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ORIGINAL RESEARCH PAPER

APPLICATION OF SILICA RICE HUSK ASH FOR CELLULASE IMMOBILIZED BY SOL-GEL ENTRAPMENT

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Abstract: Biosilica from rice husk ash can be used to immobilize enzyme by sol-gel entrapment method. Cellulase was chosen as the probe enzyme to test the feasibility of this material for such purpose. This research aimed to know the optimum condition of cellulase immobilization process by sol-gel entrapment, the optimum *p*H and temperature for immobilized enzyme, and the reusability of the immobilized enzyme, as concluded from its activity. The research was divided into 3 steps (1) sol-gel immobilization of cellulase, (2) protein and activity experiments to test immobilized enzyme by varying *p*H and temperature, and (3) reusability test. The result showed optimum condition gave 38.48 % recovery of enzyme activity with protein content of 0.42 $mg \cdot mL^{-1}$, the best *p*H and temperature were 7 and 55 °C respectively. The system could be reused for three times.

Keywords: *biomaterial, enzyme, immobilization, optimum condition, reusability*

INTRODUCTION

Silica is a porous material with wide surface area depending on the conditions while it is made. The surface contains active groups in the form of silanol (-SiOH) and siloxane (Si-O-Si) groups. There are moist to in the outer surface of silica particles, giving hydroxyl groups as active sites too. Its porosity causes silica can act as high-grade adsorbent. The active silanol (-SiOH) group allows silica to interact with other molecules through hydrogen bonds or van der Waals forces as well as dipole-dipole interactions. Silica material is actually common, since it can be found everywhere on earth. Silica, beside lignin and cellulose is also taken and accumulated by plants and used to cover the hard-material parts of the plants, for instance stems, skin, husk. One source of bio-silica is from rice husk, the ash of it contains high silica material. The silica from rice husk can be obtained through alkaline extraction process. Silica/SiO_2 content is around 90 %, the price is cheap and the material is abundant [1]. Silica can be extracted using alkaline solution because the solubility of silica increases at *p*H greater than 10 [2]. Silica was reported to be used as adsorbent for dyes and other heavy metals [3]. The material can be combined with cellulose to make an adsorbent [4, 5] to alter polarity of its surface. In a porous media such as silica there would be solid-gas interface or solid-liquid interface, that the adsorption and desorption occur simultaneously according to the equilibrium constant of each adsorbate [6]. Recently the material can be used as a support material in immobilization either for enzymes or bacteria [7, 17]. Immobilization is the process when the movement of enzyme molecules is held in a certain place (support material/matrix).

Immobilization of enzymes can be classified into two types, physical and chemical immobilization [8]. Both types of methods have their respective strengths and weaknesses. The advantage of chemical immobilization is that the bonds between enzymes with matrices are not easily separated, while the disadvantages are high costs and immobilization processes tend to be difficult. Chemical immobilization is covalent bond immobilization. Meanwhile, physical immobilization is a relatively low cost and simple in the immobilization process, while the disadvantage is that enzymes are easily released from the matrix due to weak interaction with the surface. Adsorption and entrapment are example of physical immobilization. Entrapment immobilization provides many advantages such as uniformity, high purity, bio-compatibility, thermal and chemical stability of the matrix, low processing temperature, easy control of the morphology and amount of entrapped enzymes [11, 12]. The effectiveness produced by immobilized enzymes can be seen from the repeated use cycle. The more cycles of use of immobilized enzymes the more effective the system. The process of immobilization involves a matrix, which is able to adsorb the enzyme so that it can be used repeatedly. Therefore, the immobilization process has a high economic value. As in a study conducted by Ungurean et.al., [9] silica with TMOS (tetramethyl orto silicate) precursors was used as a matrix to immobilize cellulase enzymes using the sol-gel entrapment method. It showed that the immobilized enzyme tends to be stable against changes in temperature and *p*H, and could be used up to five cycle times. The use of silica as a matrix using sol-gel technique has the advantage of being biocompatible [10].

