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Characterization of α-Amylase Activity from Thermophilic Bacteria Isolated from Bora Hot spring, Central Sulawesi

Karakterisasi Aktivitas α-Amilase dari Isolat Bakteri Termofilik dari Mata Air Panas Bora, Sulawesi Tengah

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ABSTRACT

α-Amilase (EC 3.2.1.1) is one of the most important enzyme in biotechnology. This enzyme had been implemented in various sector of industry, such as detergen, textile, paper, bread, alcohol, and starch. Thermostability of α-Amilase is one of main property which is needed in starch degrading industry. A Thermostable α-Amilase could be obtained from thermophilic bacteria in which naturally could be isolated from hot spring. Bora hot spring is one of the most potential habitat in isolating thermophilic bacteria as it possessed a highly water temperature up to 90,1°C. The present study was concerned to the characterization of α-Amilase activity from indigenous thermophilic bacteria isolated from Bora hot spring, Central Sulawesi. There were 13 (out of 18) thermophilic bacterial isolates from Bora hot spring which could produce α-Amilase in which succesfully isolated from sediment samples. Among those 13 bacteria, 3 selected isolates were described throughout this paper, they were BR 001, BR 006, and BR 012. These 3 isolates had a character of: amylolytic index value in range 0.78 – 1.44; α-Amylase activity in range 3.67 U/ml – 7.6 U/ml; protein concentration in range 0.02 mg/ml – 0.14 mg/ml; α-Amylase crude enzyme specific activity in range 54.83 U/mg – 308.45 U/mg; α-Amylase which active in temperature range 32°C – 80°C and had an optimum temperature in range <32°C and >80°C; and α-Amylase which active in pH range 5 – 7 and had an optimum pH in range 6.5 – 7. The presence of enzyme activity at high temperature from α-Amilase produced by indigenous thermophilic bacteria from Bora hot spring indicated a suitable potential in the application of local enzyme to industrial sector in the future, particulary to the starch processing industry.

Keywords: Enzyme Activity, α-Amylase, Thermophilic Bacteria, Hot Spring

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ABSTRAK

α-Amilase (EC 3.2.1.1) merupakan salah satu jenis enzim yang sangat penting dalam bioteknologi. Enzim ini sering diaplikasikan pada berbagai sektor industri, seperti industri deterjen, tekstil, kertas, roti, alkohol, dan pati. Termostabilitas dari α-Amilase merupakan sifat utama dari enzim yang dibutuhkan pada industri pemerosesan pati. α-Amilase dengan sifat termostabil dapat diperoleh dari bakteri termofilik yang secara alami dapat diisolasi dari mata air panas. Mata air panas Bora merupakan salah satu habitat yang paling berpotensi dalam mengisolasi bakteri termofilik karena memiliki suhu air yang sangat tinggi hingga mencapai 90,1°C. Penelitian ini bertujuan untuk mengetahui karakter aktivitas α-Amilase dari bakteri termofilik lokal isolat mata air Bora, Sulawesi Tengah. Terdapat 13 (dari 18 isolat) bakteri termofilik isolat mata air panas Bora yang mampu menghasilkan α-Amilase yang berhasil diisolasi dari sampel sedimen. Dari 13 isolat tersebut, 3 isolat terpilih dideskripsikan pada tulisan ini, yakni BR 001, BR 006, and BR 012. Ketiga isolat tersebut memiliki karakter: nilai indeks amilolitik berkisar 0,78 – 1,44; aktivitas α-Amilase berkisar berkisar 3,67 U/ml – 7,6 U/ml; konsentrasi protein berkisar 0,02 mg/ml – 0,14 mg/ml; aktivitas spesifik enzim kasar α-Amilase berkisar 54,83 U/mg – 308,45 U/mg; α-Amilase yang aktif pada kisaran suhu 32°C – 80°C; dan α-Amilase yang aktif pada kisaran pH 5 – 7 dan memiliki pH optimum pada kisaran 6,5 – 7. Adanya aktivitas enzim pada suhu yang tinggi dari α-Amilase yang diproduksi oleh isolat bakteri termofilik lokal dari mata air panas Bora menunjukkan adanya potensi dalam aplikasi enzim lokal pada sektor industri di masa depan, khususnya pada industri pemerosesan pati.

