The effect of purification on Snail (*Achatina fulica*) cellulase enzyme characteristic

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Abstract. Snail is an organism that able to produce cellulase enzyme due to its ability to use cellulose for energy source. The purposes of this study were to obtain best concentration of ammonium sulphate in increasing cellulase enzyme activity, optimum temperature, and cellulase enzyme specific substrate from snail. The snail cellulase enzyme was concentrated using ammonium sulphate. The study was designed as Completely Randomized Design consisting seven treatments (30-90% of ammonium sulphate) and three replicates. The result showed that the activity of crude cellulase enzyme was 0,123 U/ml. The optimum cellulose enzyme activity was at 50% ammonium sulphate as 0,609 U/ml and the specific activity was 0,970 U/mg. Optimum temperature of the concentrated cellulose enzyme was at 50°C and the highest activity was in CMC substrate at 0,396 U/ml.

1. Introduction

Cellulose is the most abundant biomolecules in nature and is the main constituent of plant structure. It is estimated about 1011 tons of cellulose are biosynthesized every year. Some of the many cellulosic compounds found are dry leaves containing about 10-20%, wood 50%, and cotton 90 % [1]. Recently, agriculture and forestry by product such as straw, wheat, and rice, corn cobs, corn germ, and others have not been used optimally. Previously cellulase enzyme is an enzyme that able to decompose cellulose by breaking the glucose polymer bonds in cellulose which has β-1,4-glycosidic bonds to become simple sugars and derivatives that involve endoglucanase, exoglucanase, and β-glucosidase [2].

Cellulase enzymes are not only used widely in the textile, detergent, pulp and paper industries but are also used in processing foodstuffs. The use of cellulase enzymes in foodstuff processing is for glucose production from materials containing cellulose waste. For example, the application of cellulase enzymes in citrus products especially for flavors extraction. In this regards, cellulase enzymes act as compression of vegetable fibers [3].

Previous study, [4] has applied cellulase and xylanase enzymes to decompose fiber components in flour germ and corn grits. Flour germ and corn grits are by-products of the corn milling process. The use of cellulase enzyme in food industry, resulting in more enzyme demand thus its necessary to find alternative source of cellulase enzymesuch as from snail. Snail (*Achatina fulica*) is kind of animal that able to use cellulose for energy source. Cellulase enzymes derived from snail were found in the
hepatopancreas whose channels drain into the digestive tract \[5\]. Isolation of cellulase enzymes from snail is relatively simple, easy, inexpensive, didn’t require much time, and the enzymes can be stored for 4 months at \(-15^\circ\)C. Cellulase enzymes in snail are produced from bacteria in the digestive tract \[6\]. The microbes which were commonly found is Bacillus, Brevibacillus, Paenibacillus, Agrobacterium, Pseudomonas and Zymomonas \[7\].

Cellulase hydrolysis of *Ananas comucus* pineapple pulp with cellulase enzymes from snail (*Achatina fulica*) has been reported before \[8\]. That results showed a specific activity of cellulase enzymes in hydrolyze cellulose from pineapple pulp is 0.7727 U/mg protein. Other study \[9\] also conducted a study on Carboxy Methyl Cellulose (CMC) hydrolysis with cellulase enzymes from snail for ethanol production using *Zymomonas mobilis*. The results showed that the activity of cellulase enzyme crude extract from snail was 2.75 U/ml and its specific activity was 2.85 U/mg protein.

Cellulase enzymes derived from snail have not been concentrated, but its crude cellulase enzyme activity have been tested \[9\]. Therefore, it is necessary to concentrate the snail cellulase enzymes to obtain pure cellulase enzymes. According to former report \[10\], enzyme purification can be done using several methods such as ion exchange chromatography, electrophoresis, gel filtration, and concentration with ammonium sulphate. The often method applied was the use of ammonium sulphate salt in enzyme concentration process. This due to it has high solubility even though it is below room temperature, non-toxic, low price, tends to stabilize enzymes, and the protein stability can last for years.

