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Immobilization of Candida Rugosa Lipase in PVA-Alginate-Sulfate Beads for Waste Cooking Oil Treatment

Nor Badzilah Hasan[a], Tan Wei Yie[a], Nor Azimah Mohd Zain[a]*, Mohd Suardi Suhaimi[b]

[a]Department of Biotechnology and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia
[b]Deparment of Chemical Engineering,Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

*Corresponding author: azimah@fbb.utm.my

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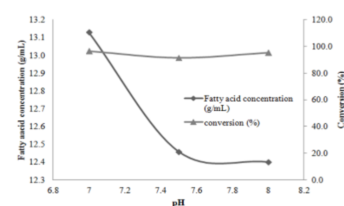
Graphical abstract



Abstract

Hydrolysis of waste cooking oil (WCO) using immobilized *Candida rugosa* lipase (CRL) was studied. PVA-Alginate-Sulfate beads were used to immobilize CRL. During the transesterification process, three parameters were considered: pH, temperature and enzyme concentration. The degree of hydrolysis as well as the rate of the hydrolysis were also determined. The morphology of the beads was analyzed using Fourier Emission Scanning Electron Microscopy (FESEM). It was found that the operating conditions, pH = 7.00, temperature = 50°C, and bead loading of 8 g, were most favourable for the hydrolysis of WCO by immobilized CRL to yield maximum fatty acid production and hydrolysis conversion. It was also found that the rate of hydrolysis by immobilized CRL is higher than that of free enzyme which is 96.50% and 68.75%, respectively.

Keywords: Immobilization; transesterification; waste cooking oil; fatty acid, pva-alginate-sulfate beads; *candida rugosa* lipase

Abstrak

Hidrolisis sisa minyak masak (WCO) menggunakan lipase *Candida rugosa* (CRL) tersekatgerak adalah dikaji. Manik PVA-Alginate-Sulfate telah digunakan untuk menyekatgerak CRL. Semasa proses transesterifikasi, tiga parameter diambilkira: pH, suhu dan kepekatan enzim. Darjah hidrolisis dan kadar hidrolisis juga dikenal pasti. Morfologi manik adalah dikaji mengunakan Fourier Emission Scanning Electron Microscopy (FESEM). Didapati pada keadaan, pH = 7.00, suhu = 50°C dan berat manik = 8 g, merupakan yang terbaik untuk hidrolisis WCO oleh CRL tersekatgerak untuk menghasilkan asid lemak dan penukaran hidrolisis yang maksimum. Dari kajian ini juga didapati, hidrolisis oleh CRL tersekatgerak adalah lebih tinggi berbanding enzim bebas, iaitu masing-masing sebanyak 96.50% dan 68.75%.

Kata kunci: Tersekat gerak; transesterifikasi; sisa minyak masak; asid lemak; manik PVA-Alginat-Sulfat; lipase Candida rugosa

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■1.0 INTRODUCTION

Enzyme has been used for ages due to its high specificity in catalytic reaction. Enzymatic hydrolysis is an advantageous approach as it can be performed at mild condition and exhibits high specificity which leads to products with high purity and less by-products [1]. Moreover, it is more energy efficient and cost effective compared to conventional chemical processes [2].

Enzymes can be used in free or immobilized form. Immobilization of enzymes can be defined as physical confinement or localization of enzymes molecule with retention of its catalytic activity which enables it to be used repeatedly [3]. Immobilization offer great numbers of advantages over free enzymes as they enhance activity and stability of enzymes, permit reusability of enzymes, hence facilitate continuous and large scale used of enzymes at industrial scale. It also allows the ease in product separation [4]. These suggest that immobilization of enzymes is economically viable.

There are various methods that have been developed to immobilize enzymes. Enzymes can be adsorbed onto an inert solid support, entrapped in porous polymeric matrices or gels or encapsulated in beads. Besides that, enzymes can be covalently bonded to solid support via chemical bonding methods [5, 6]. In recent years, polyvinyl alcohol (PVA) which is a cheap and non-toxic synthetic polymer has been widely used for enzymes immobilization due to its advantages compared to alginate beads. Enzymes immobilized in PVA beads show higher activity and

higher thermal stability with repetitive use. PVA beads also provide stronger mechanical strength due to its elasticity [7].

