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Cover Page Footnote

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Characterization of Protease from *Bacillus licheniformis* F11.1 as a Bio-Detergent Agent

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Abstract

Proteases are among the most important enzymes in both food and non-food industries taking up almost 60% of the world enzyme market. This enzyme has been used for many industrial processes, especially in the detergent industry. The purpose of this study was to characterize the protease from *Bacillus licheniformis* F11.1 as a bio-detergent agent. An enzyme assay of protease activity was used to assess and characterize the protease enzyme from *B. licheniformis* F11.1. It showed that the highest pH protease activity for alkaline protease occurred at pH 8.0 with a value of 35.00 U/mL. Under incubation temperature, the protease had the highest activity at 50 °C with a value of 24.46 U/mL. Protease activity was inhibited by Ca²⁺, Mn²⁺, K⁺, and Na⁺ ions at concentrations of 5 mM. Protease activity can be enhanced by these ions at concentrations of 2 mM. Protease stability can be measured from half-life. Under an incubation temperature of 50 °C, the half- life of the protease at pH 8, 9, and 10 was 108 min, 114 min, and 98 min, respectively. The assay for enzyme stability with an incubation temperature of 60 °C showed half-lives of 92 minutes, 56 minutes, and 61 minutes for pH 6, 9, and 10, respectively. This enzyme was found to be stable with the addition of detergent compounds such as sodium dodecyl sulfate (SDS), Triton X-100, ethylenediaminetetraacetic acid (EDTA), and hydrogen peroxide; all under low concentrations. Determination of the molecular weight using SDS- PAGE and zymogram found the molecular weight was 32.90-35.16 kDa. These results showed that the alkaline protease from *B. licheniformis* F11.1 can be used as a bio-detergent because of its tolerance to various detergent compounds.

Abstrak

Karakterisasi Protease dari *Bacillus licheniformis* F11.1 sebagai Agen Biodeterjen. Protease merupakan salah satu kelompok enzim industri yang paling penting untuk industri pangan dan non-pangan karena menguasai sekitar 60% dari total penjualan enzim di dunia. Enzim tersebut telah digunakan untuk proses industri, khususnya industri deterjen. Tujuan penelitian ini adalah untuk melakukan karakterisasi protease *Bacillus licheniformis* F11.1 sebagai kandidat biodeterjen. Pengujian aktivitas protease untuk menentukan ciri-ciri enzim protease dari *B. licheniformis* F11.1 menunjukkan bahwa pH tertinggi aktivitas protease pada kisaran pH basa, yaitu pH 8,0 dengan aktivitas sebesar 35.00 U/mL. Pengaruh suhu inkubasi memberikan informasi bahwa protease yang dihasilkan memiliki aktivitas tertinggi pada suhu 50 °C dengan nilai sebesar 24.46 U/mL. Aktivitas protease dihambat oleh penambahan ion Ca²⁺, Mn²⁺, Na⁺, dan K⁺ dengan konsentrasi 5mM. Konsentrasi ion Ca²⁺, Mn²⁺, Na⁺, dan K⁺ sebesar 2 mM dapat meningkatkan aktivitas protease. Stabilitas protease dapat diketahui melalui waktu paruh yang dihasilkan. Waktu paruh protease *B. licheniformis* F11.1 pada kondisi inkubasi 50 °C dan pH 8,9, dan 10 berturut-turut ialah 108 menit, 114 menit, dan 98 menit. Pengujian stabilitas enzim dengan suhu 60 °C menunjukkan waktu paruh berturut-turut ialah 92 menit, 56 menit, dan 61 menit untuk masing-masing pH 8,9, dan 10. Enzim tersebut ditemukan stabil dengan penambahan komponen deterjen stabil seperti sodium dodesil sulfat (SDS), Triton X-100, etilediamina asam tetraasetat (EDTA) dan hidrogen peroksida pada konsentrasi rendah. Melalui penentuan bobot molekul dengan menggunakan SDS-PAGE dan zimogram didapatkan bobot molekul berkisar antara 32.90-35.16 kDa. Karakterisasi enzim yang didapatkan menunjukkan bahwa alkalin protease dari *B. licheniformis* F11.1 dapat digunakan sebagai biodeterjen karena memiliki ketahanan terhadap komponen deterjen.

Keywords: protease alkalin, stabilitas, zimogram

Introduction

Detergents are one of the most important industrial products used in daily life, especially for household and industrial purposes. Detergents used in cleaning stains on clothes can be in liquid, paste, or powder form. These contain active ingredients such as surfactants which help increase the repelling force between dirt and stains. Powder detergents contain active ingredients on its surface and other additives. The use of powder detergents in Indonesia has been increasing rapidly in the 1990s along with an increasing population and the development of textile industries. The use of powder detergents in Indonesia has increased significantly by around 10% per year [1].