In this experiment, silica matrix from rice husk ash was used to immobilize celullase enzyme. Cellulase is one type of enzyme used in the process of cellulose hydrolysis. This enzyme consists of three components that play important roles in the hydrolysis process, namely endoglucanase, cellobiohydrolase, and cellobiase [13]. The enzymatic

process of hydrolysis of cellulose on a large scale, can provide a loss in terms of costs. The price of such enzymes is expensive, and also single used. To overcome this problem, an enzyme immobilization technique is carried out. The main benefits of the enzyme immobilization technique are reusable and can be separated from the solvent [14].

Celullase was chosen as the probe enzyme to give information about interactions in the surface. Its activities were followed by visible spectroscopy, at working wavelength of 540 nm. The activity of the immobilized enzyme was showed by the production of reducing sugar. It also described the lost activity during experiments. In this case, carboxy-methyl cellulose (CMC) was used as the substrate for cellulase. CMC is a water-soluble cellulose ester that is produced by partial substitution of the cellulose hydroxyl groups with ionic hydrophilic moieties (Ginkel, 2007). Because in the cellulase enzyme there is an endoglucase that has a high affinity for CMC (CMC-ase), then CMC is suitable as a substrate for cellulase enzyme to produce glucose.

Lowry method was used to know the protein content in immobilized enzyme, the pH and temperature optimum of the immobilized enzyme was also studied. For the effectiveness of the immobilized enzyme can be showed by the reusability tests. In the previous research, silica from rice husk ash has never been used as a matrix for immobilization, so in this study the silica ash used came from rice husk ash and for the process of immobilisation, sol-gel entrapment method was used. Optimization is needed to find out the optimum condition of the enzyme can be bound to the matrix so that it strengthens the enzyme immobilization steps and provide good enzyme activity profiles.

MATERIALS AND METHODS

Chemicals and instrumentation

The chemical were obtained from Sigma Aldrich and E.Merck and all bench activities used pyrex glassware. Cellulase of *Trichoderma viride* was chosen as the enzyme and Carboxymethyl Cellulosa (CMC) as the substrate. Bovine serum albumin (BSA) was chosen for standard solution of protein and Glucose for standard solution. All system made was stored in refrigerator.

The instruments used for this study were XD-1700 M, Nabertherm, dan Thermolyne furnace for ashing the rice husk, magnetic stirrer of NESCO LAB MS-H280-Pro or Thermoscientific Cimarec for gelling reactions, EYELA oven, type WFO-450ND, and *UV-Visible Type Pharmaspec 1700* (Shimadzu), UV-Vis to measure the activity of enzyme and protein content.

Material preparation and characterization

Preparation was done by washing rice husk then burned to obtain charcoal grey-black color. The ash was heated again in a furnace for 1 hour. This process was intended to obtain silica in an amorphous form it has a larger surface area than crystalline silica. The silica extraction process was done using NaOH to dissolve the silica in alkaline solutions. The yellow extract was clear and contains sodium silicate. *Method I*: The obtained filtrate would be combined with H_2SO_4 before adding cellulase 1 %. The result

of this process was white gel. *Method II*: As same as the first method, but the gelling process was doing in ice bath. *Method III*: Filtrate of sodium silicate was poured into a tube contain a cellulase 1 %, before adding the H_2SO_4. All gel was dried in refrigerator and would turn into white powder form. The core procedure of making silica-cellulose matrix was already granted Indonesian Patent Number IDP000049626, issued on 14th February 2018. The chemical reaction can be written as follow:

$$SiO_2(s) + 2\ NaOH(aq) \rightarrow Na_2SiO_3(aq) + H_2O(l)$$
$$Na_2SiO_3(aq) + H_2SO_4(aq) \rightarrow SiO_2(s) + Na_2SO_4(aq) + H_2O(l)$$