Kata kunci: Aktivitas Enzim, α-Amilase, Bakteri Termofilik, Mata Air Panas

INTRODUCTION

α-Amylase (E.C. 3.2.2.1.) is a hydrolase enzyme belongs to family 13 (GH-13) of glycoside hydrolase, which catalyzes the hydrolysis of internal α-1,4-glycosidic bonds in starch to yield products like glucose, maltose, and maltotriose units (Sivaramakrishnan *et al*., 2006; de Souza and e Magalhães, 2010). These enzymes can be produced by plants, animals, and microbes (Sundarram and Murthy, 2014). The most widely used α-Amylase in industrial application is α-Amylase coming from microbes (Reddy *et al*., 2003). It possesses some advantages such as cost effectiveness, large productivity, consistency, less time and space needs for production, and also easy to modify and optimize the process of production (de Souza and e Magalhães, 2010).

α-Amylase plays an important role in biotechnology field, particularly for industrial needs (Pandey *et al*., 2000). It constitutes a class of industrial enzymes having approximately 25-30% of the world enzyme market (Deb *et al*., 2013). Various industry has been known to use α-Amylase in their industrial process ranging from starch, detergent, fuel alcohol, food, textile

to paper industry (de Souza and e Magalhães, 2010).

The most widespread applications of α-Amylase are in the starch industry (de Souza and e Magalhães, 2010). Starch industry needs a thermostable α-Amylase in the starch processing as the process of gelatinization, liquefaction, and saccharification are performed in high temperature (Mehta and Satyanarayana, 2016). Thermostable α-Amylase could be isolated from mesophilic microbes, thermophilic microbes as well as hyperthermophilic microbes. These microbes possess unique properties of α-Amylase, including specificity, themostability, and pH response which are a critical properties for industrial use (Vieille *et al*., 2001). 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 (Asgher *et al*., 2007).

Hot spring is one of the habitat of thermophilic bacteria which offers great potential on isolation of thermostable α-Amylase (Madigan and Marrs, 1997; Mehta and Satyanarayana, 2016). Bora hot springs is one of the hot spring located at Central Sulawesi with water temperature up to 90,1ºC (Idral and Mansoer, 2015). 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 character of the α-Amylase produced by indigenous thermophilic bacteria in Indonesia, particularly in Central Sulawesi. The presence of enzyme activity at high temperature from α-Amilase produced by indigenous thermophilic bacteria from Bora hot spring indicated a suitable potential in the application of local enzyme to industrial sector in the future, particulary to the starch processing industry.

MATERIAL AND METHODS

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} to dilute 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. Screening of amylase-producing thermophilic bacteria

Starch agar plate was used as a media for screening of amylase-producing thermophilic bacteria. The pure isolates 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% I_2 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 isolates. The bacterial isolates which produced clear zone at starch agar plate were further characterized.

3. Amylolytic index value determination

Amylase producing bacteria colonies were sub-cultured at Luria Bertani Broth media and incubated at 55°C for 24 hours. After incubation, 10 µl of bacterial culture were 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% I_2 and 0.2% KI) and the amylolytic index value of each isolates was determined based on the ratio of clear zone diameter (cm) formed by the bacterial colony and its diameter of colony (cm) (Jamilah *et al*., 2009). Bacterial isolates with high amylolytic index value were further characterized.

4. Characterization of selected amylase-producing thermophilic bacteria

Several characterization consist of cells morphology, gram staining character, colony morphology, and biochemical assay including various sugar fermentation (glucose, lactose, and sucrose) from the selected amylase-producing thermophilic bacteria were studied in this works.

5. Crude enzyme production

Three isolates were initially subcultured at basal medium containing 1% starch according to Ashger *et al*. (2007) and incubated at 55°C for 48 hours without shaking. After incubation, 5 ml of the cultures were 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 isolates 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.

