Thermostable $\alpha$-Amylase Activity from Thermophilic Bacteria Isolated from Bora Hot Spring, Central Sulawesi

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Abstract. $\alpha$-Amylase is one of the most important enzyme in biotechnology field, especially in industrial application. Thermostability of $\alpha$-Amylase produced by thermophilic bacteria improves industrial process of starch degradation in starch industry. The present study were concerned to the characterization of $\alpha$-Amylase activity from indigenous thermophilic bacteria isolated from Bora hot spring, Central Sulawesi. There were 18 isolates which had successfully isolated from 90°C sediment samples of Bora hot spring and 13 of them showed amylolytic activity. The $\alpha$-Amylase activity was measured qualitatively at starch agar and quantitatively based on DNS (3,5-Dinitrosalicylic acid) methods, using maltose as standard solution. Two isolates (out of 13 amylolytic bacteria), BR 002 and BR 015 showed amylolytic index of 0.8 mm and 0.5 mm respectively, after being incubated at 55°C in the 0.002% Starch Agar Medium. The $\alpha$-Amylase activity was further characterized quantitatively which includes the optimum condition of pH and temperature of $\alpha$-Amylase crude enzyme from each isolate. To our knowledge, this is the first report on isolation and characterization of a thermostable $\alpha$-Amylase from thermophilic bacteria isolated from Central Sulawesi particularly from Bora hot spring.

1. Introduction
$\alpha$-Amylase (E.C. 3.2.2.1.) is a hydrolase enzymes belongs to family 13 (GH-13) of glycoside hydrolase, which catalyzes the hydrolysis of internal $\alpha$-1,4-glycosidic bonds in starch to yield products like glucose, maltose, and other oligosaccharides units [1,2]. These enzymes can be produced by plants, animals, and microbes [3]. The most widely used $\alpha$-Amylase in industrial application is $\alpha$-Amylase coming from microbes [4]. It possesses some advantages such as cost effectiveness, large productivity, consistency, less time and space needs for production, and easy to modify and optimize the process of production [1].

$\alpha$-Amylase plays an important role in biotechnology field, particularly for industrial needs [5]. It constitutes a class of industrial enzymes having approximately 25-30% of the world enzyme market [6]. Various industry had been known to use $\alpha$-Amylase in their industrial process ranging from starch, detergent, fuel alcohol, food, textile to paper industry [1].

The most widespread applications of $\alpha$-Amylase are in the starch industry [1]. Starch industry needs a thermostable $\alpha$-Amylase in the starch processing as the process of gelatinization, liquefaction, and saccharification are performed in high temperature [7]. Thermostable $\alpha$-Amylase could be isolated from mesophilic microbes, therophilic microbes as well as hyperthermophilic microbes. These microbes possess unique properties of $\alpha$-Amylase, including specificity, thermostability, and pH response which...
are a critical properties for industrial use [8]. Screening of microbes with high α-Amylase activity, high thermostability, proper optimum condition of pH and temperature could therefore facilitate the discovery of newly suitable α-Amylase for industrial application [9].

Hot spring is one of the habitat of thermophilic bacteria which offers great potential for isolating a thermostable α-Amylase [7,10]. Bora hot spring is one of the hot spring located at Central Sulawesi with water temperature up to 90,1ºC [11]. Therefore, it is mostly possible to isolate the bacteria which could produce highly thermostable α-Amylase from that hot spring.

Little is known about the properties of the α-Amylase produced by indigenous thermophilic bacteria from Indonesia. As far as our concern, this is the first report regarding the isolation and characterization of α-Amylase activity produced by indigenous thermophilic bacteria isolated form Bora hot spring, Central Sulawesi. Hence, it is necessary to know character of these local α-Amylase and its potential to be implemented in industrial sector.

2. Methods

2.1. Sample collection and Isolation of thermophilic bacteria
Sediment samples were collected from Bora hot spring, Central Sulawesi, Indonesia. Sediment samples were homogenized by continuously mixing 10 g of sediment samples with 90 ml of 0.9% NaCl solution in 150 rpm of orbital shaker at 55°C for 30 minutes. A serial dilution technique was performed up to 10^{-9} in diluting the samples. Luria Bertani Agar was used as an isolation and cultivation media of thermophilic bacteria isolates which incubated at 55°C for 24 hours to obtain the colonies.

