Protease activity from bacterial isolates of *Nepenthes maxima* reinw. ex nees

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**Abstract.** Protease is an enzyme that applied in many industrial sectors. Protease can be produced by proteolytic bacteria. Many of these bacteria were isolated from Nepenthes that lives in nutrient-poor areas. The life of Nepenthes is depend on the insects that trapped and degraded by proteolytic bacteria inside the pocket of Nepenthes. Sulawesi has a high diversity of Nepenthes, there are many species of Nepenthes that endemic to Sulawesi. However, there is no report for isolation and characterization of proteolytic bacteria from Nepenthes, especially in *Nepenthes maxima* Reinw. Ex Nees. This study was aimed to characterize the protease from bacterial isolate of *N. maxima* Reinw. Ex Nees. The methods that used in this study were screening of samples from Nepenthes and protease activity assay at temperature 37ºC with pH 7. The result showed that proteolytic index of BN1 was 0.64 mm, higher than other isolates. While the proteolytic index of BN2, BN3, BN4 and BN5 were 0.51 mm, 0.24 mm, 0.13 mm; and 0.54 mm, respectively. Isolate of BN2 were able to produce the highest protease activity (0.067 U/ml) and the lowest protease activity was produced by isolate of BN3 (0.008 U/ml). Protease activity of BN1, BN4 and BN5 were 0.015 U/ml, 0.023 U/ml and 0.055 U/ml, respectively. The result indicates that proteolytic bacteria from *N. maxima* Reinw. Ex Nees has the potential to produce protease that can be used in large-scale industrial sectors.

1. **Introduction**

Proteases are needed in the plants, animals and microorganisms to catalyze the peptide in proteins. Protease not only involved in cellular metabolic processes, but also have an important role in industry, such as in the manufacture of detergents and therapeutics [11].

Protease can be isolated from microorganisms such as fungi, algae and bacteria. Bacteria that able to degrade proteins with an extracellular protease are called proteolytic bacteria. Proteolytic bacteria can be isolated from a variety of places including landfills, hot springs, tofu waste, soil and Nepenthes. Nepenthes lives in nutrient-poor area [8], therefore it requires nutrient intake from insects that trapped in the cup of Nepenthes. Insects were trapped and degraded by proteolytic bacteria. There are around 80 species of Nepenthes in the world [10], and they increased to 87 species in 2001 [1]. Until 2012, the species continues to grow to 139 species. A total of 68 types of Nepenthes are live in Indonesia, 59 of them are endemic. *Nepenthes maxima* Reinw. ex Nees is one of the most widely spread from Maluku, Papua, Sulawesi, to Papua New Guinea.

Sulawesi has a high diversity of Nepenthes but there is no report for isolation and characterization of proteolytic bacteria from Nepenthes in Central Sulawesi, especially in *N. maxima* Reinw. Ex Nees.
Therefore, this study was aimed to determine the protease activity from bacterial isolate of \textit{N. maxima} Reinw. ex Nees.

2. Materials and methods
The materials used were the liquid samples from \textit{Nepenthes maxima} Reinw. ex Nees, Skim Milk Agar (SMA) media, casein, pospat buffer, 0.5 M Na2CO3, Folin-Ciocalteu reagent and TCA 0.4 M. The research was begun with bacterial culture screening, then the protease activity assay both in qualitative and quantitative assay.

2.1 Screening samples
Bacterial screening was done by inoculating bacteria on skim milk agar media and incubated at 37°C for 2x24 hours. Proteolytic bacteria are able to grow and form clear zone in media that contain protein.

2.2 Observation of morphology of colonies
The morphology of colonies was carried out by macroscopic and microscopic observations. Macroscopic observation was done by directly observing the characteristics of isolate colonies which included the shape, margins, elevation, and pigmentation of the colony. Microscopic observations was done by observing the shape of bacterial cells that stained through the Gram staining [6].

