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Optimizing of Protease Purification from Bacillus cereus TD5B by Ammonium Sulfate Precipitation

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The aim of this research was to optimize the protease purification from Bacillus cereus TD5B by ammonium sulfate precipitation. The material used was protease enzyme from Bacillus cereus TD5B. It was produced and purified in a liquid medium using ammonium sulfate precipitation. The optimization was performed using various levels (50, 60, 70 and 80 %) of ammonium sulfate as precipitation agent of crude microbial enzymes. Separation process of precipitate and supernatant was conducted by 3,500 rpm centrifugation for 30 min at 4 °C. The specific activity of protease enzyme, and protein content was observed on precipitate and supernatant of each levels treatment of the ammonium sulfate. Based on specific enzyme activity and protein content, the measurement showed that 70 % ammonium sulfate level treatment has the highest specific enzyme activity (78.296 U/mg) and highest protein content (2.689 mg/mL). This specific enzyme activity was three times higher compared to specific enzyme activity before purification treatment (24.12 U/mg). In conclusion, the purification of crude Bacillus cereus TD5B enzyme by ammonium sulfate purification can increase the specific enzyme activity.

1. Introduction

The need to enzyme has developed very rapidly. Recorded in 2015, enzyme consumption about 4.4 billion dollars with an increase in the production growth of 7.6 % every year which dominated by protease enzymes (Yadav et al., 2015). Protease enzyme has a vital role to the development of various industries (Mothe and Sultanpuram, 2016). Each protease enzyme produced by the bacterium has high catalysis ability (Peña-Montes et al., 2008). Singh et al. (2004) mentions that of the many enzymes produced, the proteases produced by Bacillus spp are one of the best enzymes. Protease enzyme production is obtained through bacterial metabolism, including Bacillus cereus TD5B. Bacillus cereus TD5B can produce a keratinase enzyme in medium supplemented with chicken feathers and showed degraded the feathers at about 65 h (Wandita et al., 2015). However, each protease enzyme derived from microbes has a different specification. The presence of non-enzyme components present in a crude enzyme often inhibits enzyme activity. Therefore, purified enzyme has the higher enzyme activity than a crude enzyme. Purification of enzymes is a technique to isolate certain enzymes from crude enzyme extracts that still contain cell microorganisms or other components (Sattayasai, 2012). There is no data about purification of Bacillus cereus TD5B. Therefore, it is necessary to optimize the protease purification from Bacillus cereus TD5B by ammonium sulfate precipitation.

1.1 Literature review

Bacillus cereus TD5B is a isolate microbes from the soil around odorous farm area at Yogyakarta which has protease activity was successfully obtained and confirmed to produce extracellular protease (Fitriyanto et al., 2014). The catalysis power and specific activity of the crude enzyme are determined by the level of purity of the enzyme. The presence of non-enzyme components in crude enzyme has significant effect on volume, purity, stability and catalysis power of enzyme (Miyaji et al., 2005).

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Such as ammonium sulfate salts, sodium chloride or sodium sulfate is called fractionation process. The addition of electrolyte compounds into the solution containing the protein can lead to the process of protein deposition. The process of precipitation of the protein is affected by the ionic strength of the solution. The high of ionic bounds of protein and water caused greater solubility of an enzyme or called salting in, but if added salt to reach a certain point, where the higher salt content will cause protein solubility decreases and occurs protein deposition process. It happens because of the stronger bonding of salt with water ion (Hao and Sun, 2015). This protein precipitation method is called salting out. In the strength of low salt ions, the protein will be ionized so that interaction between proteins will decrease and solubility will increase. The increasing of ionic strength can increase the water content attached to the ion, and if the interaction between protein ions is strong, its solubility decreases because of stronger protein interactions and decreased solubility.
One of the electrolyte compounds often used to precipitate proteins is ammonium sulfate. The advantages of ammonium sulfate compared to other electrolyte compounds are having high solubility, not affecting enzyme activity, having an effective precipitating effect, stabilizing effect on most enzymes, can be used at various pH and low cost (Hao and Sun, 2015). The concentration of ammonium sulfate used determines the amount and activity of the enzyme produced. Best ammonium sulfate concentrations used to precipitate the alkaline protease enzyme are range from 50 % until 80 % and could increase the purity of the enzyme to 1.2 times higher. Other researchers such as (Miyaji et al., 2005) reported that the use of ammonium sulfate with higher concentration was able to precipitate and increase the activity of enzyme using ammonium sulfate 80 % and increase the purity of enzyme to 6.7 times higher.

