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Enhancing Biodiesel Production: Comparative Study of the Catalytic Activity of Free and Immobilized Candida Rugosa Lipases

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ABSTRACT
The existing problem of diminishing petroleum reserves has attracted attention as a potential threat to resources sustainability if large-scale use remained on going. Biodiesel fuel is better than the conventional diesel fuel in terms of its renewability, improved exhaust emissions, and biodegradability. While the production at industrial level favoured alkali-catalyzed process, it has inherent downsides, including difficulty in the recovery of glycerol and salt catalyst as well as the energy intensive nature of the process. Previously we reported the immobilization of Candida rugosa (Type VII, 1176 units/mg) lipase in PVA-AlginateSulphate beads with entrapment and cross linking method.In this study, enzyme assay of Candida rugosa (Type VII, 1176 units/mg) was conducted usig p-NPP as substrate for both free lipase and the immobilized form over 3 hours of reaction using spectrophotometer, absorbance read at 410nm. Immobilized lipase in general maintained their activity after an hour of the reaction. At 0.4 and 0.5 M concentrations, both the free and the immobilized showed a steep fall with time. Reusability test of the immobilized lipase was also conducted in terms of biodiesel production by transesterification and found capable of maintaining a production of five consecutive cycles. A field Emission Scanning Electron Microscopy (FE-SEM) analysis was also carried out to study the immobilization matrix morphology before and after the reusability cycles which showed sign of lipase denaturation after 5 cycles.

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INTRODUCTION

The existing problem of diminishing petroleum reserves has attracted attention as a potential threat to resources sustainability if large-scale use remained on going. Biodiesel fuel is better than the conventional diesel fuel in terms of its renewability, improved exhaust emissions, and biodegradability (Shuhara *et al*., 2024). Biodiesel is a renewable fuel that can be synthesized from comestible and noncomestible vegetable feedstock, and waste cooking oil. The propelling geared towards the use of waste cooking oil in biodiesel production is that it stands as a solution between the demand for feedstock for food and for the biofuel. Conflict of food vs. feedstock has for long being retarding the acceptance of biofuel as a solution to the diminishing petroleum resource because of the competition and the cost that will accrue on the final fuel.

Numerous methods have been developed for biodiesel production involving transesterification by chemical or supercritical alcohol treatment or by enzymatic catalysis. While the production at industrial level favoured alkali-catalyzed process, it has inherent downsides, including difficulty in the recovery of glycerol and salt catalyst (usually potassium or sodium), the need for catalyst elimination, and the energy intensive nature of the process. Farouk *et al*. (2024) reviewed the acids use in biodiesel,

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although solid acids can solve the corrosion and environmental problem of liquid acid but yet, the former cannot replace the latter as it can concurrently conduct esterification and transesterification, but may require high temperature due to the slower reaction. The high pressures, temperature and methanol requirement of supercritical fluid technology requires excessive energy and capital input expenses, a problem that makes its industrialization worrisome (Chen *et. al*., 2009). The conversion rate of waste cooking oil via enzymatic methanolysis produced high yield and less waste water (Shimada *et al*. 2002).

As an organic catalyst, enzymes show a number of advantages such as a high order of catalytic efficiency with high degree of selectivity, comprising the chemical, regio and stero selectivities (Palomo *et al*., 2004). Lipases are enzymes that hydrolyze the ester bonds in mono-, di- and triacylglycerols. The alkyl esters of fatty acids, namely fatty acid methyl ester or ethyl ester (Biodiesel) can be produced by alcoholysis of vegetable via lipase catalyze process. Lipase catalyzed reaction at mild condition, can proceed with or without organic solvent (Pizarro & Park, 2003).

Lipase catalyzed production of biodiesel from waste cooking, sunflower, soybean oil, mixture of vegetable oils, grease and tallow, palm oil kernel and coconut oil, and rice bran oil. Yet, there exist some functional problems in the course of enzyme use, for instance, high market cost and instability in reaction. The duo problems make the biodiesel production unwise economically, nonetheless, can be overcome by immobilization and reusability. Immobilizing enzyme onto insoluble or solid matrix has been accepted as a suitable strategy to enhance enzymes thermal and operational stability, enable large amounts production with recoverability and reusability (Cao, 2005; Ye *et al*.,2006). Therefore, immobilization prior to enzymatic transesterification of triglycerides can over shadow the use of the conventional chemical processes.

In our previous study we reported successfully the immobilization of *Candida rugosa* lipase in PVA-alginate sulfates beads, and the ideal parameters to obtained biodiesel from 2-level factorial design using the beads. This study investigated the reusability cycle of PVA Alginate-Sulfate-Beads Immobilized *Candida rugosa* Lipase in biodiesel production and evaluate the immobilized matrix in terms of enzyme activity within the beads and the immobilization.

