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Screening and Identification of Thermostable Amylase-Producing Bacteria from Selayang and Hulu Langat Hot Springs, Selangor

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Abstract Thermophilic amylase-producing bacteria were isolated from water sample from Hulu Langat and Selayang hot springs at 70°C and 50°C, respectively. A total of 25 bacterial isolates, 10 from Hulu Langat and 15 from Selayang, were successfully isolated. Qualitative amylase test demonstrated 7 isolates were amylase positive that able to form clear zone around bacterial colonies grown on Nutrient Agar plates supplemented with 2% (w/v) starch. Quantitative amylase assay using DNS method indicated sample 05 of Selayang and sample 07 of Hulu Langat hot springs produced highest amylase activity. Furthermore, morphological and biochemical studies including catalase, oxidase and oxygen requirement, indicated both samples were characteristically similar to the genus *Bacillus* sp.

Key words: Amylase, Hot spring, Thermostable, *Bacillus* sp.

1. Introduction

Amylases are enzymes that hydrolyse the catalysis of starch into simple sugars such as glucose and maltose. Bacteria and fungi are among the most known amylase producing microorganisms. Amylases possess wide varieties of application including paper and textiles. In addition, amylases are also used in food and beverage industries such as in production of bread, fruit juices, sweeteners, alcoholic beverages and glucose and fructose syrup (Haq, 2010) due to their ability to utilize a wide spectrum of substrates, high stability towards extreme temperature and pH (Amutha *et al.*, 2011).

Thermostable enzymes are defined as enzymes that produced by thermophilic organisms with unique characteristics in temperature and pH stability (Bruins *et al.*, 2001) that can maintain longer activity in industrial processes. Thermophilic microorganisms, on the other hand, have received great deal of attention due to their special characteristics that they can survive at high temperature and are more versatile with respect to industrial purposes. The ability of thermophilic microorganisms to withstand high temperature provides advantageous including increased substrate solubility, low viscosity of the medium or lowered risk of microbial contamination (Kuchner & Arnold, 1997).

Many thermostable amylases are produced by different *Bacillus* sp. such as *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Bacillus amyloloquefaciens and Bacillus megaterium* have been applied in starch industry due to their inherent stability (Demirjian *et al.*, 2001). Thermostable enzymes were observed to have higher stability to organic solvents, acidic and alkaline pH detergents (Vieille *et al.*, 1996). In additions, enhancement of rate of reaction constant, increasing the reaction rate as the medium viscosity decreases with an increasing temperature are other benefits of thermostable enzymes (Kumar & Swati, 2001). Compared to mesophilic

bacteria, the protein of the cellular component of the thermophilic bacteria is more stable.
Besides, they possess high metabolic rates, physically and chemically stable enzymes (Iraj *et al*., 2008). Due to the unique macromolecular properties and the ability to grow in high temperature, the thermophilic bacteria have special feature that consists of novel enzymes and biochemical pathways (Herbert, 1992).

2. Materials and Methods

2.1 Isolation of Thermostable Amylase-Producing Bacterial

Water samples were collected in a sterile thermos flask from two different locations, namely Hulu Langat Hot spring and Selayang Hot spring at 75˚C and 50˚C, respectively. One millilitre of the water sample was inoculated in Nutrient Broth (NB) and was incubated at temperature resembling to the temperature of the isolation site for 24h. On the next day, a loopful of the microbial culture was streaked on Nutrient Agar (NA) plate supplemented with 2% (w/v) starch.

2.2 Qualitative Amylase Determination

For qualitative amylase determination, the bacterial samples were grown on Nutrient Agar (NA) plates supplemented with 2% (w/v) starch at temperature resembling to the isolation site for 24h. On the next day, the plates were flooded with iodine solution for qualitative determination of the amylase enzyme.

2.3 Quantitative Amylase Determination

As for quantitative enzyme determination, 1 ml of the bacterial sample was inoculated in 100 ml of Nutrient Broth (NB) supplemented with 2% (w/w) starch and incubated at temperature resembling to the isolation site for 24h. The extracellular enzyme was then assayed using DNS method (Bernfeld, 1955).

2.4 Morphological Identification of the Samples

Gram's staining

A dropful of sterile distilled water was dropped on the centre of the glass slide and a loopful of the bacterial culture was transferred aseptically onto the surface of a glass slide. The bacterial culture and the water droplet were mixed and spread into a thin film and then allowed to air dry at room temperature. The glass slide was then heat-fixed and was stained with Crystal Violet for 1 minute followed by washing off in running tap water. After that, the smear was flooded with an iodine solution for 1 minute followed by washing off with running tap water. The smear was decolorized with 95% (v/v) alcohol followed by washing off with water immediately. Finally, the slide was counterstained with Safranin for 30 sec followed by washing off with running tap water. The slide was left to dry before it was examined under microscope and the morphology of the bacteria was observed.