2. Methods and materials

2.1 Production of protease enzyme Bacillus cereus TD5B

The production of enzymes using the modified method (Moradian et al., 2009). The initial procedure is to make a stock solution by mixing 1 g of meat extract; 1 g microbiological peptone; 0.5 g NaCl and 70 mL of distilled water into beaker glass, then stirred until homogeneous. The medium is set at pH 7.2 (if too acid add NaOH and if too alkaline add HCl), and then add distilled water to medium until 100 mL volume. Preparation of agar medium by taking 1 mL of stock solution, 1.5 g agar, and 99 mL of distilled water then poured in Erlenmeyer. The agar medium heated in hot plate stirrer, then wait until warm and poured in a petri dish. Furthermore, the agar medium that has hardened can be inoculated with Bacillus cereus TD5B using ose and incubated for 24 h. After growing, Bacillus cereus TD5B stored in a refrigerator at temperature 4˚C.
The pre-culture was prepared by 5 mL of stock solution and sterilized by autoclave at 121 ° C. for 15 min, then cooled in laminar air flow. Next, one use of pure isolate Bacillus cereus TD5B has been grown on the agar medium put into 5 mL of pre-culture solution and incubated in the shaker for 24 h. When media becomes cloudy can be concluded that bacteria have grown perfect in pre-culture. The growth of inoculum in the media with the enzyme substrate begins by preparing a stock solution of 100 mL, 1.5 g skim and 5 mL pre-culture that has been overgrown with Bacillus cereus TD5B. After that, the medium is overgrown Bacillus cereus TD5B in the shaker with speed 120 rpm for 24 h. On the next day, it is observed that a change of medium color becomes yellow, so it can be assumed that there has been a reaction of enzymatic activity, then separated enzyme with its bacterial cell wall by centrifuging at 3,500 rpm for 15 min at 4 °C. After centrifugation, there is a separation between supernatant and pellet. The supernatant is considered a crude enzyme and then can be followed by further testing, while the pellet is removed because it is a cell wall of Bacillus cereus TD5B.

2.2 Purification by Ammonium Sulfate

Determination of the best ammonium sulfate concentration for purification was by treatment of ammonium sulfate addition of 50 % (29.1 g (NH4)2SO4 / 100 mL crude enzyme), 60 % (36.1 g (NH4)2SO4 / 100 mL crude enzyme), 70 % (47.6 g (NH4)2SO4 / 100 mL crude enzyme) and 80 % (51.6 gr (NH4)2SO4 / 100 mL crude enzyme) (Asker et al., 2016). After all concentration of ammonium sulfate are weighed, the enzyme mix slowly with stirrer during an overnight at 4 °C until homogeneous. Then centrifugation process with speed 3,500 rpm at 4 °C for 30 min. After the centrifugation process is complete, the pellets and supernatant are separated to fit in different conical tubes and then the enzyme activity level is measured using Bregmeyer and Grassl method (Bergmeyer et al., 1983) and the protein content is measured by Lowry method (Lowry et al., 1951).

2.3 Observation of activity enzyme

Measurements of enzyme activity were performed in three stages including measurement of blanks, standard measurements of tyrosine and sample measurements (Bergmeyer et al., 1983; Walter et al., 1984; Yuratmoko et al., 2010). The blank measurements were carried out by mixing 0.5 mL of buffer pH 7, plus 0.5 mL casein

and 1 mL of sterile aqueous were inserted in the test tube. The blank incubates at 37 ° C for 10 min, then add TCA 10 % by 1 mL, incubated for 10 min at room temperature then centrifuged for 15 min. The supernatant was taken as much as 0.75 mL, added 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 done at 578 nm.
Standard tyrosine measurements were performed by mixing 0.5 mL of buffer pH 7, plus 0.5 mL casein and 1 mL of tyrosine were fed in the test tube. Standard tyrosine incubates at 37 °C for 10 min, then add TCA 10 % by 1 mL, incubated for 10 min at room temperature then centrifuged for 15 min. Supernatant was taken as much as 0.75 mL, added 2.5 mL of Na_2CO_3 (0.5 M), 0.5 mL of folin reagent and was allowed to stand for 15 min at room temperature, then absorbance reading was done at 578 nm.
The sample measurements were made by mixing 0.5 mL of buffer pH 7, plus 0.5 mL of casein and 1 mL of enzyme inserted in the test tube. The sample incubates at 37 °C for 10 min, then add TCA 10 % by 1 mL, incubated for 10 min at room temperature then centrifuged for 15 min. The supernatant was taken as much as 0.75 mL, added 2.5 mL of Na_2CO_3 (0.5 M), 0.5 mL of folin reagent and was allowed to stand for 15 min at room temperature, then absorbance reading was done at 578 nm.