Detergents with enzymes addition are widely used today for various types of liquid, paste, and powder detergents; as well as soaps. For synthetic detergents, the synergistic action between various components in providing a balanced activity related to the desired properties is emphasized in the development of detergent formulations. Enzyme-based detergents that are effective in removing stains are known as green chemicals. Formulations with enzymes added in the detergent can be potentially applied to the laundry and textile industries [2].

Protease is one of the enzymes that hydrolyze the peptide bonds in proteins into oligopeptides and amino acids. Protease consists of serine proteases, cysteine or thiols, aspartates, and metals. Protease is an enzyme that is widely used in the detergent industry as a bio-detergent agent. Currently, the most popular proteases used in detergents are serine from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, as well as strong alkaline enzymes of *Bacillus* species, such as *B. lentus* [3].

B. licheniformis F11.1 has a relatively high proteolytic activity compared to the wild type. This bacterium strain was originally isolated from shrimp leather waste in Palembang, South Sumatra. This study is a part of collaboration between the Bio-industry Laboratory, Agency for Assessment and Application of Technology (BPPT) and the University of Hamburg, Germany within the Indonesia-Germany Biotechnology (IG Biotech) scheme in phase 3 [4]. This study aimed to assess the suitability of the protease enzyme from *B. licheniformis* F11.1 for use in bio-detergents.

Methods

The preparation of the starter and inoculum was based on the modified procedure done previously by Junianto [5]. The starter culture was prepared by taking one ose bacterium *B. licheniformis* F11.1 from an existing culture stock inoculated into a 250 mL Erlenmeyer flask containing 50 mL of Luria Bertani (LB) liquid medium. The LB media consisted of 1% (w/v) peptone, 0.5%

(w/v) yeast extract, 0.5% (w/v) NaCl in distilled water at pH 7.3. Furthermore, incubation was done at 55 °C on a reciprocal shaker at 180 rpm (Kuhner, Switzerland).

The production of enzymes was induced in the bacteria using methods based on Junianto [5]. In order to produce proteases, fermentation was carried out in a 1 L Erlenmeyer flask. The starter culture had an optical density (OD at 620 nm) of 0.8. The OD value is gauges the density of cells read on the spectrophotometer, thus higher values corresponds to higher number of bacteria. 20 mL of the starter culture was then added into an Erlenmeyer containing 180 mL of fermentation medium. The fermentation medium consisted of 1 g/mL peptone, 0.5 g/mL yeast extract, NaCl 0.5 g/mL, and the protein casein 1 g/mL. The crude extract enzyme (EEK) was obtained via a batch fermentation process incubated over 16 h to observe its enzymatic activity. The incubation temperature used in the fermentation was 55 °C with an initial pH of 7.3.

A unit of enzyme activity is the number of enzymes that liberate amino acids and peptides. In this case, the value corresponds to the number of enzymes liberating 1 µg of tyrosine per minute (from casein) under test conditions (U/mL). Measurement of protease enzyme activity was based on the method used by Amano [6]. A sample containing crude extract enzyme was centrifuged at 4 °C at a rate of 11.000 x g for 5 min. This measurement was performed by observing the absorbance values (at 660 nm) for the EEK samples, control solutions, standard solutions, and blanks.

Protein content was determined using the method of Bradford [7]. Bovine Serum Albumin (BSA) Fraction V was used as a protein for preparing the standard solutions. 1 mg/mL BSA was dissolved into 0.05 M phosphate buffer at pH 7.5. It was then diluted into a series of concentrations (i.e., 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/mL). Then a series of standard solutions were made using the BSA stock solution. 30 µL of each sample solution was prepared in each tube with the addition of 1500 µL of Bradford's reagent. After around 20 min, each reaction mixture was measured for absorbance (at 595 nm) in a spectrophotometer.

The measurement of optimum pH for protease enzymes was performed using the modified method used in Amano [6] using the crude extract enzyme in the fermentation medium. Measurement of enzyme activity was carried out at pH 7, 8, 9, 10, and 11. K-phosphate buffer was used for pH 7, Tris-HCl buffer was used for pH 8 and 9, and Glycine-NaOH buffer was used for pH 10 and 11. 625 µL casein hydrolysate and around 125 µL crude extract enzyme was added to enzyme samples in the microtube. Incubation was carried out at 55 °C for 10 min. Then, around 625 µL of 10% (w/v) TCA solution was added followed by incubation for 15 min. The

supernatant was recovered via centrifugation at 11,000 xg for 5 min. About 300 μL of the supernatant was treated with 750 μL of Na_2CO_3 and 150 μL of folin. Incubation was carried out at 55 $^\circ\text{C}$ for 15 min at the same agitation rate. The enzyme activity was measured using a spectrophotometer (at 660 nm).

