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Immobilization of Lipase from *Candida rugosa* on Chitosan Beads for Transesterification Reaction

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Abstract: This study is aiming to evaluate the performance of immobilized lipase on a chitosan support in transesterification reactions. Immobilized lipase improved the enzyme stability, allowing reusability, continuous operation and the possibility of better control of reactions. Chitosan offers several advantages as immobilization carrier such as versatility of available physical forms (flakes, porous beads, gel, fiber and membrane), low biodegradability and easy handling. In this study, porous bead of chitosan was used for immobilization of lipase from *Candida rugosa*. Lipase was immobilized by physical adsorption on chitosan beads. The ability of the immobilized lipase to catalyze transesterification of cooking oil was investigated. The important parameters like reaction time and oil to methanol molar ratios were studied to identify the performance comparison between free lipase and immobilized lipase on transesterification reaction. From the study it was found that maximum conversion of ester using immobilized lipase and free lipase were 73 and 77%, respectively. These results are obtained at optimum conditions of 1:4 molar ratios and reaction time of 48 h. As a conclusion, the chitosan beads were appear as a suitable support for immobilized lipase on transesterification reaction even though the ester conversion was lower than free lipase.

Key words: Lipase immobilization, physical adsorption, chitosan beads, transesterification

INTRODUCTION

Lipases are widely used in industrial applications due to the wealth of reactions they catalyze. It is an important enzyme in biological systems, where it catalyzes the hydrolysis of triacylglycerol to glycerol and fatty acids (Ricardo and Kumar, 1989). Besides their natural substrates, lipases has unique characteristics such as catalyzing reactions involving insoluble organic and aqueous phases and ability to preserve their catalytic activity in organic solvents, biphasic system and in micellar solutions (Hung *et al*., 2003). Versatility of lipase catalyzed reactions made them a unique heterogeneous catalyst for transesterification reactions.

For various applications, lipase enzymes are preferably used in an immobilized state in order to ease separation of the catalyst from the product stream (Pereira *et al*., 2003). With immobilized lipases, improved stability, reusability, continuous operation, the possibility of better control of reactions and hence more favorable economical factors can be expected (Frense *et al*., 1996). Lipases have been immobilized on various supports either by physical adsorption, covalent binding, ionic interactions or by entrapment (Nadir *et al*., 2009; Vaidya *et al*., 2008; Pires-Cabral *et al*., 2009). Various methods for enzyme immobilization can be divided into two general classes; chemical methods, where covalent bonds are formed with the enzyme and physical methods, where weak interactions between support and enzyme exist (Chiou and Wu, 2004). Selections of immobilization method will influence the properties of biocatalyst. The decrement levels in activity and diffusion limitations occurring with immobilization are mainly dependent on the properties of support material and the immobilization method. Support materials playing an important role in the usefulness of an immobilized enzyme should be low-cost and provide adequate large surface area together with the least diffusion limitation in the transport of substrate and product for enzymatic reactions (Nadir *et al*., 2009).

The objective of this study is to produce immobilized lipase by using chitosan beads as support and further aiming its application on transesterification reaction. During the experiment study, effect of reaction time and oil to methanol molar ratio for both free and immobilized lipase were studied.

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MATERIALS AND METHODS

Chitosan beads formation: Chitosan powder, acetic acid, sodium hydroxide and ethanol were used for formation of chitosan beads. Lipase enzyme from Candida rugosa type VII was used with hexane as a solvent during the immobilization on chitosan beads. Chitosan beads of 3% (w/v) were formed by dissolving the chitosan powder in 1% acetic acid. Spherical beads with diameter between 1-2 mm were produced by adding the chitosan solution droplet into a coagulant bath consisting of 1 M NaOH with 26% (v/v) ethanol under stirring condition. The mixture was allowed to rest overnight before the spherical beads were removed by filtration and washed with deionized water until neutrality. The beads were stored in deionized water at ±40°C until further use.

Immobilization of lipase: Lipase was immobilized by physical adsorption on chitosan beads following method developed by Carneiro da Cunha *et al*. (1999). Chitosan beads (18 g) were firstly soaked in hexane under agitation (150 rpm) for 1 h. Excess hexane was removed, followed by the addition of 5% (w/v) of lipase dissolve in distilled water. The mixture was left for 3 h at room temperature under agitation (150 rpm) and another 18 h under static conditions at ±4°C. Finally, the immobilized enzyme was filtered and thoroughly rinsed with hexane.