After the preparation, the matrix was treated to know the optimum method of immobilization by Somogy Nelson method and Lowry method. For Somogy Nelson experiment, the immobilized cellulase was used to produce glucose by hydrolysis 1 % CMC substrate, by using 0.25 gram of matrix. All was done at *p*H 7 and temperature 45 °C for one hour except the effect of the temperature the hydrolysis process was done in 40 minutes due to some technical problems. Produced glucose will be calculated through a linear regression equation from a standard solution (40, 80, 120, 160 and 200 ppm). For Lowry method, 1 mL of native enzyme was used to know the absorbance at the maximum wavelength and the converted to protein content by linear regression equation from a standard solution of BSA (0.2, 0.4, 0.6, 0.8 and 1 $mg \cdot mL^{-1}$). The material was also used to determine the effect of *p*H and temperature. The effect of *p*H was studied in the range of 4 to 8, while the temperature range was 40-60 °C. For the last step, the immobilized enzyme was used to know the effectiveness of immobilization by using the enzyme repeatedly till it lost its activity.

RESULTS AND DISCUSSION

Immobilization of cellulase by sol-gel entrapment methods

The results of enzyme immobilization suggest that the second and third methods provide high activity values and protein levels compared to the first method. This first method was carried out at room temperature. This indicates cold temperature (in an ice bath) cellulase enzyme immobilization in silica matrix from rice husk ash was preferable. The effect of H_2SO_4 with high concentration during process made the system hotter and causes some enzymes to be denatured [18]. The data of immobilization process optimization can be seen in Table 1.

Table 1. *Optimization of immobilization process*

Method	Absorbance [at λ = 540 nm]	Glucose content [ppm]	% Immobilized enzyme activity	Protein content [$mg \cdot mL^{-1}$]	% protein in immobilized enzyme
1	0.159	38.45	22.66 %	0.27	32.53 %
2	0.236	61.51	36.50 %	0.41	49.39 %
3	0.247	64.84	38.48 %	0.42	50.60 %
Native Enzyme	0.589	168.48		0.83	

The second and third methods provide a fairly high value of enzyme activity which is equal to 36.50 and 38.48 % which means that in 1 mL substrate, CMC 1 % the immobilized enzyme can hydrolyse 35-40 % of the substrate to yield glucose. Immobilized enzymes can still provide activation because the enzymes are trapped in the matrix, the trapping process occurs during the gelling process, ie during the condensation reaction between silanol groups which results in the formation of siloxane polymers (Si-O-Si). The viscosity of the solution increased rapidly until gelation process occurs. When the enzyme was located in between small particles and then inside the cavities/pores of the matrix, is was then trapped within the newly formed matrices.

For this reason, the activity of the enzymes produced tends to decrease when compared to the free enzymes. During process of immobilization, the 3D structure of the enzyme differed to greater extent, some of which would lower its activity. The excessive use of relatively concentrated sulfuric acid for promoting gelling process would alter the interior sites of the porous materials as well as the active sites of the enzyme. The silica active sites, the silanols, would be covered by hydrogen ions as well as water molecules would cover the surface while at the same time the enzyme bigger molecules must find their place. Less than optimal hydrolysis process was the direct consequences. In addition, the limited matrix space for substrate distribution also caused lower activity detected. In addition, protein folding in cavity decreased enzyme activity in immobile stage. The existence of enzymes in the matrix was determined by calculating the protein content. The results exhibited that the percentage of proteins in the matrix with the second and third methods of immobilization process, were both about 50 %. Consequently, the activity of immobilized enzymes was smaller too if compared to free enzymes.

A Sol-gel entrapment in inorganic matrix is actually flexible, allowing selection for the best immobilization condition. Method I & II was used to ensure homogeneity of the system meanwhile the method III was used to protect the enzyme during the immobilization process. The immobilization procedures were developed based on previous studies, for adsorption immobilization of cellulase enzyme and *zymmomonas mobilis* bacteria [7, 15]. As the previous research, cellulase was immobilized to the silica matrix by an adsorption method with the highest protein content was 62,5 % from 10 mL enzyme and the highest immobilized percentage of *Zymmomonas mobilis* bacteria was 38 %. Compare to the result of entrapment method of cellulase the highest protein content was 50.60 % from 1 mL enzyme.

