Effect of temperature and pH on polygalacturonase production by pectinolytic bacteria *Bacillus licheniformis* strain GD2a in submerged medium from Raja Nangka (*Musa paradisiaca* var. *formatypica*) banana peel waste

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**Abstract.** The aim of this research were to determine the effect of temperature (45°C, 55°C, 65°C) and pH (5.0; 6.0; 7.0) on the increase of total cell count and polygalacturonase enzyme activity produced from raja nangka banana (*Musa paradisiaca* var. *formatypica*) peel waste by pectinolytic bacterial *Bacillus licheniformis* strain GD2a. This research applied two sample repetition and one analysis repetition. The result showed temperature and pH affect total cell count. The total cell count on 45°C and pH 7 recorded the highest number at 9,469 log cell/ml. Temperature and pH also affected pectin concentration at the end of fermentation. The lowest pectin concentration recorded at 45°C and pH 7 was 0.425 %. The highest enzyme activity recorded at 65°C and pH 7 was 0.204 U/ml. The highest enzyme protein concentration was recorded at 65°C and resulted as 0.310 mg/ml on pH 6. The highest specific activity was 19.527 U/mg at 65°C and pH 7. By this result, could be concluded that optimum condition process on polygalacturonase production was at 65°C and pH 7 because it gave highest enzyme activity result (0.204 U/ml).

**1. Introduction**

Polygalacturonase (EC 3.2.1.67) was an enzyme that catalyzed hydrolysis of α-1,4-glycosidic bond on polygalacturonic acid chain into monomers. This enzyme could be produced by fermentation on raja nangka banana peel waste medium with 0.134 U/ml as the highest enzyme activity [23].

Two factors influenced enzyme activity on fermentation process were temperature and pH. Optimum condition on polygalacturonase production by *Bacillus sp.* was on 40°C of temperature and pH 9 [13]; 50°C and pH 7 [26], 50°C and pH 9 [16] and 37°C and pH 6.5 [5]. The rising of temperature would affect the solubility of protein, so that enzyme activity increased. If temperature was too high, it would denature the enzymes thus decreased activity [4; 28]. Incompatible pH could inhibit the growth of microorganism, so that inhibited the enzymes production and decreased enzyme activity [12].

*Bacillus licheniformis* strain GD2a pectinolytic bacterial had been isolated from traditional market vegetable waste. It was chosen in polygalacturonase production since capability to produce enzyme...
with the highest activity of 0.209 U/ml and it was enzyme gave the best ability to clarify Garut orange juice. The morphology of these isolates were rod-shaped, chain form, Gram positive and 0.5 μm in size [9].

Production of polygalacturonase by Bacillus licheniformis strain GD2a isolate [23] were incubated at 55°C at 48 hours without any initial pH adjustment. Based on this study, further research was needed to determine the effect of temperature and pH during fermentation to enzyme activity. Various temperature used as 45°C, 55°C and 65°C and variation of pH were 5.0; 6.0 and 7.0. From this research, the best temperature and pH to produce polygalacturonase would be selected by the highest activity of enzyme.

2. Methods
2.1. Material Preparation
Raja nangka banana peel waste as the main material was collected from UPKKS Bhakti Kencana Karanganyar, Indonesia and Bacillus licheniformis strain GD2a bacterial isolates from food microbiology and biotechnology laboratorium Department of food science and technology collection Universitas Sebelas Maret.

2.2. Banana Peel Pectin and Protein Total Assay
Raja nangka banana peel waste were collected and sorted. Sample was washed, dried and grinded to powder. Method by [25] was used to determine pectin and Kjeldahl method [2] was used to determine total protein on banana peel sample.

2.3. Subcultures and Starter Preparation
Subculture made by one loopful of Bacillus licheniformis strain GD2a culture collection isolate was streaked on pectin agar media. It was incubated (SELECTA) at 55°C for 24 hours. After that, starter was prepared as one loopful of Bacillus licheniformis strain GD2a subculture dispersed into the pectin broth media then incubated at 55°C for 24 hours until it was 10⁶ cell/ml amount [23].

2.4. Medium Preparation
Banana peel was washed, blanched and crushed. It was taken 13.18 g of slurry and diluted with distilled water into 100 mL (5% media pectin based on pectin assay result). This media added with 0.5 % of ammonium sulfate [23] and pH was adjusted on 5.0; 6.0; and 7.0 by using hydrogen chloride (HCl) 2 M and natrium hydroxide (NaOH) 3M. Media were sterilized by autoclaving (SELECTA) for 15 minutes at 121°C.

