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ORIGINAL RESEARCH

PRELIMINARY STUDY OF REDUCING SUGAR PRODUCTION FROM COCONUT HUSK BY ENZYMATIC HYDROLYSIS USING CHITOSAN IMMOBILIZED CRUDE AND COMMERCIAL CELLULOSE

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Abstract

The objective of this research was to study the production of sugar from coconut husk using immobilized crude and commercial cellulose, including temperature and mixing speed during immobilization. The enzyme from Aspergillus Niger was immobilized on chitosan alone and cross-linked with Glutaric Dialdehyde (GDA). Coconut husk waste was grinded and chemically pretreated using NaOH 1% (w/v). Fourier Transform Infrared Spectroscopy (FT-IR) measurement revealed that enzyme was covalently bonded to the support. Cellulose immobilized on chitosan cross-linked with GDA produced more sugar than immobilized on chitosan alone. Both the crude and commercial enzyme had their yield decreased after immobilization. Despite its less enzyme coupled on micro-sized chitosan, reducing sugar yielded by an immobilized enzyme on micro-sized chitosan had a competitive result with macro-sized chitosan. This may due to decreasing mass transfer resistance when using a smaller size of chitosan. Several important factors such as temperature, mixing speed, and purity of enzyme responsible for the performance of sugar produced from insoluble cellulose using cellulose immobilized on insoluble support was thoroughly discussed.

KEYWORDS:

Coconut Husk, cellulose, Crude, Immobilized, Reducing Sugar

1 | INTRODUCTION

Reducing sugar has the potential to be processed into various products such as biofuel, lactic acid, 5-hydroxymethylfurfural, and levulinic acid[1]. Coconut husk consists of 26.68% cellulose, 17.87% hemicellulose dan 41.2% lignin, which can be hydrolyzed to form reducing sugar. Enzymatic hydrolysis provides high selectivity, high yields, low energy costs, and mild operating condition. Free cellulose is utilized in this process due to the accessibility of this soluble enzyme to the cellulosic substrates[2]. However, the price of the free enzyme is the main obstacle for its applications. The immobilization of the enzyme could be an effective strategy to enhance its stability and enable the enzyme to reuse and reduce the price[3].

Polymeric substrates have been commonly utilized as support of immobilization because they have various functional groups and can be easily modified chemically. One polymeric support for enzyme immobilization is chitosan. Chitosan is a cheap, inert, and hydrophilic support. So that it is attractive for enzyme immobilization. The presence of amino groups enables covalent attachment of enzymes[4]. There were two general immobilized methods: chemical methods, where covalent bonds are established between the enzyme and support, and physical methods, where simple physical adsorption procedure is involved. It is one of the most used immobilization methods, but it has drawbacks in the weak interactions between support and enzyme[5].

Immobilization of cellulose on chitosan is affected by temperature and mixing speed during the process. Enzyme operates at its optimum temperature since it becomes idle when the temperature is too low and is denaturized when the temperature is too high. Mixing speed is vital to provide good contact and reaction during immobilization, but vigorous mixing speed makes the enzyme denatures due to shear[6].

Many studies have been conducted to immobilize cellulose in chitosan[7, 8]. But the substrate used in those studies was generally soluble cellulose, such as carboxymethyl cellulose (CMC). There was no comprehended study about the effect of immobilization conditions such as temperature and mixing speed. The utilization of crude enzymes and the addition of cross-linking substances have not been studied. This study outlines immobilization of enzyme into chitosan and chitosan cross-linked with GDA, hydrolysis of CMC and coconut husk waste and using the immobilized and free enzyme and investigated the effect of Several important factors responsible for the performance of sugar produced, i.e. temperature, mixing speed, and purity of enzyme.

