Incubation period and agitation speed alter the activity of crude amylase produced by thermophilic bacteria isolated from Pulu Hotspring

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Abstract. The purpose of this study was to determine the optimum condition in yielding crude amylase enzymes with the highest activity produced by PL-16, a thermophilic bacteria isolated from Pulu hot spring. The study was carried out using a completely randomized design with a varied incubation time of 0, 6, 12,18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 hours, and agitation speed of 60, 90, 120, 150, 180, and 200 rpm. The activity of extracted crude amylase enzyme was determined using the DNS method and bacterial growth by measuring its Optical Density (OD600). The results showed that optimum amylase activity was at 48 hours incubation time with OD of 2.823 and activity of 0.437 U/mL. Furthermore, agitation speed of 180 rpm produced the highest amylase activity of 0.498 U/mL with an OD of 2.435. Pearson correlation test exhibited a positive and considerable strength of association between enzyme activity and bacterial growth. Our crude amylase showed higher activity after optimizing its production condition with 48 hours incubation and agitation speed of 180 rpm.

1. Introduction
Amylases are types of hydrolyzing enzymes that convert complex carbohydrate molecules into simple and small sugar molecules. They are classified into three main types; Alpha-amylase (found in animals, humans, microbes, and plants), Beta-amylase (found in plants and microorganisms), and Gamma-amylase (found in animals and plants)[1]. Among them, alpha-amylase, an extracellular enzyme that converts complex starch into simpler di- or trisaccharide molecules, is favorably produced from bacteria due to its cost and production time [2].

Amylases hold a prominent role in industries of paper, textiles, fuels, detergents, and foods. Its use in the food industry, for example, is in producing glucose syrups during the liquefaction process [3]. This process requires a high temperature; thus, finding suitable thermophilic amylase is highly recommended [4]. Recently, the search for thermophilic bacteria producing hydrolases from local hot springs has been intensive. Several places, like Bora and Pulu, were reported to have potential thermophilic amylases [5,6].

Following screening thermophilic amylase from the local hot spring, finding out the best condition in producing amylase with high activity is important. Some approaches were performed to optimize amylase production from thermophilic bacteria, such as selecting proper fermentation media and
adjusting pH, temperature, incubation period, and aeration. A suitable incubation period and aeration lead to better amylase production [7]. Our previous study reported that amylase from PL-33 isolated from Pulu hot spring was a prospective enzyme for industrial applications [6]. In the present study, we pursue the best incubation period and agitation speed to produce high amylase activity.

2. Experimental procedure

2.1. Production of crude amylase with varied incubation time

Production of crude amylase was performed using an MSM medium enriched with 1 % starch as previously described [6]. PL-33 culture was incubated at 180 rpm, 60 °C for 18-24 hours, then moved into a new medium for 24 h production with a varied incubation time of 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 hours. The crude enzyme (supernatant) then collected after centrifuged at 8000 rpm and a temperature of 4 °C. Enzyme activity was determined using the Bernfield method [8].

Production of crude amylase with varied agitation speed

After determining the best incubation time for producing crude amylase, we varied agitation speed in the range of 60, 90, 120, 150, 180, and 200 rpm to determine the best shaking condition for our enzyme production.

2.2. Calculation of growth of thermophilic bacterial (OD600)

The culture of thermophilic bacteria PL-33 were collected every 12 hours during the production of a crude enzyme in both varied incubation time and agitation speed. Optical Density was measured at 600 nm.

2.3. Pearson correlation coefficient

Pearson correlation analyses were performed by plotting data between bacterial growth (x) versus enzyme activity (y) for both varied incubation and agitation speed and calculated using Pearson Correlation - Free Statistics Software (Calculator) Version 1.2.1 [9].

3. Results and discussion

We have previously reported the optimum pH and temperature of our thermophilic amylase produced by PL-33 bacteria [6]. To further characterize our crude enzyme, we determined its best incubation time and agitation speed. The incubation time was varied from 6 to 72 hours. Results showed that enzyme activity was gradually increased following the increase of incubation time and reached its maximum activity of 0.44 U/mL at 48 h (Fig.1). It was further rising in incubation time ended in lessening enzyme activity. Prolonged incubation time leads to denaturation, decomposition, and inhibition of an enzyme [10, 11]. Similar results about amylase isolated from B. licheniformis ATCC 12759 and Bacillus isolates reported that continued incubation time over 48 hours influenced amylase activity [12,13].

To evaluate the effect of agitation speed on our thermophilic amylase, we varied the shaking condition from 60 to 200 rpm, and subsequently incubated the culture based on our results above. Amylase activity progressively increased concomitant with an escalation of agitation speed. It started from 0.3 U/mL at 60 rpm, reached maximum activity of 0.5 U/mL at 180 rpm, and finally decreased slightly to 0.47 U/mL at 200 rpm. It was probably caused by the damage of the bacterial cell wall when stirred at extremely high speed. Agitation speed altered the production rate of amylase, thus affected enzyme activity [14]. Our amylase produced the highest activity when stirred at 150-200 rpm, which was similar to amylases from Bacillus species and Bacillus sp. BCC 01-50 [15,16].

The growth of PL-33 producing amylase elevated steadily following extended incubation time (Figure 1). Furthermore, it rapidly increased from an agitation speed of 90 rpm to 150 rpm, then insignificantly changed between 150 to 200 rpm (Figure 2). Our results indicated that bacterial growth affected the activity of amylase. The highest activity of amylase was observed at 42 - 54 h of incubation period and 150 to 180 rpm when the bacteria reached the peak of its growing phase. This finding was consistent with thermophilic amylase from Lejja, Purwekerto, and Larijan [16,17,18].
Figure 1. Effect of incubation time against enzyme activity (blue line) and growth of thermophilic bacteria PL-33 producing amylase (orange line). Data were expressed as mean ± standard error.

Figure 2. Effect of agitation speed against enzyme activity (blue line) and growth of thermophilic bacteria PL-33 producing amylase (orange line). Data were expressed as mean ± standard error.

Figure 3. Scatter plots data of incubation time (a) and agitation speed (b) with Pearson correlation coefficients (r).
Table 1. Pearson product moment correlation

|                | Incubation time | Agitation speed |
|----------------|-----------------|-----------------|
| r              | 0.7583          | 0.8592          |
| 95% confidence interval | 0.5724 to 0.8701 | 0.6552 to 0.9464 |
| R squared      | 0.5751          | 0.7383          |
| P-value        |                 |                 |
| P (two-tailed) | <0.0001         | <0.0001         |
| P-value summary|                 |                 |
| Significant? (alpha = 0.05) | Yes         | Yes             |

To support our results, we analyzed the correlation between bacterial growth and amylase activity using Pearson correlation analysis tools (Fig. 3)[9]. Pearson product-moment correlation (r) is frequently used to analyze the correlation between two random and normally distributed variables [19]. A number of methods have been introduced to unravel the correlation coefficient into descriptors or labels and categorized as “weak”(0.1-0.39), “moderate”(0.4-0.69), “strong”(0.70-0.89), or “very strong”(0.90-1.00) relationship [20]. As visually displayed in Figure 3 and seen in Table 1, there were strong correlations (r = 0.70 – 0.89) between bacterial growth and enzyme activity for both incubation time and agitation speed experiments.

4. Conclusions

Our current reports suggested that crude amylase produced from thermophilic bacteria PL-33 has an elevated amylase activity when adjusted to fit incubation time and agitation speed. Further research on characterizations of crude amylase, like the effect of substrate concentration, substrate preferences, enzyme stability, etc. is necessary to understand the enzyme better.

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