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# PURIFICATION BY ION EXCHANGE CHROMATOGRAPHY AND ENZYME CHARACTERIZATION OF POTENTIAL DE-HAIRING ALKALINE PROTEASE FROM *BACILLUS CEREUS* LS2B

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## ABSTRACT

**Objective**: Effort to develop an environmental-friendly oriented method of animal leather tanning, especially de-hairing process, becomes a concern of this research. The aim was to purify the alkaline protease from *Bacillus cereus* LS2B using a set of methods including ion exchange chromatography with DEAE Sepharose matrix. The enzyme was considered for having a significant potential in the de-hairing process in tanning industries.

**Method:** The step of alkaline protease enzyme purifications including enzyme productions, ammonium precipitation, membrane dialysis and ion exchange chromatography was performed in this research. The total proteins and specific activity of every fractionation resulted from ion exchange chromatography were measured in this research. Identification of molecular weight by SDS-PAGE of each fraction and measurement of optimum pH and temperature of the purified enzyme was also described.

**Result:** The data showed that there was difference enzyme activity in every fraction obtained from the chromatography indicated the position of the enzyme. Alkaline protease from *Bacillus cereus* LS2B purification using ion exchange chromatography with DEAE Sepharose matrix has resulted in 35 fractions, with each fraction containing about 1.5 ml enzyme. The research was performed with a flow rate of 1.5 ml $min^{-1}$. Each enzyme fraction has different specific activity. The highest activity is shown at the 15$^{th}$ fraction that confirmed 64.4 U/mg. The balance condition between protein concentration and specific activity was observed at the 21$^{st}$ fraction (45.5 U/mg). The 21$^{st}$ fraction was become interesting due to the total of enzyme protein was almost same as the total enzyme activity. The correlation was considered becomes one of the indications of the effectiveness and efficiency of an enzyme purification. The result of SDS-PAGE determination showed that the 15$^{th}$ fraction has 3 bands of protein enzyme with a molecular weight of 72 kDa, 20 kDa, and 13 kDa. The 21$^{st}$ fraction has smaller protein bands, which are observed 20 kDa and 13 kDa.

**Conclusion:** Each fraction has dominant pure protein molecule around 20 kDa. Moreover, it is assumed that the molecular weight of alkaline protease enzyme specific protein of *Bacillus cereus* LS2B is 20 kDa. Optimal temperature and pH of the purified enzyme were 40°C and pH 8, respectively.

**Keywords**: Alkaline Protease, *Bacillus cereus* LS2B, Ion Exchange Chromatography, SDS-PAGE

## INTRODUCTION

The leather industry faces challenges all over the world because of pollution-related problems, though it has been contributing significantly to economic development and employment and export potentials in many developing countries such as Indonesia. Leather processing involves four different unit operations: pre-tanning or beam house, tanning, post-tanning or wet finishing and finishing operations. The pollution is generated in various form from every unit operations, and the pre-tanning contribute the most in some total dissolved solids, chemical and biochemical oxygen demand (Sundararajan *et al*., 2011; Bhat., 2011). The main objective of liming is to remove hair and flesh. It also will open up the fiber structure to get the excellent properties of the final leather. Hence, liming is the most important pre-tanning operation in the leather processing. The conventional liming process involves the application of lime and sodium sulfide ($Na_2SO_4$) which results in the destruction of the skin hair and other proteinous materials impact in high pollution load regarding the biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Moreover, sulfide is more toxic, and a significant amount of lime used to create a huge number of solid waste (Kanagaraj *et al*., 2006; Qureshi *et al*., 2009). Furthermore, to reduce the impact of tanning processes on the environment, many researchers on this subject have been actively involved in the observation and development of enzyme based de-hairing process; defleshing and fiber opening processes to replace the conventional processes using lime and sodium sulfide (George *et al*., 2014).

The enzyme is a biological catalyzer which has a complex role in the development of industries, such as detergent, pharmacological, food, silk, cosmetic, protein hydrolysate and tannery industries

(Mothe and Sultanpuram., 2016). The critical role of the enzyme has a positive correlation with the level of enzyme production and catalysis ability of enzyme. Enzyme production, especially alkaline protease enzyme, can be performed by using the metabolism process of living things, such as bacteria. Bacteria have a significant advantage compared with animals, fungi, and plants in enzyme production. One of the bacteria types which can produce alkaline protease enzyme is *B. cereus* LS 2B ability is isolated from odorous farm soil at the tropical area (Fitriyanto *et al*., 2004). The enzyme produced by *Bacillus cereus* LS2B must have high catalysis ability if used as a commercial standard. The catalysis ability of enzyme heavily depends on the level of enzyme purity. The level of catalysis power or specific enzyme activity is partly determined by the level of purity and character of enzyme protein (Miyaji *et al*., 2005). The presence of a non-enzyme component in a crude enzyme strongly influences volume, purity, stability and catalysis power on the substrate. The usage of ion exchange chromatography method with sepharose matrix is a possible purification method.