2.3 Carbohydrate fermentation assay
Carbohydrate fermentation were glucose, lactose and sucrose assay. Bacteria cultures that incubated for 24 hours were inoculated one each into the carbohydrate fermentation media. Then incubated at 37°C for 24 hours. The results were positive if there is a change in color and gas on the fermentation media.

2.4 Qualitative Assay of Protease Activity
Protease activity assay was carried out by inoculating 20 µl of bacterial isolate on skim milk agar using the well diffusion agar method. Then incubated at 37°C for 24 hours. Protease activity is indicated by the presence of a clear zone around the colony on the surface of the media.

2.5 Tyrosine standard curve
A standard tyrosine curve was made with 1.10 mM tyrosine solution using variations of concentration 6,875; 13.75; 27.5; and 55.0 μM. absorbance measurements using a UV-Vis nanodrop spectrophotometer at 650nm. The absorbance value was obtained to make the calibration curve, by plotting the absorbance value against tyrosine concentration.

2.6 Quantitative assay protease activity
The crude extract of the enzyme was determined by the enzyme activity based on the method previously reported by[2], which has been modified. A total of 50 µl enzyme solution was added with 50 µl of 0.05 M phosphate buffer pH 7, then added 50 µl substrate (2% casein in 0.05 M phosphate buffer solution pH 7), incubated at 37 °C for 10 minutes. The reaction was stopped by adding 100 µl of 0.4 M of Horooracetic acid (TCA). The mixture then centrifuged at 10000 rpm for 10 minutes. The supernatant was determined by its colorimetric level of tyrosine.

Each of the 50 µL supernatant was added 250 µL Na2CO3 0.5 M then vortexed for 10 minutes. Subsequently the mixture was added 50 µL of the Folin- Ciocalteau reagent and then the mixture was left for 30 minutes. The solution was read its absorbance using a UV-Vis nanodrop spectrophotometer at λ650nm. The protein concentration in the sample then used to determine protease activity.

3. Results and Discussion
There were five bacterial isolates that successfully isolated from \textit{Nepenthes maxima} Reinw. ex Nees and coded BN1, BN2, BN3, BN4 and BN5. The isolated bacteria were proteolytic bacteria that grow on media by the formation of clear zones around the colonies. The results of observations of isolation of
proteolytic bacteria can be seen in Figure 1. Furthermore, bacterial characterization was carried out by macroscopic and microscopic observation. This observation was aimed to determine the morphology of isolates. Based on macroscopic observations showed that the five bacterial colonies have distinctive colony characteristics. Macroscopic observations can be seen in Figure 2.

**Figure 1.** (a) Isolation code for BN1 - milky white, (b) Isolation code for BN2 - yellow, (c) Isolation code for BN3 - milky white, (d) Isolation code for BN4 - milky white, (e) Isolation code for BN5 - milky white

**Figure 2.** (a) BN1 in irregular shape, raised elevation, undulate margin and white pigmentation; (b) BN2 in circular shape, convex elevation, entire margin and yellow pigmentation; (c) BN3 in irregular form, raised elevation, undulate margin and milky white pigmentation; (d) BN4 in circular shape, convex elevation, entire margin, yellow pigmentation; (e) BN5 in irregular shape, raised elevation, undulate margin, milk white pigmentation

Microscopic observation was carried out by gram staining. The mechanism of gram staining was based on the structure and composition of bacterial cell walls [9]. The results of gram staining showed that the five isolates were gram-positive bacteria in the form of bacilli and coccus. These results are directly proportional to the study conducted by [12] explained that isolates bacteria from Nepenthes were gram-positive bacteria in the form of bacilli. Observation of microscopic morphology can be seen in Figure 3.

The bacterial characterization then carried out with a simple biochemical assay with the carbohydrate fermentation (Table 1). This treatment was aimed to confirm that the five isolates were the different isolates clearly, and determine the ability of bacteria to ferment the sugars. The fermentation of sugar was indicated by a color change in the fermentation medium (red to yellow), which means that the bacteria produce acid from the fermentation of substrate by lowering the pH in the medium. The
formation of froth in the fermentation medium indicates that the fermentation is in the form of gas (CO2).

