Preliminary Study of Immobilized of Cellulase in Silica from the Rice Husk Ash to Hydrolysis Sugarcane Bagasse

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Abstract: Cellulase in the production of bioethanol from sugarcane bagasse is used to hydrolyze cellulose into reducing sugars. Cellulase needs to be mobilized in a matrix to improve its efficiency because it can be used repeatedly. The purpose of this study was to conduct a preliminary study of potential cellulase immobilized on silica to hydrolyze sugarcane bagasse including the effect of contact time (15, 45, 60, and 75 minutes) and agitation speeds (50, 100, 150, and 200 rpm) on % immobilization and immobilized cellulase activity against sugarcane bagasse, and also decreased activity of immobilized cellulase after repeated use. Contact time and agitation speeds do not affect % immobilization. The optimum contact time and agitation speeds of immobilized cellulase formation based on its activity were at 15 minutes and 100 rpm. Immobilized cellulase activity in cycles II and III decreased to 75.2% and 58.8% compared to the first cycle. Therefore, immobilized cellulase in silica is good enough to hydrolyze sugarcane bagasse and has the potential to be applied as continue system in the production of bioethanol from sugarcane bagasse.

Keywords: cellulose, immobilized, silica, sugarcane bagasse

1. Introduction

Cellulase is an enzyme that can hydrolyze glycosidic bonds in β-1,4 cellulose [1], and used in various industries, namely the textile, detergent, paper and pulp, food, animal feed, and bioethanol industries [2]. Cellulase also plays an essential role in the production of bioethanol from lignocellulose materials. Bioethanol production from lignocellulose involves pretreatment, hydrolysis, fermentation, and separation. The hydrolysis stage is the most crucial stage. Chemically using lignocellulose hydrolysis is less effective than enzymatically. Chemically hydrolysis of cellulose must be uses concentrated acids and produce several inhibiting compounds for fermentation processes such as acetate, hydroxybenzaldehyde, 5-hydroxymethylfurfural, syringaldehyde, acetate, furfural, and vanillin. Enzymatically hydrolysis using cellulase has a higher conversion efficiency than chemical (acid) hydrolysis because the production of inhibiting compounds is reduced, there is no loss of substrate, low cost does not require acid recovery, and favorable conditions such as low temperature and neutral pH [3]. It makes the technology of lignocellulose hydrolysis for the production of bioethanol using cellulase is widely used.
Cellulase hydrolyzes cellulose in lignocellulose into reducing sugar, and then the reducing sugar is fermented into bioethanol [4]. Sugarcane bagasse is one of the most widely studied lignocellulosic materials for use as a raw material for bioethanol [5–7]. Unfortunately, the use of cellulase is still in the form of free crude enzymes. The crude enzymes of cellulase are contacted directly with sugarcane bagasse during the hydrolysis process so that the enzymes can only be used once. Of course, this is economically inefficient. Increased efficiency of the use of cellulase can be attempted by using cellulase immobilization by limiting the movement of cellulase in a space. The use of immobilized cellulase can be carried out more than one process so that it can increase the effectiveness of the use of cellulase.

Various methods have been developed to produce immobilized cellulase which can be used repeatedly. [8] showed cellulase immobilization by covalent bonding method using a mixture of chitosan, L-glutamic acid and glutaric dialdehyde (1%), a combination of chitosan and 4-aminobutyric acid and glutaric dialdehyde (1%) and a combination of chitosan and glutaric dialdehyde (1%) is good enough to immobilize cellulase. The weakness of this method is the high possibility of enzymes becoming inactive because of the possible covalent bond between the mixture and the active center of the enzyme. [9] stated that cellulase immobilized using encapsulation method using binary and tertiary mixtures of tetramethoxysilane effectively converts cellulose to glucose. But, this method is quite expensive for development to industrial scale. [10] used the adsorption method to immobilize cellulase in activated carbon. This method is relatively simple; unfortunately, the price of activated carbon which is increasingly expensive causes many researchers to explore other materials for immobilization of cellulase in an absorptive manner, one of which is silica.