Lipase (triacylglycerol acylhydrolase, (EC 3.1.1.3) is an enzyme which has the ability to catalyze the hydrolysis of fats, oils and triglycerols to fatty acid and glycerol [8]. Versatility of lipase in catalytic reaction has made it a unique industrial biocatalyst in environment conservation, foods, pharmaceutical, and cosmetics industry [9]. Lipase produced from *Candida rugosa* is widely used and documented due to its high activity in hydrolysis, esterification, transesterification and aminolysis [10]. By using an appropriate choice of immobilization method the operational cost of industrial process involved can be significantly reduced as the enzymes can be used continuously and repeatedly [11].

Malaysia and Indonesia currently contributes 87% of world palm oil production for 2011 and 2012 [12]. Cooking palm oil is a vegetable oil which predominantly consists of triglycerols. Thus, it can be hydrolyzed by lipase to produce fatty acid and glycerol. Palm oil is considered one of the best oils for frying due to the ability in resisting high temperatures and does not produce unpleasant smells. On the other hand, non-edible uses of palm oil include soaps and detergent, oleochemicals, cosmetics, lubricating greases for machinery used and biodiesel production [13]. Furthermore, with the huge varieties of oil produced worldwide, management and disposal of the waste oil are the major challenge facing the production because of possible contamination of water bodies and land resources [14]. The hydrolysis of oils provides possibilities of overcoming these problems. Fatty acids and glycerol are valuable chemical intermediates with a variety of end uses. These include coatings, adhesives, specially lubricating oils, shampoos and other personal care products [4]. Hydrolysis of oil depend on different kinds of catalyst can be divided into the following methods: acid method; alkali saponification (NaOH and KOH); without catalyst and lipase catalysis. Among of these methods, lipase catalysis shows promising way in term of low energy consumption (reaction can be carried out in mild condition), less solvent, and give cleaner products attributes of green chemistry [15]. Before this, PVA-Alginate-Sulfate has been successfully used to hydrolyse liquid pineapple waste [11] and transesterified Jatropha oil [16]. In this study, PVA-Alginate-Sulfate beads were used to immobilize *Candida rugosa* lipase (CRL) then it was used to hydrolyse the waste cooking oil from palm oil. By using the aforementioned immobilized method, value-added-products such as fatty acid and glycerol could be produced.

■2.0 MATERIALS AND METHODS

2.1 Materials

Candida rugosa lipase (3.1.1.3) (Type 1176 U/mg) was purchased from Sigma Aldrich (Japan). Polyvinyl-alcohol (PVA) 60,000 MW and boric acid were purchased from Merck Schuchardt OHG, Darmstadt, Germany. Sodium alginate was obtained from FlukaChemie GmbH, Buchs, sodium sulfate from GCE Laboratory Chemicals and calcium chloride from R&M Marketing, Essex, UK. Iso-octane with 99.84% assay was purchased from Fisher Chemicals (UK). Other reagents used were analytical reagent grade and used without further purification including phosphate buffer solution pH 7.5 or otherwise stated.

2.2 Pretreatment of Waste Cooking Oil

Waste cooking oil (WCO) was obtained from food stall near Universiti Teknologi Malaysia (UTM). For a successful reaction, the oil must be free of water and other impurities. Initially the samples of waste cooking palm oil were filtered to remove any suspended food particle. Then, the waste cooking palm oil was heated at 105°C for 1 h to remove its water content. After that, titrimetry method with NaOH was used to determine the free fatty acid (FFA) content in the WCO [17].

2.3 Preparation of Lipase Enzyme Solution

The pH of the phosphate buffer solution (PBS) was adjusted to pH 7.5. *Candida rugosa* Lipase (5 g) was dissolved in 100 mL of PBS. Then the enzyme solution was filtered using 0.45 µm nylon syringe filter to sterilize the enzyme. The sterilized enzyme solution was stored at 4 °C until further used.

