Stability studies of immobilized lipase on rice husk and eggshell membrane

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Abstract. Lipase immobilization for biodiesel production is gaining importance day by day. In this study, lipase from Burkholderia cepacia was immobilized on activated support materials namely rice husk and egg shell membrane. Both rice husk and eggshell membrane are natural wastes that holds a lot of potential as immobilization matrix. Rice husk and eggshell membrane were activated with glutaraldehyde. Lipase was immobilized on the glutaraldehyde-activated support material through adsorption. Immobilization efficiency together with enzyme activity was observed to choose the highest enzyme loading for further stability studies. Immobilization efficiency of lipase on rice husk was 81% as compared to an immobilization efficiency of 87% on eggshell membrane. Immobilized lipase on eggshell membrane exhibited higher enzyme activity as compared to immobilized lipase on rice husk. Eggshell membrane also reported higher stability than rice husk as immobilization matrix. Both types of immobilized lipase retained its activity after ten cycles of reuse. In short, eggshell membrane showed to be a better immobilization platform for lipase as compared to rice husk. However, with further improvement in technique of immobilization, the stability of both types of immobilized lipase can be improved to a greater extent.

1. Introduction
The arising of global dilemmas such as depletion of fossil fuels, increasing fuel prices and global warming has caused a great concern and led the search for alternative, sustainable, renewable, efficient and cost-effective resources [1]. Biofuels are renewable energy resources produced from biomass material which can be used to replace fossil fuels [2]. Biodiesel is an attractive liquid biofuel which can replace existing fossil derived diesel to a greater extent. Lipase catalyzed biodiesel production is an attractive, environmental friendly technique. However, the high cost of lipase serves as a main hurdle for its commercialization. This can be overcome to a greater extent by immobilizing the lipase in a suitable matrix thus increasing its reusability [3].
Immobilization can be done through a number of ways. Out of these the main methods are adsorption, covalent binding, entrapment and cross-linking [4]. The methods mentioned can be coupled together to further increase the stability of the enzyme. Immobilizing an enzyme is proven to be more efficient than normal enzymes because of their properties. It is more stable as it is bound to a solid support which may increase its stability. Moreover, immobilized enzymes can be reused [5].

The carrier matrix used must be cheap or affordable. However, it must have certain characteristics for it to be an ideal support [6]. A carrier must be coupled with proper immobilization technique for better design of an immobilized enzyme [7-8].

Rice husk is able to serve as a good matrix for immobilization due to its properties of resistance towards fungi and microbial growth. Rice husk is one of the major agricultural wastes that have been produced around the globe especially from countries such as China, India, Indonesia, Malaysia, Bangladesh and Vietnam. The proper disposal of rice husk is a main challenge in waste management. Furthermore handling rice husk and rice husk ash takes a lot of work because of its density [9].

On the other hand, eggshell membrane (ESM) is a thin white film located between the eggshell and albumin of eggs. The eggshell membrane consists of crosslinked collagen, glycosaminoglycans, egg white protein and eggshell matrix protein. The membrane is not permeable to water as it is made up of disulfide bonds which gives it an advantage in certain field of application. It has a large surface area, high porosity and contain amino acids. It is reported that the content of eggshell membrane is acidic [10]. Eggshell membrane is highly adsorbent and it is used as a material in removing heavy metals from aqueous solution [11-12].

The objective of this research is to utilize two important waste materials namely rice husk and eggshell membrane as a matrix for immobilization of lipase for biodiesel production. However before it can be applied for production, it is very important to study the stability of the immobilized lipase. Thus, the main stability parameters were studied and characterized for design of an immobilized lipase for its application to biodiesel production.

2. Materials and methods

2.1. Materials. Rice husk was collected from a local rice mill in Tuaran, Sabah, Malaysia. The egg shells were collected from our faculty’s cafeteria. *Burkholderia cepacia* lipase was kindly gifted by Amano Enzymes, Japan. All other reagents and chemicals used were of analytical grade and was obtained commercially.

