Growth and Enzyme Production of Proteolytic Bacteria from Mangrove Sediment

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Abstract. Accumulation of organic component in mangrove ecosystem results in diversity of microorganisms producing extracellular enzymes. Two species of proteolytic bacteria had been isolated and identified from sediment of mangrove ecosystem in the Dumai Marine Station of Riau Province, Indonesia. This research aimed to assess the activity and enzyme production of the proteolytic bacteria, i.e. *Bacillus manliponensis* (isolate code P.Az6) and *B. toyonensis* (P.Az20). The growth of bacteria in tryptic soy broth (TSB + skimmed milk) was observed by using spectrophotometer at λ 610 nm and by analysis the total plate counts on Zobell Marine Agar at 0, 6, 12, 18 and 24 hours. Production of enzyme protease was measured from the absorbance values of bacterial supernatant, then were converted to the protease concentration by using the Bovine serum Albumin Standard. The highest bacterial growth and optimal enzyme production was observed at 6 hours incubation, and period after that showed decrease in growth enzymatic activity. *Bacillus toyonensis* showed higher bacterial growth and enzyme production than *B. manliponensis*.

1. Introduction

High productivity and accumulation of organic compounds in the sediment of mangrove result in variety of organisms found in the ecosystem including bacteria. The organic compounds originate from the mangrove litter production [1]. Some bacteria from the mangrove ecosystem have an ability to produce extracellular enzymes such as cellulase, amylase and protease. Enzyme are considered as environment-friendly (green) chemicals for various manufacturing processes, thus promising sustainable production and manufacturing [2].

Protease enzymes, found in all living organisms, play important roles in living things, mainly in breaking down long chain molecules of proteins into shorter fragments, and are essential for cell growth and differentiation. Among all forms of life, bacterial proteases are most important source due to their enormous industrial application [3] such as food, feed, leather, textile, detergent, pharmaceutical industries and bioremediation [4,5]. In addition, proteases from microbial sources have also been used in the management of industrial as well as household wastes. Similar to the animal feed industry, protease can potentially be used to improve the quality of fish feed ingredients in aquaculture [6]. Microbial enzymes are widely used in industrial processes due to their low cost, large productivity, chemical stability, environment protection, plasticity and vast availability [7]. Proteases constitute of 60% of the global industrial market due to their huge application potential in diverse industries [2].

Fungi and bacteria are the main source of microbial proteases which are frequently found in coastal ecosystems. Many researchers have found proteolytic bacteria in mangrove ecosystem. Forty five bacterial isolates producing protease have been isolated from sediment in Mangrove Forest of Chanthaburi, Thailand of which the majority of the isolates were identified into *Bacillus* sp. [8]. Subagiyio et al [9] found seven bacterial isolates which were able to produce protease, and three of them showed enzyme activity above 300 mm²/mL. Two species of bacteria, *Yersinia enterocolitica* and *Enterobacter agglomerans* were found in mangrove sediment samples from Gunung Anyar, Surabaya, East Java [10]. While, *Bacillus oceanisediminis*, *B. aquimaris*, *Halomonas aquamarina*,
Acinetobacter pittii and Salinicola salaries were found from sediment mangrove in Cilacap, Rembang and Karimunjawa of Central Java [11].

Our previous study identified proteolytic bacteria, namely Bacillus manliponensis and B. toyonensis from mangrove sediment of Dumai Marine Station. Therefore, current research aims to observe the bacterial growth and to determine optimum time of protease production during 24 hours incubation.

2. Material and Methods
This experiment research had been conducted from April until July 2020. Bacterial growth and optimum time of enzyme production were analysed in the Marine Microbiology Laboratory of the Faculty of Fishery and Marine, University of Riau, Indonesia.

2.1 Preparation of Bacteria
Proteolytic bacteria used in the experiment were isolated and identified from sediments of Marine Ecosystem in Dumai Marine Station of Riau Province [12]. The isolates were collected in the Marine Microbiology Laboratory. Two isolates used were B. manliponensis (P.Az6) and B. toyonensis (P.Az20). The bacteria were sub-cultured in Zobell Marine Agar medium added with skim milk by pour plate technique, then were incubated at 30°C for 48 hours. Grown colonies were indicated by clear zone surrounding the colonies.

2.2 Growth of Bacteria
A volume of 10 mL suspension of proteolytic bacteria was poured into bottles containing 100 mL TSB (Tryptic Soy Broth) medium with 1% skim milk. The suspensions were incubated in a shaker water bath at 37°C. The bacterial growth was observed at hours 0, 6, 12, 18 and 24 of incubation time by measuring the absorbance in spectrophotometer at wavelength (λ) 610 nm. At the same time, the total plate counts on the Zobell Marine Agar were also analysed.