MATERIALS AND METHODS

Sample Collection and Characterization

Waste cooking oil was collected locally from KB Delight, a restaurant at Taman U, Skudai, Johor Bahru, Malaysia.

Lipase Assay

The assay of both free lipase and immobilized lipase was determined according to the assay described in Ye *at al*., (2007) with a slight modification. In the standard conditions, the reaction mixture was composed of 1.0 ml ethyl alcohol containing 0.1 to 0.5 M concentration *p*-NPP and 1.0 ml PBS (pH 7.5, 50 mM) in a flask. The reaction started by the addition of 0.10 mL free lipase (7.23 gram immobilized lipase). Both mixtures were incubated at 37 °C under equal agitation at 120 rpm. After 5 min of reaction, agitation was halted; the reaction was then terminated by adding 2.0 ml of 0.5 N Na_2CO_3 followed by centrifuging for 10 min (10,000 rpm using KUBOTA 5922 Japan). The supernatant 1 ml was used was read at 410 nm, via Shimadzu UV–Visible spectrophotometer (2450) against a blank without both free and immobilized lipase. The activities of both lipases were determined by the release of p-nitrophenol (pNP)/min. One IU was defined as the amount of biocatalyst liberating 0.1 µmol of p-NPP/min.

Enzymatic Transesterification

A 9.65 g of waste cooking oil was mixed with immobilized lipase at oil to methanol ratio of 1:4. The experiment was conducted at a room temperature under agitation of 200 rpm. After every 24 hours, the reaction mixture was collected and centrifuged at 10,000 rpm to remove the organic phase while the immobilized lipase was washed with Isopropanol and reintroduced to

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another experiment (Yagiz *et al.*,2007). Finally, the upper layer of the samples was analyzed for Fatty acid methyl ester (FAME) using Gas chromatography (GC).

PVA-Alginate Beads Characterization

The FE-SEM was used to observe the surface morphology and cross-sectional structure of the PVA-alginate sulfate beads. The beads were patted dry and cut with surgical blade. The sections were placed on a stand and the cross-sectional images were observed using the FE-SEM, Model Ziess SUPRA 35 VP FE-SEM.

Gas Chromatography Setting

The samples were analyzed by using GC, Perkin Elmer Autosystem XL using Nukol™ 15x 0.53 mmm I.D., 0.5µm (25326) DB 23 column with a flame ionization detector operational. Helium served as a carrying gas at a flow speed of 20 mL/min. The temperature of column programmed was from 110°C to 220 °C with the increase of 8 °C /min. The injector (7683B) and detector being monitored at 250 °C and the amount of sample injection was 0.2 µl (direct injection). The presence of biodiesel was based on the comparison of retention time and the peak area of the sample with the standard values.

RESULTS AND DISCUSSION

Lipase Assay

Absorbance at different substrate concentration, 0.1-0.5 M of p-NPP was determined as an indication of release of p-NP per minute. The absorbance against time is plotted in Figure 1. In this figure, high rate of degradation of p-NPP was experienced at 30 min of reaction with an activity of 832.7 IU free lipase, indicating high release of p-NP. After that a drastic fall was seen after an hour indicating loss of activity. A lower peak of 407.2 IU was experienced in the initial of the reaction for the immobilized enzymes, this is showing a less degradation of substrate with a corresponding decrease in the release of p-NPP as compared to the free enzyme. But subsequent activities even though both are falling nevertheless the immobilized ones are more striving than the free ones with the time and tend to have a more uniform level than the free ones.

This trend is implying that if the reaction was to continue for assuming 4 hours the free lipase would not be able to withstand substrate degradation. In a study of Biochemical properties of free and immobilized *Candida rugosa* lipase by Yeşiloğlu and Şit (2011), a similar scenario was observed where free lipase peaks early (832.7 IU) but loses activity, while immobilized lipase sustains performance. Thus, free lipase expressed higher initial activity, but immobilized one had improved stability and retained activity longer.

At 0.2 M concentration in Figure 2, it has almost the same case with the previous graph, the only difference is that the free lipase strives till two hours of the reaction before it falls below the immobilized lipase after 180 mins corresponding to an activity of 35.51 IU free and 38.1 IU immobilized. Figure 3 shows a steep fall in activity at time 30 min, this is somewhat similar case with Figure 1 and 2. From the graph it can be seen that the immobilized lipase tends to perform better in an hour time than the free lipase, which its activity goes below the immobilized. Metin (2023) in a comparative study between free and immobilized Candida rugosa lipase onto hydrophobic group carrying polymeric support shows lower initial degradation (lipase activity, 407.2 IU) has lower initial degradation nonetheless more uniform activity over time.