2.4 Immobilization of Candida rugosa Lipase (CRL)

CRL solution with the volume of 10 mL, 5% (v/v) was mixed with 90 mL PVA-Alginate solution. The mixture was mixed comprehensively and introduced as drops by using a rotary pump into a 100 mL mixed solution of saturated boric acid 5% (w/v) and calcium chloride 2% (w/v). The beads were stirred gently for 30-50 min to complete the solidification. Then, the PVA–Alginate beads were stored at 4°C for 24 h. After 24 h, the mix solution of boric acid and calcium chloride was discarded and replaced with 7% (v/v) boric acid solution. The beads were stirred in boric acid solution for half an hour and the solution was then replaced with 0.5 M of sodium sulfate solution and stirred for another 30 minutes. Then, the beads were kept at 4°C until further used [18].

2.5 PVA-Alginate-Sulfate Beads Characterization

The FESEM was used to observe the surface morphology and cross-sectional structure of the PVA-Alginate-Sulfate beads. The PVA-Alginate-Sulfate beads was pattered dry with tissues and cut with a sterile surgical knife to obtain the cross section of the beads. Then the beads were placed on a stand and the beads cross section were observed using FESEM [7].

2.6 Waste Cooking Palm Oil Hydrolysis

A Conical flask of 250 ml was initially filled with 3 g of cooking palm oil and 30 ml of iso-octane solvent. PBS (30 mL, pH 7.0) (unless otherwise stated) was added into the conical flask so that the ratio of oil to aqueous (buffer solution) is 1. The mixture formed two layers. Three other identical mixtures as above were prepared. To start the reaction, 0.3 g of CRL was added to three flasks of reaction mixtures and one was left without the CRL for control measurement. The mixtures were agitated in the orbital shaker at 45°C at 200 rpm. Samples were withdrawn from the oil every 30 min. The same procedure was carried out using immobilize lipase [19]. To determine the effect of parameters on the hydrolysis of waste cooking oil, three variables were taking into consideration which is pH, temperature and enzyme concentration. The pH was varied from 7 to 8. The temperature was varied from 30 °C to 50°C and enzyme loading was varied from 2 g to 8 g of beads which correspond to 96.43 U/mL to 385.73U/mL enzyme.

2.7 Determination of Degree of Hydrolysis (Conversion) and Rate of Hydrolysis

The degree of hydrolysis was determined by titration of the oil phase samples with 0.1 M sodium hydroxide (NaOH). To each samples, 5 mL of the oil phase was dissolved in 5 mL ethanol: diethyl ether (1:1% v/v). The amount of 0.1 M NaOH required to neutralize the acid was noted. A blank titration was done as a control sample. Phenolphthalein was used as an indicator. The degree of hydrolysis, X is calculated as below [17].

$$X\ \% = \frac{(ml\ NaOH\ used)(molarity\ of\ NaOH)(avarage\ molecular\ weight\ of\ fatty\ acid)}{10(weight\ of\ sample)}$$

2.8 Fatty Acid Concentration Determination

All samples collected were analyzed gas chromatography (Perkin Elmer Autosystem XL equipped with a flame-ionization and a Nukol™ 15m x 0.53mm i.d. column coated with 0.5 µm (25326) column) method [20]. Helium served as a carrier gas at a flow rate of 20 mL/min. The column temperature was programmed from 110°C to 220°C with the increase of 8°C/min. The injector and detector was monitored at 250°C and the amount of sample injection was 0.2 µL with direct injection. The presence of fatty acid was based on the comparison of retention time and peak area of the sample with oleic acid as standard.

■3.0 RESULTS AND DISCUSSION

3.1 Field Emission Scanning Electron Microscopy

Figure 1 shows the immobilized enzyme in PVA-Alginate-Sulfate beads. PVA-Alginate-Sulfate beads produced were examined using FESEM with a magnification of 2.50kX. This image was captured after dissecting the beads with surgical blade thereby revealing the native *Candida rugosa* globular shape as depicted in Figure 1. This similar image was also reported in the work of Zarei *et al.* [16] with magnification 2.0kX.