2.3 Enzyme Production
Optimum time of protease production was determined following the procedure of Melaty [13]. 10 mL of bacterial suspension was poured into bottles of TSB medium containing 90 mL and 1% skimmed milk. The suspensions were incubated in a shaker water bath at 37°C. The enzyme production was measured at 0, 6, 12, 18 and 24 hours of incubation. One mL of each culture was centrifuged at 9,000 rpm for 15 minutes in order to separate the supernatant and the bacterial pellet. Absorbance of the supernatant containing protease was measured in a spectrophotometer at λ 660 nm. The absorbance values were converted to production values by using linear regression formula (Y=0.0175x + 0.0249) obtained from the absorbance values of the Bovine Serum Albumin (BSA) standard.

2.4. Data Analysis
The bacterial growth was recorded from the observation on the absorbance value of broth culture and the total plate counts. Data of the bacterial growth and enzyme production were presented in tables and were analysed descriptively. The results were compared to previous related and similar researches.

3. Results and Discussion
3.1. Bacterial Growth
The growth of two tested proteolytic bacteria in broth culture (TSB + 1% skim milk) was observed from the absorbance value in spectrophotometer (λ = 610 nm) every 6 hours for 24 hours incubation, and from analysis of the total plate counts. Data on the growth of B. manliponensis (P.Az6) and B. toyonensis (P.Az20) were presented in Table 1. Development of the bacterial growth during the incubation time was as shown in Figure 1.
Table 1. Absorbance value of the proteolytic bacteria growth in TSB for 24 hours

| Bacterial code | Replication | Absorbance value at incubation (hours) |   |   |   |   |
|---------------|-------------|----------------------------------------|---|---|---|---|
|               |             | 0 | 6 | 12 | 18 | 24 |
| P.Az6         | 1           | 0.061 | 0.458 | 0.436 | 0.420 | 0.390 |
|               | 2           | 0.042 | 0.428 | 0.396 | 0.398 | 0.378 |
|               | 3           | 0.052 | 0.424 | 0.422 | 0.380 | 0.330 |
| Average       |             | 0.052 | 0.437 | 0.418 | 0.399 | 0.366 |
| ± St. dev.    |             | ±0.010 | ±0.019 | ±0.020 | ±0.020 | ±0.032 |
| Average growth|             | - | 0.385 | -0.019 | -0.019 | -0.033 |
| P.Az20        | 1           | 0.071 | 0.450 | 0.428 | 0.414 | 0.408 |
|               | 2           | 0.062 | 0.454 | 0.438 | 0.432 | 0.389 |
|               | 3           | 0.053 | 0.477 | 0.374 | 0.326 | 0.325 |
| Average       |             | 0.062 | 0.460 | 0.413 | 0.391 | 0.374 |
| ± St. dev.    |             | ±0.009 | ±0.015 | ±0.034 | ±0.057 | ±0.043 |
| Average growth|             | - | 0.398 | -0.047 | -0.022 | -0.017 |

Note: P.Az6 = Bacillus manliponensis  P.Az20 = B. toyonensis

Figure 1. Development of absorbance of bacterial growth within 24 hours incubation

Figure 1 indicates an increase in absorbance values of proteolytic bacterial growth from 0 hour to 6 hours incubation. The increase of B. toyonensis is higher (0.398) than B. manliponensis (0.385). Development of the absorbance values is in line with the total bacterial count as shown in Table 2 and Figure 2, that the highest bacterial counts was found at 6 hours incubation. The data indicates that total plate counts of B. toyonensis \((1.52 ± 0.162 \times 10^8 \text{ cfu/mL})\) is higher than that of B. manliponensis \((1.36 ± 0.161 \times 10^8 \text{ cfu/mL})\). The growth of the two bacteria decreased from 12 hours until 24 hours incubation.
Table 2. Total plate count of bacterial growth on Zobell Marine Agar for 24 hours

| Bacterial code | Replication | Total counts at incubation (hours) (×10^8 cfu/mL) |
|---------------|-------------|-----------------------------------------------|
|               | 0 6 12 18  24 |                                              |
| P.Az6         | 1 0.86 1.52 1.38 1.17 0.94 |                                             |
|               | 2 0.79 1.44 1.24 1.01 0.78 |                                             |
|               | 3 0.69 1.21 1.19 0.97 0.68 |                                             |
| Average ± St. dev | 0.78 ±0.085 1.39 ±0.161 1.27 ±0.098 1.05 ±0.106 0.80 ± 0.131 |
| P.Az20        | 1 1.01 1.68 1.57 1.29 1.16 |                                             |
|               | 2 0.86 1.52 1.32 1.10 0.98 |                                             |
|               | 3 0.74 1.36 1.25 0.97 0.83 |                                             |
| Average ± St. dev | 0.87 ±0.135 1.52 ±0.160 1.38 ±0.168 1.12 ±0.161 0.99 ±0.165 |