Determination of the optimum temperature was carried out via incubation at varying temperatures (i.e., 40 $^\circ\text{C}$, 50 $^\circ\text{C}$, 60 $^\circ\text{C}$, 70 $^\circ\text{C}$, 80 $^\circ\text{C}$, and 90 $^\circ\text{C}$). Casein hydrolysate substrate of 625 μL (pH 8) was added to 125 μL of the enzyme, and was incubated for 10 min with varying temperature. 625 μL TCA solutions was reacted with the mixture and subsequently re-incubated for 15 min under a temperature range from 40 $^\circ\text{C}$ to 90 $^\circ\text{C}$. Supernatant was obtained through centrifugation for 5 min at 11,000 x g. 300 μL of the supernatant was treated with 750 μL of Na_2CO_3 . 150 μL of foline reagents were added and incubated at varying temperatures for 15 min. Proteolytic activity measurements were performed using a spectrophotometer (at 660 nm). The protease activity was determined via the modified method used in Amano [6].

The effect of metal ions on enzyme activity was carried out using a modified method based on Baehaki *et al.* [8]. Ca^{2+} , Mn^{2+} , K^+ , and Na^+ metal ions in salt form from CaCl_2 , MnCl_2 , KCl , and NaCl , respectively, were tested. Determination of each ion as an activator or inhibitor to protease activity was carried out by combining the enzyme solution with casein hydrolysate substrate 0.6% (w/v). Metal ions were added at concentrations of 2 mM and 5 mM. A blank control treatment with no addition of metal ions was used. Enzyme activity was measured using the modified method of Amano [6].

The stability of the enzyme at various pH levels was tested under three pH substrates (i.e., 8, 9, and 10). Determination of the stability of this enzyme was carried out by pre-incubating the enzyme at 50 $^\circ\text{C}$ for 2 h at intervals of 15 min as the value of residual activity. The protease activity was then measured every 15 min. This protease activity was determined using the modified method. The procedure for protease stability was carried out with incubation conditions at 60 $^\circ\text{C}$ while the pH used was pH 8, 9, and 10. Determination of this protease stability was performed by preincubation for 2 h in interval of 15 min for testing protease activity. The modified Amano [6] method was used to test the protease activity.

The effect several surfactants and oxidizing agents commonly present in detergents on protease enzymes was tested using methods based on Nascimento and Martins [9]. Incubation of enzymes was done using Triton X-100 (1%–5% v/v), sodium dodecyl sulfate (SDS) (0.1%–0.5%), hydrogen peroxide (2.5% and 5.0%) and EDTA (1, 2, 5, and 10 mM) at 60 $^\circ\text{C}$, pH 9 after 0, 15, and 30 min. A control treatment without the added detergent components was included.

Determination of molecular weight was performed using SDS-PAGE [10] and zymogram [11] was has been modified. For the measurement via SDS-PAGE, staining was done using the PAGE Blue method and the marker used was Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). A 12% separating gel and 4% stacking gels were used in the polyacrylamide gel. The gel was poured in a mold, and the gel was mounted by a perforated comb, which was left until it hardened. Electrophoresis was carried out by pouring 1 \times running buffer and adding 12 μL of sample with 3 μL of 5 \times sample buffer. The sample solution was boiled in boiling water for 10 min, and then inserted into the well in the gel. The installed electrode used for electrophoresis process had a voltage of 200 V, 15 mA, run for 60 minutes on a Bio-Rad electrophoresis machine. Finally, staining using a protein staining solution was used to determine which bands were formed. The appearance of blue bands ended the electrophoresis process. The zymogram procedure is carried out using acrylamide gel (12%) copolymerized with casein substrate (1%) under gel electrophoresis. The gel was soaked with Triton X-100 (2%) for 20 min and incubated in 50 mM Tris-HCl buffer, pH 8 for 20 min with 50 $^\circ\text{C}$ temperature. The gel was immersed in 10% TCA for 15 min. Then, it was stained for 30–60 min. The enzyme hydrolysis bands in the casein substrate appear as white patches after soaking with the de-staining protein. Determination of enzyme specificity was carried out by adding 50 mM Tris-HCl buffer with various detergent components (0.1% SDS, 2.5% H_2O_2 , and 2 mM EDTA). The electrophoresis gel was immersed in 2% Triton X-100 (0.2 mL of 2% Triton X-100 and 3.8 mL dH_2O) for 20 min. The gel was incubated at room temperature for 20 minutes. Triton X-100 contained in the gel was replaced with 50 mM Tris-HCl buffer with a mixture of detergent components for 15 min at 50 $^\circ\text{C}$. Tris-HCl buffer was removed and replaced with 10% TCA solution, and then the gel was re-incubated for 15 minutes at room temperature. The TCA solution was then removed, and the gel was stained with protein staining solution for 30–60 minutes. Enzyme protein degradation can be observed by gel immersion with de-staining protein.