2.5 Bacterial Identification via Biochemical Tests

Oxidase Test

Four pieces of filter papers are prepared. Using a sterile loop, a single bacterial colony was picked and streaked on the filter paper for sample 1. Immediately, one drop of

oxidase was dropped on the bacterial colony in the filter paper. The colour changes were observed and the result was recorded.
The same method was repeated for another three samples of Hulu Langat. The same procedure was carried out for other four samples of Selayang in another filter paper.

2.6 Catalase Test

A clean glass slide was prepared. A small amount of the bacterial colony was transferred on the surface of glass slide using a sterile cooled loop. With a Pasteur pipette, one drop of hydrogen peroxide was dropped over the smear. The fluid over the smear and the formation of bubbles for 15 sec was observed.

3. Optimum temperature

Based on amylase assay, the sample which shows highest absorbance was recorded and the sample were used for the production of enzyme at different temperature. The sample were inoculated from glycerol stock and incubated in 50°C for Selayang sample and 75°C for Hulu Langat sample in different incubators for 24h. The samples were then inoculated in Nutrient Broth (NB) containing 2% (w/v) starch and incubated in incubator shaker for 24h at 121 rpm at temperature resembling to the isolation sites. On the next day, 1 ml of the sample was transferred into 100 ml of Nutrient Broth (NB) containing 2% (w/v) starch and was incubated at different temperature range from 30°C – 80°C for 24h. After 24h incubation, 1 ml of the bacterial culture was taken and was centrifuged. The activity of the enzyme was determined using DNS method [10].

4. Results and Discussion

Isolation of Thermophilic Amylase-Producing Bacteria

A total of 25 bacterial isolates, 10 from Hulu Langat and 15 from Selayang Hot springs, were successfully isolated as indicated in Figure 1 and Figure 2. The appearance of turbidity when isolates were cultured in Nutrient Broth (NB) supplemented with 2 % starch after 24h incubation has preliminary anticipated the bacterial isolates were successfully grown.

Figure 1: Isolation site at Hulu Langat Hot spring at 70°C

Qualitative Amylase Determination

Three bacterial isolates from Hulu Langat and four bacterial isolates from Selayang hot springs were amylase positive by forming clear zone. Sample 05 of Selayang hot

spring and sample 07 of Hulu Langat hot spring were found to form largest diameters of clear zone.

Figure 2: Isolation site at Selayang Hot spring at 50°C

The diameters of the clear zone formed by bacterial samples were found directly proportional to the amount of amylase produced. Hence, these two samples were selected for further studies. Different diameters of the clear zone formed by different bacterial isolates are shown in Table 1 and Table 2.

Table 1: Diameter of the clear zone formed by bacterial isolates from Selayang Hot spring

Sample	Diameter (mm)
2	$0.85^{a} \pm 0.05$
4	$1.20^{a} \pm 0.02$
5	$1.44^{a} \pm 0.04$
6	$1.34^{a} \pm 0.04$

All analysis is the mean of duplicate measurements ± standard deviation. Means not sharing the same letter in the same column were significantly different at $p<0.05$

Table 2: Diameter of the clear zone formed by bacterial isolates from Hulu Langat Hot spring

Samples	Diameter (mm)
7	$1.88^{b} \pm 0.03$
8	$1.38^{a} \pm 0.08$
9	$1.18^{a} \pm 0.08$

All analysis is the mean of duplicate measurements ± standard deviation. Means not sharing the same letter in the same column were significantly different at $p<0.05$

5. Quantitative Amylase Determination

The quantitative amylase determination using DNS method (Bernfeld, 1955) indicated all seven bacterial isolates were amylase positives. Different amylase activities of different bacterial isolates are indicated in Table 3 and Table 4.

Table 3: Quantitative amylase activity produced by isolates from Selayang Hot spring

Sample	Amylase Activity (U/ml)
2	$0.035^{a} \pm 0.002$
4	$0.042^{a} \pm 0.001$
5	$0.075^{b} \pm 0.002$
6	$0.065^{b} \pm 0.001$

All analysis is the mean of duplicate measurements ± standard deviation. Means not sharing the same letter in the same column were significantly different at $p<0.05$

Table 4: Quantitative amylase activity produced by isolates from Hulu Langat Hot spring

Sample	Amylase Activity (U/ml)
7	$0.093^{c} \pm 0.001$
8	$0.066^{b} \pm 0.002$
9	$0.047^{a} \pm 0.001$

All analysis is the mean of duplicate measurements ± standard deviation. Means not sharing the same letter in the same column were significantly different at $p<0.05$

6. Morphological Characterization of the Isolates

Morphologically, both bacterial isolates were observed as yellow colour and circular shape on the surface of Nutrient Agar (NA) media. Microscopic observation with 1000X magnification indicated that both bacterial isolates were long rod shape and Gram's positive that appeared to be the same characteristics of *Bacillus* sp. (Figure 3).