Note: P.Az6 = Bacillus manliponensis  P.Az20 = B. toyonensis

Figure 2. Development of total plate count of proteolytic bacteria within 24 hours incubation

The decrease of bacterial counts could be due to protein contained in skim milk as sole of carbon and nitrogen sources were used for growing in the first six hours incubation. It was therefore, the availability of carbon and nitrogen from hours 12 until 24 decreased. Skim milk agar has been used for the isolation of protease production bacteria by many researchers [14; 15]. Nitrogen sources is a nutritive factor influencing protease production, that is metabolized to produce primarily amino acid, nucleic acid, and protein and cell wall components [16].

3.2. Enzyme Production

Proteases are a group of enzyme found in living materials. The enzyme is found in bacteria such as Bacillus spp. In this research Bacillus manliponensis (P.Az6) and B. toyonensis (P.Az20) can produce protease as it was measured from the conversion of absorbance values of the supernatant containing enzyme (Table 3).
Table 3. Concentration of crude protease-contained in bacterial supernatant

| Bacterial code | Replication | Concentration (mg/mL) at observation time (hour) |
|----------------|-------------|-------------------------------------------------|
|                |             | 0  | 6  | 12 | 18 | 24 |             |
| P.Az6          | 1           | 0.0255 | 0.0258 | 0.0253 | 0.0253 | 0.0250 |           |
|                | 2           | 0.0256 | 0.0257 | 0.0254 | 0.0252 | 0.0251 |           |
|                | 3           | 0.0254 | 0.0260 | 0.0257 | 0.0254 | 0.0251 |           |
|                | Average     | 0.0255 ± 0.0001 | 0.0258 ± 0.0001 | 0.0255 ± 0.0002 | 0.0253 ± 0.0001 | 0.0251 ± 0.0001 |
|                | ± SD        | 0.0001 | 0.0001 | 0.0002 | 0.0001 | 0.0001 |           |
| P.Az20         | 1           | 0.0258 | 0.0264 | 0.0257 | 0.0254 | 0.0252 |           |
|                | 2           | 0.0257 | 0.0260 | 0.0259 | 0.0254 | 0.0251 |           |
|                | 3           | 0.0254 | 0.0263 | 0.0261 | 0.0252 | 0.0251 |           |
|                | Average     | 0.0256 ± 0.0002 | 0.0262 ± 0.0002 | 0.0259 ± 0.0002 | 0.0253 ± 0.0001 | 0.0251 ± 0.0001 |
|                | ± St. dev.  | 0.0002 | 0.0002 | 0.0002 | 0.0001 | 0.0001 |           |

Note: P.Az6 = Bacillus manliponensis  P.Az20 = B. toyonensis

Table 3 indicates that the optimum enzyme production of the proteolytic bacteria occurred at 6 hours incubation. Period after that the enzyme production decreased. This could be due to decrease in the bacterial growth as described before. Previous studies found that maximum protease production by Bacillus sp. occurred at 48 hours; after that the production gradually decreased [16]. In order to obtain maximum enzyme production, other factors influencing such as inoculum size, temperature, pH, carbon and nitrogen sources of growing medium can be optimized [17]. In this research, bacterial growth and enzyme production were only based on incubation time which was in short period (i.e. within 24 hours).

In this research, the maximum enzyme production performed by B. toyonensis occurred at 6 hours incubation. This finding was different from that reported by Elzabalawy et al. [18] who found maximum protease production by B. toyonensis occurred at 24 hours incubation while by B. cereus and B. subtilis occurred at 48 hours incubation. The B. toyonensis shows higher ability in extracellular enzyme production than B. manliponensis. The Bacillus species can be found in various sources and exhibit enzymatic activities. Bacillus toyonensis isolated from stingless bee, Heterotrigona itama showed high proteolytic and cellulytic activities [19]. While, B. toyonensis PNTB1 isolated from coastal area of Arabian Sea shows lipase, CM-cellulase and chitinase activities. B. toyonensis BCT-7112 originated from soil [20] and was known as Toyocerin® which had been used as probiotic for animal feed [21]. Meanwhile, Bacillus manliponensis is a new member of Bacillus cereus Group. This species and its enzymatic activities is very few reported. The type strain, BL4-6T was isolated from oil-contaminated tidal flat sediment from Malipo in the Yellow Sea coastal region of Tae-An, Republic of Korea [22, 23].

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