Transesterification reaction: Transesterification reaction was conducted following a suggested method by Devanesan *et al*. (2007). Different period of transesterification reaction was studied which were 24, 48 and 54 h. Experiment were carried out at temperature ±40°C. Two gram of immobilized cell was mixed with 50 mL of oil and methanol mixture (1:4 and 1:6 molar ratio of oil to methanol and 3 mL of n-hexane) which react as solvent. The produced ester and by product glycerol were separated using separating funnel. Transesterification reaction utilizing free lipase was also been conducted as the control using the same condition as above. Quantitative analysis of ester produced was carried out by using thin layer chromatography method.

Lipase activity was determined by calculated the percentage of ester produced from transesterification process. It was determined by using Thin Layer Chromatography (TLC) method. The solvent system used was a mixture of hexane and chloroform at 1:1 molar ratio. Protein assay was determined based on Bradford's method by using Bovine Serum Albumin (BSA) and commosive blue reagent. Determination on the enzyme rate of reaction during the transesterification process cannot be performed due to lack of facilities to do so.

RESULTS

Concentration of lipase was determined with BSA standard curve at 595 nm wavelength. The amount of bound enzyme was determined indirectly from the difference between the amount of enzyme introduced and the amount of enzyme remained in the solution, which is shown in Table 1.

Previously prepared immobilized lipase had been used as a catalyst for transesterification reaction to study its activity on particular reaction on comparison with free lipase to form ester and glycerol. Result shows in Table 2 indicate the ester conversion of each parameter which has been studied on transesterification reaction for both immobilized lipase and free lipase.

Table 1: Lipase concentration and bound lipase

Enzyme used	OD at 595 nm	Concentration (μg lipase mL^{-1})	Bound lipase (μg g^{-1}-chitosan)
Immobilized lipase			
Lipase introduce	2.000	2131.00	-
Lipase remain	1.645	1736.33	-
Immobilized lipase	-	394.67	21.93
Free lipase			
-	1.856	1970.78	-

Table 2: Transesterification reaction results

Reaction time (h)	Catalyst	Oil to methanol molar ratio	Highest peak of spot	Height of plate	Ester conversion (%)
24	Immobilized lipase	1:4	4.80	8	60.00
		1:6	4.50		56.25
	Free lipase	1:4	5.10		63.75
		1:6	4.80		60.00
48	Immobilized lipase	1:4	5.70	8	71.25
		1:6	5.00		62.50
	Free lipase	1:4	6.12		76.50
		1:6	5.70		71.25
54	Immobilized lipase	1:4	5.60	8	70.00
		1:6	4.80		60.00
	Free lipase	1:4	6.00		75.00
		1:6	5.50		68.75

DISCUSSION

Lipase immobilization: Based on the result in Table 1, the initial concentration of lipase introduce was 2131 μg mL^{-1} and the free lipase remain in the solution after immobilized process was 1736.33 μg mL^{-1}. Concentration of immobilized lipase on the surface of chitosan was defined as the different between lipase concentrations introduce and free lipase remain in the solution after immobilization process. Therefore, the lipase concentration has been bound on the chitosan beads (immobilized lipase) was 394.67 μg mL^{-1}. It is mention earlier, that each parameter will use 2 g of immobilized lipase beads. During the preparation of immobilized lipase, all the parameter was standardize to the same amount of lipase bonding on each gram of chitosan, which was 21.93 μg lipase g^{-1}-chitosan. The enzymatic transesterification reaction between immobilized and free lipase will be discussed later.

Enzymatic transesterification reaction by immobilized lipase: Based on Table 2, the highest conversion of ester was achieved at 48 h reaction time with oil to methanol molar ratio of 1:4 given the value of 72%. At 24 h reaction time, the conversion of ester is 60% and at 54 h reaction time the ester conversion is 70%. Meanwhile, 63% conversion was the highest conversion achieved in 1:6 molar ratios system after 48 h reaction time. The ester conversion at 24 and 54 h reaction time for 1:6 oil methanol molar ratio systems were 56 and 60%, respectively.

