Immobilization of Cow Rumen Fluid Cellulase by Entrapment in Calcium Alginate Beads

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Abstract :

Cellulases represent a class of enzyme which provide a key opportunity for achieving tremendous benefits of biomass utilization. Cellulase can be produced by fungi, bacteria or Actinomycetes. Cow rumen fluid is a potential source of cellulase since cow rumen fluid is contain various kind of cellulolytic bacteria. Industrial application of enzyme is often hampered by a lack of long-term operational stability and difficult recovery and re-use of the enzyme. These drawbacks can be overcome by immobilization of the enzyme. In this research, we isolated enzyme from cow rumen fluid and immobilized it in calcium-alginate beads. The aim of the experiments was to investigated the effect of process parameters towards the cellulase activity. The process parameters are include: sodium alginate concentration (1%-4%), and temperature (40-55 ^{0}C). The immobilization was conducted in various condition and the free and immobilized enzyme were assayed by using DNS method. The research result showed that the activity of free cellulase was recorded 5,2 unit/ml. It was found that concentration of 3 % (w/v) sodium alginate solution gave the highest cellulase acivity, 67.41 unit/ml. While the optimum temperature for immobilization of cow rumen cellulase was 55 ^{0}C. Immobilization of cow rumen cellulase was able to increase the thermal stability of the enzyme.

Keywords: alginate, beads, cellulase, cow rumen fluids, immobilization.

1. Introduction

Cellulase, a multicomponent enzyme, consisting of three different enzymes (endocellulase, cellobiohydrolase and ß-glucosidase) is responsible for the depolymerization of cellulose into fermentable sugars **[1,2]**. Thus, cellulase provide a key opportunity for achieving tremendous benefits of biomass utilization.

Cellulase has been used in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textile, and laundry **[3,4,5,6]**. One of the potential utilization of cellulases is the extraction of natural compound through cell wall degradation **[7,8,9,10,11]**. Today, these enzymes account for approximately 20% of the world enzyme market **[12,13]**.

The utilization of enzyme offer various advantages including highly specific, mild operation condition, high reaction rate, only small amount of enzymes are needed, the reactions are easily controlled, and reduce the impact of manufacturing on the environment by reducing the consumption of chemicals, water and energy, and the subsequent generation of waste **[14]**. But, despite of all the advantages descibed above, the use of enzymes has been limited by some factors, mainly their stability, high cost of production, availability in small amounts, non-reusability, high sensitivity to several denature agents, and expensive to recover them from reactor effluents at the end of the catalytic process because of their solubility **[15]**. Many of these undesirable constraints may be removed by the use of immobilized enzymes.

Immobilized enzyme are widely used in different industries. It is offer several advantages including processes can be readily controlled and operated continuously, provides higher purity and product yields, greater pH and thermal stability, product inhibition is less apparent, effluent problems are minimized, products are easily separated, greater flexibility in reactor design and catalyst can be reused **[15]**.

Commonly, enzyme immobilization is carried out by three principle means, matrix assisted entrapment of enzyme, adsorption on a solid support, ionic or covalent binding. Entrapment is a physical method to immobilize or physical enclosure of enzymes in a small space. Enzyme remains free in the solution, but restricted in movement by the lattice structure of a gel. Pore size of the gel lattice is controlled so that the structure is tight enough to prevent enzyme leakage while allowing free movement of substrates and products **[16]**. Entrapment is considered as the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability in microenvironment of matrix, and protects enzyme from microbial contamination. Entrapment of the enzyme in calcium alginate is one of the important methods of immobilization. Entrapment

within insolubme calcium alginate gel is recognized as a rapid, nontoxic, inexpensive and versatile methods for immobilization of enzyme **[17-19]**.