Many researchers are able to purify enzyme using ion exchange chromatography method, including purification of the alkaline protease of *Bacillus sp*. HR-08 which was successfully purified 20.2 times from crude enzyme using DEAE sepharose (Moradian *et al*., 2009). The ability of column ion exchange chromatography, an especially with DEAE sepharose matrix in purifying enzyme can't be separated from enzyme protein content and matrix content. The electrostatic interaction of various types of charged ligand in a matrix with a group which can ionize in protein produces to separation mechanism. Exchange of positively charged anion binds an acid molecule, while the exchange of negatively charged cation produces separation mechanism for wet molecule (Wu *et al*., 2013). Based on various references on ion exchange chromatography with a column filled with sepharose matrix in purifying enzyme, the purified alkaline protease enzyme from *B. cereus* LS2B is expected to have increased activity and show profiles of alkaline protease specific protein bands from data of molecular weight confirmation of protein using SDS-PAGE.

## MATERIALS AND METHODS

**Culture condition and enzyme production:** Enzyme production was performed using a modified method from Moradian *et al*., 2009. The preliminary procedure was started by making the stock solution medium using 1 g of meat extract, 1 g of microbiological peptone, and 0.5 g NaCl with 70 ml of distilled water into a beaker glass, continued by stirring into homogenous. The medium was then set to pH 7.2 followed by adjusted with distilled water to a volume of 100 ml. Solid agar medium is made from 1 ml of stock solution, 1.5 g agar and 99 ml of distilled water in 250 ml Erlenmeyer flask. Agar medium is heated with a hot plate stirrer, and then wait until quite cold and poured in a petri dish. Furthermore, when agar medium has hardened, by using ose, bacteria were streak and incubated for 24 hours. Once bacteria have grown, it stored in a refrigerator at 4°C. Liquid medium was made with a mixture of 90 ml of distilled water and 10 ml of stock solution continued by sterilization by autoclaving at 121°C for 15 minutes, then cooled in the Laminar Air Flow (LAF). One ose of the pure isolate was grown on agar or cultivated by putting into 5 ml stock liquid medium and incubated on a shaker for 24 hours. The growth of bacteria was confirmed by observing the turbidity of the medium (Moradian *et al*., 2009; Pouryafar *et al*., 2015)

To produce an alkaline protease enzyme, 3 ml of overnight-grown pre-culture bacteria were grown in a liquid medium having 100 ml of stock solution and 3 ml of skim milk. The pH of the medium was same as the pH of the stock solution. The inoculated medium was then incubated for 24 h at 30°C at 120 rpm in an orbital shaker. The crude enzyme was separated from bacterial cell by centrifugation at speed 3500 rpm for 15 min at 4 °C and continued by stored in refrigerator temperature 4°C for further observation.

**Precipitation using ammonium sulfate:** The amount of 100 ml of cell-free extract was put into Erlenmeyer flask, and it was mixed with 80% ammonium sulfate slowly with stirrer and kept at 4°C condition for overnight. In order to separate the precipitated protein enzyme, it was centrifuged at 3500 rpm for 30 minutes at 4°C. The components separated during the centrifugation process, i.e. pellet and supernatant were separated. The pellet was considered as protein enzyme and supernatant was discarded. Membrane dialysis process was performed to remove ammonium sulfate content in the component of protein enzyme (Moradian *et al*., 2009; Ghosh *et al*., 2008; Abidi *et al*., 2007).