Results and Discussion

The protease enzyme produced by *B. licheniformis* F11.1 is an extracellular enzyme. The production of this enzyme was done by incubating *B. licheniformis* F11.1 for 16 h. Activity measurements were performed after 0,

Table 1. Optical Density (OD) and Protease Activity for Each 2 Hour Period

Time (hour)	OD 620 nm	Activity (U/mL)
0	0.079	0.38
12	1.826	1.86
14	1.636	2.81
16	1.617	2.72

12 and 16 hours with a two-hour interval of protease activity testing. Table 1 shows the value of protease activity after 0 to 16 hours. OD values increased over time from 0 to 12 hours. After which, OD values decreased from the 12 h to 16 h. The protease activity after 16 hours was 2.72 U/mL. The protease enzyme was to be harvested after 16 hours in order to obtain the enzyme under high activity. However, after 16 hours the enzyme was in the stationary phase.

Junianto [5] found that the bacteria *B. licheniformis* F11.1 grown in fermentation medium undergo an exponential phase between 0 to 12 hours. The stationary phase of the bacteria was reached after 12 hours to 24 hours. The death phase occurs after 24 hours until the end of the fermentation process. The adaptation phase was not visible because this phase occurs very quickly (i.e., less than 12 hours). The protease enzyme can generally be produced in high numbers by *B. licheniformis* F11 bacteria in the stationary growth phase. The amount of enzyme produced depends on the number of bacteria in the stationary phase. Much of the scientific literature explains that protease production occurs at the end of the log phase and the beginning of the stationary phase. Some researchers also confirm that extracellular enzymes can be produced early in the stationary phase. The enzyme was harvested and had produced a protease activity of 4.62 U/mL. The protease was concentrated 6 times using PEG 2000 to be used in enzyme characterization testing.

The protease activity was measured at 55 °C. Figure 1 shows the effect of pH on protease activity *B. licheniformis* F11.1. The results showed protease enzyme activity is the highest at pH 8 (35.00 U/mL). At this pH level, the enzyme was conducive to bind the substrate so that the resulting protease activity is higher. The stability of the three-dimensional structure of the enzyme was also maintained. The pH conditions above or below pH 8 caused the enzyme activity to decrease due to the denaturation of enzyme proteins. The speed of catalyzing by an enzyme is determined by the environmental pH because the concentration of hydrogen ions can affect the three-dimensional structure of the enzyme. Each enzyme has an optimum pH that causes the enzyme to bind the substrate effectively. If the concentration of hydrogen ions changes from the optimal concentration, the enzyme may not be functional and will affect the activity [3]. At pH 7, the activity of protease *B. licheniformis* F11.1 was at 28.93 U/mL. The protease activity decreased to 29.11 U/mL at pH 9. At pH 10 and 11, protease activity further decreased with activity values of 26.61 U/mL and 11.79 U/mL, respectively (Figure 1).

Environmental pH may affect enzymatic reactions. Environmental pH is required in catalyzing a reaction. Alkaline proteins have highest activities under alkaline

conditions. The acidic degree of protease produced by various species of *Bacillus* varies from pH 7–9 [12]. Protease activity can also be affected by other physical parameters such as temperature. Temperature must be high enough to allow the enzyme activity to reach the optimum rate.

In general, most commercially available alkaline proteases have optimum activities ranging from 50 °C–70 °C. The decrease in protease activity of *B. licheniformis* F11.1 at high temperatures (60 °C–90 °C) is due to the enzyme being damaged through alteration of its three-dimensional structure (Figure 2).

This damage negatively affects the enzyme's ability to bind the substrate. Increasing temperature causes the increase of substrate molecule energy to increase the rate of enzyme reactions. Under elevated temperatures, the substrate conformation changes so that the substrate active site experiences obstacles when entering the enzyme's active site, which causes the decrease in enzyme activity [13].

Metal ions are required by enzymes in the form of monovalent and divalent cations that act as activators or inhibitors. The protease activity may be affected by the addition of metal ions. At the lower ion concentration of 2 mM, the enzyme produced higher activity than the control. This demonstrates that at low levels (e.g., 2 mM) the ions act as activators. With the addition of the Ca^{2+} ion, the enzyme activity increased to a value of 25.09 U/mL, whereas the control treatment had a value of 13.23 U/mL. The protease activity also increased with the addition of Na^+ , K^+ , and Mn^{2+} ions (Figure 3). Protease activity of *Bacillus* sp. can be stimulated with the addition of Mn^{2+} and Ca^{2+} ions. These results showed that

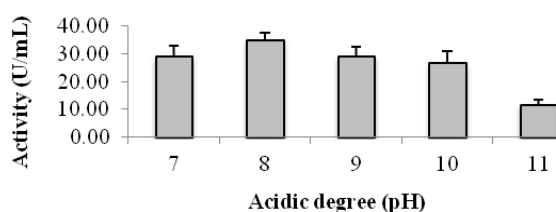


Figure 1. Effect of Different pH on Protease Activity *B. licheniformis* F11.1 Measured at 55 °C