Moreover, cellulases can be produced by fungi, bacteria or Actinomycetes **[20,21]**. But currently, most commercial cellulases are produced by Trichoderma species and Aspergillus species **[3]**. Finding new sources of cellulases in an important point in improving the cellulase production. Cow rumen fluid is reported as a rich source of cellulolytic bacteria **[22]**. Thus, we can isolate cellulases from it. Cellulases of cow rumen fluid are offer various advantages over commercial enzyme including more stabile at higher temperature, higher specific activity, higher optimum pH and inexpensive **[23]**. The present study deals with the immobilization of cow rumen fluid cellulase by entrapment in calcium alginate beads. The conditions of entrapment like concentration of sodium alginate and temperature were studied.

2. Material and Methods

2.1. Sample collection

The main material in this study was rumen fluid of local Indonesian cow from the Penggaron Slaughtering House, Semarang. Rumen fluid sample was obtained from gut right after cow's death by filtering into a prewarm (39°C) thermos flask **[24]**. Oxygen was removed from gas by filling CO_2 into flask and covering it with a sterile butyl rubber stopper.

2.2. Isolation of cow rumen fluid cellulase

Cow rumen fluids were collected and centrifuged at 10.000 g for 10 minutes at 4 ^{o}C in order to separate the supernatan from the cell and cell debrix. The supernatan then taken as crude enzyme. The supernatan was reacted with 60% ammonium sulphate and agitated in a magnetic stirrer for an hour and stored for a night at 4 ^{0}C. The supernatan was centrifuged at 10.000 g for 15 minutes at 4 ^{o}C. Phosphat buffer pH 7.0 was added into the sedimen of enzyme in 1:10 ratio.

2.3. Immobilization of Cellulase

The enzyme solution was mixed with sodium alginate solution (1%) in 1:2 ratio. The cellulase-alginate mixture was added dropwise into calcium chloride (0.2 M) solution with continuous shaking at 4 ^{0}C. As soon as the drop of cellulase-alginate solution mixed with $CaCl_2$ solution, Na^+ ions of sodium alginate were replaced by the Ca^{2+} ions of $CaCl_2$ solution, which finally formed Ca-alginate beads. The beads thus formed were washed 3-4 times with deionized water and finally with 50 m Tris HCL buffer of pH 7.5. These beads were dried for further studies.

2.4. Enzyme Assay

The activity of Cellulase was assayed using DNS method **[25]**. The total reaction mixture of 2 ml contained 1 ml of 1 % (w/v) CMC solution in phosphat buffer (50 mM, pH 7) and 1 ml of the free enzyme or immobilized enzyme made from 1 ml of free enzyme. The reaction mixture was incubated at 50 °C for 30 min. After incubation, the enzyme activity was stopped by adding 3 ml DNS reagent; tubes were then placed in a water bath at 90 °C for 15 minutes, 1 ml of sodium potassium tartrate was added to each tube before cooling. The adsorbant of samples was immediately measured at 575 nm using spectrophotometer.

The enzyme activity was then calculated based on formula:

$$\text{enzyme activity} = \frac{\text{glucose concentration x dilution factor}}{\text{mass molecule of glucose x incubation time}}$$

One unit of cellulase activity is the amount of enzyme that release µmol of glucose in 1 minute of the assay. The dilution factor was 1, the mass molecule of the glucose was 180 and the incubation time was 30 minutes.

2.5. Effect of sodium alginate concentration

For studying the effect of sodium alginate concentration, the enzyme assay was carried out at four different sodium alginate concentrations (1,2,3 and 4 %).

2.6. Effect of temperature

For investigating the effect of temperature, the enzyme assay was carried out at four different temperatures (40, 45, 50, 55 and 60 °C).

3. Result and Discussion.

3.1. Effect of sodium alginate concentration

Sodium alginate is a polymer (long molecules made by attaching one after the other a large number from one or several small molecules) made of two carbohydrates. This polymer comes along with sodium ions. Alginates are able to produce gels with cations. The most suitable divalent cation is calcium due to its low toxicity. The introduction of calcium chloride into a solution of sodium alginate can cause a gel or precipitate instantaneously. Typically, alginate beads are made by dripping aqueous sodium alginate solution into a solution of calcium ions. The calcium ions induce a cooperative effect between G-blocks of alginate to form a 3D network which is known as the "egg-box mode (Fig. 1) **[26]**. Then, Ca-alginate beads are accordingly produced after the gelation reaction **[27]**.