2.4 Observation of protein content

The total enzyme protein measurement was based on the Lowry method using BSA (Bovine Serum Albumin) as the standard protein (Lowry et al., 1951). The first step in the process of measuring the total protein is by making some reagents: A reagent (2 % Na_2CO_3 in 0.1 N NaOH), B reagent (0.5 % $CUSO_4$ in 1 % K. Na-Tartate), C reagent (50 mL of reagent A mixed with 1 mL of reagent B then homogenized) and reagent E (1: 1 between foline and aquadest). The work begins by mixing 0.2 mL of enzyme mixed with 1 mL of C reagent, then incubated at room temperature for 10 min. After that a quantity of 0.1 mL of E reagent was mixed until homogeneous and incubated at room temperature for 30 min. Furthermore, the absorbance rate of the sample was measured using spectrophotometry with wavelength λ = 750 nm then calculated total protein obtained.

3. Results and findings

The enzyme is produced from Bacillus cereus TD5B by growing bacterial culture on a medium containing skim milk as a substrate. Purification steps include collecting enzyme crude by centrifugation, a crude enzyme obtained then precipitated using ammonium sulfate $(NH_4)_2SO_4$. The importance of determining the best concentration of ammonium sulfate was caused it determines the amount and activity of the enzyme obtained and used for the next purification step. Preliminary sample testing was done to obtain preliminary data to know the ability of the purification process through ammonium sulfate precipitation. Activity data and protein content of enzyme can be seen in Figure 1, based on data from the test of enzyme activity and protein level can be determined the specific activity of the enzyme in Figure 2.