2.5. Enzyme Production
One mL of Bacillus licheniformis strain GD2a starter was added into 100 mL of production media. Incubation was carried out at 45°C [13], 55°C [17] and 65°C [26] in incubator shaker at 144 rpm of speed [29] for 48 hours [13].

2.6. After Fermentation Assay
Haemocytometer (NEUBAUEUR ASSISTANT) bacterial count method [8] was used to count cell amount on media. Final pectin on fermentation media was assayed by modified [25] method. It was taken 20 g of fermentation media and analysed same as [25] method [23].

2.7. Enzyme Isolation and Partial Purification
75 mL of production media was centrifuged (HETTICH) at 6000 rpm for 15 minutes at 4°C [29] to separate supernatant and pellet. Supernatant precipitated with ammonium sulfate at 50% saturation [9] for 24 hours at 4°C. This precipitate was centrifuged at 12,000 rpm at 4°C for 10 minutes [29] to get the pellet. This pellet was dissolved in 0.05 M acetate buffer pH 5.2 (1: 1). Dialysis membrane MWCO 12 KDa was used to dialysis the pellet. It was soaked in 300 mL 0.05 M
acetate buffer solution pH 5.2 in a 600 mL of glass beaker. Magnetic stirrer was used to homogenize this process for 24 hours in a cool chamber, at 4°C [29]. Buffer solution was replaced every six hours.

2.8. Partial Purificated Enzyme essay
Partial purificated enzyme activity was measured with DNS methods [15; 3]. Protein enzyme concentration was measured using the method of Lowry [24]. From the results of enzyme activity and protein concentration could be determined the specific activity of the enzyme to determine the purity of the enzyme [30].

2.9. Data Analysis
Data was analyzed by One Way Analysis of Variance (ANOVA) by using SPSS 16.0 software. If there was a difference in response between the treatment then continued by Duncan Multiple Range Test (DMRT) at α = 5%.

3. Result and discussion
3.1. Banana Peel Pectin and Protein Total Assay
The analysis showed that average pectin content of raja nangka banana peel samples were 37.924 ± 1.250%. This result was lower than the content of pectin in raja nangka banana peel samples on [23], it was 68.47%. This different of pectin content caused of banana ripening level [1]. While the total protein content of raja nangka banana peel samples was 4.742 ± 0.196%. These results were higher than [6] was 2.15% on a kepok banana peel sample. The difference was caused of different types of banana [14].

Analysis of pectin and total protein content of banana peels were important to determine. Pectin as a carbon source and protein as source of nitrogen which would be utilized during the production polygalacturonase. Both of these compounds were the main energy source of the pectinolytic bacteria during the fermentation process.

3.2. After Fermentation Total Cell Assay

Table 1. Effect of Temperature and pH Variation to Total Cell Count

| Temperature (°C) | End Cell Count (log cells / ml) | pH 5  | pH 6  | pH 7   |
|-----------------|---------------------------------|-------|-------|--------|
| 45              | 8.763 ± 0.03<sup>ab</sup>       | 9.005 ± 0.10<sup>bii</sup> | 9.469 ± 0.03<sup>CB</sup> |
| 55              | 8.351 ± 0.10<sup>aA</sup>       | 8.802 ± 0.03<sup>bA</sup> | 8.975 ± 0.16<sup>bA</sup> |
| 65              | 8.665 ± 0.02<sup>ab</sup>       | 8.878 ± 0.03<sup>bAB</sup> | 9.111 ± 0.01<sup>cA</sup> |

Description: Different notations in the same line and different capital letters notation in the same column indicate significant differences at α = 5%.

Table 1 showed data after fermentation cell count (log cells/ml) after fermentation process. Statistically, the difference in pH variations gave a significant effect (α≤5%) against the after fermentation cell count on the variation of temperature of 45°C, 55°C and 65°C. Increasing in pH was followed increase the number of cells at all temperatures. The highest cell count at pH 7.

Differences in temperature variations gave a significant impact (α≤5%) of the after fermentation cell count at various pH 5, pH 6 and pH 7. Data showed that the highest number of cells count in general at a temperature of 45°C in each variation of pH. These results were supported by [22] which explained that there was a positive relationship between enzyme activity and cell growth during fermentation.

3.3. After Fermentation Pectin Content Assay
Determination of pectin of the after fermentation process was intended to determine the yield of the pectin in the fermentation media. Initial Pectin content of the fermentation medium was 5%. By
knowing the pectin contained in the media, could be attributed the microbial activity to degrade pectin media.