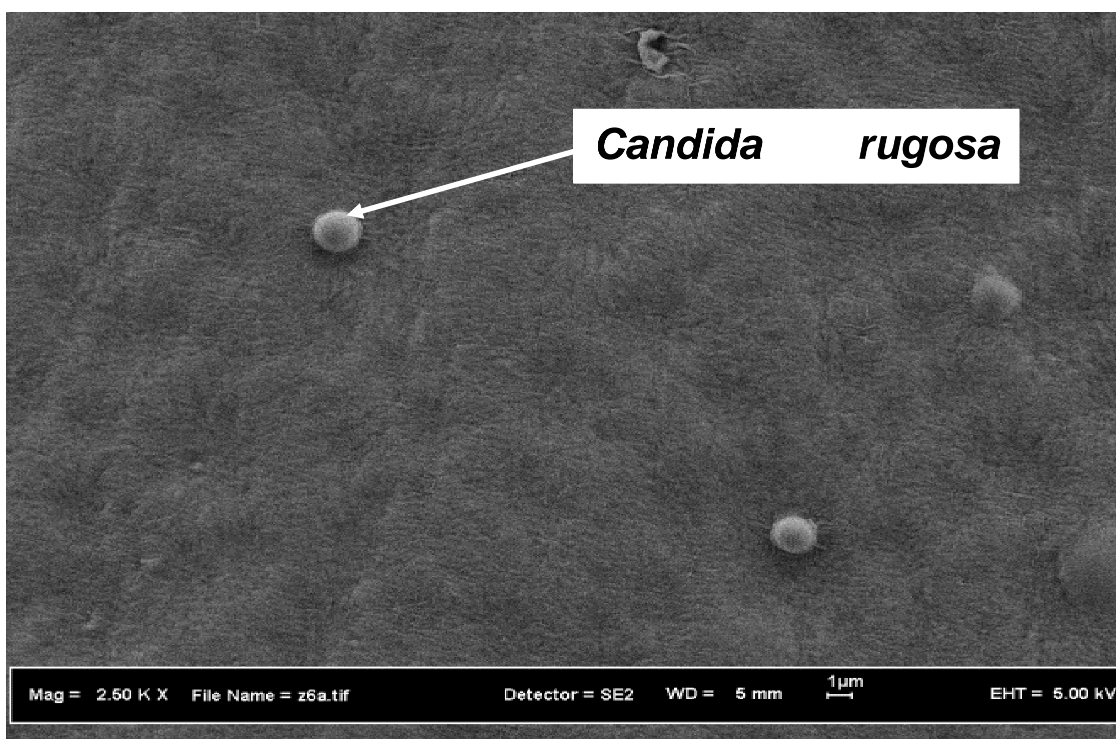

Figure 1 Image of lipase immobilized in PVA-alginate beads taken using FESEM with the magnification of 2.50kX.

3.2 Effect of pH

Enzyme is very sensitive to pH changes. Changes in pH will alter the ionization states of the enzyme and thus affect its selectivity and activity [17]. Therefore, the pH for fatty acid production and hydrolysis conversion from WCO using immobilized CRL were studied. The pH range was varied from 7.0 to 8.0 with other parameters such as temperature (30ºC-50 ºC), enzyme loading, (2 g-8 g) and agitation (200 rpm) were fixed.

Figure 2 shows that the best pH was found to be 7.0 with highest fatty acid production and highest hydrolysis conversion. Subsequent decrease in fatty acid production and hydrolysis conversion were observed as pH was increased. Similar observation was also reported by Garcia *et al*. [21] when lipase from *C. rugosa* was immobilized by adsorption on flat sheets made of micro-porous polypropylene for the hydrolysis of milk fat triglyceridesan. Kang and Rhee [22] also observed that pH of 7 is the best pH for their hydrolysis when using *C. rugosa* lipase immobilized by adsorption on swollen Sephadex for the hydrolysis of olive oil. However, Santos *et al*. [23] obtained an optimum pH of 8 when a lipase from *C. rugosa* was immobilized on poly(N-methylolacrylamide) by physical adsorption. Apparently, the working pH depends mainly on the method of immobilization and the interaction between enzyme and support [24].

