Preliminary studies on immobilization of lipase using chicken eggshell

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Abstract. A few advantages of enzyme immobilization are reusability of expensive enzyme, improvement of stability and activity compared to crude enzyme. Various organic components can be used as carrier for enzyme immobilization such as chicken eggshell. It can be used as a carrier for immobilization as its mineral component mostly contains of calcium carbonate. In the present study, Tributyrin method was used to test enzyme activity of Rhizomucour Miehei, Candida Antarctica and Candida Rugosa. Rhizomucour Miehei shows the highest enzyme activity (360.8 mol/min/mL lipase) and was used in further experiment. Experiment was continued to study incubation time for lipase immobilization on eggshell (1-4 hours) and reaction time of esterification of sugar ester (0-72 hours). Two hours incubation time for lipase immobilization was observed and gives the highest yield of sugar ester (78.13%). Fructose and stearic acid as substrate was used for the production of sugar ester. The highest percentage of sugar ester production was shown at 36 hours of reaction time.

1. Introduction
Nowadays, there are many methods by researcher to improve the characteristic of an enzyme and also to ensure the stability of the enzyme. One of the methods is by immobilizing the enzyme. Enzyme immobilization is widely used method in industry [1]. In 1976, it was reported as the first industrial use of enzyme immobilization where they develop the immobilization of Aspergillus oryzae amino acylase in order to resolute synthetic racemic D-L amino acid [2]. In order to choose the carrier for immobilization, certain properties have to be followed: hydrophilicity, ability to resist microbial attack, biocompatibility and availability at low cost [3]. Egg is one of the common foods consumed by humans. According to statistics by Penang Veterinary Services, 360,641.98 metric tonnes of chicken eggs was produced in 2014 [4]. The yolk inside the egg is only the part that can be eaten meanwhile the shell is considered as a solid waste. The cuticle on the outer surface, a spongy calcereous layer and an inner lamellar layer are the three layered structure that made up the hen eggshell [5]. All avian eggshells are made up from the same mineral component, calcium carbonate (CaCO₃) also known as calcite [6].

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One of the main problems in enzyme immobilization is the cost of the carrier. As the eggshell can be obtained at zero cost, it can be used as the carrier for enzyme immobilization. On top of that, eggshells have a great mechanical strength where it helps in resisting against microbial attack [7]. There are many methods for enzyme immobilization and adsorption is the most common method used. Only weak physical interaction occurred between the carrier and enzyme such as Van Der Waals forces, hydrogen bonding and ionic interactions. This weak binding maintained the native structure of the enzyme [8].

There are a wide range of applications sugar ester in industry such as in food, cosmetics and surfactants in pharmaceuticals [9]. The efficiency of the enzyme lipase for esterification process depends on the choice of substrates [10]. Different lipases favor different selectivity either has high selectivity on short, branched fatty acids or long and medium chain fatty acid [11]. According to Saultani et al. [12], 100% of fructose conversion yield obtained when C18 fatty acid was used as acyl donor. In this present study, parameter affecting immobilization of lipase on chicken eggshells was studied together with parameter affecting synthesis of fructose fatty acid ester.

2. Materials and Method

2.1 Materials
Lipase from Rhizomucour Miehei, Candida Antarctica and Candida Rugosa used in this study were purchased from Sigma. All chemicals used were analytical grade.

2.2 Lipase activity assay
Tributyrin method was used to determine the lipase activity following method suggested by Chattopadhyay and Sen [13]. 1mL of 0.02M NaOH is equivalent to 100 mol fatty acid liberated per minute. The lipase activity was expressed as fatty acid produced per minute per mg lipase. For immobilized lipase on eggshell, 1g of eggshell was used.

2.3 Lipase Immobilization
Before lipase immobilization, the eggshells used were pretreated. Eggshells were boiled in a 0.1% Sodium dodecyl sulfate (SDS) solution for 15 min. The shells were washed thrice with distilled water to remove residual SDS. Next, eggshells were washed with acetone thrice to remove water. Acetone were removed by drying at 60°C for 5 h. Dried eggshells were then crushed and sieved through a mesh to generate uniform size. Lipase was added into conical flask containing eggshells and incubated in incubator shaker at 37°C. After incubation, the conical flasks were stored at 4°C for 16 h. The enzyme-immobilized matrices were then washed with phosphate buffer (50mM, pH7) three times to remove unbound enzyme.