Table 2 showed data of the final pectin content (%) after fermentation. Statistically, difference in pH variations gave a significant impact (α≤5%) against after fermentation pectin content at a temperature of 65°C. While at a temperature 45 °C and 55°C, pH variation did not give effect to pectin content. The highest level of pectin was pH 5. While the temperature variations gave a significant effect (α≤5%) against after fermentation pectin content at pH 5 and pH 7. Variations in temperature of 45°C, 55°C and 65°C did not give effect to final pectin content at pH 6. The highest pectin content in general at a temperature of 55°C.

**Table 2. Effect of Variations in Temperature and pH to Pectin Content (%)**

| Temperature (°C) | pH | After Fermentation Pectin Content (%) |
|------------------|----|--------------------------------------|
| 45               | 5  | 0.565 ± 0.1 1 aA                      |
|                  | 6  | 0.472 ± 0.1 3 aA                      |
|                  | 7  | 0.425 ± 0.01 aA                       |
| 55               | 5  | 0.632 ± 0.00 abA                      |
|                  | 6  | 0.615 ± 0.04 aA                       |
|                  | 7  | 0.605 ± 0.01 abB                      |
| 65               | 5  | 0.570 ± 0.0 2 bA                       |
|                  | 6  | 0.428 ± 0.01 aA                       |
|                  | 7  | 0.540 ± 0.04 bB                       |

**Description:** Different notations in the same line and different capital letters notation in the same column indicate significant differences at α = 5%.

From initial pectin content 5% and after fermentation pectin content data, could be calculated the pectin that degraded during fermentation process. Hydrolyzed pectin number on this study was about 4.36% to 4.57%. It was not much different from the research by [23], where the levels of pectin degraded in treatment variations pectin 5% with 0.5% ammonium sulfate was 4.790%.

3.4. Partial Purified Enzyme Assay

**Table 3. Effect of Temperature and pH Variation to Partial Purified Enzyme Activity (U / ml)**

| Temperature (°C) | pH | Enzyme Activity (U / ml) |
|------------------|----|--------------------------|
| 45               | 5  | 0.129 ± 0.00 bc          |
|                  | 6  | 0.084 ± 0.01 db          |
|                  | 7  | 0.150 ± 0.0 5 abA        |
| 55               | 5  | 0.066 ± 0.00 bB          |
|                  | 6  | 0.050 ± 0.00 aA          |
|                  | 7  | 0.082 ± 0.00 cA          |
| 65               | 5  | 0.029 ± 0.00 aA          |
|                  | 6  | 0.026 ± 0.01 aA          |
|                  | 7  | 0.204 ± 0.01 bB          |

**Description:** Different notations in the same line and different capital letters notation in the same column indicate significant differences at α = 5%.

Table 3 showed that variation of different pH gave a significant impact (α≤5%) at the production temperature 55°C and 65°C. However, at 45°C, pH variation did not give effect to polygalacturonase activity. The highest enzyme activity was at pH 7. The lowest enzyme activity was at pH 6.

The results of this study supported by [11], [7] and [18] which stated that the optimum pH conditions to produce polygalacturonase by Bacillus sp. was at pH 7. The highest enzyme activity at pH 7 with regard to the after fermentation cell count (table 1.) which also showed the highest cell count at pH 7. [22] revealed that the high activity of the enzyme related with number of microbes existing and further described by [19] that microbes had an optimum pH for growth. If the pH is not appropriate, microbial metabolism would be disturbed and its growth would be stopped. But in this study, after fermentation pH measurements needed to know to determine the pH change media that might be affected to enzyme activity.

Table 3 also showed significant effect (α≤5%) variations in production temperature 45°C, 55°C and 65°C to the activity of an enzyme produced in pH 5, pH 6 and pH 7. On temperature 45°C produced the highest activity and temperature of 65°C resulted in the lowest activity at pH 5 and pH 6 during production. While at pH 7, incubation temperature 65°C produced the enzyme with the highest activity and temperature of 55°C produced an enzyme with lowest activity.
Temperature was a factor that very influential in the production of enzymes. [18], explained if the temperature was too low, the cell metabolism would run slower, but if the temperature was too high, it would damage the enzymes cell growth, which killed the cell itself. The result of this study were generally at temperature 45°C produced the highest activity, in accordance with the number of cells in table 1, which stated that the highest number of cells also recorded at 45°C.

However at 65°C activity of the enzyme gave low result generally, except at pH 7. This phenomenon occurred due environmental factors related to the bacterial enzyme. pH environments and extreme temperatures damaged the enzyme secreted microbial cells. [22] revealed that the production of the enzyme pectinase influenced by the environment.

