

## Conference Paper

# Production and Characterization of Extracellular Protease from *Bacillus* sp. 140-B Isolated from Pineapple Plantation in Lampung, Indonesia

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## Abstract

*Bacillus* sp. being industrially important organisms produces a wide variety of extracellular enzymes including protease. *Bacillus* sp. 140-B isolated from rhizosphere area of pineapple plantation in Lampung Province, Indonesia was tested for production of protease on skim milk agar medium. The aims of this study were to characterize and investigate some properties of protease activity from *Bacillus* sp. 140-B. *Bacillus* sp. 140-B showed protease activity qualitatively by clear zone diameter of 15.0 mm and the highest protease activity was 35.02 Unit · mg<sup>-1</sup> protein at 14 h after incubation. Some properties of protease activities from *Bacillus* sp. 140-B including effects of temperature, pH and several metals were observed in this experiment. The protease activity from *Bacillus* sp. 140-B had optimum pH of 7,0 and the optimum temperature was 60°C. Several metals which were evaluated on protease activity showed that Mn could increase protease activity, while other metals (Ca, Co, and Hg) showed decreasing protease activity of *Bacillus* sp. 140-B.

**Keywords:** *Bacillus* sp. 140-B; enzyme activity; enzyme properties; protease.

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## 1. Introduction

Proteases are enzymes that hydrolyze protein to short peptides or amino acids. Protease execute a large variety of functions and represent the most important industrial enzymes of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale [1-3]. They have a wide range of industrial application, particularly in detergents, food, dairy, leather, weave, textile tanning, pharmaceutical, chemical and bioremediation processing industries [4-7].

Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are found in a wide diversity of source such as plants, animals and microorganisms, but they are mainly produced by microorganisms like bacteria and fungi [3]. Microorganisms serve as a preferred source

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of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [7]. Several species of bacteria especially from *Bacillus* species including *Bacillus subtilis*, *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megatterium*, *B. licheniformis*, *B. firmus*, *B. alcalophilus*, *B. amyloliquefaciens*, *B. proteolyticus* and *B. thuringiensis* were reported to produce protease [8–11].

Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium. The effect of environmental conditions of the fermentation medium play an important role on the growth and protease production from microbial. The most important among these are the medium, incubation temperature and pH [3]. The aims of this study were to produce and characterize some properties of extracellular protease from *Bacillus* sp. 140-B.

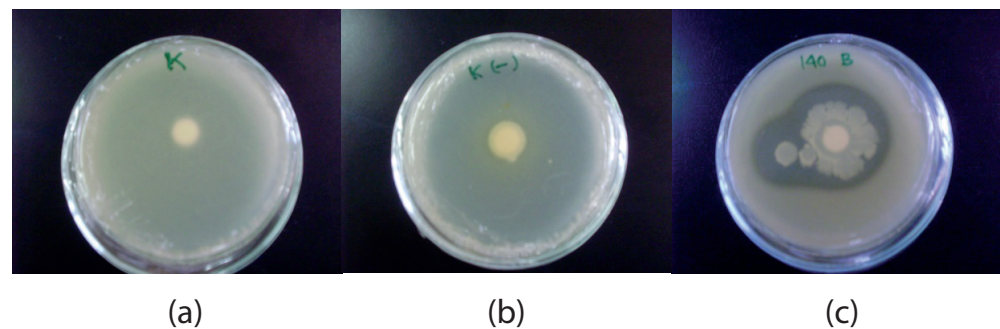
## 2. Material and Methods

### 2.1. Isolation and cultivation of bacteria for protease production

*Bacillus* sp. 140-B was isolated from soil sample from pineapple plantation (PT. GGPC), Lampung Province, Indonesia. Protease activity was determined by inoculating isolate *Bacillus* sp. 140-B onto protease medium that have been added with skim milk using disc blank method and was incubated for 24 h at 30°C. Protease activity was detected by the presence of a clear zone that formed around disc blank which was previously immerse in bacterial culture. The bacteria was cultivated in 50 mL exoprotease medium and incubated for 24 h at 30°C. Exoprotease medium contained 10 gr · L<sup>-1</sup> peptone, 5 gr · L<sup>-1</sup> yeast extract, 100 mL · L<sup>-1</sup> BS solution and 900 mL · L<sup>-1</sup> aquadest and had a pH value of 7.2. Composition of BS solution were 70 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 2 mM FeSO<sub>4</sub>, 7.5 mM Na<sub>2</sub>SO<sub>4</sub>, and 50 mM KH<sub>2</sub>PO<sub>4</sub>. Two ml of bacteria culture were taken and centrifugated at 12 000 rpm (1 rpm = 1/60 Hz) for 10 min at cool temperature of 4°C. About 1 mL of the supernatant was used for the analysis of protease enzyme activity and specific activity, effect of temperature, pH and some metals on protease activity as much as 1 mL of the supernatant.