2 | MATERIAL AND METHOD

2.1 | Material

Coconut husk was gained from Sulawesi, Indonesia. Coconut husk was pretreated mechanically and chemically. It was grinded, dried, and sieved to 100-120 mesh. Then, it was pretreated with NaOH 1% (w/v) for 16 h at 80° C. After that, it was washed using hot water and dried again for 24 hours at 60° C Aspergillus niger strain was from Biochemical Laboratory, ITS Surabaya, Indonesia. Potato Dextrose Agar, Yeast extract, Glucose, Bovine Serum Albumin, Coomassie Briliant Blue, Sodium citric, NaOH, Citric acid, CH3COOH, CH3COONa, Glutaric dialdehyde (GDA) were obtained from Merck, Germany. Chitosan flake technical grade was from PT. KIMINDO, Surabaya, Indonesia. Dinitro salicylic acid, Chitosan, CMC (carboxymethyl cellulose), Magnetic powder (Fe3O4), Commercial cellulose from Aspergillus niger were purchased from Sigma-Aldrich, Germany. Crude cellulose was produced based on procedure introduced in[9] using rice straw as a substrate.

2.2 | Preparation of Microparticle and Nanoparticle

Chitosan magnetic microparticles were prepared using the method[10] with some modification. Four grams of chitosan and 8 grams of magnetite particles were added in 200 mL of 0.2 M acetic acid. After they were completely dissolved, 1 M NaOH was added excessively to convert the solution into an insoluble magnetic chitosan microparticle. Chitosan nanoparticle was made as to the same concept of microparticle[11].

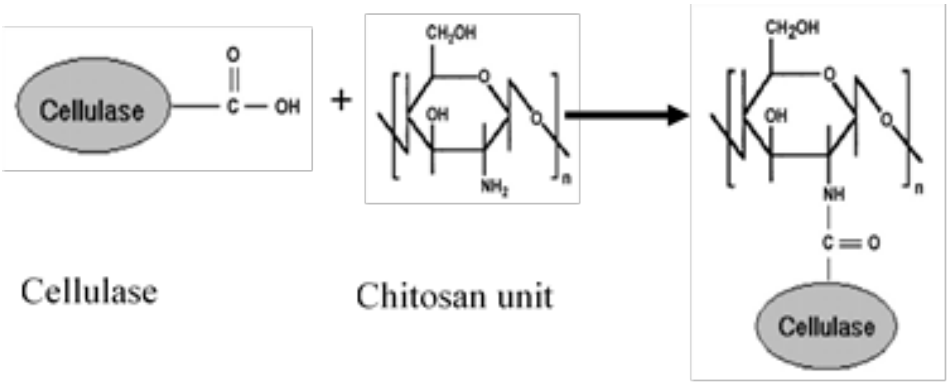

FIGURE 1 A covalent bond formed between cellulose and chitosan.

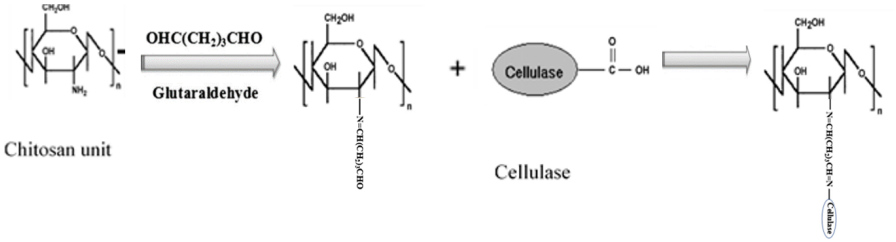

FIGURE 2 A covalent bond formed between cellulase and chitosan + Glutaral dialdehyde (GDA).

2.3 | Preparation of GDA + Chitosan

The 0.1 g Chitosan was added to a 10 ml 1% (v/v) GDA solution. It was kept in shaking incubator at 25°C, 125 rpm for 4h, and left at 25°C without shaking for 12h. The chitosan that had been cross-linked was filtered and washed with phosphate buffer pH 7.

2.4 | Immobilization of Enzyme

The 0.1 g Chitosan or Chitosan+GDA were supplemented to the enzyme solution, and the immobilization reaction was executed for 24 h at (20,30,37°C) in incubator shaker (100, 125, 175 rpm). The precipitates were separated and washed with phosphate buffer (pH7). They were stored at 4°C until use.