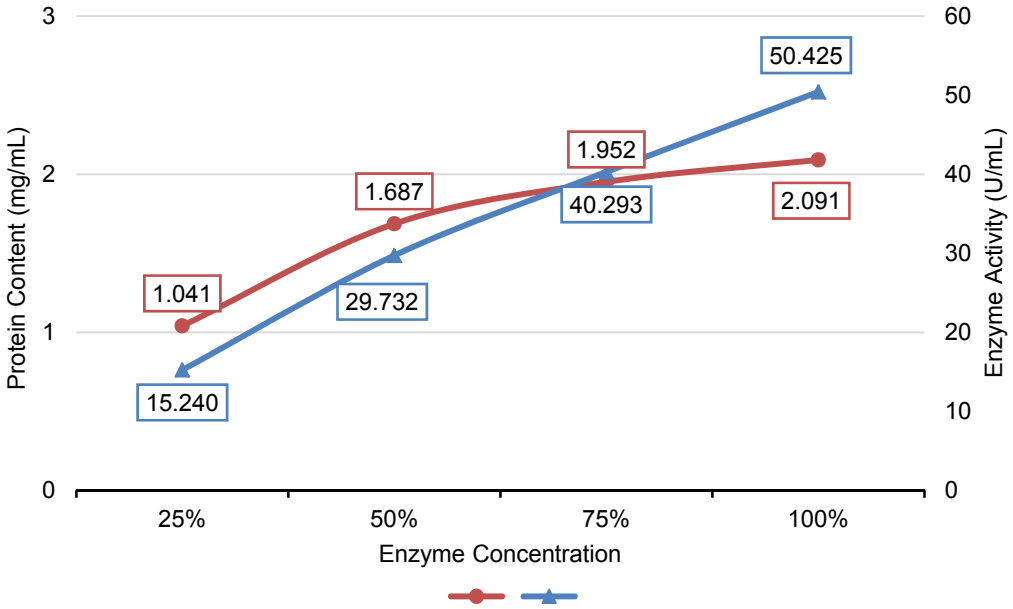

Figure 1: Crude enzyme of Bacillus cereus TD5B before precipitation

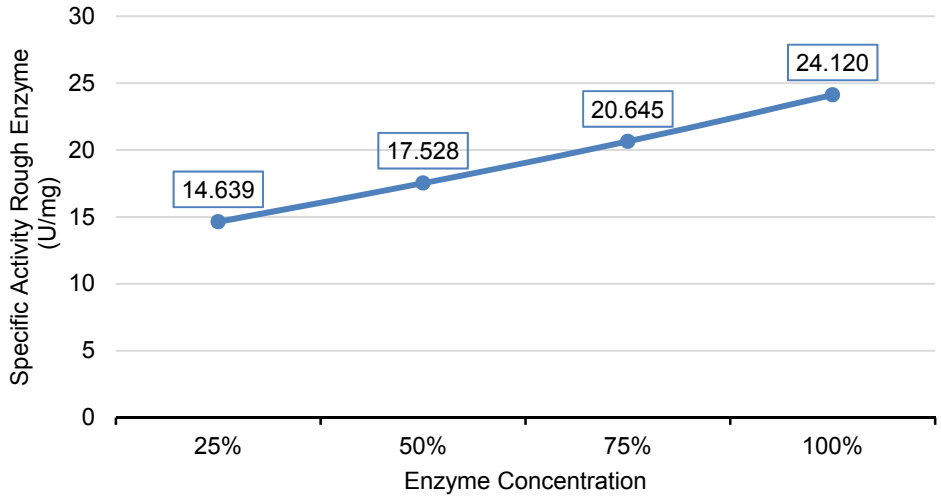

Figure 2: Specific activity of crude enzyme of Bacillus cereus TD5B before precipitation

The addition of ammonium sulfate with a suitable concentration of enzyme character can produce maximum specific enzyme activity. Many of the references mentioned that the protease enzyme character is very well precipitated with ammonium sulfate with concentrations ranging from 50-80 %. The result data of various ammonium sulfate concentration on the centrifuge that separates pellet and supernatant can be seen in Figure 3.

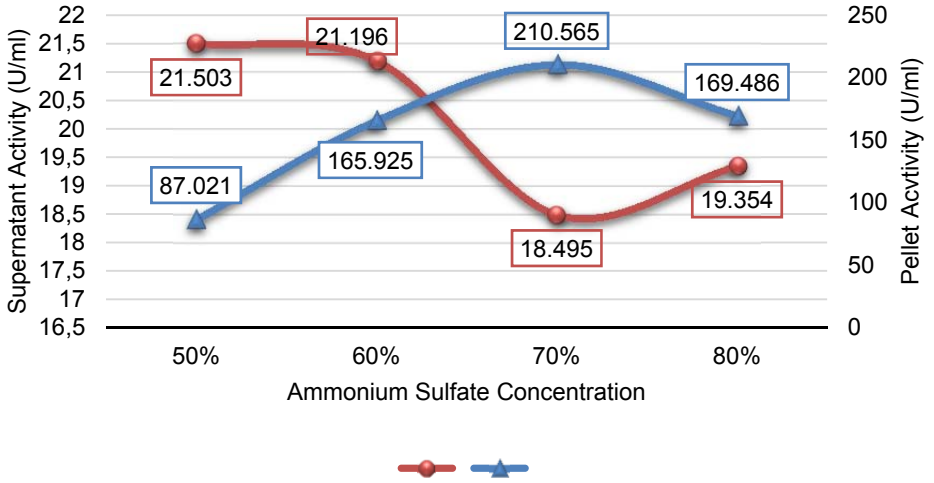

Figure 3: Enzyme activity of Bacillus cereus TD5B after precipitation

Enzyme activity obtained on enzyme pellet with 70 % (210.565 U/mL) ammonium sulfate concentration was higher than concentration of 50 % (87.021 U/mL), 60 % (165.925 U/mL) and 80 % (169.486 U/mL), and inversely proportional to activity in the supernatant, where the lowest activity of the enzyme supernatant was obtained at a concentration of 70 % (18.495 U/mL). Based on the comparison of activity on pellet (210.565 U/mL) and supernatant (18.495 U/mL), it can be concluded that the process of precipitation of Bacillus cereus TD5B enzyme protein is very good at 70 % ammonium sulfate concentration. The importance of the purification process is essential for increasing enzyme activity.

The data obtained from the protein content test of pellet from centrifuge after treatment were also in accordance with the data obtained from enzyme activity test, the highest protein content was at ammonium sulfate level of 70 % (2.689 mg/mL), compared with the ammonium level Sulfate 50 % (2.351 mg/mL), 60 % (2.476 mg/mL), and 80 % (2.195 mg/mL), while the protein content data on the supernatant of the 70 % treatment was the lowest (1.687 mg/mL) as can be seen In Figure 4.