Silica is a porous material that has a wide surface area, composed of active groups in the form of silanol (-SiOH) and siloxane (Si-O-Si). Its porosity causes silica to act as a high-grade adsorbent, besides that the active silanol (-SiOH) group in silica allows silica to interact with other molecules through hydrogen bonds [11,12]. Silica can be extracted from rice husk ash as its abundant and contains a lot of silica (almost 90%). It has been studied how the potential of silica to immobilize cellulase including the effect of contact time and agitation speeds of immobilization proceed on % immobilization and immobilized cellulase activity against sugarcane bagasse, and decreased the activity of immobilized cellulase after repeated use.

2. Methods

The materials used were Cellulase of *Trichoderma viride* from Merck (219466), rice husk ash from Karangploso-Batu Balittas, NaOH pa (Merck), H₂SO₄ pa (Merck), NH₄OH pa (Merck), pH 7 phosphate buffer, aquades, Na₂CO₃ pa (Merck), Rochelle salt, NaHCO₃ pa (Merck), Na₂SO₄ pa (Merck), CuSO₄ pa (Merck), arsenumolobdate reagent, Carboxymethyl Cellulosa (CMC), ammonium molybdate pa (Merck), Na₃HAsO₄.7H₂O pa (Merck), filter paper rough, and Whatman filter number 1.

Sugarcane bagasse is commonly referred to *blotong*, the waste which comes from the end of the extortion of sugarcane bagasse in sugar mills, located in the PG Kebon Agung at Highway Pakisaji-Malang. The material was stored in a plastic bag at a temperature of about 5 °C. Before the process began, the treated bagasse was washed and squeezed in the filter cloth. The washing process was repeated several times to remove residual soil and other impurities, to obtain clean dry bagasse (48% of the weight before squeeving). The silica was extracted from the rice husk ash by dissolving the ash into a strong base before back gelation by strong acid with some modification refers to [13].

As much as 0.25 g of the silica matrix was contacted with 10 mL of enzyme solution (0.25% w/v). The mixture was then homogenized using a shaker with a speed of 100 rpm for a time variation of 15 minutes, 45 minutes, and 60 minutes to determine the effect of contact time on % immobilized and immobilized cellulose activity [10]. The mixture was filtered to separate the filtrate from the residue. Free enzyme protein levels and protein levels in the filtrate were determined by the Lowry method. The % immobilization value was determined by comparing the difference between free protein content and protein content in the filtrate with free protein levels. The residue produced at the separation was the immobilized cellulase. Determination of the activity of immobilized cellulase enzymes based on
Reducing sugars produced in the enzymatic process between sugarcane bagasse and immobilized cellulase refers to [14]. The positive control was the free enzyme, and the negative control was silica. The effect of stirring speed on % immobilized and immobilized cellulase activity was carried out in the same way but by varying the stirring speed of 50 rpm, 150 rpm and 200 rpm. The data obtained were analyzed statistically with ANOVA methods. The decrease in immobilized cellulase activity against sugarcane bagasse was observed by measuring the activity of immobilized cellulase against bagasse in three cycles. The decrease in activity was determined relative to activity in the first cycle.