2.5 | Hydrolysis

The 0.1 g immobilized cellulose was added to 1 g pretreated coconut husk in 20 ml 0.1 M citric buffer pH 3. Hydrolysis was done in shaker incubator 35°C, 125 rpm for 48 hours. The same method was also used for 2 ml 1% Carboxymethyl Cellulose (CMC) but only for 10 minutes.

2.6 | Determination of Enzyme Stability

The 0.1 g immobilized cellulose and free cellulose containing the same amount of protein as the immobilized one was mixed with 2 ml of 1% CMC. It was reacted at pH (3, 4, 5.5, 7 and 10) and (15°, 30°, 35°, 45°, 50°, 60 °C) temperature.

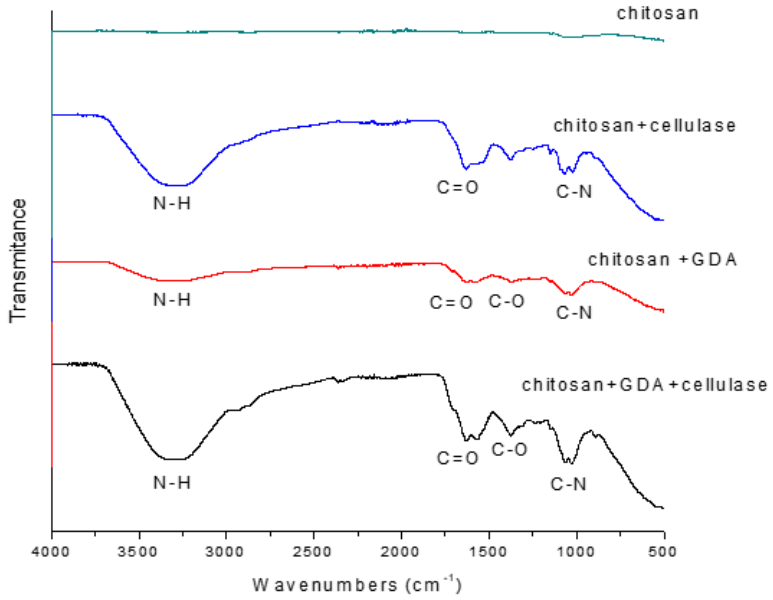

FIGURE 3 FTIR spectra of chitosan immobilized cellulose.

2.7 | Analytical Method

Using the Bradford method, the unbound enzymes were determined using Bovine Serum Albumin (BSA) as a standard solution. Reducing sugar was analyzed by Dinitro salicylic acid (DNS) method to obtain the concentration of sugar. FT-IR spectra are measured using FT-IR spectrometer (Thermo Scientific, US)

3 | RESULTS AND DISCUSSION

3.1 | Characterization of The Immobilized Enzyme by FT-IR

From previous research conducted[5], the reaction between chitosan and cellulose is described as in Figure 1 and chitosan+GDA in Figure 2 . It can be seen from the figure that amino groups of chitosan were bonded directly with the carboxylic terminal residue in the enzymes.

This reaction is also confirmed with Fourier Transform Infrared (FT-IR) spectra, as shown in Figure 3 . spectra from chitosan and enzyme immobilized on chitosan. From those figures, there was a significant change of the peak in wavelength 3272.53 cm-1 that is characteristic of the amino group (N-H), wavelength 1632.67 cm-1 that is characteristic of C=O, and wavelength 1080 cm-1 that is characteristic of (aliphatic amide) C-N[12]. These indicate that enzymes have been immobilized on chitosan.

FT-IR spectra of enzyme immobilized on chitosan-GDA can be seen in figure 3 . In that figure, there are also some significant change of peak in wavelength 3267.54 cm-1 (N-H), 1629.15 cm-1 (C=O), 1027.66 cm-1 (C-N) which is same as cellulose-chitosan and wavelength 1376.0 cm-1 (C-O) that indicated GDA as cross-linking agent and spacer arm in covalent bonding.