[20] explained that incompatible temperature would be caused denaturation of enzymes. Denaturation of the enzyme was due to the release of covalent and hydrogen bond in the structure of the enzyme protein. The liberation of these bonds results in the disintegration of the enzyme protein folds that caused enzyme inactivation. While the influence of pH, described by [27] related with pH balance of ionization on active site enzyme. Ion balance affected the bonding of enzyme molecules. If there was no balance of ions, the 3D-shape of active site on enzyme structure would be disturbed so that the substrate could not be bounded in to the enzyme. Such conditions would lead to a decrease in the activity of enzymes.

3.5. Enzyme Protein Concentration Assay

Table 4 showed the enzyme protein concentration data produced at various pH and temperature. pH variation did not give effect to the protein concentration of the enzyme at each temperature of incubation except at 45°C. In general, the highest enzyme concentration was at pH 6. For a given temperature variation, there was significant effect (α≤5%) for enzyme protein concentration at each pH production. The highest protein concentration at temperature of 55°C produced at all pH conditions given.

| Temperature (°C) | Partial Purificated Enzyme Protein Concentration (mg / ml) |
|-----------------|----------------------------------------------------------|
|                 | pH 5 | pH 6 | pH 7          |
| 45              | 0.014 ± 0.00 aA | 0.140 ± 0.04 bA | 0.075 ± 0.00 ab |
| 55              | 0.279 ± 0.07 ab | 0.310 ± 0.07 ab | 0.227 ± 0.01 ac |
| 65              | 0.021 ± 0.01 A  | 0.022 ± 0.01 A  | 0.013 ± 0.01 A  |

Description: Different notations in the same line and different capital letters notation in the same column indicate significant differences at α = 5%.

Concentration of the enzyme protein associated with the enzyme purity. [10] conducted a purification of enzymes decreased concentration of soluble protein. The decreasing concentration of this protein occurred during purification process, the protein would be cleaned.

On this study, there was also a relationship between amount of protein and enzyme activity, which on table 4, the production conditions of temperature 65°C and pH 7 resulted in a lower protein concentration. However, on table 3 under the same conditions gave high enzyme activity. This indicated that production on 65°C and pH 7, polygalacturonase purity level was high.

| Temperature (°C) | Specific Enzyme Activity (U / mg) |
|-----------------|----------------------------------|
|                 | pH 5 | pH 6 | pH 7          |
| 45              | 9.360 ± 2.44 bB | 0.636 ± 0.26 aA | 2.018 ± 0.69 aA |
| 55              | 1.561 ± 0.39 abA | 1.062 ± 0.20 aA | 2.333 ± 0.22 ba |
| 65              | 1.453 ± 0.37 aA | 1.154 ± 0.14 aA | 19.527 ± 13.19 aA |

Description: Different notations in the same line and different capital letters notation in the same column indicate significant differences at α = 5%.
From table 5, it could be seen that pH variation gave significant effect (α≤5%) to the specific activity of the enzyme in the production temperature 45°C and 55°C. However, the pH did not give effect to the specific activity of the enzyme at 65°C. pH 5 resulted in the highest specific activity of enzyme at 45°C and pH 7 recorded highest specific enzyme activity value at 55°C. While the temperature variation gave significant effect (α≤5%) to the specific activity enzyme only on pH 5, and the highest was at 45°C. Meanwhile at pH 6 and pH 7, temperature variations did not give effect to the specific activity of the enzyme.

The highest specific activity of polygalacturonase was at 65°C and pH 7 in the amount of 19.527 U/mg. This result was smaller compared to [21] which produced polygalacturonase with specific activity of 1.015 U/mg. This difference caused by bacteria activity and media used in fermentation. [21] used *Bacillus licheniformis* KIBGE IB-21 with a fermentation medium of 1% apple pectin.

Selection of the optimum conditions for enzyme production polygalacturonase treatment based on temperature and pH which gave a significant impact on the parameters of the final number of cells, the levels of pectin, enzyme activity and protein enzyme and specific activity of enzyme. But the main point in this study was activity polygalacturonase enzyme. So that the optimum conditions for produced polygalacturonase was at a temperature of 65°C and at pH 7 because it gave highest enzyme activity (0.204 U/ml).

4. Conclusion

Temperature and pH gave an effect to the production of polygalacturonase, cell counts, pectin content, enzyme activity, enzyme protein concentration and specific activity of the enzyme polygalacturonase. There was a relationship that in conditions of high cell counts, enzyme activity polygalacturonase was high too. Selected Condition fermentation process for producing polygalacturonase was at 65°C and pH 7, due this condition produced enzyme polygalacturonase with the highest activity at 0.204 U/ml.

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