As the reaction time was increased from 0 to 48 h, the percentage of ester conversion also increase and thereafter decreases until it reach 54 h of reaction time. This is happen because of the depletion of lipase activity on the substrate due to extended operation time. Further increase in the reaction time (more than 54 h) does not increase the production of ester. Therefore, in this research, the optimum reaction time for transesterification reaction using immobilized lipase was 48 h.

Another important parameter affecting the yield of ester in transesterification process is the molar ratio of oil to alcohol. Excess amount of alcohol was needed in order to bring the reaction towards the desired product which is the ester. The ester conversion for immobilized enzyme on transesterification reaction using 1:4 molar ratio systems was higher than 1:6 molar ratio systems. The yield of ester was decreased as the oil to methanol molar ratio was increased beyond 1:4. It may be due to the inhibition of excess methanol which reduces the enzyme activity.

Transesterification reaction by free lipase: Based on Table 2, in 1:4 molar ratio systems, the highest conversion of 77% was achieved after 48 h reaction time and the lowest conversion was achieved at 24 h reaction time which is 64%. The ester conversion was slightly decreased to 75% as the reaction time increased to 54 h. Similar trend was also been observed for 1:6 molar ratio system, where the highest ester conversion was achieved at 48 h reaction time with 71% conversion. At 24 and 54 h reaction time, the ester conversions are 60 and 69%.

Free lipase activity was also increased by increasing the reaction time. Unfortunately, the conversion was decreased as the reaction time continues to 54 h and beyond. By increasing the reaction time, the lipase activity was being reduced due to the low survival of free lipase operating in longer period of reaction. By increasing the operating time (more than 54 h) will only reduced the ester conversion. Thus in this study, optimum reaction time for transesterification reaction using free lipase was 48 h.

Comparison between immobilized lipase and free lipase on transesterification reaction: Based on the result in Table 2, oil to methanol molar ratio of 1:4 gave better ester conversion compared to 1:6 oil methanol molar ratios system on transesterification reaction when using both using free and immobilized lipase. The ester conversion for all three reaction time (24, 48 and 54 h) in 1:4 molar ratio systems was higher than in 1:6 oil methanol molar ratios system up to 11, 14 and 16% conversion, respectively. This might due to low survival of lipase on excess methanol that inhibits their activity during transesterification reaction. Hence, excess methanol (more than 1:4 molar ratio) seems to give side effect to the ester production on transesterification reaction using lipase as catalyst.

Based on the results, the highest conversion of ester was obtained by using free lipase at 48 h reaction time and 1:4 molar ratio systems with the value of 76.50%. Even though the lowest conversion of ester was observed in immobilized lipase with difference of 20%, but still, it managed to catalyzed the reaction. Generally, the conversion of ester for free lipase is higher than immobilized lipase on transesterification reaction for all studied parameters.

Higher ester conversion obtained in free lipase transesterification probably due to its higher activity compared to the immobilized lipase. The interaction between the enzyme and its substrate is usually by weak forces. In most cases, van der Waals forces and hydrogen bonding were responsible for the formation of enzyme-substrate complexes. The weak linkage established between enzyme and support has little effect on catalytic activity. Regeneration of the immobilized enzyme is often possible. However, because of the bonds

were so weak, the enzyme can easily be desorbed from the carrier. Therefore, in this study the activity of immobilized lipase was lower than free lipase due to the easily desorbed of lipase from the chitosan beads. Further experimental work on studying the rate of the enzyme reaction along the transesterification reaction shall be conducted to clarify this matter.

CONCLUSION

Immobilization of lipase on chitosan beads was achieved by adsorption method using hexane as a solvent. The experimental results showed that immobilized lipase has an optimum reaction time of 48 h with oil to methanol ratio of 1:4. The optimum reaction time for free lipase was also the same as the immobilized one. However, the conversion of ester for free lipase is 7% higher than the immobilized lipase. In this study, the chitosan beads can be an appropriate support for immobilized lipase for transesterification reaction even though the ester conversion was lower than free lipase. On the other hand, immobilized lipase managed to provide several advantages such as easy separation from the product and has high potential to reuse. Further studies on reusability of immobilized lipase may benefit the potential of this support in order to increase the ester productivity in transesterification reaction.

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