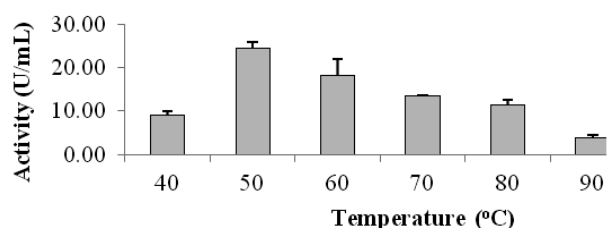


Figure 2. The Effects of Temperature on Protease Activity of *B. licheniformis* F11.1 at pH 8

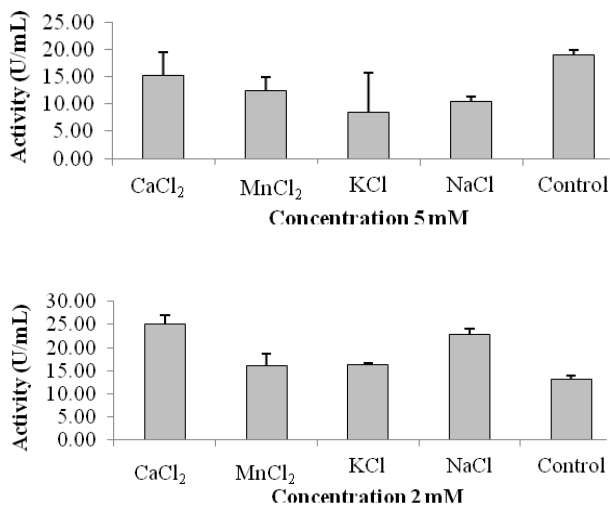


Figure 3. Influence of Metal Ions on Protease Activity of *B. licheniformis* F11.1 at pH 8 and Temperature 50 °C

metal ions can protect the protease enzyme against temperature denaturation and play an important role in maintaining the active site conformation of the enzyme under higher temperatures [14]. Cations such as Ca²⁺, Mg²⁺, Mn²⁺, and Cu²⁺ generally increase protease activity whereas Hg²⁺ and Zn²⁺ may inhibit enzyme activity. However, the presence of Na⁺, K⁺, Al³⁺, and Cd²⁺ are often found to be appropriate for enzymes added in detergents. The protease enzyme from *B. licheniformis* RP1 was enhanced by the addition of 2 mM Ca²⁺ ions [15].

At a concentration of 5 mM, metal ion caused inhibition of enzyme activity, where the enzyme activity was observed to be lower than control (Figure 3). Most of the metal ions can inhibit protease activity at higher concentrations, which is related to ionic strength. This is because ionic strength can influence the site conformation or the three-dimensional structure of the enzyme proteins or substrate proteins [8].

The stability of the enzyme is influenced by environmental factors. Appropriate environmental conditions can improve the stability of the enzyme. In order to determine the stability of an enzyme, it is necessary to determine the half-life of an enzyme. The half-life of the enzyme was reached at 127 min under pH 8 at 50 °C. These results indicate that the enzyme incubated at pH 8 lost 50% of its activity after 108 min. The half-life of the enzyme was 114 min at pH 9, and was 98 min at pH 10 (Figure 4). This indicates that the resulting enzyme is able to remain stable for more than 1 hour. The stability of protease can be determined by the pre-incubation of enzymes in different buffers with different pH levels. During the 2 hour pre-incubation period, the enzyme was stable at pH 8–11. At 50 °C, the half-life of the enzyme Subtilisin Carlsberg and Subtilisin BPN were

3.4 min and 2.4 min, respectively [16]. This indicates that the proteases of *B. licheniformis* F11.1 have higher stability. The protease enzyme was found to be stable over a wide range of pH (6.5 to 12); and higher stability was observed at pH range of 7.5–8.5 [17].

The stability of the enzyme under varying pH and temperature is useful in determining the nature and the properties of the enzyme, in which can help determine the suitability of the enzyme for use in bio-detergents. The stability of enzymes incubated at 60 °C under pH 8, 9, and 10 demonstrated enzyme half-lives of 92, 56, and 61 min, respectively (Figure 5). These results show that the half-life of enzymes can be achieved when the enzyme was incubated for more than 1 hour, even up to 2 hours under incubation. Previous reports of thermostable proteases showed half-lives of 2–22 min at 60 °C for serine proteases [16]. The results show that the tested proteases have higher stability, and thus have greater potential for use in the laundry detergent industry.