**Separation of nonprotein compound using dialysis method:** The precipitated protein enzyme using ammonium sulfate might bonded with the molecules of an ammonium salt. Thus, dialysis is the appropriate method to separate salt content from the protein solution (Sattayasai, 2012). Dialysis was performed with dialysis buffer pH 8

that contained 0.58 g NaCl, 1.38 g $NaH_2PO_4$, and 1.78g $Na_2HPO_4$ in 1000 ml distilled water. Enzyme sample was put inside dialysis membrane sack (cut off membrane <10 kDa) and diluted by dialysis buffer pH 8 for 5 times of dilution (enzyme 1: 4 buffer). Dialysis process started by inserting dialysis membrane sack containing the enzyme and buffer into 1000 ml of dialysis buffer solution at 4°C while stirring using stirrer at low speed. In every 4 hours, buffer solution outside of membrane sack was replaced. It was replaced three times, and at the last replacement, the sample was dialyzed overnight (Li *et al*., 2016; Papagianni and Sergelidis., 2014).

**Molecular fractionation of enzyme protein using Ion exchange chromatography column:** Alkaline protease from *Bacillus cereus* LS2B that has been dialysised was then purified using ion exchange chromatography column with DEAE. Sepharose matrix. The volume of the column was 25 ml, with dimension 14.5 cm in length and diameter 1.5 cm. The preliminary implementation stage was calibrating column by feeding it with 0.05 M pH 8 of phosphate buffer for one night. Enzyme purification was performed in three stages. In stage 1, the column was fed to pH 8 equilibration buffer with mixture ($KH_2PO_4$ 0.1 N and NaOH 0.2 N) 4 times of column volume with 1.5 ml $min^{-1}$ flow rate. The amount of fraction in one running was 52.5 ml, which was stored in 35 tubes. It was followed by feeding enzyme sample to a number of column volumes. After all enzyme samples had been saved in the column, it was fed with elution buffer, which consisted of a mixture of pH 8 equilibration buffer ($KH_2PO_4$ 0.1 N and NaOH 0.2 N) and 4 times the column volume 0.5 N NaCl. Every released elution after being fed with elution buffer was stored in Eppendorf tube and labeled according to the order of elution (Mothe and Sultanpuram., 2016; Maruthiah *el al*., 2013; Joo and Chang 2005). The processing produced 52.5 ml elution per running with a total of 35 fractions. Every elution fraction kept was stored in the freezer if not immediately used. The fractions were then measured for a specific activity, total protein, and protein profile.

**Measuring alkaline protease enzyme activity of every fraction:** The activity of enzyme was measured from each fraction using modified method of Bergmeyer, 1983 (Bergmeyer *et al*., 1983). The test stage included measurements of blank, standard tyrosine and sample. Blank was measured by mixing 0.5 ml of pH 7 buffer added with 0.5 ml of casein and put into a test tube and incubated at 37°C for 10 minutes. 1.0ml of 10% TCA and 0.75 ml of enzyme were added, incubated for 10 minutes at room temperature and centrifuged for 15 min. 0.75 ml of supernatant was collected and added with 2.5 ml of $Na_2CO_3$ (0.5M), 0.5 ml of folin reagent and allowed to stand for 15 min at room temperature; then absorbance reading was performed at 578 nm. Standard tyrosine measurement was carried out by mixing 0.5 ml of pH 7 buffer, 0.5 ml of casein and 1 ml of tyrosine and inserting them into a test tube. It was incubated at 37°C for 10 minutes, then added with 1 ml of 10% TCA, incubated for 10 minutes at room temperature and then centrifuged for 15 min. 0.75 ml of supernatant was collected, added with 2.5 ml of $Na_2CO_3$ (0.5 M), 0.5 ml of Folin reagent and allowed to stand for 15 minutes at room temperature then absorbance reading was measured at 578 nm. The sample was measured by mixing 0.5 ml of pH 7 buffer, and 0.5 ml of casein for each fraction and 1.0 ml of the enzyme from each fraction was put into test tube labeled according to fraction order. It was incubated at 37°C for 10 minutes, then added with 1.0ml of 10% TCA, incubated for 10 minutes at room temperature. Each fraction was then put into Eppendorf tube and centrifuged for 15 min. 0.75 ml of supernatant was collected, added with 2.5 ml of $Na_2CO_3$ (0.5 M), 0.5 ml of Folin reagent and allowed to stand for 15 min at room temperature. Absorbance reading was then performed at 578 nm.

**Measuring total alkaline protease protein of each fraction:** Total enzyme protein was measured using the Lowry's method (Lowry *et al*., 1951) using BSA (Bovine Serum Albumin) as a standard protein. The total protein was measured using 0.2 ml of the enzyme from each fraction with 1.0 ml of biuret reagent in each enzyme fraction, then incubating it at room temperature for 10 minutes. Afterward, 0.1 ml of reagent E (1:1 of Folin to distilled water) was mixed until it was homogenous in each fraction and incubated at room temperature for 30 minutes. Then, the absorbance level of each sample fraction was measured using spectrophotometry with wavelength $\lambda = 750$ nm, and then total protein was calculated.

