bacillus* sp. have been reported to produce amylase at temperature of 37-60°C (Mishra and Behera, 2010; Sivaramkrishnan *et al.*, 2006), amylase from *Bacillus licheniformis* was produced at 70°C (Alkando and Ibrahim, 2011).

Amylase was successfully produced by the bacteria isolated from the hot spring. This condition was observed from the formation of a clear zone around the colony with the iodine test in starchy medium (Figure 1). A clear zone indicated that the starch has been hydrolyzed by the amylase produced by bacteria, whereas purple zone was the results of starch and iodine reaction.

The genus of the amylase-producing bacteria was identified by observation of the morphology and some biochemistry reactions described below (Table 1) (Holt *et al.*, 1994). The genus proposed of the amylase-producing bacteria obtained was *Thermus* sp. This bacteria have been reported for amylase production (Egas *et al.*, 1998; Shaw *et al.*, 1995). Among bacteria used as a source of amylase, the genus *Bacillus* is the most widely used bacteria to produce thermostable amylase and includes *B. subtilis* (Ensari *et al.*, 1995; Konsula and Liakopoulou-Kyriakides, 2004), *B. stearothermophilus* (Manning and Campbell, 1961), *B. licheniformis* (Ashraf *et al.*, 2003; Saito, 1973), and *B. Amyloliquefaciens* (Demirkan *et al.*, 2005).

After amylase-producing bacteria identification had been performed, the amylase production time was optimized.

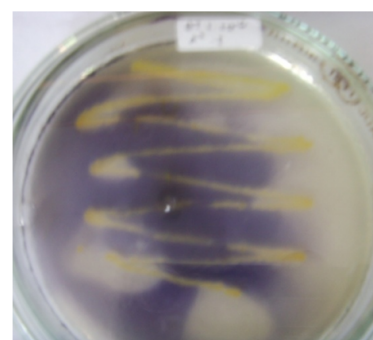


Figure 1. Clear zone observed around amylase-producing bacteria. The bacteria were grown on a nutrient agar medium contains 0.1% soluble starch, incubated at 52°C for 48 hours.

Table 1. Morphological and biochemical characteristic of amylase-producing bacteria

No	Examination	Description
1	Colony characteristic	Yellow, translucent, circular, uneven edge
2	Cell shape	Rod
3	Cell arrangement	mostly solitary, some duplex
4	Growth on NB	Aerobic, microaerophilic
5	Endospores	Negative
6	Catalase reaction	Positive
7	Oxidase reaction	Positive
8	Nitrate oxidase	Positive
9	Gram staining and 3% KOH	Negative
10	Idols	Negative
11	Motility	Non motile
12	pH	7
13	Temperature	30–50°C
14	Maltose	Negative
15	Genus proposed	Thermus

Note : *Escherichia coli* and *Bacillus subtilis* were used as control on each test

The optimum production time was determined by isolating the enzymes produced in the incubation period of 0-48 hours and observing every three hours. Data determining the optimal production time (Figure 2) showed optimum protease production for amylase-producing bacteria was at 39 hours.

3.2 Amylase optimum substrate concentration

Determination of the concentration of substrate was performed using various substrate concentrations between 2% - 14% with an interval of 2%. Optimum substrate concentration (Figure 3) was found at the concentration of 10%. Substrate concentration was increased until a certain amount of enzymatic reaction accelerated the reaction until it reached the optimum condition. Addition of the substrate concentration exceeding the maximum concentration will not increase the speed of enzymatic reactions, because the active enzyme has to bind all of the substrate, so the addition of the substrate concentration will not affect the reaction rate, because it has reached the maximum rate when the enzyme has been saturated by the substrate (Lehninger *et al.*, 2005).

3.3 Amylase optimum pH

The optimum pH was determined in the range of pH 4 to 10 on amylase crude extract (Figure 4). Amylase activity was increased with pH until it achieve the optimum pH and the activity was decreased after reaching the optimum pH. Changes in pH alter the amino acid side chain ionization on the active side of the enzyme and therefore the enzyme will be in the best conformation at the optimum pH. This three-dimensional structure at the best conformation of the enzyme can be caused by binding and processing of substrates with a maximum speed resulting in maximum product (Garrett and