The protease enzyme to be incorporated into the detergent formula must have characteristics that are active and stable under alkaline pH conditions, and have good activity and stability at relatively high temperatures (40 °C–50 °C or more). The protease enzyme produced by *B. licheniformis* F11.1 is stable under basic pH conditions and washing temperatures, thus it is suitable as a bio-detergent agent. The addition of EDTA as a detergent component may affect protease activity. Higher EDTA concentrations can inhibit the protease

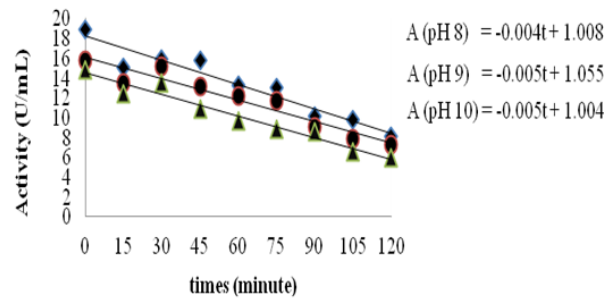


Figure 4. Enzyme Stability at 50 °C Incubation Temperature; Incubation pH 8 (♦), pH 9 (●), pH 10 (▲)

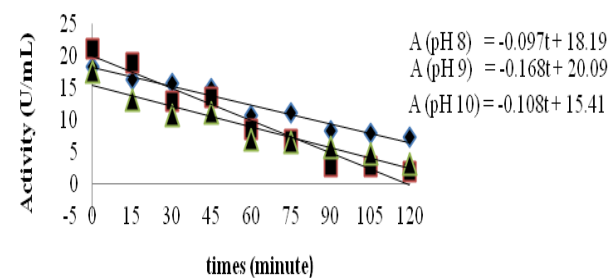


Figure 5. Enzyme Stability at 60 °C Incubation Temperature; Incubation pH 8 (♦), pH 9 (■), pH 10 (▲)

activity of *B. licheniformis* F11.1. This was observed from the decrease in relative activity with increasing EDTA concentration (Table 2). The same was also observed with the addition of Triton X-100, SDS, and H₂O₂. Metallic protease can be inhibited by 40% by EDTA addition when compared to the control treatment [18]. In addition, inhibition of the enzyme by EDTA will increase along with its concentration level [19]. The addition of SDS and H₂O₂ led to a dramatic decrease in protease activity. Some detergent enzymes are partially inhibited by SDS. Increased concentrations of SDS may decrease protease activity because it is a strong surfactant,

which is a moderate inhibitor. The addition of Triton X-100 above 2% causes a decrease in protease activity. In comparison, the protease activity of *B. licheniformis* KBDL4 decreased by 7 and 14% with the addition of 1% and 5% Triton X-100, respectively. Generally, the detergent enzyme is stable in the presence of oxidizing agent and bleach. The protease enzyme has resistance to bleaching and oxidizing agents such as H₂O₂ during washing. At 2.5 % H₂O₂ with incubation times of 0 min, 15 min, and 30 min; the yield relative activities were 115%, 90%, and 57%, respectively. The H₂O₂ concentration did not significantly affect the protease activity

Table 2. Influence of Various Surfactants on Protease Activity

Surfactant	Relative Activity (%)		
	t (minute)		
	0	15	30
Control	100	100	100
EDTA 1 Mm	114	82	97
EDTA 2 Mm	91	80	67
EDTA 5 Mm	84	67	67
EDTA 10 Mm	72	49	45
Triton X-100 1%	113	147	151
Triton X-100 2%	142	167	145
Triton X-100 3%	123	91	70
Triton X-100 4%	92	58	52
Triton X-100 5%	49	43	24
SDS 0.1%	83	22	9
SDS 0.2%	57	69	18
SDS 0.3%	54	15	8
SDS 0.4%	35	8	16
SDS 0.5%	32	44	26
H ₂ O ₂ 2.5%	115	90	57
H ₂ O ₂ 5%	47	26	24

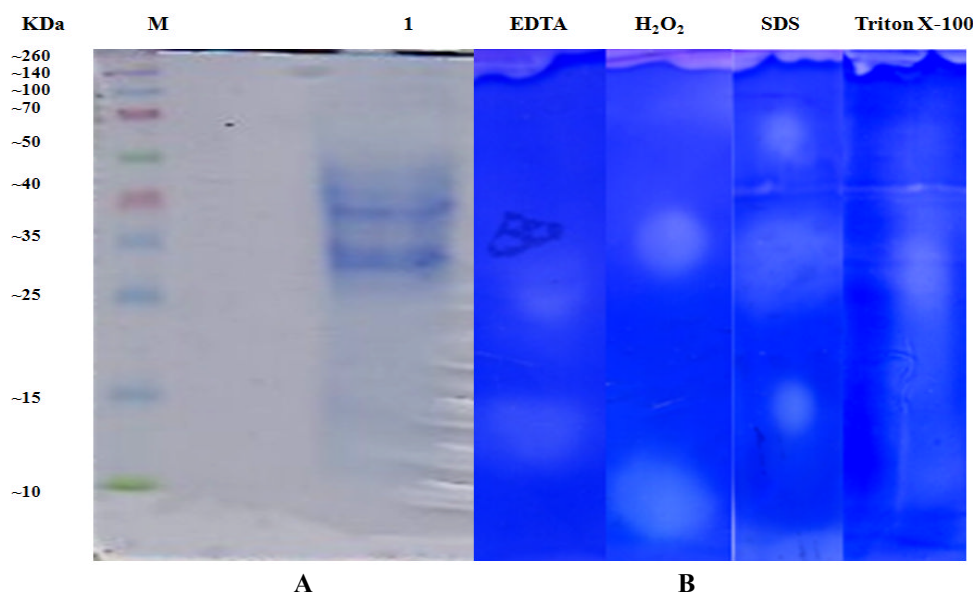


Figure 6. SDS-PAGE and Zymogram
A. SDS-PAGE; B. Zymogram; M = Marker, 1 = Enzim protease *B. licheniformis* F11.1

