Optimizing the immobilization of lipase enzyme (*Aspergillus oryzae*) in the silica and silica-cellulose matrix by adsorption method

C A Maharani¹, S Suharti¹, and S Wonorahardjo¹²,*

¹ Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Malang, Indonesia
² Center of Advanced Material and Renewable Energy (CAMRY), State University of Malang, Indonesia

*Corresponding author: surjani.wonorahardjo@um.ac.id

**Abstract.** Silica and silica-cellulose were safe biomaterials used as the matrix for immobilized enzyme or any bigger molecule. Some enzymes play role in some processes to biofuel production. In this study, the enzyme used was the lipase enzyme, to test the ability of the matrix as carrier. This research aimed to know the ability to immobilize lipase enzyme using adsorption method. Spectroscopy methods were involved. The research was divided into 4 steps (1) preparation of silica and silica-cellulose matrices, (2) characterization of silica and silica-cellulose, (3) immobilization of lipase enzyme on the silica and silica-cellulose which included activity test: optimum temperature, optimum pH, and optimum stirring speed, and (4) effectiveness of immobilized lipase enzyme on the silica and silica-cellulose. The result showed silica porous material as a matrix was more effective than silica-cellulose as matrix for lipase enzyme.

1. Introduction

In the era of material science, the topic energy is popular since many put effort form making materials related to renewable energy. Some biocatalysts in renewable energy routes are enzymes to convert biomass to biofuels. However, such biocatalyst is usually high cost and irreversible, until scientist managed to make it immobilized. For this purpose, some biomaterials were made to support immobilization without too much dropping the activities. There are some adjustments can be made during sol gel processing.

Enzymes are organic biocatalysts produced by living organisms in the protoplasm, which consist of proteins or a protein-bound compound. One enzyme that is widely used is the lipase enzyme that catalyzes the hydrolysis of long-chain triglycerides into short chains, free fatty acids, and glycerol. Lipase commonly used in industrial process is immobilized lipase. [1] The high cost of lipase, makes the enzymatic manufacturing process, less desirable. The use of lipase immobilization is a possible solution to this problem because enzymes can be recovered from the product and reused. Some of the advantages of immobilized lipase are the increased activity and stability of the enzyme, and the ease of obtaining the immobilized enzyme at the end of the reaction [2].

In this study, silica is used as a material used to carry enzymes. Silica is a porous material that can be used as an adsorbent because it consists of active groups in the form of silanol (-SiOH) and siloxane (Si-O-Si) [3–5]. The active Si-OH group in silica allows silica interaction with enzymes through
hydrogen bonds. In addition, silica has a wide pore size and surface area which make the silica often used in the adsorption process. The source of silica that being used was obtained from rice husk ash. Rice husk ash was chosen because it has a high enough silica content. According to Kalapathy, et.al [6] the silica content contained in rice husk ash is 89-91%. Another advantage of silica from rice husk ash is relatively low cost, with active sites of silanol and reactive siloxane with low surface activity. Moreover, rice husk is actually agro-waste that can be made functional materials, a higher level of functional material. The function of used biosilica has also been carried out by Maharani, et.al [7] by modifying silica with carrageenan as adsorption of Pb\(^{2+}\) and Cd\(^{2+}\) as disruptive ions, or with cellulose for dyes separation [8,9]. In addition, cellulose also used as a modification to silica surface. The merging of this matrix is based on the change in the polarity of silica surface [9–11]. The cellulose was obtained through the hydrolysis process of nata de coco. Nata de coco was chosen because it has cellulose with high purity, it is easy, inexpensive, and is biodegradable [12].

Previous research has been carried out on silica-cellulose [3] that applied silica-cellulose as an absorber of chlorophyll and curcumin compounds which is proven to be more effective at absorbing chlorophyll by the batch method. This research develops the potential of silica-cellulose as a matrix for immobilization of the lipase enzyme. Free lipase enzymes are difficult to separate from the solution so that it is only disposable, for that we need to do a way to solve the problem. One method that can be used is immobilization. Immobilization is the process of moving an enzyme molecule that is held in a certain place (matrix). Immobilization process itself is divided to physical and chemical immobilization. Adsorption and entrapment constitute physical immobilization, whereas covalent bonding and cross-linking constitute chemical immobilization.

In this study, physical immobilization was used, named the adsorption method. It does not cause changes in enzyme conformation and does not interfere with functional groups in the active center of the enzyme so that the enzyme can still work well. The success of enzyme immobilization can be seen from the repeated use of enzymes, the higher number of repetition, the more effective it is. Immobilized lipase has been discussed and modelled [13] as well as used for other support materials [14,15].