6. Soluble Protein Estimation

Extracellular soluble protein in culture filtrate was estimated by the Lowry's method using bovine serum albumin (BSA) used as standard solution (Lowry *et al*., 1951). 70 µl of analytical reagent was added to 50µl crude enzyme solution. The mixture was mixed well and allowed to stand for 20 min at 55°C in the dark condition. Then 10 µl of the folin-ciocalteau reagent was added and shaken to mix well and incubated for about 30 min to 60 min at at 55°C in the dark condition. Optical density of the reaction mixture was measured at 750 nm, against a blank prepared with 50 µl sterile basal medium with 1% starch (Ashger *et al*., 2007). A standard curve was constructed with each experiment using bovine serum albumin as a known protein. The amount of the soluble protein was calculated from the standard curve of as mg protein per ml of test samples.

7. Enzyme activity 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). 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 enzyme 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 with dH_2O. 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. At last, the α-Amylase specific activity (U/mg) could be determined from the ratio of α-Amylase activity with its protein concentration.

8. Effect of temperature and pH on α-Amylase activity

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.

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RESULT AND DISCUSSION

Eighteen bacterial colonies were successfully isolated from sediment samples, collected from Bora hot spring, Central Sulawesi, Indonesia. Among these 18 isolates, 13 isolates showed amylolytic activity at 0.002% starch agar plates. Three isolates consist of BR 001, BR 006, and BR 012 were described throughout this paper.

All these three isolates were rod-shaped bacteria or called bacil with 2.5 – 3.2 µm long and 0.9 – 1.1 µm wide, and stained as purple which were classified as a Gram positive bacteria (Figure 1). Several rod-shaped Gram positive bacteria, particularly from *Bacillus* spp. were reported as good α-Amylase producers which had high α-Amylase production with high enzyme activity and possess high thermostability (Reddy *et al*., 2003).

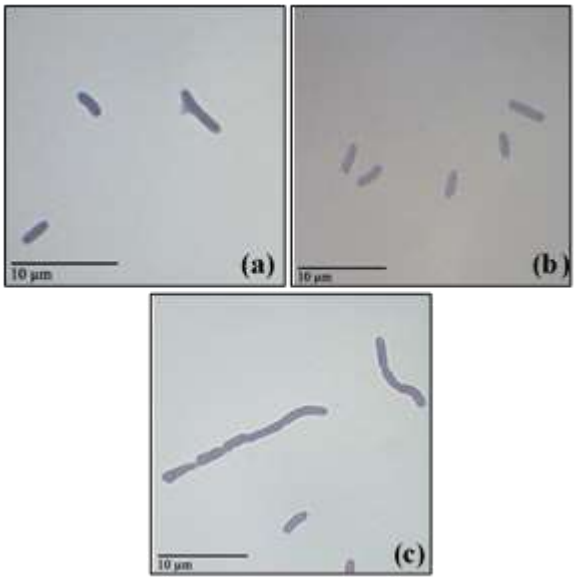

Figure 1. Cells morphology and gram staining character from (a) BR 001; (b) BR 006; (c) BR 012 (1000x magnification)

The character of colony morphology was determined based on size, pigmentation, shape, margin, and opacity. BR 001 showed a white colony which were irregular in shape and had a lobate margin (Figure 2a). BR 006 showed a small milky white colony which were filamentous in shape and margin (Figure 2b). BR 012 showed a small white colony which were irregular in shape and had an undulate margin (Figure 2c). All these three isolates colony showed opaque character in colony opacity. On the other hand, BR 001 and BR 006 showed similar biochemical character with no fermentation process were observed in any sugar test, whereas BR 012 showed a positive test in glucose and sucrose test, but not in lactose test.