The effect of *p*H on immobilized enzyme activity

The *p*H variation was carried out to determine the optimum *p*H possessed by cellulase enzymes in immobilized conditions to provide activities with high glucose levels. The *p*H measurements were done from *p*H 6 to 8 with 0.5 intervals. The results can be seen in Table 2 and described in Figure 1.

Figure 1 shows that the optimum *p*H for cellulase enzymes after immobilization was 7, as indicated by the concentration of glucose produced. The glucose level decreased as the *p*H of the solution increased, suggesting that large concentration of OH- ions would disrupt the hydrolysis process of cellulase.

Table 2. *pH effect*

*p*H	Absorbance [at λ = 540 nm]	Glucose [ppm]
6	0.189	47.27
6.5	0.213	54.54
7	0.245	64.24
7.5	0.226	58.48
8	0.207	56.33

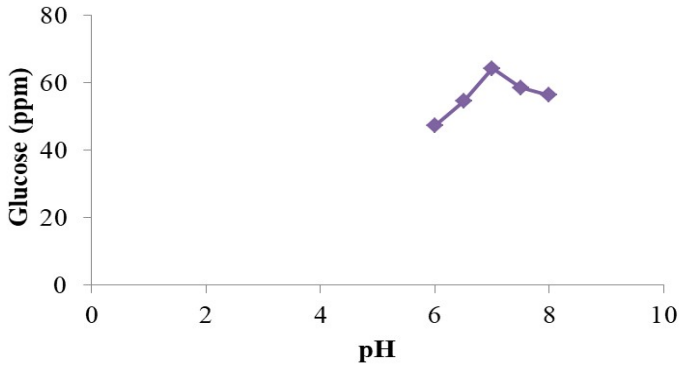

Figure 1. *Effect of pH*

One research conducted previously using cellulase enzyme from *Tricodherma Viride* provides optimum conditions at lower *p*H (*p*H between 5 to 6). The optimum *p*H difference of free enzymes and immobilized enzymes was expected since the immobilized enzyme would undergo great conformational changes in restricted condition. In addition, environmental situation from different matrices might cause shift from the optimum *p*H, which generally ranges between 4.5 to 7. When the highest activity was obtained within this *p*H range, the enzyme could be expected as having good stability. In this case, it can be assumed that the immobilized cellulase enzyme in was in stable condition.

The effect of temperature on immobilized enzyme activity

Temperature is one of the major factors that influence enzyme activity. In this study temperature variations were carried out to determine the effect on glucose levels produced on cellulase enzymes that had been immobilized. This is also to get an idea about the effect of restriction condition to enzyme mobility inside cavities.
Based on the glucose level data produced in Table 3, Figure 2 was drawn. It can be concluded that the optimum temperature of the immobilized enzyme was 55 °C.

Table 3*. Temperature effect*

Temperature [°C]	Absorbance [at λ = 540 nm]	Glucose [ppm]
40	0.152	36.33
45	0.184	46.03
50	0.243	63.90
55	0.256	67.84
60	0.207	53.00