**Analysis of profile of enzyme protein of each fraction using SDS-PAGE:** Sodium dedocyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method (1970). The relative molecular weight of the purified protein was estimated by comparing its mobility to standard protein size marker (Bio-Rad., 2014)

**Measuring pH and optimum temperature of fraction with the highest activity:** The optimum temperature of the pure enzyme was determined using temperature range 20-70°C with 30 minutes

of enzyme incubation time. Maximum pH was determined by mixing enzyme with 0.2M phosphate buffer. The pH ranges used were 6-12. Phosphate buffer was first made with pH 8. Low pH 6 and 7 were made by adding 0.1 M HCL, and high pH 9-12 were made by adding 0.1 M NaOH to phosphate buffer. The enzyme was mixed with a buffer of varying pH then stored at 37°C for 30 minutes, the activity of the enzyme was measured as reported modified method of Asker *et al*., (2013).

## RESULT AND DISCUSSION

**The result of ammonium precipitation and dialysis before purification with ion exchange chromatography column:** Enzyme protein deposition obtained considerable number of 10 ml with specific activity 0.5 (U/mg) than specific activity from the crude enzyme just with 0.4 (U/mg). For the release of salt residues from the ammonium sulfate was dialysis process with a semi-permeable membrane (cellophane) pore 10 <kDa. An increase in specific activity was quite good from the dialysis is 1.8 (U/mg), so based on that protein enzymes successfully separated with residual salt and residues of other ions, thus the enzyme results of dialysis can be used for fractionation using ion exchange chromatography column.

**Purification of alkaline protease enzyme resulted from ion exchange chromatography column with matrix filled with DEAE sepharose:** Alkaline protease from *Bacillus cereus* LS2B is categorized as an extracellular enzyme with very diverse enzyme solution component. A non-protein component in enzyme indicates low enzyme specific activity. Enzyme protein is a protein containing anion or cation, so the enzyme protein should be purified using compound or matrix which can bond with the charge, such as DEAE sepharose. Purification using ion exchange chromatography exchanges anion and cation of DEAE Sepharose which binds negatively or positively charged protein based on the density of its protein content (Hosseini and Nasiri, 2015).

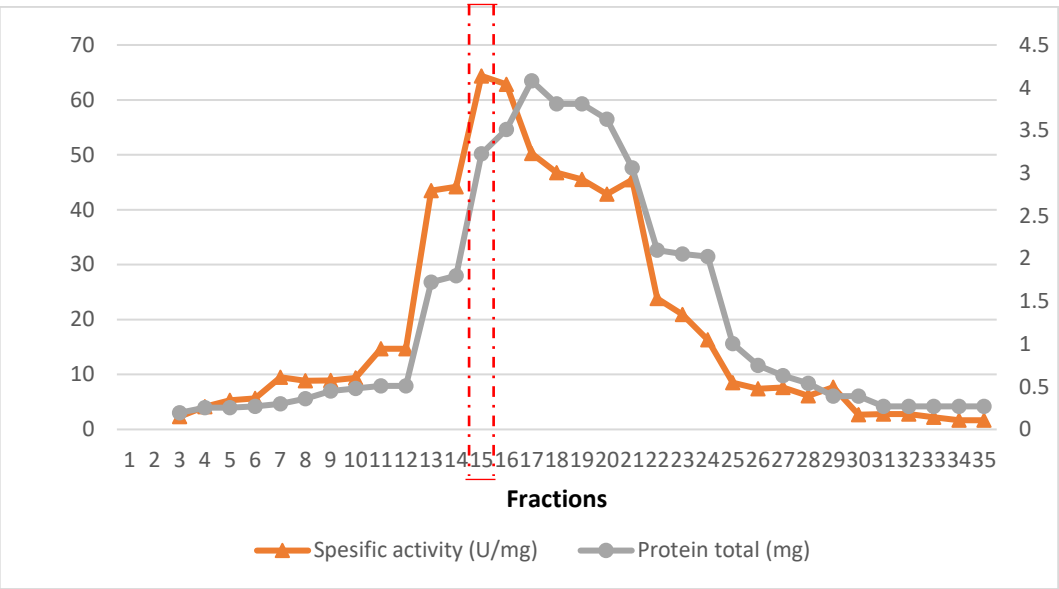
Based on the collected data (Table 1) alkaline protease enzyme of *Bacillus cereus* LS2B was successfully purified using ion exchange chromatography column with DEAE sepharose matrix. The results of ion exchange chromatography were 35 fractions where each enzyme fraction has different enzyme specific activity. Fractions 3-12 have low specific activity level from 2.3 (U/mg) to 14.7 (U/mg). Fractions 13-21 have very high specific activity level compared with other fractions, i.e. from 44.2 to 64.4 U/mg. Fractions 22-35 have significantly declined activity from 23.9 to 1.7 U/mg. The illustration of enzyme specific activity level shows the highest activity in the 15$^{th}$ fraction 64.4 U/mg with purity level up to 27.9 folds, the 16$^{th}$ fraction 62.8 U/mg with purity level up to 27.2 folds, and the 17$^{th}$ fraction 50.3 U/mg with purity level up to 21.8 folds. Increased purity to >20 folds was supported by data from various researchers such as Mothe and Sultanpuram (2016). Mothe and Sultanpuram (2016) who purified alkaline protease enzyme of *Bacillus caseinylticus* using DEAE sepharose where the purification fold is 20.74. Purification of alkaline protease from *Bacillus sp* HR-08 using DEAE sepharose resulted in 20.02 folds of purity level from crude enzyme (Moradian *et al*., 2009).