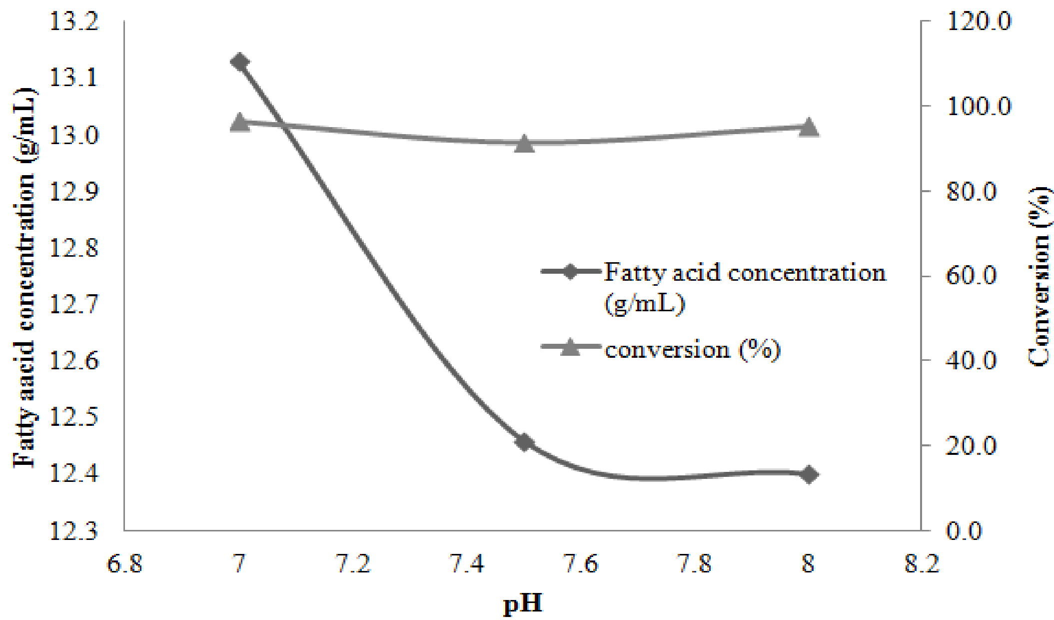

Figure 2 Effect of pH on fatty acid production and hydrolysis conversion by using immobilized *Candida rugosa* lipase. (Temperature = 50 °C, enzyme loading = 8g of immobilized beads (385.73U/mL); 200 rpm)

3.3 Effect of Temperature

Temperature plays an important factor in hydrolysis reaction in order to achieve optimum production of fatty acids and hydrolysis conversion. As reported by most of the studies [25-27], an increased in temperature will speed up the enzyme-mediated reaction to a certain optimum temperature. However, enzymes will be denatured when heated beyond the optimum temperature causing the reaction rate to decrease sharply. In addition to the denaturation of the enzymes, the presence of inactive enzymes at the interface has blocked the accessibility of active enzyme to penetrate the interface, resulted further decrease in the reaction rate [28].

Figure 3 explains that the increase of temperature will also increase fatty acid production and hydrolysis conversion. The highest fatty acid concentration and hydrolysis conversion were achieved at 50°C which is 13.13 g/mL and 96.5%, respectively.