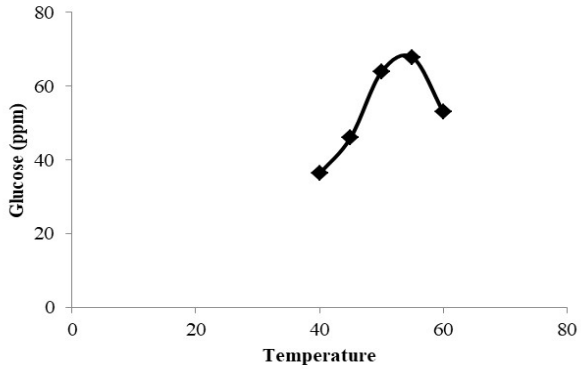

Figure 2. *Effect of temperature*

The Figure 2 shows that immobilized cellulase has different optimum temperature compared to free enzyme. In general, free cellulase enzymes will reach optimum conditions at temperature of 40-50 °C. Inside restricted gels the enzymes undergo higher rigidity level and more energy from the surroundings needed. The increased optimum temperature is then explained for those activities inside porous matrices. Immobilized enzymes have a fairly high level of thermal stability, compared to free enzymes. In this case free enzymes are denatured more easily than immobilized enzymes. The same discussion was explained by Dinçer & Telefoncu [16], that the presence of enzymes in the matrix will improve its thermal stability up to 60-80 °C. In this study, immobilized enzyme activity will decrease again if the temperature exceeds 55 °C. Cellulase will experience denaturation at high temperatures, as well as the silanols and hydroxyl groups would be more pronounced and make the surface displacement greater. This is also the sign of enzyme physical adsorption occurring in the system, and not chemisorption with silica matrices [3]. The active site of the enzyme is also affected by the additional kinetic energy from the environment. Is the difference of glucose content for this process at 45 °C and *p*H 7 because of the hydrolysis time.

Sol-gel immobilized cellulase reusability

The effectiveness of cellulase enzymes immobilization can be seen from repeated use of it. The reusability of cellulase immobilized by sol-gel method was investigated in hydrolysis reaction of CMC, at *p*H 7 and 55 °C, the obtained optimum condition from previous investigation. Immobilized cellulase entrapped in silica in fact could be used up to 3 times under same circumstances, with lower hydrolyzed glucose from cycle to cycle. As the previous research [11], the reusability of immobilized cellulase by adsorption method could be up to 3 cycle usage. Explanation of the decrease of enzyme activity is attributed to the released of enzyme from the matrices. As can be seen at Figure 3 the glucose level produced in each cycle gets less in concentration with repetition up to three times. The amount of enzyme was not the same anymore on the second use, beside the fact that the enzyme was also undergo denaturation during incubation. The third cycle showed the lowest glucose concentration produced at 20.6 ppm with a percentage of 24.9 % compared to glucose levels from the first cycle. After the third cycle do not provide activity as indicated by the absence of glucose produced and zero absorbance. During the last hydrolysis process the matrices experienced

leaking, and the enzymes to wash away from the porous media. Another study from this research group for similar systems shows better values, up to 5-10 times repetition (private communication).

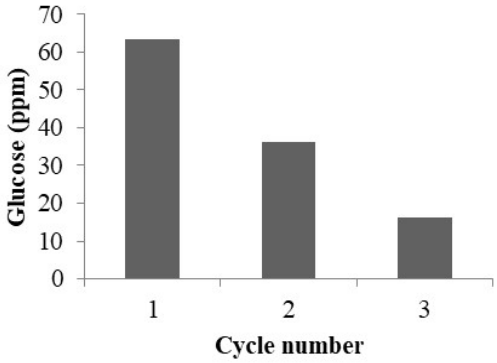

Figure 3. *Repeated use of immobilized cellulase*

For additional information about the system, a series of experiments by aging system were also done. The good storage time of immobilized cellulase enzymes is 14 days, when used after that day then the enzyme does not provide activity. The information can be seen in Table 4.

Table 4*. Storage time of immobilized cellulase in silica by entrapment*

Day	Absorbance
1	0.247
7	0.222
13	0.163
15	~0 (no absorbance)

This result showed also the decaying process of the system occurred and might due to many possibilities. During storage in laboratory refrigerator the system damaged by contamination as well as out of controlled temperature. The enzyme inside porous media was not kept in optimum condition. Further attempt must follow this problem in the future as the good aim is to make good immobilized enzyme for better use.

CONCLUSION

Cellulase enzyme immobilization in the silica matrix by sol-gel entrapment method was done. The systems gave optimum results for immobilization process that was carried out at cold temperatures (using an ice bath). The percentage of immobilized enzyme activity produced was 38.48 % and level of protein was 0.42 $mg \cdot L^{-1}$. Immobilized cellulase enzymes provided high activity at the best temperatures of 55 °C and *p*H 7. Immobilized cellulase enzymes in the silica material from rice husk ash using sol-gel entrapment method can hydrolyze cellulose to glucose with repeated use cycles of 3 times and with the lowest glucose levels of 16.33 ppm. Further experiments will be

carried out to investigate the immobilized enzyme to produce glucose with other types of immobilization as well as storage time.

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