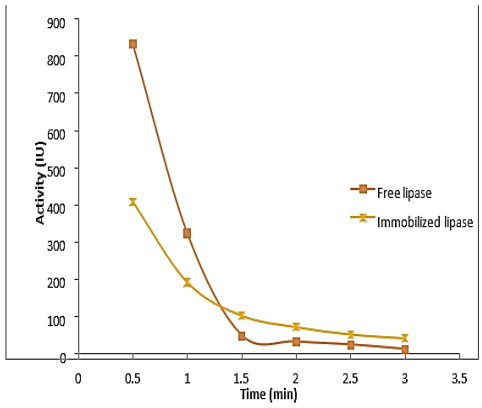

Figure 1 Comparison of the activity of free and immobilized lipase at 0.1 M p-NPP

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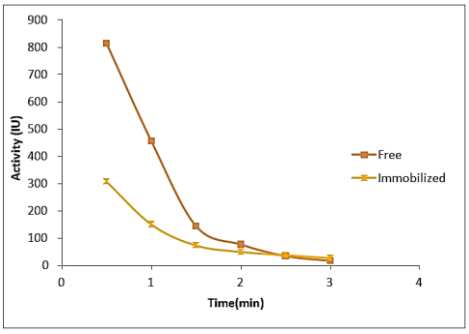

Figure 2 Comparison of the activity of free and immobilized lipase at 0.2 M p-NPP

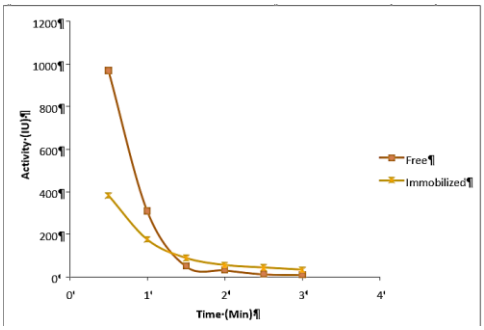

Figure 3 Comparison of the activity of free and immobilized lipase at 0.3 M p-NPP

From Figure 4 and 5 especially the latter, totally different phenomena contrary to above concentrations were observed. Both the free and immobilized showed a drastic fall, and subsequently lost their activities at nearly the end of the reaction, immobilized one falling even much lesser than the free one.

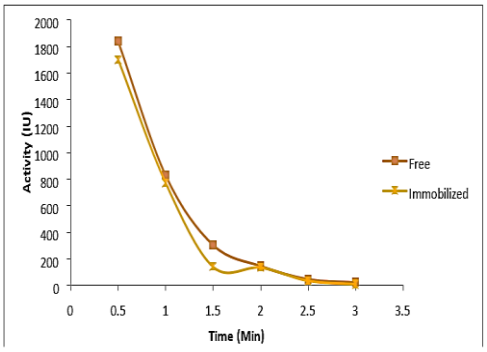

Figure 4. Comparison of the activity of free and immobilized lipase at 0.4 M pNPP

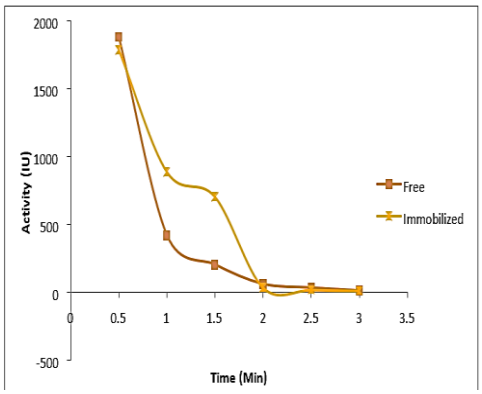

Figure 5. Comparison of the activity of free and immobilized lipase at 0.5 M p-NPP

Krakowiak *et al*. (2003), reported difficulty in accessing immobilized lipase on a carrier by large molecules of substrate present in a reaction mixture particularly palmitate. According to Murty, Bhat *et al*. (2002) entrapment technique has the inevitable disadvantage that the support will act as a barrier to mass transfer, thereby resulting frequently in transfer limitations of substrate to the enzyme active site (Murty, *et al*., 2002). Therefore, small substrate must be used in order for the substrate to reach the enzyme active site.