3.2 | Effect of Temperature, Solubility, and Mixing Speed on Immobilization Process

Table 1 and Table 2 exhibit the sugar resulted from CMC and Coconut Husk as the substrate with immobilized cellulose prepared in several operating conditions. Immobilized cellulose prepared at 30°C yielded more Sugar from CMC as a substrate than the one that prepared at 20°C. The effect of mixing speed was studied under the same 30°C to 37 °C[6]. The study shows that it loses its biocatalytic activity[13]. Thus, the enzyme inactivation and denaturation caused by shear did not happen at 175 rpm.

TABLE 1 Reducing sugar result by cmc using cellulase immobilized on chitosan.

Immobilization Condition	Reducing Sugar (mg/L)
20 0C, 100 rpm	102.278
30 0C, 100 rpm	162.715
30 0C,125 rpm	357.973

TABLE 2 Reducing sugar result by coconut husk as substrate.

Support	Immobilization Condition	Reducing Sugar (mg/L)
chitosan	37 0C, 175 rpm	387.53
	30 0C, 125 rpm	144.119
chitosan GDA	37 0C, 175 rpm	1559.69
	30 0C, 125 rpm	288.238

3.3 | Effect of Enzyme Purity in Sugar Production

TABLE 3 The yield of reducing sugar from free enzyme and immobilized enzyme that is made in 37°C and 175 rpm.

Enzyme Type	Reducing Sugar (g/L)
Free crude enzyme	1.290
crude immobilized on chitosan	0.453
Commercial cellulase	4.130
Commercial cellulase immobilized on chitosan	0.570

TABLE 4 Reducing sugar produced from different size of chitosan.

Immobilized Enzyme	Bonded Enzyme (mg)	Reducing Sugar (mg/L)
Macro sized chitosan	38.35	0.57
Micro sized chitosan	5.50	0.507
Nano sized chitosan	4	0.49

Various types of enzymes were applied as a biocatalyst to convert coconut husk to reducing sugar. Table 3 shows the sugar yielded by each type of enzymes. A free crude enzyme produced less sugar than the commercial cellulose. This is because the enzyme contained substances other than celluloses. The absence of celluloses allows the enzyme to hinder hydrolysis. After the enzymes were immobilized, the immobilized crude enzyme was able to maintain 35% of its productivity. It was more significant than the immobilized commercial cellulose, which could only maintain its activity at 12%. This result indicated that the crude enzyme made in our laboratory has an excellent prospect to applied as immobilized enzymes. The drawback of crude enzyme application on immobilization was its composition, which contained not only a single type of enzymes, which made it challenging to observe the substance that was immobilized on a support.

3.4 | Effect of Particle size of The Support

Chitosan, as support for immobilization, was modified to be macro-sized, micro-sized, and nano-sized. As described in the material and methods section, the microparticles were synthesized by dissolving and magnetite chitosan in acetic acid. NaOH was then added to the solution, which transformed it to be chitosan microparticles, which magnetite entrapped in it. The nanoparticles had the same concept as microparticles. The difference was in the size of the magnetite used. Chitosan macroparticles did not have a particular way of creating them. The microparticles had <500 μm in diameter size, while macroparticles had >1000 μm and the nanoparticles had <20nm. Table 4 shows the performance of cellulose immobilized on different sizes of chitosan. The competitive result was shown although the micro-sized and the nano-sized had less coupled. It was due to smaller size particle provide larger surface area and numerous active sites available for the cellulose molecules to be coupled[10, 14].

3.5 | Temperature and pH Stability of The Immobilized Enzyme

To study the stability of the enzyme, a commercial cellulose from Aspergillus Niger immobilized on chitosan nanoparticle, and its free form was used. The cellulose was used for hydrolysis carboxymethyl cellulose (CMC). Figure 4 shows that immobilized cellulose had optimum activity at pH 5.5, but the other pH did not have any difference inactivity. The free enzyme shows maximum activity at pH four but shows big differences with the other pH. The same pattern also applied to temperature stability. The immobilized enzyme had more wide range temperature than the free enzyme. This was caused by the transformation