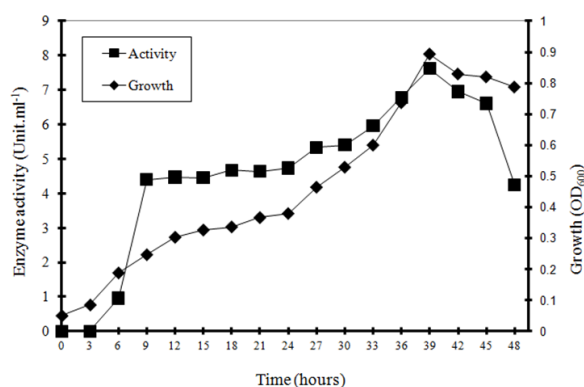


Figure 2. Amylase was produced at different stages of bacterial growth, and amylase activity was estimated in the culture supernatant. The bacteria were grown using a medium contain 0.2 % (b/v) of soluble starch and were incubated at 52°C and pH of 8.0. The growth was monitored by measuring its optical density at 600 nm.

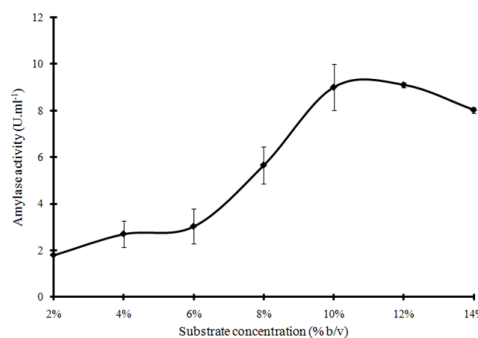


Figure 3. Substrate concentration effect on amylase from *Thermus* sp. activity. The enzymatic reaction was performed at 45°C for 30 minutes using various substrate concentrations in a buffer pH of 8.0.

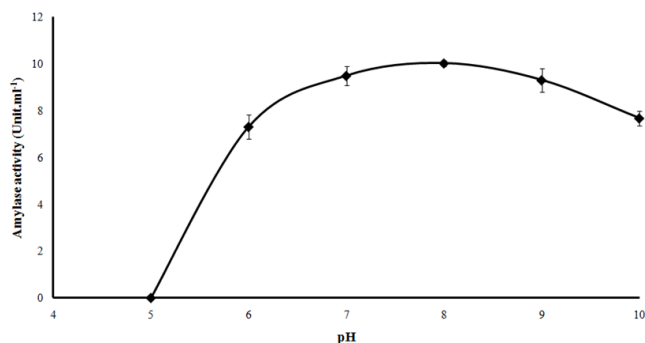


Figure 4. Effect of pH on amylase from *Thermus* sp. activity. The enzymatic reaction was performed using 10 % (b/v) of starch substrate in various buffers pH at 45°C for 30 minutes.

Grisham, 1999). The optimum pH of the amylase studied was 8.0 and this optimum pH was higher compared to that of amylase from *Thermus* sp. of 5.5-6.5 (Shaw *et al.*, 1995) and *Thermus filiformis* of 5.5-6.0 (Egas *et al.*, 1998). In similar studies amylase from *Bacillus* KSM-K38 had an optimum pH of 8 (Hagihara *et al.*, 2001). Other studies have reported some of the bacterial amylase has an optimum pH in the range 5.0–10.5 (Sivaramakrishnan *et al.*, 2006).

3.4 Amylase optimum temperature

The optimum temperature of enzymatic reaction was determined by the incubation of the amylase crude extract at temperature range of 45-75°C (Figure 5). Enzyme activity increases with the increasing temperature up to the optimum temperature, due to increasing kinetic energy that increases the chances of collisions between enzyme and substrate resulting higher substrate-product catalysis. After reaching the optimum temperature, increase in temperature decrease the amylase activity. The high temperature will break the secondary, tertiary and quaternary bonds that maintain the enzyme in its natural structure. Enzyme denaturation would lead to conformational changes of enzyme active side (Lehninger *et al.*, 2005). The amylase produced showed its optimum activity at 60°C. This high optimum temperature of the amylase provides some advantages in the industrial process such as reducing the risk of microbial contamination, reduce the cooling cost, and better solubility and lower viscosity of the substrate at high temperature (Burhan *et al.*, 2003). However, the optimum temperature of amylase produced in this work was lower compare with amylase produce from *Thermus* sp. of 70°C (Shaw *et al.*, 1995) and *Thermus filiformis* of 95°C (Egas *et al.*, 1998).

Other bacteria produce amylase with the same temperature as this amylase such as *Thermobifida fusca* NTU22 (Yang and Liu, 2004) and *Scytalidium thermophilum* (Aquino *et al.*, 2003). Several studies have reported bacterial amylase active at medium to high temperatures, in the range of 50-100°C (Sivaramakrishnan *et al.*, 2006).