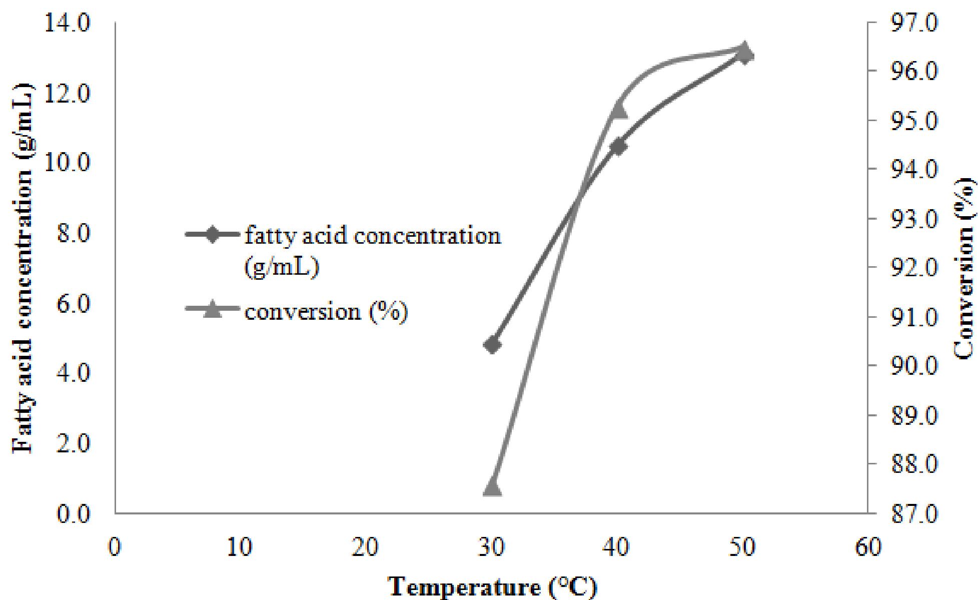

Figure 3 Effect of temperature on fatty acid production and hydrolysis conversion by using immobilized *Candida rugosa* lipase. (pH = 7.0, enzyme loading = 8g of immobilized beads (385.73U/mL), 200 rpm)

Previously, a study done by Dave and Madamwar [29] also gave the same result of the optimum temperature for CRL. The best temperature for CRL that was immobilized in PVA-Alginate-Sulfate beads was found to be 50ºC. Similarly, the best temperature of 50°C was also reported by Santos [23] when using CRL immobilized by covalent attach on polysiloxane–polyvinyl alcohol for the hydrolysis of olive oil. This indicates that the immobilization process did affect the optimization temperature for that particular immobilized enzyme. Therefore, previously reported values supported the finding of this study.

3.4 Effect of Enzyme Loading

The amount of enzyme loaded has a large effect on lipase hydrolysis reaction. In this study, the enzyme loading was varied from 96.43U/mL to 385.73U/mL which corresponded to 2g to 8g of immobilized beads. In general, as the enzyme concentration increased with constant substrate concentration, there is a corresponding increase in reaction rate.

As shown in Figure 4, an increase in enzyme loading led to a subsequent increase in the fatty acid production and hydrolysis conversion. The best enzyme loading was found to be 385.73 U/mL which equal to 8 g of immobilized beads under condition of pH of 7.5, temperature of 40ºC and 200rpm rate of agitation. However, Ozturk [30] found that the enzyme activity reached the highest activity when the *C.rugosa* lipase concentrations immobilized on chitosan beads is 160U/mL. The best enzyme loading needed for reaction also depends on the interaction between support and enzyme.

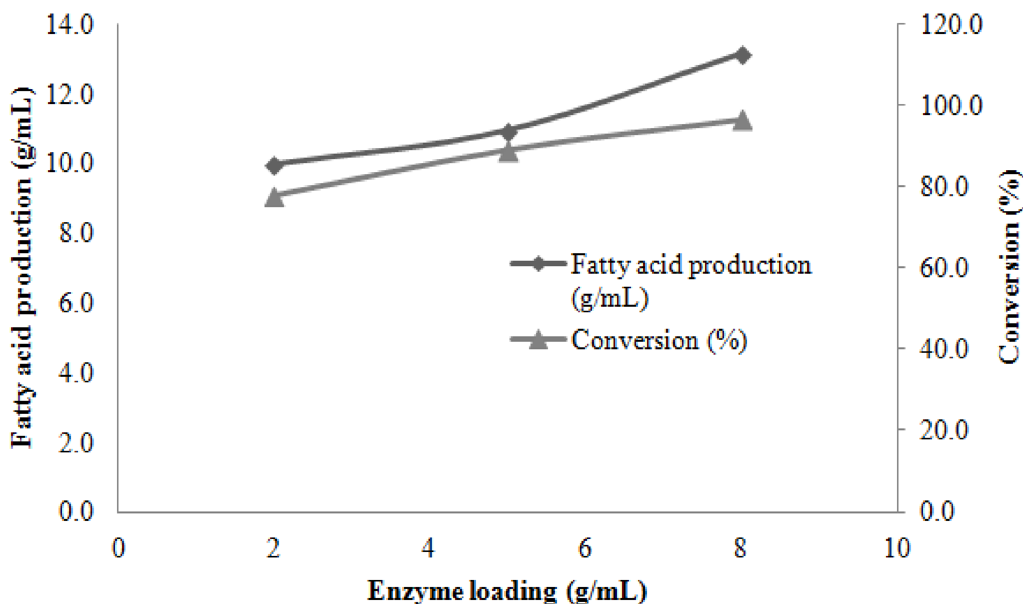

Figure 4 Effect of enzyme loading on fatty acid production and hydrolysis conversion by using immobilized *Candida rugosa* lipase. (Temperature = 50 °C, enzyme loading = 8g of immobilized beads, 385.73U/mL; 200rpm)