In this research, to get optimum results, it is necessary to optimize the enzyme immobilization process. The optimization includes optimization of temperature, pH, and stirring speed during the immobilization process. This optimization is the initial part to find out the optimum conditions in the lipase immobilization process in silica and silica-cellulose matrices.

2. Methods

2.1. Chemicals and Instrumentation

The tools used in this research are aluminum foil, evaporation cup, stirring rod, pyrex and iwaki brand glassware, pyrex and iwaki brand elrenmeyer, pyrex and iwaki brand test tubes, mortar, pastel, porcelain crucible, watch glass pyrex and iwaki, pyrex and iwaki brand elrenmeyer, pyrex and iwaki brand test tubes, mortar, pastel, porcelain crucible, watch glass pyrex and iwaki, elrenmeyer brands pyrex and iwaki clamps, blenders, pycnometers, IR-Prestige-21 (SHIMADZU) spectroscopy, SEM, UV-Vis spectrophotometers, spectronic-20, furnaces, stirrers, magnetic stirrers, buchner, ovens and analytical balance. The materials used include rice husk ash, dry powder nata de coco, aquades, NaOH pa, H\(_2\)SO\(_4\) pa, NH\(_4\)OH pa, I\(_2\) pa, KI pa, KIO\(_3\) pa, Na\(_2\)S\(_2\)O\(_3\) pa, amyllum indicator, oleic acid, copper (II) acetate, lipase enzymes (Aspergillus oryzae), phosphate buffer, olive oil, HCl pa and n-hexane pa.

2.2. Material Preparation and Characterization

First, burning rice husk to get the ash as the source of silica to 350ºC to produce amorphous silica. The ash was heated again in the furnace for 1 hour prior to addition. The risk husk was dissolved in NaOH solution, stirred using a magnetic stirrer for 1 hour. Then it was allowed to stand overnight before filtering it. The filtrate containing silica in the form of sodium silicate (Na\(_2\)SiO\(_3\)) can be used to make silica and silica-cellulose as a matrix. To make silica gel as a matrix, the sodium silicate filtrate was added with sulfuric acid to produce an acidic pH. After that, NH\(_4\)OH solution was added to neutral pH.
The colloids obtained are filtered and drying in an oven until dry. Furthermore, dry residue was crushed using mortar and pestle to produce silica powder.

Preparation of cellulose colloid is done by adding nata de coco powder to \( \text{H}_2\text{SO}_4 \) solution accompanied by stirring them in magnetic stirrer to form of a purple gel. The filtrate from the extraction of silica was combined to the nata de coco colloid until the gelling started. The mixture was added a solution of concentrated NH\(_4\)OH until pH rises slowly. The formed gel was filtered and washed before drying in an oven. Dry residue pulverized to obtain silica-cellulose powder used as a matrix for immobilization of lipase enzyme. The overall reaction of silica making can be seen in equations below.

\[
\text{SiO}_2(s) + 2 \text{NaOH}(aq) \rightarrow \text{Na}_2\text{SiO}_3(aq) + \text{H}_2\text{O}(l)
\]
\[
\text{Na}_2\text{SiO}_3(aq) + \text{H}_2\text{SO}_4(aq) \rightarrow \text{SiO}_2(s) + \text{Na}_2\text{SO}_4(aq) + \text{H}_2\text{O}(l)
\]

After the preparation, the matrix was the characterized, covering moisture level, density, ash level, and the iodine adsorption value. Identification of surface topography and functional groups of this adsorbent were done with SEM and FT-IR. For application, the material of silica and silica-cellulose were used in immobilization.

2.3. Immobilization of Lipase Enzymes

Immobilized Lipase Enzyme Activity Test with the Kwon & Rhee Method also done. 1 gram of immobilized enzyme added 4.5 mL of phosphate buffer pH 6.5 and then add 1 mL of olive oil. The mixture is then stirring with a speed of 150 rpm within 30 minutes. Next, the mixture was centrifuged for 5 minutes at a speed of 1000 rpm. The mixture is allowed to stand until it forms 2 perfect phases. The upper phase (oil) is taken and added with n-hexane 2.25 mL and 1.5 mL copper (II) acetate 5%. The mixture is shaken vigorously to form 2 phases. The upper phase taken then measured using a UV-Vis spectrophotometer at a wavelength of 615 nm. The next step is to determine the optimal condition of the immobilized enzyme, including temperature, pH, and stirring speed optimization by Kwon & Rhee Method.