The data shows that there is great enzyme purification in the 15$^{th}$ fraction and the 21$^{st}$ fraction, where total protein in fraction 15 isn't as high as fractions 16, 17, 18, 19 and 20 but has the highest specific activity of all fractions. It indicates that enzyme protein in the 15$^{th}$ fraction is pure alkaline protease protein which can perform catalysis process many times. Fractions 16-20 have the highest total protein but have specific activities below fraction 15. It's interpreted that alkaline protease enzyme protein in the 16-20$^{th}$ fractions are still mixed with non-enzyme protein compounds or even bacterial cell protein, substrate protein and ammonium salt compound using in enzyme production, although dialysis has been performed. Non-enzyme protein compounds in enzyme component are a lipid, metal, phosphate and other apoenzyme parts, so the components support total enzyme protein captured by spectrophotometer wave so that the quantity of sample solution solid is higher (Zhang *et al*.,2016; Venkatanagaraju and Divakar., 2015). The 21$^{st}$ fraction is kind of interesting to note because the total enzyme protein is almost the same as the total enzyme activity, so the correlation is considered an indication of effectiveness and efficiency of an enzyme of each protein unit in catalyzing a milligram of the substrate. This is supported by data of confirmation of molecular weight protein that the band formed in gel well row is fully clear.