### 2.2. Protease activity assay

Protease activity was determined using Azocasein as substrate. In brief, 250 μL Azocasein substrate in 50 μL 1 M Tris-HCl buffer pH 8 was mixed thoroughly with 1 mL diluted enzyme sample. The mixture was incubated at 40°C for 30 min. The reaction was terminated by the addition of 1 mL HClO<sub>4</sub> 7%. Subsequently, the mixture was centrifugated at 12 000 rpm for 10 min. The supernatant was separated from the pellet and added by 150 μL 10N NaOH and mixed thoroughly. Product of the reaction was measured by spectrophotometer at λ 430 nm wavelength. Protease activity enzyme (Unit · mg<sup>-1</sup>) was determined as 0.1 unit per 0.15 mL diluted enzyme sample. Specific activity was measured by comparing protease activity (Unit · mL<sup>-1</sup>) with protein content of the



**Figure 1:** Protease activity test qualitatively. (a) Control (aquadest); (b) Negative Control (Isolate PNK-10); (c). Isolate *Bacillus* sp. 140-B showed the clear zone in Protease medium.

enzyme ( $\text{mg} \cdot \text{mL}^{-1}$ ). The protein content was measured by Bradford method, with Bovine serum albumin (BSA) as standard.

### 2.3. Effect of temperature, pH and metals on protease activity

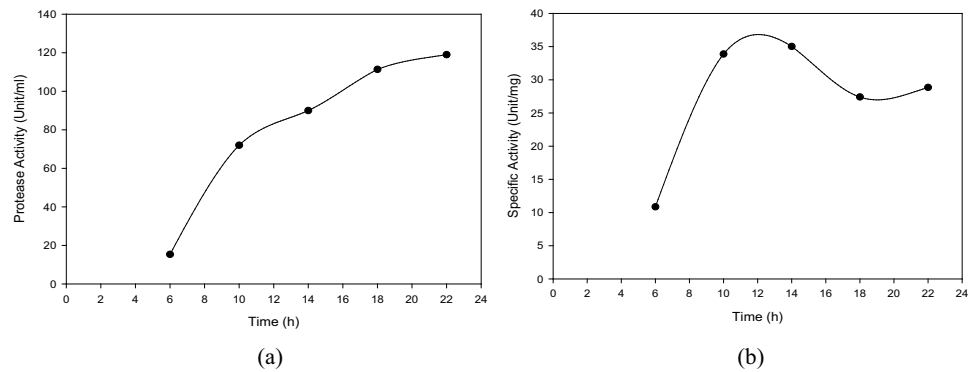
The optimum temperature on protease activity from isolate 140 B was determined in Tris-HCl buffer solution pH 8.0 at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C. The reaction was carried out at designated temperatures for 30 min. The optimum pH on protease activity from isolate 140 B was determined at 40°C in phosphate buffer (pH 6.0 to pH 7.0) and Tris\_HCl buffer (pH 8.0 to pH 10.0). The reaction was incubated for 30 min. Effect of some metals on protease activity was determined by incubating enzyme sample at 40°C in Tris-HCl buffer pH 8.0 for 30 min containing various metals such as  $\text{MnCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{HgCl}_2$  and  $\text{CoCl}_2$ .

## 3. Result and Discussion

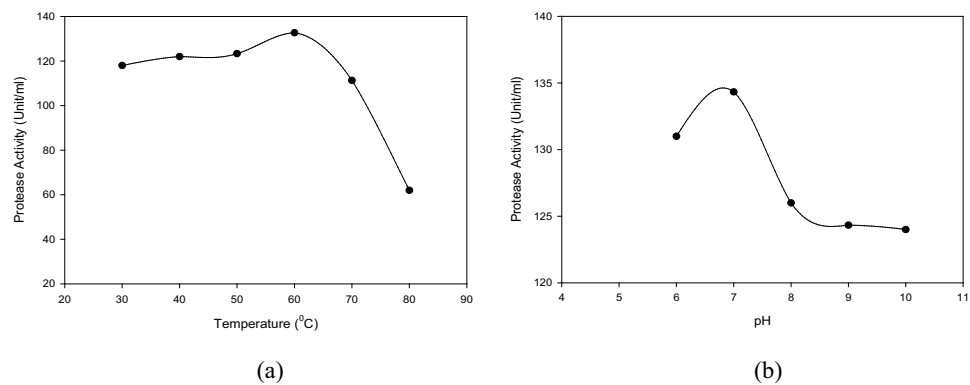
*Bacillus* sp. 140-B was inoculated in Protease medium showed a clear zone around the colony with 15.0 mm of diameter after 24 h incubation (see Figure 1). A clear zone indicated that the protein near the colony was utilized by the bacteria. A disc blank which was previously immersed in a sterile aquadest was used as control, while the negative control was a disc blank immersed in PNK-10 bacteria culture which was known to be lacking of protease activity.