Concentrated enzymes have a more specific activity compared to crude extracts \[11\]. Therefore, it is important to obtain concentrated snail cellulase enzymes by precipitation method using ammonium sulphate aiming to have higher cellulase activity for breaking cellulose into glucose. For that this study aims to obtain the best concentration of ammonium sulphate in increasing cellulase enzyme activity, optimum temperature, and cellulase enzyme specific substrate which derived for snail by concentrated the enzyme using ammonium sulphate.

### 2. Methods

#### 2.1. Extraction cellulase enzymes from hepatopancreas of snail (*Achatina fulica*)

The snail cellulase enzyme was extracted from hepatopancreas which refers to the previous protocol \[9\]. Snails were separated from their shells, hepatopancreas then obtained from their stomachs. In total of 35g of snail hepatopancreas were homogenized using 500 ml of 1% NaCl which had been cooled overnight for blending for 3 minutes at 1-4\(^\circ\)C. The homogenate obtained was then filtered using cloth into a 1.5 ml micro tube followed with 30 minutes centrifugation at 2\(^\circ\)C at a speed of 3000 rpm. After the process is complete, the supernatant as crude cellulase extract enzyme will then tested for enzyme activity. Cellulase enzyme activity will be tested for its characteristic before and after given the enzyme was concentrated.

#### 2.2. Precipitation cellulase enzymes using ammonium sulphate ((NH\(_4\))\(_2\)SO\(_4\))

To concentrate the cellulase enzymes by using ammonium sulphate ((NH\(_4\))\(_2\)SO\(_4\)), earlier protocol was referred \[12\]. The crude extract of cellulase solution (20 ml) was concentrated with ammonium sulphate at saturation 30-90%. Ammonium sulphate with various concentrations was poured little by little, along with slowly stirring using magnetic stirrer for 30 minutes, until all ammonium sulphate dissolves.

This research was carried in a non-factorial completely randomized design (CRD) experiment. A quantitative descriptive analysis was performed for enzyme optimal temperature test. This study uses a variation of ammonium sulphate concentration consisting of seven levels. Each treatment was repeated three times to obtain 21 experimental units. Measurements were carried out every day until the sixth day for several observation parameters, namely enzyme activity test, protein content test, and temperature characterization. The treatment in this study was the addition of ammonium sulphate with several concentrations as:
K1: Addition of ammonium sulphate with a concentration of 30%;
K2: Addition of ammonium sulphate with a concentration of 40%;
K3: Addition of ammonium sulphate with a concentration of 50%;
K4: Addition of ammonium sulphate with a concentration of 60%;
K5: Addition of ammonium sulphate with a concentration of 70%;
K6: Addition of ammonium sulphate with a concentration of 80%;
K7: Addition of ammonium sulphate with a concentration of 90%.

Before the precipitate was centrifuged, a mixture of enzymes and ammonium sulphate at various concentrations is left in the refrigerator at 4°C for overnight aiming to give plenty time to the enzyme to precipitate all cellulase enzymes. On the next day, the precipitation then centrifuged at 3000 rpm for 30 minutes, supernatant then precipitated by adding 0.05 M pH 5 phosphate buffer two time until the pellet produced. Then the precipitation is carried out using centrifugation at a speed of 3000 rpm to remove the remaining salt from the protein. The enzyme deposits which have been concentrated with ammonium sulphate will be calculated by concentrating cellulase enzyme activity and protein content.

2.3. Cellulase enzyme activity assay (crude enzyme, after precipitation, and characterization enzyme)
The enzyme cellulase enzyme assay was refers to previous protocol [13]. Before the crude cellulase enzyme concentration is extracted, the activity will be tested based on its absorbance. In total, 1.8 ml of CMC substrate were dissolved in a pH 5 phosphate buffer, then 0.2 ml of the enzyme crude cellulase extract was added and stirred using a magnetic stirrer, followed with incubation for 30 minutes at 50°C, and the enzyme reaction was stopped by boiling at 100°C for 15 minutes. After that, 1 ml of reaction mixture was taken and 1 ml of DNS was added, then boiled at 100°C for 15 minutes. After the solution was cold, the absorbance is measured at λ575 nm.