3. Results and Discussion

3.1. Cellulase immobilization in silica

In this experiment showed that there was a decrease of protein concentration in the free enzyme (cellulase) solution compared to the filtrate obtained after the immobilization process which was from 19.4 µg/mL to 7.7 µg/mL. On the other side, the mixture of silica and buffer as a negative control contained very low of protein as 0.1 µg/mL (See Table 1). These results indicated that the protein in free enzymes solution has interacted with silica. This opinion was supported by the activity of cellulase on sugarcane bagasse which an increase in cellulase activity of immobilized cellulase as 24.5 U/mL compared to silica 3.9 U/mL (See Table 2). Silica is composed of active groups namely silanol (-SiOH) and siloxane (Si-O-Si) which form pores with a large surface area. The FT-IR silica spectrum extracted from rice husks showed the presence of these clusters and from BET analysis known that surface area was 13.514 m²/g [13]. The presence of OH groups on silica caused silica to be an adsorbent. Silica can interact attractively with other molecules around the surface of silica such as cellulase. This interaction can be in the form of Van Der Waals forces, dipole-dipole, and the hydrogen bond between the hydroxy group on silica and the side chain of amino acid residues on the surface of cellulase. Cellulase is a globular protein that dissolves in water. Generally, globular proteins have a hydrophilic outer surface because the side chains of uncharged polar amino acid residues (serine, tyrosine, methionine) and charged polar (glutamic acid, aspartic acid, lysine, arginine, and histidine) are exposed to the environment (water). Side chains of these amino acid residues can form hydrogen bonds and dipole-dipole interactions with silanol groups in silica.

Table 1. Comparison of the protein content of free enzyme solution, filtrate after immobilization process and silica mixture with buffer.

| The solution                          | Concentration of protein (µg/mL) |
|--------------------------------------|----------------------------------|
| Free enzyme solution                  | 19.4                             |
| Filtrate after the immobilization process | 7.7                              |
| Silica and buffer mixture             | 0.1                              |

Table 2. Changes in cellulase activity of silica and immobilized cellulase in silica

| Sample                      | Activity of enzyme (U/mL) |
|-----------------------------|---------------------------|
| Silica                      | 3.9                       |
| Cellulase immobilized in silica | 24.5                      |

3.2. Effect of contact time on percentage immobilized and immobilized cellulase activity

The contact times of 15, 45, 60 and 75 minutes did not produce a significant effect on percentage immobilized (Figure 1). It was assumed that this result was due to the equilibrium of the cellulase adsorption process on silica was reached at the 15 minutes contact time, so the contact time of more than 15 minutes was not having a significant effect on percentage immobilized. The lowest percentage immobilized value of the treatment was 54%, and the highest was 62%, but immobilized cellulase activity against sugarcane bagasse was highest in the 15 minutes contact time treatment (Figure 2). Then the optimum contact time for immobilized cellulose formation in silica is 15 minutes.
3.3. Effect of shaking speed on percentage immobilized and immobilized cellulase activity

The shaking speed of 50, 100, 150 and 200 rpm did not produce a significant effect on percentage immobilized as shown in Figure 3. The lowest percentage immobilized value of the treatment was 21.8%, and the highest was 27.83%, but immobilized cellulase activity against sugarcane bagasse was highest at a treatment time of 100 rpm (Figure 4). Then the optimum shaking speed for the formation of immobilized cellulose in silica was 100 rpm.
The reusability of immobilized cellulase in the hydrolysis of sugarcane bagasse was examined by following the change in the activity with the repeated utilization in the enzymatic hydrolysis of sugarcane bagasse. The activity in each repeated use was determined and expressed as the percentage relative to that in the first use. The results obtained with the immobilized cellulase are shown in Figure 5. Figure 5 shows a decrease in immobilized cellulase activity in cycles II and III compared to cycle I. Immobilized cellulose activity in cycle II was 75.2% compared to cycle I and cycle III was 58.8%. The decrease was thought to be due to the interaction between cellulase only occurs on the surface of the protein so that it can be relatively disconnected after repeated use. Another possibility was because enzymes undergo conformational changes during the hydrolysis process so that they were released after hydrolysis reaction. Based on the matter, the use of silica potentially replaces activated carbon as an immobilizer for cellulase enzymes.
Figure 5. Reusability of immobilized cellulose onto silica in the hydrolysis of sugarcane bagasse

4. Conclusion
The contact time between cellulase and silica and the shaking rate has no effect on percentage immobilized. Immobilized cellulase with optimum hydrolysis activity of bagasse is immobilized cellulose produced at 15 minutes contact time and shaking 100 rpm. On three cycles of repeated use, there was a slight decrease in the ability to hydrolyze bagasse to 75.5 and 58.8%. These results indicated that silica was less effective to be used to immobilize cellulose.

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