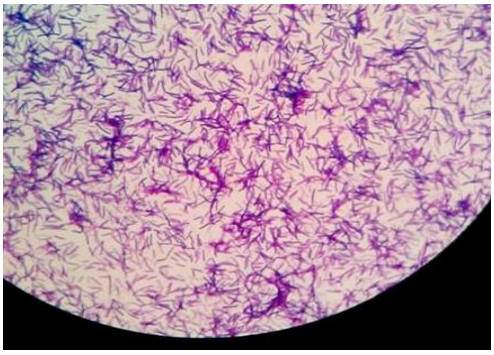

Figure 3: The microscopic observation of the sample isolated from Hulu Langat hot spring under 100x magnification indicated as Gram's positive of and rod-shaped bacteria.

7. Identification of the Isolates via Biochemical Tests

Catalase Test

Both samples, 05 and 07, were found to be catalase positive. The formation of bubbles when treated with hydrogen peroxide has proved the assumption. The formation of bubbles was explained due to the decomposition of hydrogen peroxide by catalase enzyme to produce water and oxygen. Catalase-positive bacteria have the ability to respire using oxygen as a terminal electron acceptor.

Oxidase Test

The ability of a bacterium to produce certain cytochrome c oxidases can be determined by oxidase test. Changing in the colour of the colonies into dark purple indicated the two samples were oxidase positive. The changing in the colour is explained due to the presence of cytochrome c oxidase which oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple colour while the negative oxidase test it remains colourless.

Oxygen Requirement Test

Both samples were found to grow on the surface of the slant as indicated in Figure 4, thus, suggested they were aerobic bacteria. Whilst no colonies were observed to grow inside the slant indicating they do require oxygen for their growth due to the lack of enzymes to neutralize the harmful forms of oxygen.

Figure 4: The growth on bacteria on the top of slant agar and also in the test tube which indicate that it is a facultative anaerobe.

Optimum Temperature of Amylase

Study on the optimum temperature of the amylase indicated the highest enzyme activity produced by the isolate from Selayang hot spring was 0.058 U/ml when it was incubated at 50°C for 24h as shown in Figure 5. On the other hand, the highest enzyme activity (0.048 U/ml) was detected at 60°C from the isolate of Hulu Langat hot spring after 24h incubation time as indicated in Figure 6. Therefore, result of the study indicated both amylases produced by the two isolates were proved to be thermostable enzyme since isolates that able to grow above 50°C are considered as thermophilic bacteria (Muralikrishna & Nirmala, 2005).

Conclusions

The thermostable amylase-producing bacteria were successfully isolated from two different hot springs located at Selayang and Hulu Langat, Selangor at temperature of 50°C and 70°C, respectively.

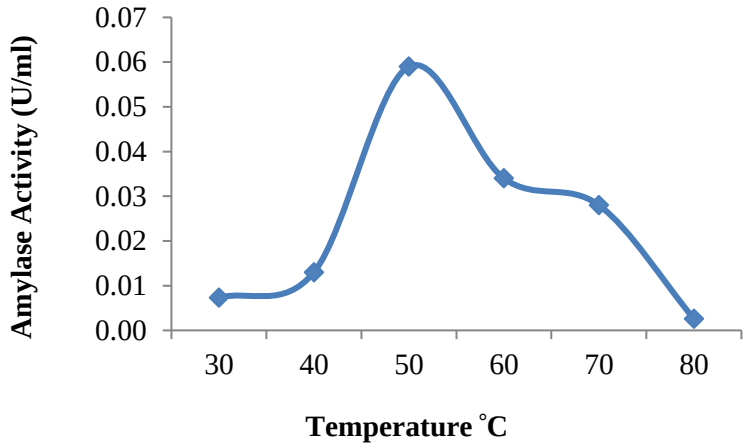

Figure 5: Optimum temperature of the amylase produced by sample 05 isolated from Selayang hot spring

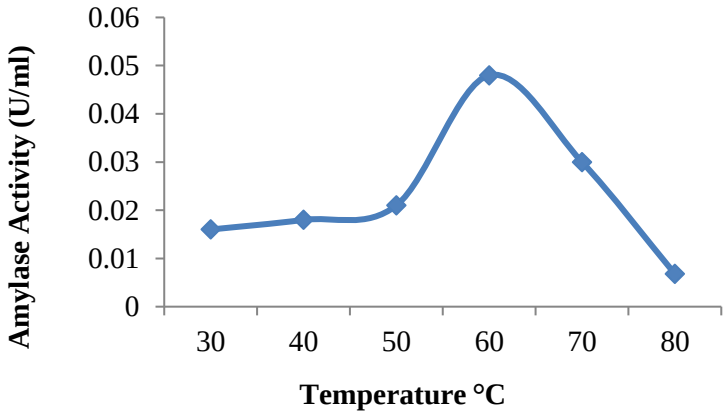

Figure 6: Optimum temperature of the amylase produced by sample 07 isolated from Hulu Langat hot spring

Out of 25 bacterial isolates, 7 samples showed amylase positive with sample 05 of Selayang host spring and sample 07 of Hulu Langat hot spring demonstrated highest amylase activities. Furthermore, biochemical analyses of the two samples showed their similarities to *Bacillus* sp. Thus, the enzyme produced by the two samples was deliberated as thermostable amylase

8. Acknowledgment

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9. References

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