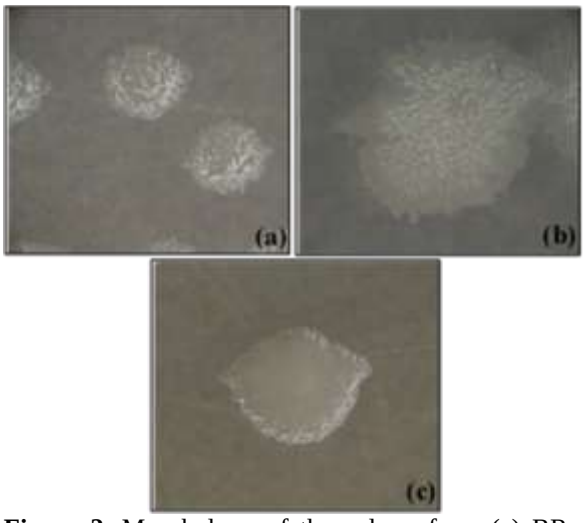

Figure 2. Morphology of the colony from (a) BR 001; (b) BR 006; (c) BR 012

These three isolates showed zone of clearance on starch agar plates (Figure 3). The amylolytic index value ± Standard Error (ratio of clear zone diameter to colony diameter) of BR 001, BR 006 and

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BR 012 were 1.44 ± 0.099, 0.87 ± 0.062, and 0.78 ± 0.034 respectively (Figure 4). These number were still lower than the amylolytic index value from *Bacillus* spp. reported by Jamilah *et al*. (2009) which were in range 2.5 – 8.3.

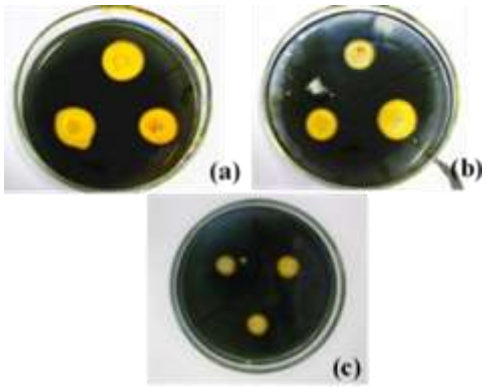

Figure 3. Zone of clearance on starch agar plates from (a) BR 001; (b) BR 006; (c) BR 012

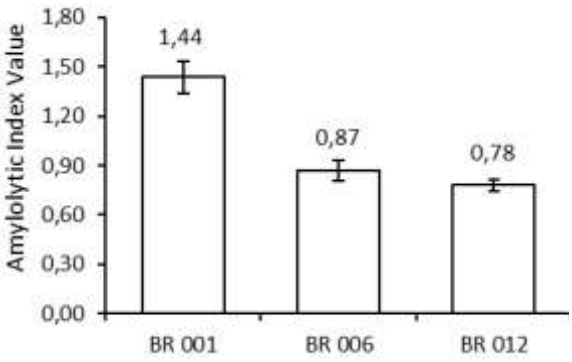

Figure 4. Amylolytic index value

The enzyme activity was further characterized quantitatively using 3,5-dinitrosalicylic acid method. α-Amylase activity ± Standard Error from BR 001, BR 006, and BR 015 were 4.79 U/ml ± 0.559, 7.46 U/ml ± 0.113, and 3.67 U/ml ± 0.113, respectively (Figure 5). These number showed higher value of α-Amylase activity compared with α-Amylase activity from *Bacillus* spp. reported by Jamilah *et al*. (2009) which only had it up to 2.5 U/ml. However, these number were still lower than the enzyme activity from several bacteria which had already implemented in industrial sector, such as *Bacillus subtilis* JS with α-Amylase activity up to 44.84 U/ml (Asgher *et al*., 2007). This data also showed that α-Amylase activity from these three isolates didn't correspond with the value of amylolytic index which would be caused by the difference of medium composition, including the presence of yeast extract, persentage of the starch, and the state of medium to be used (Asgher *et al*., 2007; Florencio *et al*., 2012). The same works were carried out by Goyari *et al*. (2014) which described the relative enzyme index on plate agar was not correspond with the enzyme activity.