Control and blank treatments are carried out simultaneously with the same methods and stages. In the control, the enzyme that will be reacted with the substrate has been inactivated by heating the enzyme for 30 minutes in boiling water. In blanks, the enzyme solution is replaced with distillate water to be reacted with the substrate.

Cellulase activity is expressed in international units, namely U/ml. One unit is the amount of enzyme needed to break 1 μmol cellulose into reducing sugar per minute under test conditions. Then cellulase activity was calculated based on pervious protocol [14]. This test was carried out for enzyme characterization at optimum temperature and enzymes activity on various substrates.

3. Results and discussion

3.1. Extraction cellulase enzyme from hepatopancreas of snail (Achatina fulica)
Cellulase enzyme extraction from hepatopancreas snail (Achatina fulica) was obtained by supernatant which is a crude brown cellulase extract enzyme and obtained as much as 450 ml of 500 ml of cellulase enzyme solution produced by centrifugation. The crude extract cellulase enzyme was then tested for activity using CMC substrate.

The activity cellulase enzyme crude extract was obtained at 0.123 U/ml. The crude extract cellulase enzyme activity is much lower than the research conducted by [9] which is 2.75 U/ml. Different values of cellulase enzyme activity can be caused because the method of testing crude extract cellulase enzyme activity in this study is different from that carried out by [9], that the incubation time of enzyme testing is much longer than the incubation time conducted in this study. However, the indicator of increased cellulose activity in both studies is influenced by the amount of sugar formed. Incubation time causes the duration of hydrolysis, which is the reaction time required by an enzyme to hydrolyse complex substrate to be simpler. The longer the hydrolysis, the higher the value of the cellulase enzyme activity produced.

The thing that really determines the low level of crude extract cellulase enzyme activity is that due to the crude extract cellulase enzyme there is still another impurity for the cellulase enzyme
concentration. One concentration that can be done is to use ammonium sulphate so that cellulase enzyme activity can be increased.

3.2. Concentration of cellulase enzymes using ammonium sulphate ((NH₄)₂SO₄)

Cellulase enzyme concentrations of crude extracts were carried out to increase cellulase enzyme activity and determine the effect of ammonium sulphate addition. Addition of ammonium sulphate with a certain concentration can increase cellulase enzyme activity. Based on the results of variance, it was found that the concentration of cellulase enzymes using ammonium sulphate had a significantly different effect (P> 0.05) on the cellulase enzyme activity produced. The average cellulase enzyme activity after further testing with DNMRT at 5% level can be seen in Figure 1.

![Figure 1. Graph of cellulase enzyme activity after precipitation using ammonium sulphate](image)

Figure 1 showed that concentration using ammonium sulphate has a significantly different effect on cellulase enzyme activity. The lowest cellulase enzyme activity was found in 30% ammonium sulphate concentration, which was 0.137 U/ml. This is because in the concentration of 30% ammonium sulphate there are many non-enzyme proteins which can interfere with cellulase enzyme activity and cellulase enzymes have not been fully deposited by ammonium sulphate. The addition of ammonium sulphate with low saturation causes the ionic salt produced to cover protein molecules and prevent the integration of protein molecules (salting in) so that the protein remains dissolved but not completely [15].

The highest cellulase enzyme activity was found in 50% ammonium sulphate concentration of 0.608 U/ml. This is because the concentration of ammonium sulphate 50% cellulase enzyme has precipitated so that the cellulase enzyme activity becomes high. The addition of high concentrations of ammonium sulphate causes an increase in the electrical charge around the protein which will attract the water mantle from the colloidal protein, the hydrophobic interaction that occurs between protein molecules will reduce protein solubility (salting out).

However, at the concentration of ammonium sulphate 60% cellulase enzyme activity decreased by 0.489 U/ml. This is because most of the cellulase enzyme activity is lost in the precipitation process with increasing ammonium sulphate concentration. The addition of ammonium sulphate with a higher concentration causes a decrease in the speed of the enzymatic reaction because ammonium sulphate acts as an inhibitor that can interfere with enzyme activity [10].