2.2. Immobilization of lipase on rice husk. Immobilization of lipase on rice husk was done by modifying the method of [13]. At first, 500µl of 25% glutaraldehyde was added into a beaker containing rice husk and incubated for 5 minutes. This was followed by addition of different lipase concentrations separately (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml) into 1g of rice husk and incubated for 20 minutes.

2.3. Immobilization of lipase on eggshell membrane. Immobilization of lipase onto eggshell membrane (1g) was performed by modifying the method of [13]. 500µl of 25% glutaraldehyde was added into a beaker containing eggshell membrane and incubated for 5 minutes. After the incubation, different lipase concentration (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml) was added into different beakers containing 1g of rice husk and incubated for 20 minutes.

2.4. Determination of lipase activity. The activity of the immobilized lipase on rice husk and egg shell membrane were determined by using titrimetric assay with olive oil emulsion as the substrate[14]. The reaction mixtures contained 10ml of olive oil emulsion (1ml of olive oil and 9ml of arabic gum at 10%), 20 ml of distilled water and immobilized lipase (1g). The reaction was carried out at 37°C for 30 minutes using orbital shaker. The amount of free fatty acid released during hydrolysis were
estimated by titration with 0.01N NaOH solution. One unit of activity is defined as the amount of enzyme, which release 1µmole fatty acid per min per ml under specified assay conditions.

2.5. Determination of pH and thermal stability. This was performed by modifying the method of [15]. The pH stability of immobilized lipase on rice husk and eggshell membrane were studied by incubating 1g of immobilized lipase in Tris HCl buffer at different pH (6-10) for 1 hour at 4°C. The hydrolytic activity were determined using olive oil hydrolysis. The thermal stability of immobilized lipase were done by incubating them at various temperatures in the range of 20-60°C for 1h. The activities were measured using olive oil hydrolysis.

2.6. Determination of organic solvent stability. Stability of immobilized lipase were tested in different solvents like ethanol, methanol, hexane, n-butanol and 1-propanol. The immobilized lipase was incubated for 1h in the above mentioned solvents at 4°C. The immobilized lipase were filtered, washed and the catalytic activities were determined using olive oil hydrolysis assay [16].

2.7. Determination of storage and reusability studies. The storage stability of immobilized lipase on rice husk and eggshell membrane were done by storing in Tris HCl buffer (pH 7) at 4°C for one week. The activity of immobilized lipase was determined on a daily basis. The immobilized lipase on rice husk and eggshell membrane were repeatedly used ten times for hydrolysis of olive oil emulsion. Followed by hydrolysis, the immobilized lipase was filtered, washed and suspended again in fresh olive oil emulsion in order to measure its lipase activity.

3. Results and discussion
3.1. Immobilization efficiency. The immobilization efficiency of lipase on rice husk and eggshell membrane are shown in Figure 1. An immobilization efficiency of 81% was achieved with a lipase concentration of 500 µg/ml on rice husk. On the other hand, a lower lipase concentration of 400 µg/ml gave highest immobilization efficiency of 87% on eggshell membrane.

Rice husk is known to be a highly adsorbent natural material because of its porosity. Wan Ngah and Hanafiah [17] has reported rice husk to be a good adsorbent material for metals and dyes. In addition to this, rice husk has greater surface area which makes it as a suitable carrier for immobilization [18]. The low lipase activity on rice husk as compared to eggshell membrane might be due to the fact that the enzyme’s catalytic site being blocked or lipase enzyme is deactivated [19].

On the other hand, immobilized lipase on eggshell membrane exhibited an efficiency of 87%. This can be attributed to the properties of eggshell membrane. The eggshell membrane surface has high permeability towards substrates and products. The amino acids present on the membrane increases its chances of getting crosslinked with glutaraldehyde which inturn leads to increased immobilization efficiency [13].
3.2. Enzyme Loading.
Figure 2 shows the results of enzyme loading at different concentrations on rice husk and eggshell membrane. It is clear that 300 µg/ml of lipase concentration gives the highest enzyme activity on rice husk while 400 µg/ml gave the highest enzyme activity for eggshell membrane. Therefore, immobilized lipase on rice husk was chosen at 300µg/ml enzyme loading and 400µg/ml enzyme loading for eggshell membrane for further investigation in stability studies.