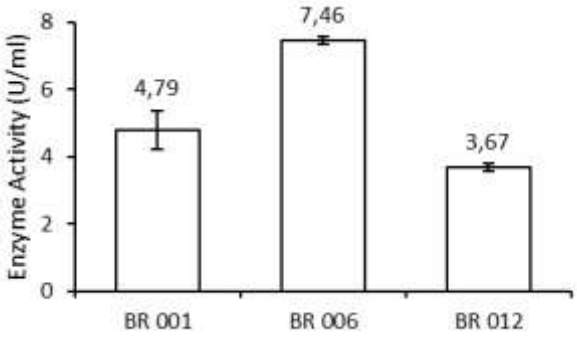

Figure 5. α-Amylase activity

Enzyme specific activity could be determined by knowing the concentration of the protein in the supernatant. Based on Lowry method as previously described, the protein concentration ± Standard Error of BR 001, BR 006, and BR 012 were 0.02

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mg/ml ± 0.016, 0.14 mg/ml ± 0.009, and 0.04 mg/ml ± 0.017 respectively (Figure 6). According to those protein concentration, the α-Amylase specific activity from BR 001, BR 006, and BR 012 were 308.45 U/mg, 54.83 U/mg, and 92.19 U/mg (Figure 7). These number showed higher value of α-Amylase specific activity compared with the α-Amylase specific activity from *Alicyclobacillus acidocaldarius* reported by Kumar *et al*. (2010) which only had it up to 398 U/mg, but significantly showed a lower value when it compared with the purified enzyme from *Alicyclobacillus acidocaldarius* which had α-Amylase specific activity up to 3239 U/mg. It suggests that the increasing number of α-Amylase specific activity was corresponding with the purity level of the enzyme.

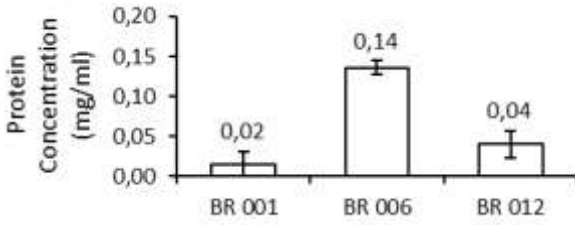

Figure 6. Protein concentration of the crude enzyme

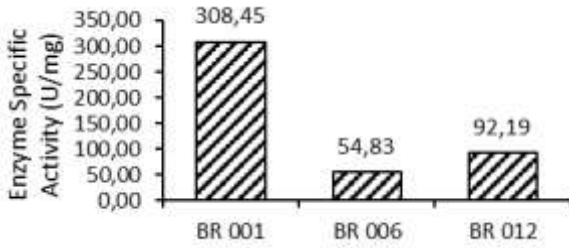

Figure 7. Spesific α-Amylase activity

The effect of temperature on α-Amylase activity was shown in Figure 8. The α-Amylase activity of BR 001 was found to decrease at temperature ranging on 32ºC to 90ºC and it suggested that the optimum temperature was below 32ºC. Meanwhile, the α-Amylase activity of BR 006 was found to decrease at temperature ranging on 32ºC to 55ºC and start to increase at temperature ranging on 55ºC to 80ºC, and it suggested that the optimum temperature was below 32ºC or above 80ºC. On the other hand, the α-Amylase activity of BR 012 was found to increase at temperature ranging on 32ºC to 90ºC and it suggested that the optimum temperature was above 80ºC. It was explained that the α-Amylase activity from all these three isolates was found to have widely range of active temperature as well as higher optimum temperature, particularly for isolates BR 012. In addition, the abbility of α-Amylase produced by all these three isolates to be able to active at high temperature, as well as the optimum temperature exceeding 80ºC from BR 012 and mostly possible BR 006 confirm the suitable character needed by most of industrial sector, specially starch processing industry as had been mentioned by Goyal *et al*. (2005)

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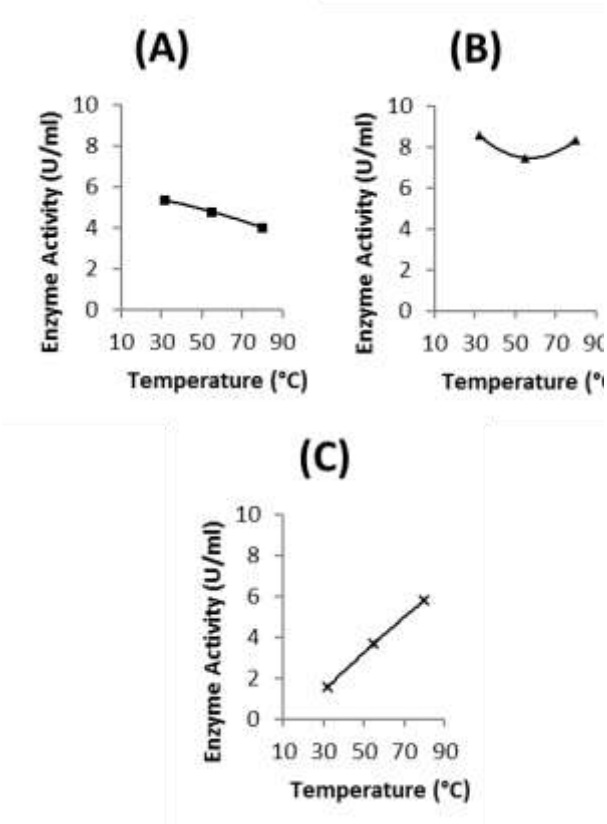