Certain hypothesis can be drawn based on the statement from above researchers, for the immobilized lipase in 0.4-0.5 M concentration, most likely this scenario cannot be attributed to mass transfer limitation since both immobilized lipases from the two graphs showed a higher activity from the initial time or the mass transfer limitation had occurred after an hour considering the graph. However immobilized lipase in 0.1-0.3 might have encountered the difficulty in the transfer. Otherwise, the inevitable disadvantage of the immobilization technique had occurred to all of them as reported (Murty *et al*., 2002).

Kinetics of Free and Immobilized Lipase

In order to know the exact rate at which the enzyme works, the enzyme kinetics become pertinent. In this work the Lineweaver-Burk method of analysis was selected for linearizing substrate-velocity data in order to determine the kinetic constants K_m and V_{max}. Secondary data

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was generated by the plot of the reciprocal of velocity and the substrate, this is seen in Figure 6 Various concentrations of p-NPP as a substrate was varied with the unvarying enzyme concentration to determine the K_m and V_{max}, i.e, Michaelis constant and velocity, respectively.

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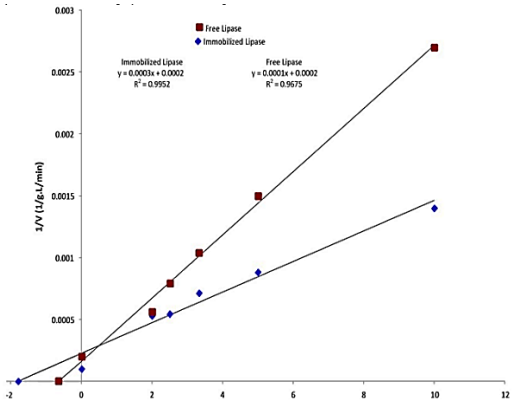

Figure 6. Lineweaver-Burk plots for Free and Immobilized lipase

K_m is (more or less) an inverse determinant of the affinity of binding between the enzyme and its substrate. Thus, the lower value of K_m, the greater the affinity, so that the concentration of should be low in order to achieve a desirable rate of reaction.(http://users.rcn.com/jkimball.ma.ultranet /BiologyPages/E/Enzyme Kinetics.html). In this case the value of K_m^{app} obtained for immobilized enzyme from the experiment is notably lower than the free enzyme, and in both cases have higher V_{max} to their K_m. By way of comparison between the free and immobilized enzyme, the likeness of enzymes to substrate is seen higher for the immobilized. The result is seen in Table 1. Zain *et al*. (2011) using the same kind of immobilization matrix reported, K_m^{app} and V_{max} of two produced beads; 12PVA-5BA and 12PVA-7BA as 4.7 x 10^{-5} (mM) and 2.8 x 10^{-5} (mM) and 2281.02 and 1751.36 and for the free enzyme, 28.7 (mM) and 75.49 (Umg^{-1}) (19).

Table 1 Kinetic parameters of free and immobilized lipase

	Free lipase	Immobilized lipase
K_m^{app} (mM)	1.60	0.56
V_{max} (U mg^{-1} enzyme)	5.33 x 10^3	5.6 x 10^3

Higher affinity of immobilized lipase towards the substrate than the free lipase was reported by Li *et al*. (2010), as PST-300-lipase- K_m^{app} 0.402(mM) and PST-100-lipase K_m^{app} 0.411(mM) with the free lipase having K_m of 0.441(mM) (Li *et al*., 2010). The difference in affinity between the free and immobilized might be linked to the increase substrate concentration in the locality of binding site of immobilized enzyme (Zain *et al*., 2011). Affinity lower than the current literature was reported on the work of (Ozcan & Sagiroglu, 2009), The K_m^{app} was 1.67 (mM) and V_{max}^{app} 22.2 (mM) for immobilized lipase.

Enzymatic Transesterification

For any immobilized biocalyst to be accepted for economic and practical productions, the recycle ability is of paramount importance to elucidate its catalytic efficiency. Since one of the several main rationales behind the immobilization is to allow the enzymes to be reused for quite a number of times, it is alongside this background that the quality of support matrix material should be determined based on the enzyme catalytic retention while within the matrix. Robles-Medina, (2009) reported that the strength of the matrix depends on its catalytic retention. The 1st, 2nd , 4th , 5th cycles of the production the production of all the fatty acid methyl esters was almost maintained as seen in Figure 7. Oleic acid methyl ester production started with 1 g/l on day one and ended with the same concentration of day 5 with the exception of day 3, 0.8 g/l. A Similar trend was followed by oleic acid methyl esters, however with different concentration. Oleic acid methyl ester was 0.4 g/l on day one and ended with the same concentration on day 5, exceptions being day 3 with 3 g/l.