3.3. pH stability.
As shown in the Figure 3, enzyme activity for rice husk is shown to be the highest at pH 8 indicating the optimum pH for immobilized lipase. As for immobilized lipase on eggshell membrane the optimum pH was found to be 7. With further increase in pH, both immobilized lipase showed a decrease in enzymatic activity. Similar results for pH studies were also reported [20] [21]. The decreased enzymatic activity with increase in pH can be attributed to the loss of H⁺ ions from side chain groups of the matrix [18]. The pH of immobilized lipase will depend to a greater extent on the properties of the carrier and also the method of immobilization employed [22].
3.4. Temperature Stability.
The enzymatic activity of immobilized lipase on rice husk and eggshell membrane at different temperatures are shown in Figure 4. The optimum temperature for both types of immobilized lipase were found to be 40°C. The activity of immobilized lipase decreased with further increase in temperature. This is due to the deactivation of the enzyme as well as disintegration of the carrier material [18]. The rice husk showed better stability at high temperature as compared to the eggshell membrane.

![Figure 4. Temperature stability of immobilized lipase on rice husk and eggshell membrane](image)

3.5. Organic solvent stability.
The stability of immobilized lipase on rice husk and eggshell membrane in different organic solvents are shown in Figure 5. It can be seen that immobilized lipase on eggshell membrane exhibited higher activity as compared to immobilized lipase on rice husk. Immobilized lipase on eggshell membrane gave higher enzymatic activity in ethanol as a solvent. On the other hand, immobilized lipase on rice husk showed maximum enzymatic activity in methanol. The stability of immobilized lipase in organic solvents is very important towards its application in industry. The stability inturn depends on the matrix used, mode of attachment of lipase to the matrix and also polarity of the solvent [23]. The higher stability of lipase immobilized on eggshell membrane as compared to the lipase on rice husk can be attributed to the better properties of eggshell membrane.

![Figure 5. Organic solvent stability of immobilized lipase on rice husk and eggshell membrane.](image)

3.6. Storage stability. The storage stability of immobilized lipase on eggshell membrane and rice husk for a period of seven days is shown in Figure 6. Both types of immobilized lipase were stored at 4°C in tris-HCl buffer (50mM, pH 7). In both cases, there was loss of enzyme activity with increase in number of days. The reasons can be attributed to leakage of enzyme from the carrier during storage.
[21]. With respect to storage stability both types of immobilized lipase showed more or less similar characteristics.

![Graph showing storage stability of immobilized lipase on rice husk and eggshell membrane at 4°C.](image)

**Figure 6.** Storage stability of immobilized lipase on rice husk and eggshell membrane at 4°C.

3.7. Reusability.
Reusability is one of the most significant parameters in design of an immobilized enzyme for industrial application. The enzyme activity of immobilized lipase on eggshell membrane and rice husk measured under the same storage conditions for over a period of ten reuses is shown in Figure 7. The enzymatic activity was shown to decrease with each cycle of reuse. After 10 number of cycles, immobilized lipase on eggshell membrane retained an activity of 13.5% and immobilized lipase on rice husk retained an activity of 11.3%. The loss of activity with each cycle of reuse can be explained in terms of enzyme leakage. Moreover, some enzymes might desorb from the matrix during the process of washing and reuse [24].

![Graph showing reusability of immobilized lipase on rice husk and eggshell membrane.](image)

**Figure 7.** Reusability of immobilized lipase on rice husk and eggshell membrane

4. Conclusion
Agricultural and domestic wastes such as rice husk and eggshell membrane were tested as lipase immobilization matrices. Lipase from *B. cepacia* was successfully immobilized on eggshell membrane and rice husk and used for stability studies mainly by hydrolysing olive oil emulsion. Both types of immobilized lipase were proven to be quite faster and cheaper. In short, this work demonstrates a simple, easy and economical method to immobilize lipase for biodiesel production by utilizing domestic wastes such as egg shell and rice husk.
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