3.5 Comparison between immobilized and free cells

The best working conditions for immobilized enzyme in this study and free enzyme [19] for hydrolysis waste cooking oil are shown in Table 1. From the results, the best working pH shifted slightly from 7.5 to 7.0 after immobilization. Such a shift of working pH could be attributed to the relatively higher proton concentration inside the beads which give a more favorable acidic environment needed for the immobilized enzyme. Therefore, the working pH for the immobilized *C.rugosa* lipase shifted from strong neutral to neutral. Similar finding was also reported by Zain *et al*. [7]. However, Liu *et al*. [31] reported that best working pH for lipase shifted from 7 to 8 during olive oil hydrolysis when it was immobilized in micron-sized-magnetic beads. On the contrary, the working pH of lipase in the hydrolysis of olive oil remains unchanged when it was immobilized on polyphenylene sulfide dendrimers. Therefore, the best pH depends mainly on the immobilization method and matrix as well as the interaction between enzyme and its support [24].

Table 1 The best working conditions for immobilized enzyme in this study and free enzyme (Serri *et al*., 2008) for hydrolysis waste cooking oil

Type of working condition	The best working condition This study	Serri *et al.* (2008)
pH	7.0	7.5
Temperature	50°C	45°C
Enzyme loading	385.73U/mL	7.64kLU/mL

An increase in the optimum temperature from 45°C to 50°C for the *C.rugosa* lipase after immobilization was also observed. After immobilization, the beads confer a more rigid structure for *C.rugosa* lipase and led to higher thermal stability. Immobilization provided a more rigid structure for lipase enzyme hence reducing the effect of temperature in denaturing lipase. Some studies [7, 29, 32] also reviewed that the thermal stability of the lipase entrapped in PVA matrices is better than free enzyme. Thus, the thermal stability of lipase could be increased via immobilization. The ability of enzyme to retain enzyme activity at higher temperature offer several advantages such as

reduced risk of contamination, improved transfer rates and improved substrate solubility [33].

Upon immobilization, the optimum enzyme loading needed for waste cooking oil (WCO) hydrolysis was much lower compared to free enzyme. This demonstrates that immobilization method could be used to increase lipase enzyme activity inside the beads. This also implies that less amount of enzyme loading is required to achieve equivalent enzyme activity of the free enzyme.

Hydrolytic activity was also performed for free enzyme under the optimum conditions determined previously for immobilized *C.rugosa* lipase. The results obtained were compared with immobilized *C.rugosa* lipase. As shown in Figure 5 and Figure 6, the immobilized *C.rugosa* lipase has a higher fatty acid production and hydrolysis conversion (at enzyme concentration of 385.73U/mL; pH 7.5; 50°C) which is 13.13g/mL and 96.5% respectively. For free lipase (385.73U/mL; pH 7.5; 50°C) only 2.9 g/mL of fatty acid production and 68.75% of hydrolysis conversion were observed. These results suggest that immobilized *C.rugosa* lipase shows higher activity compared to free lipase under the same conditions. Moreover, this has proven that the PVA-Alginate-Sulfate beads are able to enhance the activity of lipase.