3.3. Protein levels and specific activity cellulase enzyme

Determination of protein content in enzymes was measured to find out that enzyme protein is still present in every ammonium sulphate deposit (protein is not lost during concentration) with a fixed or good activity. The results of variance showed that the concentration of cellulase enzymes using ammonium sulphate had a significantly different effect (P> 0.05) on the level of protein produced. The highest protein levels were found at 30% ammonium sulphate concentration, which was 0.686 mg/ml.
This is because in the concentration of 30% ammonium sulphate there are many non-enzyme proteins and other impurity proteins besides cellulase enzyme. However, having a low cellulase-specific enzyme activity is 0.201 U/ml. This is in accordance with the opinion of [16] which stated that high protein levels with low activity levels are caused by contaminant proteins (proteins other than enzyme proteins).

The highest specific activity was found in ammonium sulphate with a concentration of 50% which was 0.970 U/mg protein with protein level was 0.628 mg/ml. This allows the cellulase enzyme protein to settle and the other proteins have been separated, so that at a concentration of 50% cellulase enzyme activity becomes high. The lowest protein content was found at 90% ammonium sulphate concentration of 0.314 mg/ml. This is due to the higher saturation of ammonium sulphate which is added causing a decrease in the speed of enzyme reaction because ammonium sulphate acts as an inhibitor which can damage the protein so that the resulting protein content becomes low. The effect of NH₄⁺ ions resulting from the deposition of cellulase enzymes can interfere with the determination of protein levels [17].

3.4. Optimum temperature of cellulase enzyme results of concentration

Temperature affects the enzymatic reaction. Determination of the optimum temperature is determined by varying various temperatures. Optimum temperature is the most appropriate temperature for a reaction that uses enzymes [18]. The effect of temperature on cellulase enzyme activity can be seen in Figure 2.

![Figure 2](image)

Figure 2. The optimum temperature of the cellulase enzyme produced by ammonium sulphate concentration

Figure 2 showed that the optimum temperature cellulase enzyme activity was 50°C with cellulase enzyme activity of 0.051 U/ml. The temperature area where the stability and activity of the enzyme is large enough is called the optimum temperature for the enzyme concerned [8]. This is consistent with research conducted by [9] which stated that the optimum temperature of cellulase enzyme activity from snail is at a temperature of 50°C. However, the same study was conducted by [8], about cellulase enzymes from snail having different results, namely cellulase enzyme activity from snail working at 37°C. This is because [8] did the dilution of the cellulase enzyme before the cellulase enzyme activity was tested so that the cellulase enzyme had an optimum temperature compared to this study.

Optimal temperature the interaction between enzyme and substrate are very effective so that the formation of enzyme substrate complexe and the resulting product increases [19]. Therefore, the activity of cellulase enzymes produced at an optimum temperature of 50°C is very high. The temperature range of 45-80°C belongs to the thermophilic enzyme group [20]. Based on this research, the cellulase enzyme from snail was thermostable (resistant to heat).

The temperature range is below the optimal temperature causing the cellulase enzyme activity to decrease. At 30°C the cellulase activity was lower than at a temperature of 50°C which was 0.012 U/ml. This is because the temperature range below the optimal temperature causes a lack of enzyme
collisions in hydrolyzing the substrate so that the resulting cellulase enzyme activity becomes low. The incubation temperature is directly proportional to the collision that occurs between enzyme molecules and the substrate. When the temperature is low, the collisions between molecules become reduced so that the substrate molecules that can bind to the active side of the enzyme become small. Therefore, the amount of substrate hydrolyzed by cellulase enzyme is also small so that the cellulase enzyme activity produced is low [21].

Increasing temperature will cause a decrease in cellulase enzyme activity. This is because the temperature is too high causes the cellulase enzyme to become damaged or denatured so that the enzyme molecules become large and break the secondary bonds that retain the enzymes in their original form so that the cellulase enzyme activity decreases. This is evidenced by the decrease in the value of cellulase enzyme activity after the optimum temperature of 50°C, at 90°C the cellulase enzyme activity decreased from 0.051 U/ml at 50°C to 0.010 U/ml. An increase in temperature can also affect hydrogen bonds or hydrophobic interactions that play a role in maintaining the enzymes of the enzyme. Changes in communication will affect the active side of the enzyme, certain heat conditions cause the hydrogen bond to break. The breakdown of a hydrogen bond will cause easy termination of the hydrogen bonds further in the peptide chain so that the enzyme protein is denatured [22].