2.2. Screening of amylase producing thermophilic bacteria
Starch Agar plate was used as a media for screening of amylase producing thermophilic bacteria. The pure isolate colonies were inoculated and streaked on 0.002% starch agar plates and incubated at 55°C for 24 hours. After incubation, the plates were flooded by Gram’s Iodine (2% I₂ and 0.2% KI) to produce starch-iodine complex which is visualized by deep blue color. No color would be appeared at starch-free zone which is known to be the zone of clearance. The appearance of clear zone at starch agar plate was used as the main indicator of the activity of starch degrading enzyme produced by the bacterial strain. The bacterial strains which produced clear zone at starch agar plate were further characterized.

2.3. Amylolytic index value determination
Amylase producing bacteria colonies were sub-cultured at Luria Bertani Broth media and incubated at 55°C for 48 hours. After incubation, 10 µl of bacterial culture was inoculated to filter paper at 0.002% starch agar plate. The agar plates were incubated at 55°C for 48 hours. After incubation, the plates were flooded by Gram’s Iodine (2% I₂ and 0.2% KI) and the amylolytic index value of each isolate was determined based on the ratio of clear zone diameter (mm) formed by the bacterial colony and its diameter of colony (mm) [12]. These were performed in triplicates and the standard error were determined based on the deviation number obtained. Bacterial isolates in high amylolytic index value was further characterized.

2.4. Crude enzyme production
Two isolate were initially sub-cultured at basal medium containing 1% starch according to Asgher et al. (2007) [9] and incubated at 55°C for 48 hours without shaking. After incubation, 5 ml of the cultures was transferred to 45 ml sterile basal medium and incubated at 55°C for 48 hours on 130 rpm of orbital shaker. The crude enzyme of each isolate was obtained after centrifugation process of 48 hours cultures. The cultures were centrifuged at 8,000 rpm on 4°C to sediment the cell. Cells free supernatant was used as the crude enzyme for enzyme assay. The enzyme assays were performed immediately after centrifugation process to prevent the lost activity of enzyme.
2.5. Enzyme assay
α-Amylase activity was determined by measuring the increase of reducing sugar as maltose formed by the hydrolysis of starch. The quantity of the releasing reducing sugar was determined by 3,5-dinitrosalicylic acid method according to Bernfeld (1955) [13]. A 1.0% (w/v) solution of starch in 50 mM Phosphate Buffer (pH 7.0) was used as a substrate. The reaction mixture (100 µl), containing 50 µl crude enzymes and 50 µl substrate was incubated at 55°C for 15 minutes. After incubation, 50 µl of 3,5-dinitrosalicylic acid was added to stop reaction. This reaction mixture was boiled for 15 minutes and diluted 4 times in dH₂O. The blank was made by adding 3,5-dinitrosalicylic acid before the crude enzyme. Also, the control was made by adding 3,5-dinitrosalicylic acid before the substrate. The absorbance was measured at 540 nm, where one unit of α-Amylase enzyme corresponded to the formation of 1 µmol reducing sugar as maltose per minute in 50 mM Phosphate Buffer (pH 7.0) with 1% soluble starch at 55°C. These were performed in duplicates and the standard error were determined based on the deviation number obtained.

2.6. Determination of optimum temperature and pH of the crude enzyme
Effect of temperature on α-Amylase activity was determined by performing enzyme assay at a various temperature ranging on 32°C - 80°C. Effect of pH on α-Amylase activity was determined by performing enzyme assay at various pH ranging on 5 - 8.6 using 50 mM citrate buffer, 50 mM phosphate buffer, and 50 mM tris-HCl buffer. The optimum condition (of both temperature and pH) was determined by polynomial regression equation with derivative = 0 using software Microsoft Excel 2016 where the peak of the curve showed the maximum point of both temperature and pH.

3. Result and Discussion
Eighteen thermophilic bacteria were successfully isolated from sediment samples, collected from Bora hot spring, Central Sulawesi, Indonesia. Out of 18 isolates, 13 isolates showed amylolytic activity at 0.002% starch agar plates. Two isolate, BR 002 and BR 015 were further described throughout this paper, due to their consistence in showing high amylolytic activity on early selection using starch agar plates (figure 1). The amylolytic indexes (ratio of clear zone diameter to colony diameter) of BR 002 and BR 015 were 0.8 mm and 0.5 mm respectively (table 1).