of *B. licheniformis* F11.1. The addition of H₂O₂ produced a relative activity of 93.54%, which indicates that the proteases of *B. licheniformis* NCIM-2042 are stable binding and bleaching enzymes, though hydrogen peroxide is a strong oxidizing agent and usually inactivates the protein via oxidation [20]. Protease activities of 95% and 86% were observed for 2.5% and 5% H₂O₂, respectively (at 60 °C for 15 min). An increase in H₂O₂ concentrations of 1% and 5% resulted in a 36% and 38% decrease in activity of *B. licheniformis* KBDL4, respectively [15].

The molecular weight of an enzyme protein can be determined using SDS-PAGE and zymogram. The result from the SDS-PAGE test showed that there were 5 bands formed following electrophoresis. The resulting bands were compared to the result from the zymogram method, which showed a degradation band of 32.90 kDa, 34.70 kDa, and 35.16 kDa. The degraded band is capable of showing the specificity of the target enzyme activity, which has the ability to hydrolyze casein substrate. It is also resistant to the addition of detergent components (Figure 6). The appearance of three bands of different sizes was due to the use of crude extract enzymes and not purified enzymes. However, each extract enzyme produced molecular weights of 30 kDa. The alkaline protease has a molecular weight of ~30 kDa and undergoes a folding of its structure characterized by the presence of α -helical, β -sheet folds, and the outer helical folds that form like folds of subtilisin [21]. Previous research on the alkaline protease enzyme from *Bacillus* produced different molecular weights, namely *B. licheniformis* RSP-09-37 (55 kDa), *Bacillus* sp. SM2014 (71 kDa), *B. cereus* MCM B-326 (45 kDa), *B. subtilis* DM-04 (16.9 kDa), *B. licheniformis* (32 kDa), and *Bacillus* sp. RKY3 (38 kDa) [22].

Conclusions

Protease produced by *Bacillus licheniformis* F11.1 has optimum enzyme activity under 50 °C and pH 8. This enzyme was activated by the presence of 2 mM Ca²⁺, Mn²⁺, K⁺, and Na⁺ with a concentration of 2 mM, respectively. At a temperature of 50 °C, the half-life of the protease was 108 minutes, 114 minutes and 98 minutes at pH 8, 9 and 10, respectively; and at 60 °C, the half-life was 92 minutes, 56 minutes, and 61 minutes, respectively. The enzyme had no resistance to the addition of 10 mM ethylene diamine tetraacetic acid (EDTA), but was resistant to 1% and 2% Triton X-100. The enzyme activity was inhibited by the addition of 0.1%–0.5% SDS. 5% H₂O₂ also inhibited protease activity. The following measurements were observed in measuring molecular weight: 32.90 kDa, 34.70 kDa, and 35.16 kDa. The protease enzymes generated by *B. licheniformis* F11.1 provides information about the optimal conditions in order to produce the best protease activity for use as candidates for bio-detergents.

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References

- [1] Rahman, A.A., Lelono, G.S. 2013. Pemanfaatan minyak goreng bekas menjadi deterjen alami melalui kombinasi reaksi trans-esterifikasi dan sulfonasi. *J. Teknol. Kim. Indust.* 2(2):84-90, <http://ejournal3.undip.ac.id/index.php/jtki/artikel/view/2612/2604>. (in Indonesian).
- [2] Keshwani, A., Malhotra, B., Kharkwal, H. 2015. Natural polymer based detergents for stain removal. *World. J. Pharm. Phar. Sci.* 4(4):490-508. <http://wjpps.com/download/article/1427864924.pdf>.
- [3] Pakpahan, R, M.Si. Tesis, Program Studi Biologi, Fakultas Matematika dan Ilmu Pengetahuan Alam. 2009. Isolasi bakteri dan uji aktivitas protease termofilik dari sumber air panas Sipoholon Tapanuli Utara, Sumatera Utara, Universitas Sumatera Utara, Indonesia. (in Indonesian).
- [4] Hoffmann, K., Daum, G., Koster, M., Kulicke, W.M., Rammes, H.M., Bisping, B., Meinhardt, F. 2010. Genetic improvement of *Bacillus licheniformis* strains for efficient deproteinization of shrimp shell and production of high-molecular-mass chitin and chitosan. *Appl. Environ. Microbiol.* 76(24): 8211-8221, <http://doi:10.1128/AEM.01404-10>.
- [5] Junianto, Dr. Disertasi, Program Studi Teknologi Industri Pertanian, Fakultas Teknologi Pertanian. 2008. Perancangan proses dan peningkatan skala ekstraksi kitin dari kulit udang secara biologis, Institut Pertanian Bogor, Indonesia. (in Indonesian).
- [6] Amano, M. 2007. Amano Enzyme. Nagoya, Jepang: Amano Enzyme Inc. pp 77-78.
- [7] Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* 72(1-2):248-254, <http://pdfs.semantic-scholar.org>.
- [8] Baehaki, A., Rinto, Budiman, A. 2011. Isolasi dan karakterisasi protease dari bakteri tanah rawa Indralaya, Sumatera Selatan. *J. Teknol. Indust. Pangan.* 22(1):37-42, <http://journal.ipb.ac.id>. (in Indonesian).
- [9] do Nascimento, W.C.A., Martins, M.L. 2006. Studies on the stability of protease from *Bacillus* sp. and its compatibility with commercial detergent. *Braz. J. Microbiol.* 37(3):307-311, <http://scielo.br/pdf/bjm/v37n3/v37n3a20.pdf>.