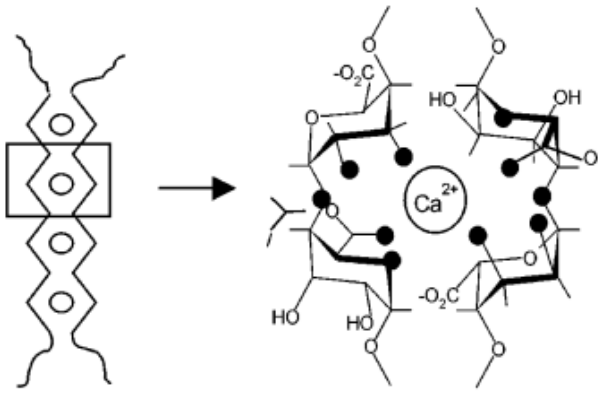

Figure 1. The "egg-box model for alginate gelation with calcium ions **[26]**

The proper sodium alginate concentration are needed to form good spherical beads. When the concentration of sodium alginate is too low, the beads cannot be obtained, but the fragments were formed. When the concentration is too high, the beads can be obtained, but the beads are in form of oval **[28]**. It also has been reported that the sodium alginate concentration effect the porosity of the calcium alginate beads **[29]**. More over beads porosity affect the stability of the alginate beads produced. So various concentrations of sodium alginate (1-4 %) were studied in order to acquire beads with greater stability.

The Figure 2 clearly showed that the cellulase activity was found to be highest (unit/ml) at concentration of 3% (w/v) sodium alginate solution. The activity of the immobilized cellulase at all point in Figure 2, were higher than the actiivty of free cellulase. The activity of free cellulase was recorded 5,2 U/ml. As seen in Figure 2, the cellulase activity at sodium alginate concentration of 1, 2 and 4 % was found lower than 3 %. It might be due to at the lower percentage sodium alginate solution the beads produced were smaller than the beads produced at concentration of 3 % (w/v) sodium alginate solution. The smaller the size of the alginate beads the lower the pore size of the beads. The smaller the pore size of the beads the more difficult for the enzyme and the substrate to diffuse in and diffuse out of the alginate gel matrix. While leakage of the enzyme might be the reason for the declining activity of cellulase in alginate beads made from concentration of 4 %, because the greater the sodium alginate concentration, the larger the pore size of the beads.

Similar result are reported by several research, including in the immobilization of rifamycin oxidase in alginate beads **[30]**. They mentioned that sodium alginate concentration of 3% was found to be the optimum concentrations for maximal biotransformation. Moreover, the immobilization of protease obtained from a newly isolated strain of Bacillus subtilis KIBGE-HAS in Ca-Alginate showed that the best beads were formed with 2% alginate concentration. 3% and 4% were too viscous, hence decreased the pores size and thus hindered the penetration of substrate in to the beads. While Maximum leakage of enzyme from beads occurred at 1% (w/v)

sodium alginate concentration owing to the larger pore size of the less tightly crossed linked fragile Ca-alginate beads **[19].**

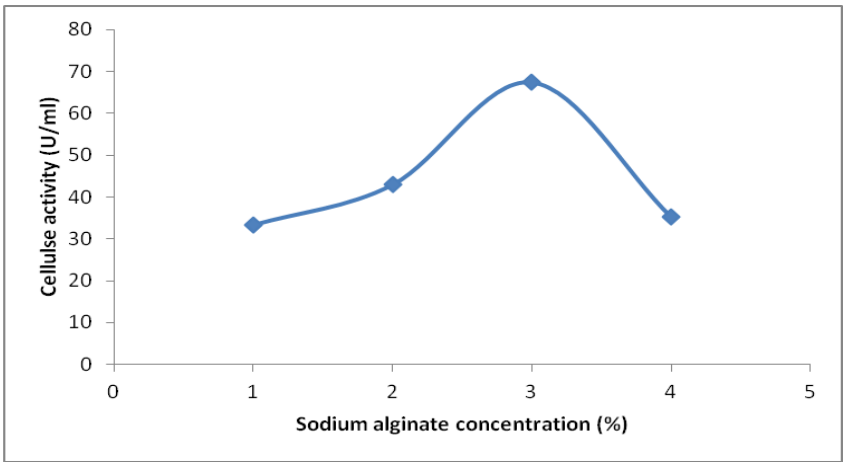

Figure 2. Effect of sodium alginate concentration on immobilized cellulase activity