![Figure 1. Zone of clearance on starch agar plates from BR 002 isolates (left) and BR 015 isolates (right).](image)

The enzyme activity was further characterized quantitatively using 3.5-dinitrosalicylic acid method. α-Amylase activity from both isolates, BR 002 and BR 015 were around 2 U/ml and 3.7 U/ml, respectively (table 1). The data showed that α-Amylase activity from both isolates didn't correspond to the value of amylolytic index. The same works were carried out by Goyari et al. (2014) [14] which described the relative enzyme index on plate agar was not corresponded to the enzyme activity.
Table 1. Comparative analysis of amylolytic activities of both thermophilic bacteria isolates.

| No. | Bacterial Isolates | Amylolytic Index (mm) ± SE\(^a\) | Enzyme Activity (U/ml) ± SE |
|-----|--------------------|----------------------------------|---------------------------|
| 1   | BR 002             | 0.8 ± 0.05                       | 2.0 ± 0.3                 |
| 2   | BR 015             | 0.5 ± 0.03                       | 3.7 ± 0.2                 |

\(^a\)Standard Error

Determination effect of temperature on α-Amylase activity was shown in figure 2. The α-Amylase activity of BR 002 was found to increase at temperature ranging on 32ºC to 52ºC and start to significantly decrease at 55ºC to 80ºC where no activity of α-Amylase was observed. The optimum temperature of the α-Amylase activity of BR 002 was around 50ºC with enzyme activity around 2 U/ml. Meanwhile, the α-Amylase activity of BR 015 was found to increase at temperature ranging on 32ºC to 69ºC and start to decrease after 69ºC. The optimum temperature of the α-Amylase activity of BR 015 is around 70ºC with enzyme activity around 6.4 U/ml. Hence, it was explained that the α-Amylase activity from BR 015 isolates was found to have broader range of active temperature as well as higher optimum temperature compare with BR 002 isolates. However, the optimum temperature exceeding 50ºC from both isolates confirm the suitable character needed by most of the industrial sector as had been mentioned by Goyal et al. (2005) [15].

![Figure 2. Effect of temperature on α-Amylase activity.](image)

The effect of pH on α-Amylase activity was shown in Figure 3. The effect of pH on α-Amylase activity of BR 002 was found to be similar with BR 015. No enzyme activity to a very slight enzyme activity was shown at pH 5 in α-Amylase from both isolates. The enzyme activity from both isolates starts to significantly increase until it reaches pH around 8.0 and begin to decrease afterwards. Hence, the optimum pH of both BR 002 and BR 015 isolates was around 8.0 with enzyme activity 2.2 U/ml and 6.5 U/ml respectively. It was suggested that, both isolates had optimum pH at slightly basic. The same works were performed by Sen et al. (2016) [16] which had characterized optimum pH of α-Amylase at slightly basic produced by thermophilic bacteria isolated from hot spring.
The α-Amylase activity of both isolates, particularly when it was in the optimum condition, showed good potential for the application in industrial sector. However, it was still far from the real implementation regarding the α-Amylase activity which was relatively low compared with the α-Amylase activity of Bacillus sp. (up to 57 U/ml) [17] or Bacillus subtilis (up to 100 U/ml) which commonly had already been implemented in industrial sector [9]. Hence, the α-Amylase activity of both isolates should reach at least more than 57 U/ml (α-Amylase activity of Bacillus sp.) when it was planned to be implemented in industrial sector. However, this limitation could be solved by performing further characterization such as enzyme purification, determination of enzyme stability against temperature and pH, effect of cofactor, effect of inhibitor, effect of substrate concentration, and optimum enzyme production as those properties would affect the activity of the enzyme. By knowing the exact properties of the enzyme, the optimization process of enzyme including the improvement in enzyme activity could obviously be performed. More than that, here we show the potential genetic material from the indigenous bacteria which could be improved on its enzyme activity by such as advance genetic engineering.

4. Conclusion
The crude α-Amylase from indigenous thermophilic bacteria isolate has high optimum temperature and pH where the other enzyme would denature. However, these characteristics were not enough to use the enzyme in industrial application because of the low of enzyme activity. Furthermore, further characterization should be performed in order to know the exact properties of the enzyme.

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