**Figure 3.** (a) BN1 in the form of bacilli, gram-positive; (b) BN2 in the form of bacilli, gram-positive; (c) BN3 in the form of coccus, gram positive; (d) BN4 in the form of bacilli gram-positive; (i) BN5 in the form of bacilli, gram-positive.

Carbohydrate fermentation were observed after incubation for 24 hours on glucose substrate. The result showed that BN1 and BN3 were able to ferment but unable to produce the gas, while the other isolates were unable to ferment and produce the gas. In sucrose and lactose substrates, all samples were unable to ferment and produce the gas. These results indicate that the five isolates have different sugar fermentation abilities, especially in the BN1 and BN3 isolates.

**Table 1.** Fermentation assay

| No. | Isolate | Glucose Fermentation | Sukrosa Fermentation | Laktosa Fermentation |
|-----|---------|----------------------|----------------------|----------------------|
|     |         | Gas                  | Gas                  | Gas                  |
| 1   | BN1     | +                    | -                    | -                    |
| 2   | BN2     | -                    | -                    | -                    |
| 3   | BN3     | +                    | -                    | -                    |
| 4   | BN4     | -                    | -                    | -                    |
| 5   | BN5     | -                    | -                    | -                    |

The protease activity was qualitatively measured by the diameter of the clear zone formation around the colonies. This treatment was aimed to determine the highest proteolytic activity of isolates. The protein that contained in medium was an inducer for the protease. The clear zone formation was the result of hydrolysis in protein substrate by protease from the isolates. The peptone and skim milk in the medium was the main carbon source for the needs of bacterial metabolism. BN1, BN2, BN3, BN4, and BN5 were able to produce protease with a proteolytic index of 0.64 mm each; 0.51 mm; 0.24 mm; 0.13 mm; and 0.54 mm, respectively. These results indicate that BN1 was the highest proteolytic index compared to the others. The proteolytic index (enzymatic index) is affected by the microbial growth (generation time) in solid media, the ability to produce and the effectiveness of enzymes [3]. The qualitative assay of protease activity can be seen in Figure 4.

In the quantitative assay of protease activity, casein was the substrate. When the protease digests casein, tyrosine are released together with amino acids and other peptide fragments. The folin ciocalteus reagent reacts with tyrosine to produce blue chromophores, which can be measured as absorbance values at 650nm with a spectrophotometer. Protein concentration from crude enzyme was used to determine the activity of enzymes. The results of protein concentration for BN1, BN2, BN3, BN4, and BN5 were
Protease activity was measured at 37°C and pH 7 with incubation for 30 minutes. The results of enzyme activity for BN1, BN2, BN3, BN4, and BN5 were 0.015 U/ml; 0.067 U/ml; 0.008 U/ml; 0.023 U/ml and 0.055 U/ml, respectively (Figure 6).

According to [4], the enzymatic index is a qualitative estimation that confirmed to be used in determining an enzyme activity. The enzymatic index depends on the method of enzyme production and not necessarily the same as quantitative enzyme activity. According to [5], reported that there is no relationship between the enzymatic index and enzyme activity. Therefore, in this study, the proteolytic index was only used for screening of protease-producing bacteria. The discrepancy between the proteolytic index and protease activity was affected by the microbial growth and the differences in the medium [7]. Skim milk agar medium has a different composition from the protease enzyme production medium. Meanwhile, protease activity was affected by the substrate concentration, pH, temperature, inhibitors, cofactors and coenzymes.

4. Conclusion
Protease activity from five isolates had a proteolytic index from 0.013 to 0.064. Proteolytic bacteria from Nepenthes have the different potential to produce protease. However, low enzyme production is a challenge in the application of enzymes in the large-scale industrial sector.

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