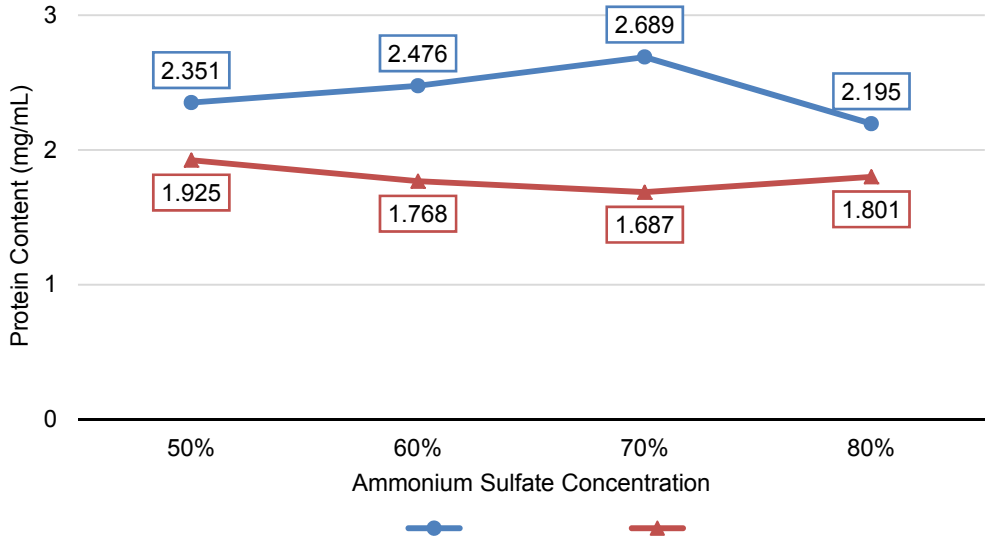

Figure 4: Protein content of enzyme after precipitation

Based on the data of activity test and enzyme protein content, it can be known that the data of specific activity of enzyme based on ammonium sulfate level as can be seen in Figure 5.

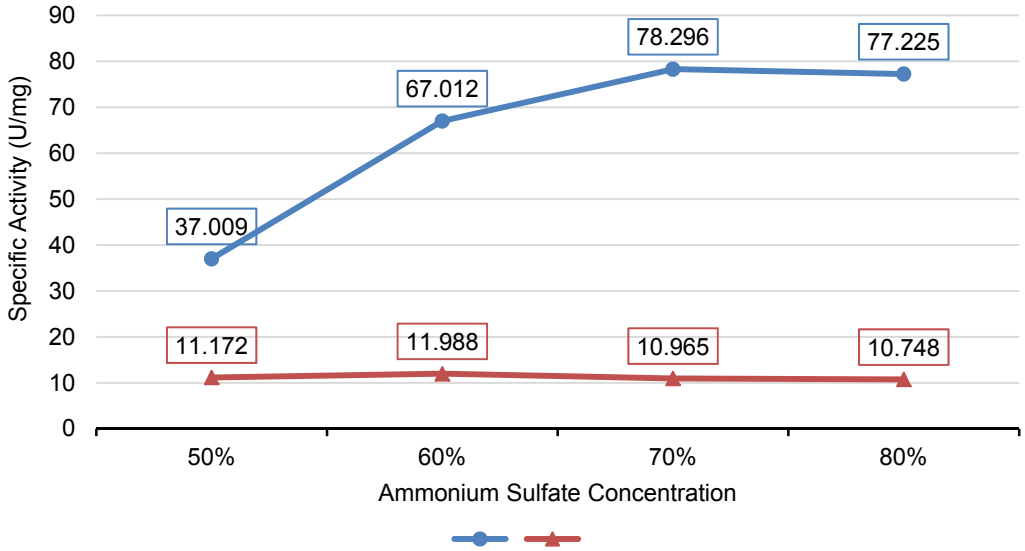

Figure 5: Specific activity enzyme after precipitation

Crude enzymes precipitated using 70 % ammonium sulfate have increased specific activity from 24.12 U/mg to 78.296 U/mg in ammonium precipitation. The increasing of enzyme activity was caused by proteins in enzyme solutions separated from water molecules caused by competition of salt ions to attract water molecules. The salt ion attracts water molecules from proteins because the salt ions have greater solubility than enzyme proteins, so the protein concentration becomes higher in the solution causing the specific activity of the enzyme to increase (Wardani and Nindita, 2013).

4. Conclusion

70 % ammonium sulfate level treatment has the highest specific enzyme activity (78.296 U/mg) and highest protein content (2.689 mg/mL). The purification of crude Bacillus cereus TD5B enzyme by ammonium sulfate purification able to increase the specific enzyme activity.

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