After optimization, the effectiveness of the enzyme test for repeated use was carried out as follows: 1 gram of immobilized enzyme was added 5 mL phosphate buffer pH 6.5 and 5 mL olive oil. The mixture was incubated at 50 °C for 15 minutes at 250 rpm. Then the mixture is centrifuged at a speed of 1000 rpm for 5 minutes. The resulting product was then tested for its activity using the Kwon & Rhee method at a wavelength of 615 nm. Subsequently the immobilized enzyme was washed with 20 mL phosphate buffer pH 6.5, filtered and dried in an oven at 40 °C for 2 hours. Furthermore, the dried enzymes are used again to determine further activities.

3. Results and Discussion

3.1. Silica and Silica-Cellulose Material Characterization

Adsorbent characterization test carried out included density test, moisture level, ash level, iodine adsorption value, Scanning Electron Microscopy (SEM), and Fourier-Transformed Infrared (FT-IR). The moisture level of the silica and silica-cellulose is low. The moisture level was conducted to determine the amount of water content of the adsorbent. The ash level of silica-cellulose was very high. The absorption of iodine in silica-cellulose was quite high too, indicating that the adsorbent’s surface is full of active sites. The physical characterization of the material can be seen in Table.1.

| Adsorbent       | Density (g/cm\(^3\)) | Moisture level (%) | Ash level (%) | Iodine Adsorption Value (%) |
|-----------------|-----------------------|--------------------|--------------|-----------------------------|
| Silica          | 2.157                 | 3.116              | 30.515       | 5.584                       |
| Silica-Cellulose| 2.516                 | 4.783              | 14.982       | 5.964                       |
Physical characterization by SEM instrument can be seen in Figure 1. Characterization of the surface structure of the adsorbent using SEM was carried out to determine the shape of the surface topology of the adsorbent. The image of silica and silica-cellulose with 80,000 times magnification shown the structure of the silica and silica-cellulose surface look uneven with globular particle shape indicating pores on the surface of silica-cellulose. The presence of pores on the surface would increase the surface area.

![Figure 1. The result of SEM of (a) Silica and (b) Silica-cellulose](image)

Surface topology analysis was also carried out on the immobilized silica and silica-cellulose matrices. It can be seen in Figure 2 if lipases are adsorbed on the matrix surface. Silica cellulose can be used as a lipase enzyme adsorbent with a magnification of 10,000 times for silica while silica-cellulose is magnified 20,000 times.

![Figure 2. The result (a) silica-enzyme and (b) silica-cellulose-enzyme](image)

The next characterization is FT-IR test aimed to identify the functional groups on the adsorbent. Spectra by FT-IR instrument can be seen in Figure 3 and 4. The comparison of silica and silica-cellulose can be seen. There was wavelength shift occurred, indicating different functional groups in the new material.

The absorption patterns that appear generally are siloxane groups (≡Si–O≡Si) and silanol groups (≡Si–OH–) contained in silica gel. The absorption band at wavenumber 457.14 cm\(^{-1}\) and 477.77 cm\(^{-1}\)
are bending vibration of the siloxane group. The area at 820-600 cm\(^{-1}\) shows the stretching vibration of Si–O symmetry in siloxane (≡Si–O≡Si) and the addition of cellulose particles caused the appearance of absorption peak at the 1633.71 cm\(^{-1}\) that indicated C–O vibration of cellulose. The similarity of this spectra indicated that only physical interaction occurs on the two material, there was no indication of new chemical bonds on silica-cellulose.

Figure 3. Spectrum IR of Silica and Silica Cellulose before immobilized and after immobilized

FT-IR test was also carried out on immobilized enzymes. In Figure 3, a frequency of 2850-2970 cm\(^{-1}\) uptake of C–H alkanes appears on the silica and cellulose silica matrices. In the presence of Amine N–H uptake, Amides appear at wave numbers 3300-3500 cm\(^{-1}\) which alter lipase enzymes through wave numbers 3100-3000 cm\(^{-1}\) due to aromatic C-H from the n-hexane solvent.