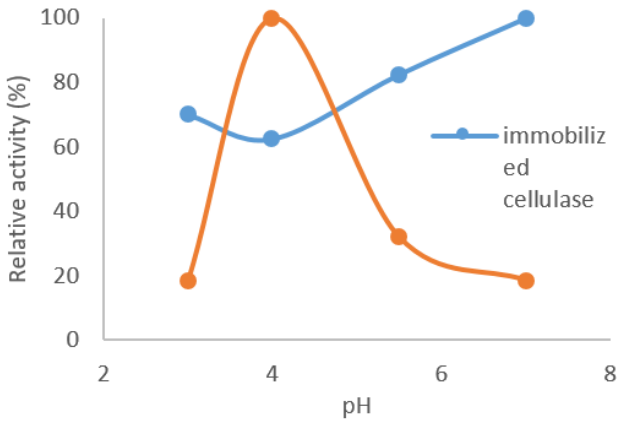

FIGURE 4 Effect pH in hydrolysis CMC.

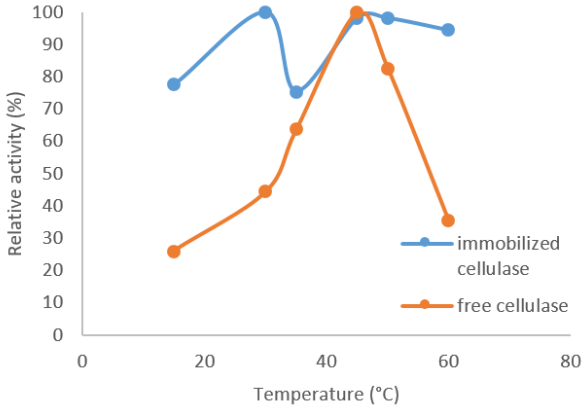

FIGURE 5 Temperature vs relative activity in hydrolysis CMC.

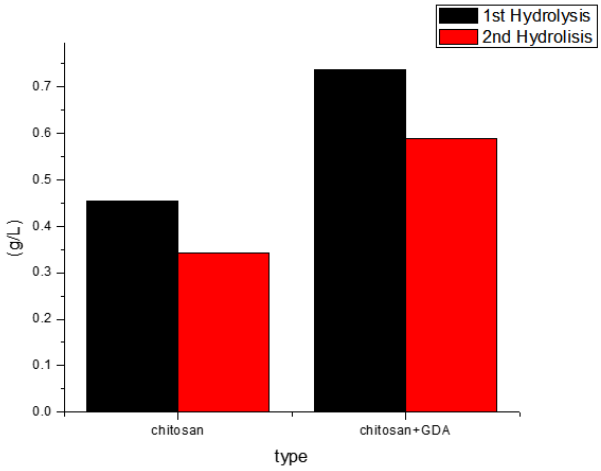

FIGURE 6 Temperature vs concentration in concentration in hydrolysis CMC.

of the physical and chemical properties of the enzyme. The covalent bond formation might also reduce the conformational flexibility and may result in higher activation energy for the molecule to re-organize the proper conformation for binding to the substrate.[5, 15, 16].

3.6 | Reusability

Reusability is the main advantage of immobilization. Figure 4 shows the reusability of immobilized cellulose on chitosan and chitosan+GDA. After two times hydrolysis, chitosan and chitosan GDA maintained 75.31% and 79.94% of its activity, respectively. This outcome proved that the immobilized enzyme could be used repeatedly to increase productivity and reduce the overall cost. Effect temperature in hydrolysis CMC can be seen in Figures 5 and 6 .

4 | CONCLUSION

These preliminary studies describe an enzyme immobilization in chitosan for hydrolyzing carboxymethylcellulose (CMC) and coconut husk (insoluble cellulose). This study also describes the critical variable of enzyme immobilization that affects the reduction of sugar production. The temperature and mixing speed of the immobilization process changed, improving enzyme binding in support. The addition of cross-linker, Glutaral dialdehyde (GDA), enhance the yield of reducing sugar. Both crude enzyme and commercial cellulose have their yield of sugar decreased after immobilization. Coconut husk, as a substrate, has good prospects in the application of immobilized enzymes in the production of reducing sugar. Despite its low sugar yield, immobilized enzymes offer their ability to be used repeatedly so that the process becomes inexpensive.

5 | ACKNOWLEDGMENT

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