**Table 1:** Summary of activity of alkaline protease enzyme of *Bacillus cereus* LS2B of each fraction

| Fraction No. | Protein concentration (mg/ml) | Activity (U/ml) | Total protein (mg) | Total activity (units) | Specific activity (U/mg) | Purification fold |
|---|---|---|---|---|---|---|
| 3 | 0.13 | 0.3 | 0.20 | 0.45 | 2.3 | 1.0 |
| 4 | 0.17 | 0.7 | 0.26 | 1.05 | 4.1 | 1.8 |
| 5 | 0.17 | 0.9 | 0.26 | 1.35 | 5.3 | 2.3 |
| 6 | 0.18 | 1.02 | 0.27 | 1.53 | 5.7 | 2.5 |
| 7 | 0.20 | 1.9 | 0.30 | 2.85 | 9.5 | 4.1 |
| 8 | 0.24 | 2.13 | 0.36 | 3.20 | 8.9 | 3.8 |
| 9 | 0.30 | 2.67 | 0.45 | 4.01 | 8.9 | 3.9 |
| 10 | 0.32 | 3 | 0.48 | 4.50 | 9.4 | 4.1 |
| 11 | 0.34 | 5 | 0.51 | 7.50 | 14.7 | 6.4 |
| 12 | 0.34 | 5 | 0.51 | 7.50 | 14.7 | 6.4 |
| 13 | 1.15 | 50 | 1.73 | 75.00 | 43.5 | 18.8 |
| 14 | 1.20 | 53 | 1.80 | 79.50 | 44.2 | 19.1 |
| 15 | 2.15 | 138.4 | 3.23 | 207.60 | 64.4 | 27.9 |
| 16 | 2.34 | 147 | 3.51 | 220.50 | 62.8 | 27.2 |
| 17 | 2.72 | 136.7 | 4.08 | 205.05 | 50.3 | 21.8 |
| 18 | 2.54 | 118.7 | 3.81 | 178.05 | 46.7 | 20.3 |
| 19 | 2.54 | 115.7 | 3.81 | 173.55 | 45.6 | 19.7 |
| 20 | 2.42 | 103.7 | 3.63 | 155.55 | 42.9 | 18.6 |
| 21 | 2.04 | 92.7 | 3.06 | 139.05 | 45.4 | 19.7 |
| 22 | 1.40 | 33.4 | 2.10 | 50.10 | 23.9 | 10.3 |
| 23 | 1.37 | 28.7 | 2.06 | 43.05 | 20.9 | 9.1 |
| 24 | 1.35 | 22 | 2.03 | 33.00 | 16.3 | 7.1 |
| 25 | 0.67 | 5.7 | 1.01 | 8.55 | 8.5 | 3.7 |
| 26 | 0.50 | 3.7 | 0.75 | 5.55 | 7.4 | 3.2 |
| 27 | 0.42 | 3.2 | 0.63 | 4.80 | 7.6 | 3.3 |
| 28 | 0.36 | 2.2 | 0.54 | 3.30 | 6.1 | 2.6 |
| 29 | 0.26 | 2 | 0.39 | 3.00 | 7.7 | 3.3 |
| 30 | 0.26 | 0.7 | 0.39 | 1.05 | 2.7 | 1.2 |
| 31 | 0.18 | 0.5 | 0.27 | 0.75 | 2.8 | 1.2 |
| 32 | 0.18 | 0.5 | 0.27 | 0.75 | 2.8 | 1.2 |
| 33 | 0.18 | 0.4 | 0.27 | 0.60 | 2.2 | 1.0 |
| 34 | 0.18 | 0.3 | 0.27 | 0.45 | 1.7 | 0.7 |
| 35 | 0.18 | 0.3 | 0.27 | 0.45 | 1.7 | 0.7 |

**Confirmation of molecular weight of enzyme protein of each fraction using SDS-PAGE method:** The SDS-PAGE result of alkaline protease enzyme fraction of *Bacillus cereus* LS2B with ion exchange chromatography DEAE sepharose column separation shows different protein band profile. Band visualization of the first 11 fractions (Fig. 1) shows low protein density but with an entire protein band. Fractions 7 and 8 have 3 protein bands with molecular weights of 55 kDa, 20 kDa and 13 kDa, fractions 9-13 have 4 protein bands with molecular weight ranges of 72 kDa, 55 kDa, 20 kDa and 13 kDa. Visualization of protein bands of the second 11 fractions has a very high density (Fig. 2), dominated by protein bands with a molecular weight of 20 kDa. Fractions 14, 15, 16, 17, 18 19, 20 have protein with molecular weights of 72 kDa, 20 kDa and 13 kDa. Fractions 21-24 have declining whole enzyme, protein band to 2 bands with molecular weights of 20 kDa and 13 kDa.



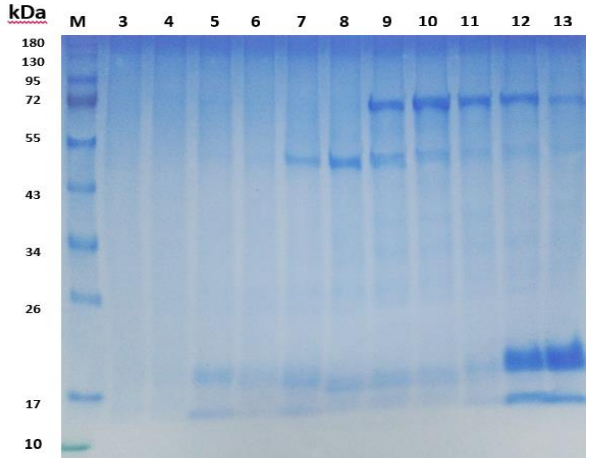




**Figure 1:** The profile of alkaline protease enzyme protein fraction from 3rd to the of ion exchange chromatography. SDS-PAGE used 12% gel concentration. **M=** Marker and number shows fractions