2.4 Esterification
In this study, fructose and stearic acid was used as substrates for the synthesis. Synthesis of sugar ester was done in a 25mL shake flask where 0.5 mol stearic acid, 0.5 mol fructose and 0.2g of immobilized lipase were added into the shake flask. 6mL of ethanol was added as solvent. The mixture was incubated for 72 hours at condition 200 rpm and 37°C. Another set of mixture which acts as a control was prepared simultaneously without lipase. This method was done according to Neta et al., [14]. To determine the content of sugar ester, volumetric method was used by calculating the amount of fatty acid left in the reaction mixture. 0.1g of aliquot was taken from the reaction mixture and added into 20mL of ethanol. Phenolphthalein was used as indicator. The sample was then titrated with NaOH (0.02M). The yield was calculated based on equation 1-3.

\[
\frac{V_{NaOH \times 0.02}}{W_{sample}} = X
\]  

(1)
Yield (%) = 100 - \frac{V_{\text{NaOH}}}{V_{\text{Control}}} \times 100 \tag{3}

V \text{ NaOH indicated the volume of sodium hydroxide, W sample was the weight of the sample and W Control was the weight of the control.}

3. Results and Discussion

3.1 Lipase activity
Lipase activity assay was done on three different lipases from Rhizomucor Miehei, Candida Antarctica and Candida Rugosa. As shown in Table 1, Rhizomucor Miehei showed the highest lipase activity that was 360.8 mol/min/mL. Based on this result, Rhizomucor Miehei was chosen to be used in further study on parameter affecting immobilization. All the experiments were done in triplicate. Previous study by Palomo et al. [15] used all three enzyme in their study but immobilized lipase from Rhizomucor Miehei showed 20 times more active than the soluble enzyme.

| Enzyme                  | Activity (mol/min/mL lipase) |
|-------------------------|-------------------------------|
| Rhizomucour Miehei      | 360.8 ± 1.6                   |
| Candida Antarctica      | 32.64 ± 1.7                   |
| Candida Rugosa          | 6.83 ± 1.4                    |

3.2 Effect of incubation time on lipase immobilization
Based on Figure 1, 2 hr of incubation time shows the highest lipase activity followed by 1 hr of incubation time. This result was supported by previous research that also acquired 2 hr as the best incubation time [16]. Lipase activity started to drop at 3 and 4 hr. Long incubation time can caused multi-layered adsorption and will decrease the enzyme activity [17].

![Figure 1](image-url) Lipase activity for eggshell immobilized with R.Miehei
3.3 Effect of enzyme loading on eggshell immobilized lipase

Enzyme loading was one of the vital parameters that should be studied as it will affect the production cost. In this study, 0.2 mL and 0.4 mL was loaded into 1g of eggshell during immobilization. After immobilization, these eggshells were used in the synthesis of sugar fatty acid ester (Figure 2). Both showed the highest yield at 36 hours where 78.13% sugar ester was produced. 0.2 mL enzyme loading showed faster conversions compared to 0.4 mL. Study by Soultani et al. [12] which used the same substrates (fructose and stearic acid) achieved 100% conversion yield at condition 60°, 200 rpm using 2-methyl-2 butanol as solvent. Although the maximum yield obtained in this present study was only 78%, lower temperature was used (37°C).

![Figure 2](image)

**Figure 2.** Esterification of fructose and stearic acid with eggshell immobilized lipase using different enzyme loading.

3.4 Reaction time of esterification producing sugar ester

Figure 3 showed the reaction time from 0-72 hours of esterification using different incubation time of lipase immobilization. The highest yield of conversion showed at 36 hr (78.13%). After 36 hr, the conversion started to drop and maintained from 48 hr to 72 hr. The same period of time was also obtained by Neta et al. [14] when synthesizing sugar fatty acid ester with the highest yield at 37.8 hr. There were quite large differences in yield of conversion between 1 and 2 hr and 3 and 4 hr incubation time (Figure 3). This may be due to a decrease of lipase activity as shown previously in Figure 1 (Section 3.2).
4. Conclusion

Based on this study, eggshells can be used as a carrier for lipase immobilization. Lipase from *Rhizomucor Miehei* which obtained the highest lipase activity was used for immobilization onto eggshells with the enzyme loading of 0.2 mL and incubated for 2 hr. 78.13% yield was obtained when the immobilized lipase was used in synthesis of fructose fatty acid ester.

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