Figure 8. Effect of temperature on α-Amylase activity from (a) BR 001; (b) BR 006; (c) BR 012

The effect of pH on α-Amylase activity is shown in Figure 9. The effect of pH on α-Amylase activity of BR 001 was found to be similar with BR 012. 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 started to significantly increase until it reached pH around 7.0 and begin to decrease afterwards. The optimum pH of both BR 001 and BR 012 isolates was pH around 7. Meanwhile, α-Amylase activity from BR 006 showed good activity at pH 5 and continued to increase until it reached pH around 6.5, then it started to significantly decrease until only a very slight enzyme activity was observed at pH 8.6. Itsuggested that the optimum pH of α-Amylase from BR 006 was pH around 6.5 or slightly acid. The same works were performed by Bajpai *et al*. (2009) and Kumar *et al*. (2010) which had characterized optimum pH of α-Amylase at slightly acid to netral produced by thermophiles.

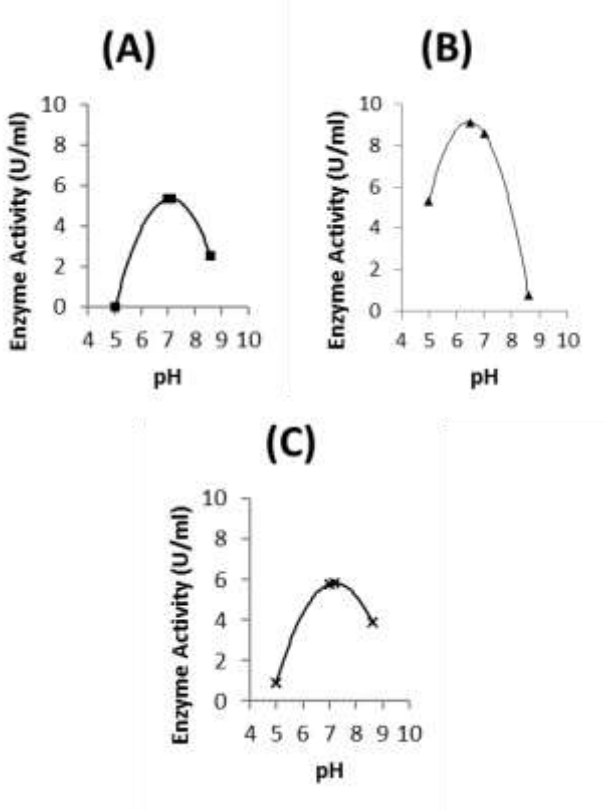

Figure 9. Effect of pH on α-Amylase activity from (a) BR 001; (b) BR 006; (c) BR 012

α-Amylase activity from all these three isolates which could active at high temperature as well as had an optimum temperature at a very high temperature, showed good potential for the application in industrial sector. However, it was still far from the real implementation regarding the α-Amylase activity which is relatively low

compare with the *Bacillus* sp. α-Amylase activity (up to 57 U/ml) (Cordeiro *et al*., 2002) which commonly had already been implemented in industrial sector. Furthermore, further detail characterization such as in enzyme purification, determination of enzyme stability against temperature and pH, effect of enzyme co-factor, and effect of inhibitor are required to know the exact properties of the enzyme.

The crude of α-Amylase from indigenous thermophilic bacteria isolates could active at high temperature 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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