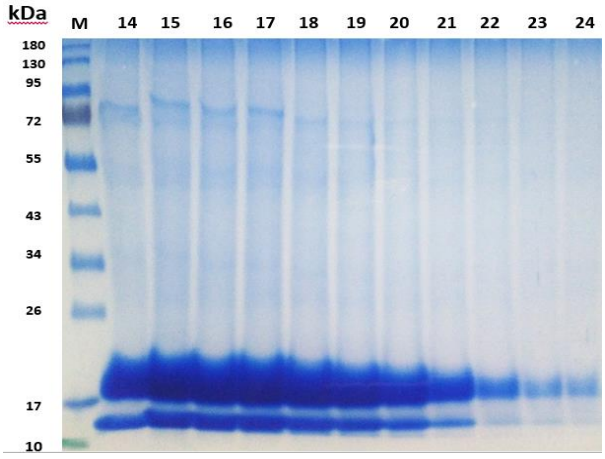



**Figure 2:** The profile of alkaline protease enzyme protein fractions from 14th to the 24th of ion exchange chromatography. SDS-PAGE used 12% gel concentration. **M=** Marker and number shows fractions

The 21st fraction is unique. The comparison of its enzyme specific activity and total enzyme protein (Table 1) is equal, so the visualization of visible protein band is also very clear, dominated by protein density with a molecular weight around 20 kDa. The statement is supported by Horikoshi (24) opinion that the efficiency of enzyme usage has correlation or equal proportion between enzyme activity and enzyme protein, so it can be assumed that every protein unit in enzyme has the same ability to catalyze one milligram of substrate compound. The visualization of protein bands of the last 10 fractions shows declining enzyme protein band. There isn't even any protein compound in enzyme solution, as shown in (Fig 3). In Fig. 5, the running enzyme sample is the 25th enzyme fraction. The lack of formed protein band indicates that there isn't any protein compound.

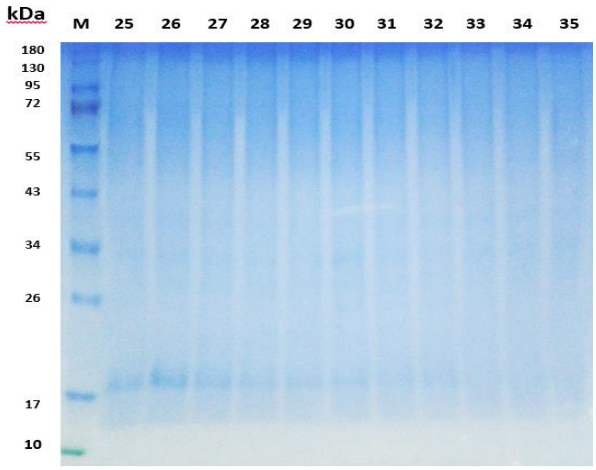



**Figure 3:** The profile of alkaline protease enzyme protein fractions from of the 25th to the 35th of ion exchange chromatography. SDS-PAGE used 12% gel concentration. **M=** Marker and numbers shows different fractions.

The protein band profile demonstrated by each fraction of fractions 3-35 shows that alkaline protease enzyme of *Bacillus cereus* LS2B has moderately good purification process using ion exchange chromatography column with DEAE sepharose matrix, where dominating protein band is found in molecular weight around 20 kDa. Miyaji *et al*., (2005) report that alkaline protease enzymes from *Bacillus sp* have the average molecular weight of 20-40 kDa. The resulting dominating protein band with molecular weight of 20 kDa is supported by the other study (Singh *et al*., 2012), who report that alkaline protease enzyme produced by *Bacillus cereus* SIU1 has a molecular weight of the protein of 22 kDa. Saxena and Singh (Saxena and Singh, 2015) report that the molecular weight of alkaline protease enzyme protein produced by *Bacillus cereus* B80 has a molecular weight of 26 kDa. Prakash, Banik (Prakash *et al*., 2005) also report research data on the molecular weight of alkaline protease enzyme from *Bacillus cereus*, which is 28 kDa. The study of Venkatanagaraju and Divakar (2015) finds the molecular weight of alkaline protease protein of *Bacillus cereus* GD55 which is a little different from the finding of this study. The molecular weight of alkaline protease protein of *Bacillus cereus* GD55 is 31 kDa.