3.5 Optimum enzymatic reaction time

Determination of incubation time was performed by measuring the amylase enzyme activity at various incubation times. Figure 6 shows that the optimum incubation time was 45 minutes with amylase enzyme activity of 14.38 Unit.ml⁻¹ and glucose that was produced at 45.31 mg.ml⁻¹. Amylase enzyme activity increased with the increasing incubation time until the optimum condition was reached. The contacts of enzyme-substrate increase so more products are released. Amylase activity tends to be stable after 45 minutes of incubation time.

3.6 Starch hydrolysis followed by fermentation into bio-ethanol

Starch hydrolysis using tapioca solid waste as the substrate was carried out enzymatically with amylase produced in this study, at the condition of the substrate con-

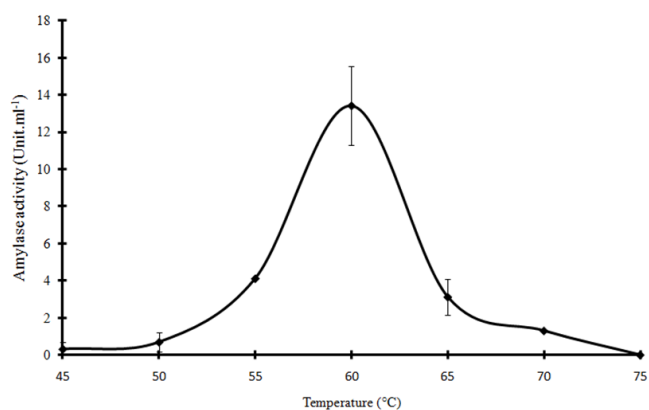


Figure 5. Effect of temperatures on amylase activity from *Thermus* sp. The enzymatic reaction was performed using 10% (b/v) of starch substrate in a buffer pH of 8.0 with various incubation temperatures for 30 minutes

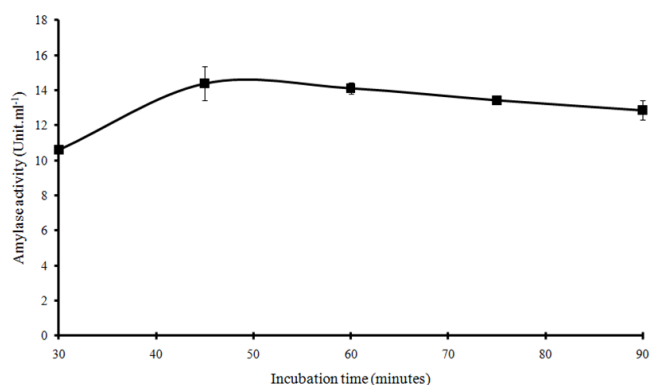


Figure 6. Incubation time effect on amylase from *Thermus* sp. activity. The enzymatic reaction was performed using 10% (b/v) of starch substrate in a buffer pH of 8.0 at 60°C with various incubation times

Table 2. Summary results of solid waste starch hydrolysis and fermentation into bioethanol

No	Treatment	Description
1.	Starch hydrolysis by amylase	Temperature 60°C, pH 8, for 45 minutes, 10% substrate concentration, enzyme substrate ratio of 1:10
2.	Fermentation of hydrolyzed starch	Temperature at 37°C for 24 hours, fermipan-substrate ratio of 11:1000 Ethanol content: 0.29% (v/v)
3.	Direct fermentation of starch without amylase hydrolysis as a control	Temperature at 37°C for 24 hours, fermipan-substrate ratio of 11:1000 Ethanol content: not detected (below 0.01%)
4.	Purifying by simple distillation	Temperature 78-80°C Ethanol content : 10,17 % (v/v)

centration 10% (w / v), temperature 60°C, and pH 8 and for 45 minutes of reaction time. Glucose obtained was fermented into bioethanol using commercial *Saccharomyces cereviceae* at the temperature of 37°C for 24 hours. The result of hydrolysis and fermentation is summarized in Table 2. Fermented ethanol content was 0.29% (v/v) and increased to 10.17% (v / v) after purification by distillation.

4. Conclusion

An amylase-producing bacterium was isolated from hot-spring water and was proposed as the genus of *Thermus* sp. Amylase was produced from the *Thermus* sp. with optimum temperature of 60°C and pH of 8.0. The optimum substrate concentration was achieved at 10% (w/w) with the optimum enzymatic reaction time of 45 minutes.

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