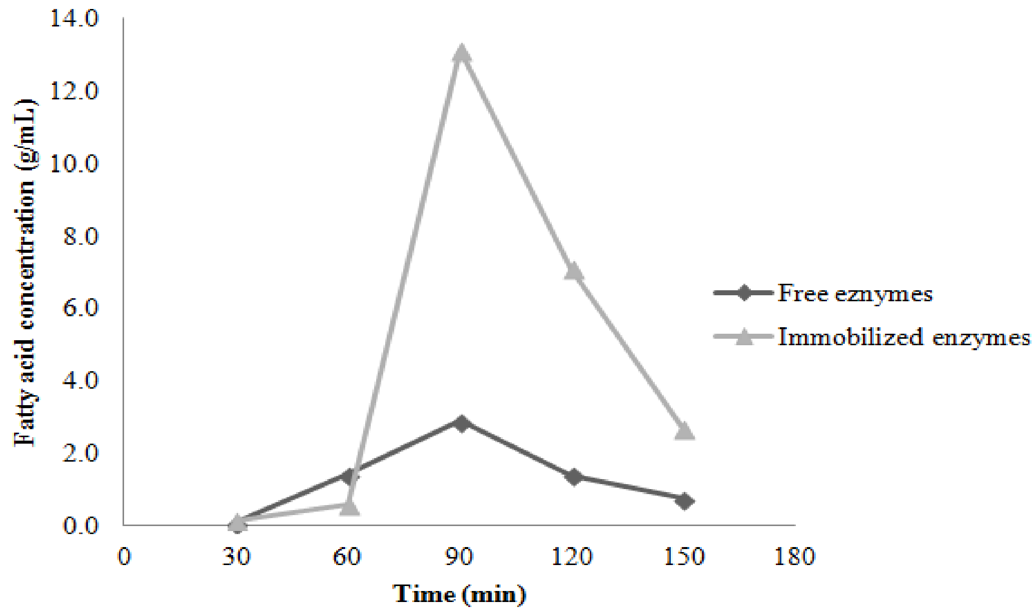

Figure 5 The fatty acid production for waste cooking oil by using free and immobilized enzyme. (Temperature = 50 °C; pH = 7.0; enzyme loading = 8g of immobilized beads, 385.73U/mL; 200rpm)

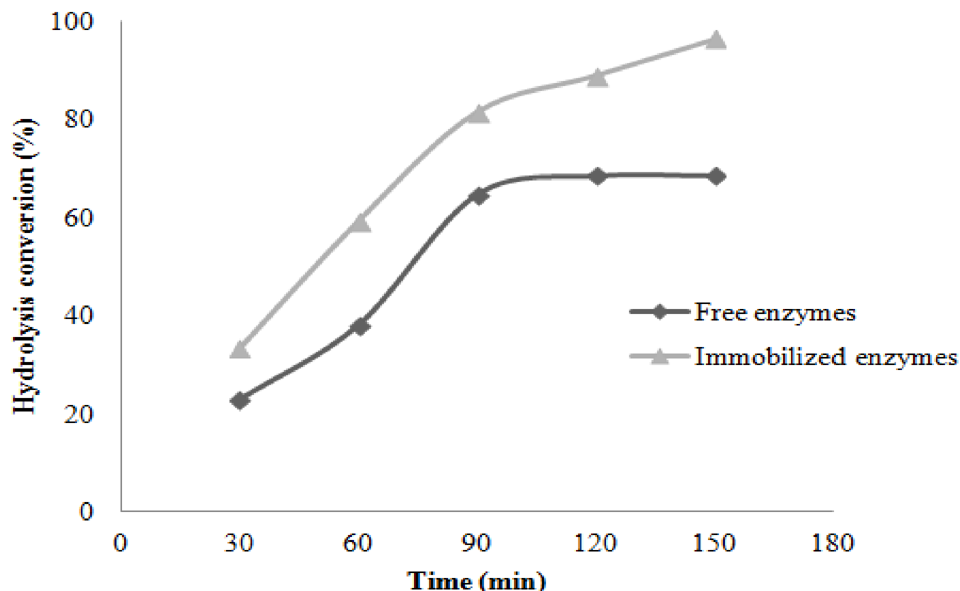

Figure 6 The hydrolysis conversion for waste cooking oil by using free and immobilized enzyme. (Temperature = 50 °C; pH = 7.0; enzyme loading = 8g of immobilized beads, 385.73U/mL; 200rpm)

4.0 CONCLUSION

This study proved that PVA-Alginate-Sulfate is a suitable matrix to immobilize *C. rugosa* lipase. The best conditions for immobilized *C. rugosa* for hydrolysis of waste cooking oil were found to be; pH of 7.0; temperature of 50°C and enzyme concentration of 385.73U/mL. By comparison, immobilized *C. rugosa* lipase shows higher fatty acid concentration compared to free enzyme. Besides that, immobilized enzyme also show higher percentage of conversion compared to free enzyme. It can be concluded that the application of lipase immobilized in PVA-Alginate-Sulfate beads is a promising method for transesterification of waste cooking oil.

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