- [10] Laemmli, U.K. 1970. Clave of structural proteins during the assembly of the head of bacteriophage T4. *Nature. Intern. J. Sci.* 227:680-685, <http://doi:10.1038/227680a0>.
- [11] Choi, N.S., Yoon, K.S., Lee, J.Y., Han, K.Y., Kim, S.H. 2001. Comparison of three substrates (casein, fibrin, and gelatin) in zymographic gel. *J. Biochem. Mol. Biol.* 34(6):531-536, <http://researchgate.net/publication/299064461>.
- [12] Kasana, R.C., Salwan, R., Yadav, S.K. 2011. Microbial proteases: detection, production, and genetic improvement. *Rev. Microbiol.* 37(3):262-276, <http://doi:10.3109/1040841x.577029>.
- [13] Tekin, N., Cihan, A.C., Takac, Z.S., Tuzun, C.Y. 2012. Alkaline protease production of *Bacillus cohnii* APT5. *Turk. J. Biol.* 36:430-440, <http://doi:10.3906/biy-1104-6>.
- [14] do Nascimento, W.C.A., Martins, M.L.L. 2004. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Braz. J. Microbiol.* 35(1-2):91-96, <http://dx.doi.org/10.1590/S1517-83822004000100015>.
- [15] Niyonzima, F.N., More, S. 2014. Detergent-compatible proteases: microbial production, properties, and stain removal analysis. *Prepar. Biochem. Biotechnol.* 45(3):233-258, <http://doi:10.1080/10826068.2014.907183>.
- [16] Adinarayana, K., Ellaiah, P., Prasad, D.S. 2003. Purification and partial characterization of thermostable serin alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS Pharm. Sci. Technol.* 4(4):1-9, <http://doi:10.1208/pt040456>.
- [17] Sarker, P.K., Talukdar, S.A., Deb, P., Sayem, S.M.A., Mohsina, K. 2013. Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis* P003. *Springer. Open. J.* 2(506):1-11, <http://doi:10.1186/2193-1801-2-506>.
- [18] Maruthiah, T., Esakkiraj, P., Prabakaran, G. 2013. Purification and characterization of moderately halophilic alkaline serin protease from marine *Bacillus subtilis* AP-MSU 6. *Elsevier. Sci.* 2(2):116-119, <http://doi:10.1016/j.bcab.2013.03.001>.
- [19] Singh, S.K., Singh, S.K., Tripathi, V.R., Garg, S.K. 2011. An oxidant, detergent and salt stable alkaline protease from *Bacillus cereus* SIU1. *African. J. Biotechnol.* 10(57):12257-12264, <http://doi:10.5897/AJB11.193>.
- [20] Bhunia, B., Dutta, D., Chaudhuri, S. 2011. Extracellular alkaline protease from *Bacillus licheniformis* NCIM-2042: improving enzyme activity assay and characterization. *Eng. Life. Sci.* 11(2):207-215, <http://doi:10.1002/elsc.201000020>.
- [21] Saeki, K., Ozaki, K., Kobayashi, T., Ito, S. 2007. Detergent alkaline proteases: enzymatic properties, gene, and crystal structures. *J. Biosci. Bioeng.* 103(6):501-508, <http://doi:10.1263/jbb.103.501>.
- [22] Yilmaz, B., Baltaci, M.O., Sisecioglu, M., Adiguzel, A. 2015. Thermotolerant alkaline protease enzyme from *Bacillus licheniformis* A10: purification, characherization, effects of surfactants and organic solvents. *J. Enzyme. Inhibit. Med. Chem.* 31(6):1-7, <http://doi:10.3109/14756366.2015.1118687>.