**Optimum temperature and pH of enzyme fraction with the highest activity resulted from ion exchange chromatography column:** Temperature and pH are two of supporting factors of the catalysis ability of an enzyme, especially in the pure enzyme. At certain temperature and pH, the chemical reaction of the enzyme in catalyzing substrate can be very fast (high activity) and slow (low activity). The data of activity of alkaline protease enzyme of pure *Bacillus cereus* LS2B at various temperatures (Fig. 4) shows that the optimum temperature of alkaline protease enzyme of *B. cereus* LS2B is 40°C. It's because the highest enzyme activity is found at 40°C. The result is in line with the research result of Liu *et al*., (2010) who report that the optimum temperature of pure alkaline protease enzyme of *Bacillus cereus* MBL13 is 40°C. However, other researchers, such as Orhan *et al*., (2005), mention that the maximum temperature of alkaline protease enzyme of *Bacillus cereus* is 60°C. However, alkaline protease enzyme still has activity at 30, 50, 60 and 70°C although with a lower percentage than 40°C, so alkaline protease enzyme of *Bacillus cereus* LS2B can be called a thermostable enzyme. Orhan *et al*., (2005) state that the temperature range of alkaline protease enzyme produced by *Bacillus cereus* is 10-70°C. The optimum pH of alkaline protease enzyme of *Bacillus cereus* LS2B (Fig. 5) is pH 8. The result is in line with the research result of Orhan *et al*., (2005) who state that the optimum pH of pure alkaline protease enzyme of *Bacillus cereus* is pH 8.0. Liu *et al*., (2010) also report the same data, that the optimum pH of pure alkaline protease enzyme which can be isolated from *Bacillus cereus* MBL13 is pH8. Analysis of optimum pH of alkaline protease enzyme is also performed by Kim *et al*., (2001) who find that the optimum pH of alkaline protease enzyme of *Bacillus cereus* KCTC 3674 is pH 8.0. Although slightly different from the results of research (Margino, 2015) of the pure protease enzyme *Bacillus sp*. TBRSN- 1 with optimum pH of 7 and a temperature of 30°C.

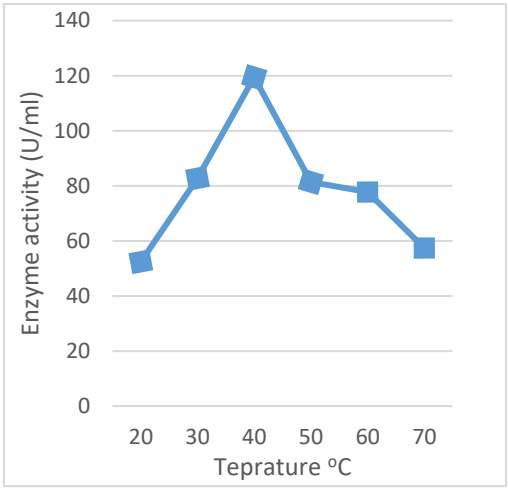



**Figure 4.** The optimum temperature of purified alkaline protease enzyme of *Bacillus cereus* LS2B

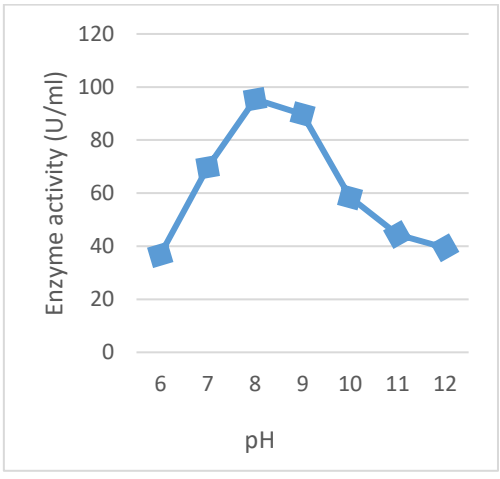



**Figure 5.** The optimum pH of purified alkaline protease enzyme of *Bacillus cereus* LS2B

## CONCLUSION

Alkaline protease from *Bacillus cereus* L2SB has been purified through fractionation process using ion exchange chromatography column with

DEAE sepharose matrix. The flow rate of the ion exchange chromatography tool is 1.5 ml $min^{-1}$ with 35 fractions, each fraction containing 1.5 ml of enzyme. Dominating enzyme specific activity is found in the 15th fraction with a specific activity of 64.4 U/mg. The molecular weight of dominant protein and interpreted as alkaline protease protein of *Bacillus cereus* LS2B resulted from SDS-PAGE determination is 20kDa. The result of SDS-PAGE also shows that the cleanest enzyme protein is the 21st fraction. The maximum temperature and pH of pure alkaline protease enzyme of *Bacillus cereus* LS2B are 40°C and pH 8.

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