3.5. **Cellulase enzymes concentration on various substrates**

Testing of cellulase enzyme activity on various substrates was carried out using various kinds of cellulose namely bagasse, banana peel, corn cobs, and CMC. The number of substrates used to test cellulase enzyme activity is to see the activity of cellulase enzymes to form glucose against agricultural waste cellulose. Testing of cellulose hydrolysis of agricultural waste substrates was carried out by adding cellulase enzymes with agricultural waste substrates. The results of cellulase enzyme hydrolysis of agricultural waste substrates can be seen in Figure 3.

![Figure 3. Effect of various substrates on activity cellulase enzyme from ammonium sulphate concentration](image)

Figure 3 showed that the highest activity on various substrate on the CMC substrate with cellulase enzyme activity of 0.396 U/ml. This showed that cellulase enzymes on CMC substrates work well in forming glucose. The high cellulase enzyme activity in hydrolyzing CMC substrate in this study is almost the same as the research that has been done by [23] cellulase enzyme activity using CMC substrate has an activity of 0.0219 U/ml. Cellulase activity using CMC substrate was once carried out by [24] where cellulase enzyme activity hydrolyzed CMC substrate by 236 U/ml. This is because the use of CMC as a substrate for hydrolyzing cellulose has much different enzyme activity compared to other substrates. CMC is a material that contains pure cellulose and has a higher viscosity than other cellulose.
The highest activity was also shown on the corn cob substrate which was 0.131 U/ml. The high cellulase enzyme activity on corn cob substrate is caused by the high cob cellulose content of corn cobs which is 65.96%, however, there is a lignin content in corn cobs which can inhibit cellulase enzyme activity in hydrolyzing corn cob substrate so that the cellulase enzyme activity is much lower than cellulase enzyme activity on CMC substrates.

The lowest cellulase enzyme activity was found in bagasse waste substrate which was 0.067 U/ml. This is because the bagasse still has lignin and hemicellulose content which can interfere with cellulase enzyme activity. This is different from the research conducted by [25] which utilizes bagasse waste as a cellulase enzyme substrate cellulase enzyme activity obtained at 2,285 U/ml. This is because in the research [25] conducted a preliminary study of bagasse substrate in the form of delignification by adding 2% NaOH solution to reduce the lignin and hemicellulose content of bagasse substrate then add nutrients and minerals in the form of concentrated solutions containing NaH2PO4, CaCl2, and KCl on the bagasse substrate which functions as a buffer in the enzyme reaction so that the cellulase enzyme activity in hydrolyzing bagasse substrate becomes high. Whereas in this study no preliminary process in the form of delignification was carried out.

The difference in cellulase enzyme activity in hydrolyzing substrate of agricultural waste is due to the different cellulose content in agricultural waste used, the higher the cellulose content, the higher the glucose level is formed. Agricultural waste that is used should have high cellulose content because it affects the glucose levels produced where if the cellulose content is high, the cellulose content produced is also high [26]. Enzyme activity depends on the content and structure of each different substrate. CMC is a pure cellulose substrate in the form of amorphous, therefore, on the substrate CMC enzyme cellulase which is active is an endo-1,4-β-glucanase cellulase enzyme [27].

4. Conclusions
Based on the results it can be concluded that the concentration of cellulase enzymes using ammonium sulphate has effect on cellulase enzyme activity. The highest cellulase enzyme activity was found at 50% ammonium sulphate concentration, which was 0.608 U/ml greater than the crude extract cellulase enzyme which was 0.123 U/ml. Optimum temperature activity of the cellulose enzyme concentrated was 50°C and the highest activity cellulase enzyme at Carboxy Methyl Cellulose (CMC) was 0.396 U/ml.

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