3.2. Effect of temperature

In order to effectively facilitate the immobilization reaction and prevent enzyme deactivation at higher or lower reaction temperatures, it is very major to investigate thermal stability of immobilized enzyme. As observed in most of the chemical reactions, an increase in temperature increases the rate of reactions but it is different for proteins because the stability of a protein decreases due to the thermal inactivation.

The effect of temperature on the activity of immobilized cellulase was determined in the temperature range of 45-60 ^{0}C. As given in (Figure.3), the optimum temperature recorded was at 55 ^{0}C. Immobilization of enzyme was reported able to increase the thermal stability of an enzyme. It was reported that free cellulase of cow rumen fluid was optimum at 50 ^{0}C **[31]**. The optimum temperature for free enzyme is varied from another. It is depend on the microorganism which produced it. Cellulase of cow rumen fluid is produced by cellulolytic bacteria found in cow rumen. The cellulolytic bacterial organism are including Bacillus subtilis, Bacillus circulans, Clostridium cellobioparum, Erwinia spp and Clostridium thermocellum **[22]**. Clostridium thermocellum recorded optimal cellulolytic activities at 50 ^{0}C and pH 6. Hence, it is proved that immobilization of cow rumen cellulase able to increase the thermal stability of the enzyme.

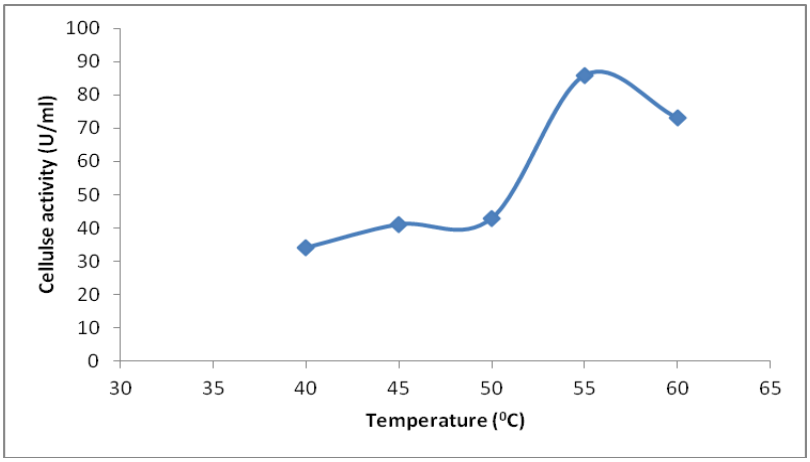

Figure 3. Effect of temperature on immobilized cellulase activity

The increasing thermal stability of an immobilized enzyme was also reported by several researchers. The immobilization of amylase was able to increase the optimum temperature from 50 ^{0}C to 57 ^{0}C **[29]**. However, the

same optimum temperature found for both free and immobilized enzyme was also reported. The optimum temperature of activity for both free invertase and immobilized invertase in alginate gel capsule was 60 °C **[32]**, and in the immobilization of protease from Newly Isolated Strain of Bacillus subtilis KIBGE-HAS, it was observed that optimum temperature of entrapped enzyme was 50°C. The result showed no change in temperature and pH of protease before and after entrapment **[19].**

4. Conclusions

Cellulase isolated from cow rumen fluid has been immobilized in alginate beads. The activity of free cellulase was recorded 5,2 unit/ml. It was found that concentration of 3 % (w/v) sodium alginate solution gave the highest cellulase acivity, 67.41 unit/ml. While the optimum temperature for immobilization of cow rumen cellulase was 55 ^{0}C. Immobilization of cow rumen cellulase was able to increase the thermal stability of the enzyme.

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