Protease production of *Bacillus* sp. 140-B grown in 50 mL exoprotease medium for 24 h period increased exponentially from 6 h and showed the highest protease activity of  $119 \text{ Unit} \cdot \text{mL}^{-1}$  at 22 h cultivation (see Figure 2a). While the highest specific activity was observed at 14 h incubation which was  $35.02 \text{ Unit} \cdot \text{mg}^{-1}$  protein (see Figure 2b). Other investigators reported the maximum protease production from *Bacillus* SNR01 was obtained at 24 h, *B. licheniformis* LBLL-11 and *Bacillus* sp. HSo8 was obtained at 48 h [7, 12, 13].

The optimum temperature of protease activity was observed at 60°C (see Figure 3a). Protease activity was increased within temperature range of 30°C to 60°C, and decreased at temperature above 60°C (70°C to 80°C). The same result was found on



**Figure 2:** (a) Protease activity; (b) protease specific activity of *Bacillus* 140-B.



**Figure 3:** (a) The effect of temperature; (b) the effect of pH on protease activity from isolate *Bacillus* sp. 140-B.

earlier research, which showed that *B. licheniformis* LBL-11 also had optimum temperature at 60°C [12, 13]. Other investigations reported that *B. aquimaris* VITP4 and *Bacillus* SNR01 had optimum temperature at 40°C, *Bacillus* sp. HSO8 at 70°C, and *Bacillus* sp. N-40 at 55°C [7, 13–15].

The optimum pH for protease activity from *Bacillus* sp. 140-B was determined by varying pH from pH 6.0 to pH 10.0 at 40°C. The optimum pH was observed at pH 7.0 (see Figure 3b). The protease activity was very low in alkaline conditions. It was also noted that the enzyme is neutral protease. In acidic condition (pH 6.0), protease activity from *Bacillus* sp. 140-B is higher than in alkaline condition. The same results showed on isolate *Bacillus* sp. N-40 and *Bacillus* SNR01 which had the highest protease activity with optimum pH of 7.0 [7, 15]. Other studies showed the protease activity from, *B. licheniformis* LBL-11, *B. aquimaris* VITP4 and *Bacillus* sp. HSO8 had an optimum pH of 8.0 [12–14].

The influence of metal ions are among many external factors that affect the catalytic activity and stability of the enzymes. They are known to play a role as cofactors for enzyme activities, and often act as salt or ion bridges between two adjacent amino acid residues. Cations are known to increase the thermal stability of protease and play a vital role in maintaining the active conformation of the enzyme. In the present study, protease activity was increased in the presence of  $Mn^{2+}$ , but the activity was reduced

Metal	Protease activity (Unit · mL <sup>-1</sup> )	Percentage increase* or decrease
Control	14.83	—
Mn <sup>2+</sup>	15.47	4.27*
Ca <sup>2+</sup>	14.43	2.7
Co <sup>2+</sup>	12.97	12.58
Hg <sup>2+</sup>	11.6	21.8

TABLE 1: The effect of metals on protease activity from isolate *Bacillus* sp. 140-B.

by the presence of Ca<sup>2+</sup>, Co<sup>2+</sup>, and Hg<sup>2+</sup>. Protease activity from isolate *Bacillus* sp. 140-B was enhanced to 4.27% after being incubated with Mn. While protease activity was decreased to 2.7, 12.58 and 21.8 after being incubated with Ca, Co and Hg (Table 1). Sevinc & Demirkan [15] reported the protease activity from *Bacillus* sp. N-40 was enhanced by Mn<sup>2+</sup> and Ca<sup>2+</sup>, and was reduced by Ba<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup>. Other investigator reported the protease activity from *B. aquimaris* VITP4 increased in the presence of Cu<sup>2+</sup>, Ni<sup>2+</sup>, and Mn<sup>2+</sup>, and was inhibited by Zn<sup>2+</sup>, Hg<sup>2+</sup>, and Fe<sup>2+</sup> [14].

## 4. Conclusion

*Bacillus* sp. 140-B have ability to produce extracellular protease, which is the highest activity was 119 Unit · mL<sup>-1</sup> (22 h incubation) and the highest specific activity was 35.02 Unit · mg<sup>-1</sup> protein after 14 h incubation. Characterization of extracellular protease from *Bacillus* sp. 140-B included the effects of temperature, pH and several metals on protease activity. The optimum temperature of protease activity was found at 60°C and the optimum pH was found at pH 7.0. We concluded that the type of protease is neutral protease. The protease activity was enhanced by Mn, and was decreased by Ca, Co and Hg.

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