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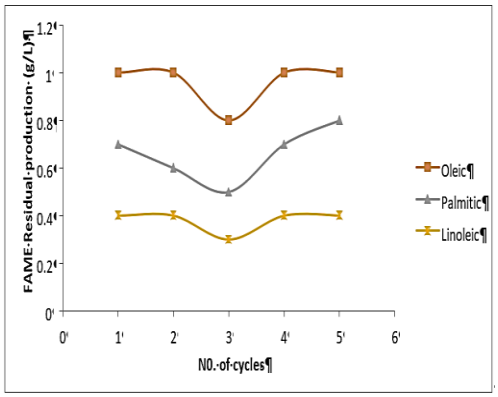

Figure 7. Reusability cycles for lipase

Figure 7 shows the production of palmitic acid methyl ester, with 0.7 g/l on day 1, 0.6 g/l, day 2, 0.5 g/l, day 3, 0.7 g/l, day 4 and 0.8 g/l, day 5. In generally, the production of fatty acid methyl ester is almost maintained throughout the reaction, indicating no depression in the activity of the immobilized lipase which is our point of interest. The slight fall experienced in cycle 3 can be hypothesized to inadequate washing of the beads thereby decreasing the access of substrate to the active site, as similarly observed by Idris *et al*. (2008). This finding attests to the fact that immobilized lipase in PVA-alginate- sulfate beads can be used for quite a number of times without significant loss in activity (Zain *et al*., 2010) and enzymes immobilized have their activity retained for a longer time and can be used repeatedly. Quite a number of researchers (Kumari *et al*., 2008; Chatterjee *et al*., 2014) have reported the use of immobilized lipase to that fold.

PVA-Alginate Sulphate Beads Characterization and Fesem- Analysis

The PVA-alginate sulphate beads produced was examined and characterised by FESEM with a magnification of 2.50 KX under EHT 5.00 KV. Figure 8 (a) reveals the *Candida rugosa* globular shape before the transesterification and the (b) showed the *Candida rugosa* lipase after 5 cycles of transesterification. Figure 8 (b) shows a sign of perforations in the lipase. Which could be related to inactivity of enzymes as a result of protein denaturation (Zhang *et al*., 2025).

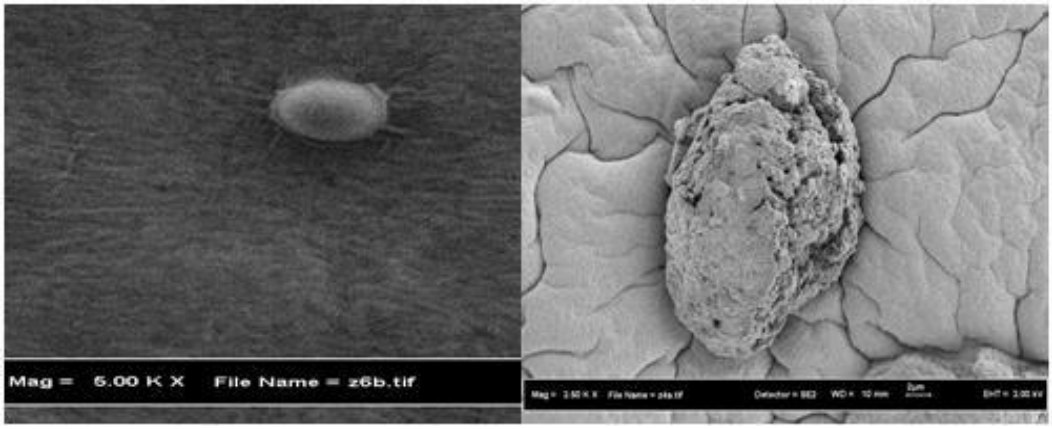

Figure 8. Immobilized *Candida rugosa* (a) before enzymatic transesterification, (b) after 5 cycles of enzymatic transesterification

From the number of cycles and the FESEM Images a deduction can be made; which is most likely that the inactivation started after the 5th cycle since the result of cycle 5 did not show decrease in production. Therefore, reusability cycles beyond 5 have become pertinent.

CONCLUSION

The findings in this research indicated that the immobilized *Candida rugosa* lipase in

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PVA alginate-sulfate beads are reusable and capable of being used systematically and productively for up to five numbers of cycles without considerable loss in activity. Certain hypothesis can be drawn with higher concentration of *p*-NPP in assay, most likely this scenario cannot be attributed to mass transfer limitation since both showed a higher activity from the initial time. Lower concentration might have been affected the difficulty in the transfer. Or the M concentration is generally high. There is need for further investigation.

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