3.2. Immobilization Lipase Enzyme with Adsorption Method

3.2.1. Optimum Temperature.
In this research, temperature optimization is carried out in order to find out the optimal temperature of immobilized enzymes. The varied temperatures are 35, 40, 45, 50, and 55 °C. Higher temperatures cause enzyme activity to increase. This is due to the interaction of the intensity between the immobilized enzymes with olive oil. As the temperature rises, enzyme activity increases because it is denatured. The stability of immobilized enzymes on the effect of temperature depends on the compatibility of the enzyme with the matrix and the content of hydrophobic groups on the amino acids that make up the enzyme. The content of hydrophobic groups produced by molecules from enzymes forms a denser structure in the composition easily and easily with silica or silica-cellulose structures. Therefore, silica and silica-cellulose matrices are able to save enzymes from increased physical bonds. The results of the study can be seen in Figure 4 below.
Based on the results of the study, the maximum immobilize lipase enzyme activity in the silica matrix occurs at 45 °C and in the silica cellulose matrix occurs at 40 °C with lipase activity of 30.970% in silica and 25.426% in silica cellulose. This is due to the presence of hidrophobic groups in enzyme molecules that can physically bind to silica and silica-cellulose matrices so that they form a more stable structural conformation in solution. This causes the matrix of silica and silica-cellulose to protect the enzyme from breaking physical bonds from heating.

3.2.2. Optimum pH.
Determination of optimum pH was carried out by aiming to determine the optimum pH of immobilized enzyme activity. In this study, the buffer used was a phosphate buffer with a pH variation of 5.5; 6.0; 6.5; 7.0; and 7.5. The results can be seen in Figure 5.
Based on the results obtained, pure lipase enzymes and immobilized lipase enzymes on silica and silica-cellulose matrices work optimally at pH 6.5. This shows that the pH variation affects the changes in the conformational structure of enzymes in the silica or silica-cellulose matrices caused by the formation or termination of ionic interactions, especially in the enzyme parts containing R−NH₃⁺ and R−COO⁻ due to environmental influences, acid or bases. Each enzyme has a different optimum pH. This results in enzymes undergoing denaturation if the pH was far from optimal conditions.

3.2.3. Optimum Stirring Speed.
The stirring speed is needed to provide energy so that the lipase enzyme is distributed evenly on the porous surface of the silica and silica-cellulose matrix and to encourage interaction between the lipase enzyme with the silica and silica-cellulose matrix. In this study the stirring speed variations were carried out, namely 50, 100, 150, 200, 250 rpm in 30 minutes. The results of the stirring speed optimization are the fatty acid levels which can be seen in Figure 6.

![Figure 6. Optimum Stirring Speed of Silica and Silica-cellulose](image)

From the results obtained, the stirring speed on the optimal silica matrix occurs at a speed of 200 rpm which is equal to 1084.5 ppm with an immobilized enzyme percent of 29.350% and decrease thereafter to 17.036%. On the silica-cellulose matrix the stirring speed optimum occurs at a speed of 150 rpm which is 1069.5 ppm with immobilized enzyme percent 28.944% and increases the decrease thereafter. This stirring causes weak bonds such as van der waals forces, hydrogen bonds, and dipoles formed between the lipase enzyme and the silica matrix and silica selica to become unstable. This is due to the effect of kinetic energy from stirring. At large stirring speeds, the given kinetic energy is large enough to release the weak bonds that occur between the enzyme and the matrix.

3.2.4. Effectiveness of Immobilized Lipase Enzyme.
If seen from the figure 7, immobilization of the lipase enzyme with silica matrix can be used as many as 5 times of repeated use with the lowest activity of 30.287%. The immobilized lipase enzyme in the silica matrix is very effective in the first cycle with 100% relative activity while in the second, third, fourth and fifth use, the enzyme activity decreases. The decrease in enzyme activity is due to the breakdown of van der waals bonds between the hydrophobic groups of the enzyme and the silica matrix. The same thing happened to lipase immobilization with silica-cellulose matrix can be used as many as 4 times of repeated use with a relative activity of 18.809%. This indicates that the immobilized lipase enzyme is more effective in silica matrices than in silica-cellulose matrices.
Figure 7. Repeated use of Immobilized Lipase Enzyme for Silica and Silica-cellulose

4. Conclusion

In this experiment, the silica and silica-cellulose materials were made, it has low moisture level, and high level of ash. The silica has 30.515% of ash level, meanwhile the silica-cellulose has 14.982% of ash level. The absorption of iodine in silica and silica-cellulose was high as well. The characterization of adsorbents using SEM showed that the structure of the adsorbents were uneven with the shape of globular particles, the pores were uniformly present and lipase can adsorbed on the matrix surface. The FT-IR spectra showed siloxane silanol groups contained in silica gel and has C-O vibration that indicated cellulose. The lipase activity with adsorption method between silica and silica-cellulose were finished. Silica as a matrix is more effective than silica-cellulose as matrix.

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