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

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Abstract: Problem statement: Further study is recommended to improve the immobilization technique and the immobilized lipase's performance as catalysis in transesterification reaction. **Approach:** To investigate the ability of immobilized lipase on chitosan beads to catalyze the transesterification of cooking oil to an ester. The porous bead of chitosan was used for immobilization of lipase from *Candida rugosa* by physical adsorption. Parameters like reaction time and oil to methanol molar ratios were studied to compare the transesterification performance between free lipase and immobilized lipase. **Results:** The experimental result showed that the maximum conversion of ester using immobilized lipase and free lipase were 72.25 and 76.5% respectively which was obtained at conditions of 1:4 molar ratios and reaction time of 48 h. **Conclusion:** Even though the conversion of ester was higher in free lipase than in immobilized lipase, it is still feasible to use immobilized lipase to catalyze the transesterification process provided it's easier to separate the end product and high potential to be reused. Immobilize lipase also tolerated the water content of oil and increases product yield by avoiding the soap formation.

Key words: Immobilization of lipase, physical adsorption, chitosan beads, transesterification, ester

INTRODUCTION

Many efforts have been made over the years to develop the catalytic activity and operational stability of industrial enzymes through the use of genetic engineering, immobilization and/or process alterations. Enzyme immobilization is the most commonly used strategy to impart the desirable features of conventional heterogeneous catalysts onto biological catalysts. Immobilization of enzyme not only enhanced its stability, it also known to offer several advantages such as reusability, ease of product separation, greater control over catalysis and process.

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[1]. Besides their natural substrates, lipases has unique characteristics such as can catalyze reactions involving insoluble organic and aqueous phases and are able to preserve their catalytic activity in organic solvents, biphasic system and in micellar solutions[2]. Versatility of lipase catalyzed reactions made them a unique heterogeneous catalyst for transesterification reactions.

Previous studies show that biodiesel can be produced enzymatically by lipase-catalyzed transesterification which has become more attractive in biodiesel production since the glycerol can be recovered easily and the purification process for biodiesel is simple[3]. In addition, the use of lipase in biodiesel production tolerates the water content of oil and increases biodiesel yield by avoiding the soap formation. In order to use the enzyme economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectivity and operational stability can be modified by immobilization.

For many applications lipase enzymes are preferably used in an immobilized state in order to easily separate the catalyst from the product stream[4]. With immobilized lipases, improved stability, reuse, continuous operation, the possibility of better control of

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reactions and hence more favorable economical factors can be expected[5].

Lipases have been immobilized on various supports either by physical adsorption, covalent binding, ionic interactions or by entrapment[3,6,7]. 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[8]. Selection of immobilization method wills influences 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[3].

In this research, chitosan has been chosen to be a support for lipase. Chitosan, which is a poly-N-acetylglucosamine, is a transformed oligosaccharide obtained by deacetylation of chitin and is the second after natural cellulosic carbohydrate polymer abundance[8]. Chitosan has many significant biological and chemical properties such as biodegradable, biocompatible, bioactive and polycatonic properties[9].

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

MATERIALS AND METHODS

Chitosan powder, acetic acid, sodium hydroxide and ethanol were used for formation of chitosan beads. The catalyst used was *Candida rugosa* type VII lipase and hexane as a solvent during the immobilization of lipase on chitosan beads. Protein assay was determined based on Bradford's method by using Bovine Serum Albumin (BSA) and commosive blue reagent to form a standard curve. Transesterification reaction between cooking oil (Sri Murni brand) and methanol, catalyzed by lipase with solvent system of hexane was performed. Hexane and chloroform were used as a solvent for enzyme activity assay by Thin Layer Chromatography method with TLC plate and Iodine pallets. All other chemical used in this study were of analytical grade. All the solutions were prepared in deionized water and distilled water.

Formation of chitosan beads: 3% (w/v) chitosan powder is first dissolved in 1% acetic acid. Spherical beads of diameter in the range 1-2 mm were produce by adding the chitosan solution dropwise into a coagulant bath consisting of 1M NaOH containing 26% (v/v) ethanol under stirring. Allowing the mixture to remain overnight, the spherical beads are removed by filtration and washed with deionized water until neutrality. The beads then are stored in deionized water at 4°C until use.

Immobilization of lipase: Lipase is immobilized by physical adsorption on chitosan following a previously developed methodology by[10]. Chitosan beads (18 g) had been previously soaked in hexane under agitation conditions (150 rpm) for 1 h. Then, excess hexane has removed followed by the addition of 3 g of powder lipase dissolved in 60 mL of distilled water. The lipase was on the support under agitation (150 rpm) for 3 h at room temperature followed by an additional period of 18 h under static conditions at 4°C. The derivative is filtered (Whatman filter paper 41) and thoroughly rinsed with hexane.

Transesterification reaction: Transesterification reaction is conducted following a suggested method by Devanesan *et al*.[11]. Effect of time on transesterification reaction of immobilized lipase is studied by conducting experiments with different periods of 24, 48 and 54 h. Experiment were carried out at the optimum temperature 40°C, immobilized cell concentration of 2 g beads and substrate concentration of 50 mL of oil (1:4 and 1:6 molar ratio of oil to methanol) with n-hexane (3 mL) as solvent. After a period of reaction time, the reaction is stopped and the immobilized cells are removed from the reaction mixture. The producer ester and byproduct glycerol are separated using separate funnel. Transesterification of free lipase is repeated with the same reaction condition. Quantitative analysis of ester product is carried out by using thin layer chromatography method.

Analytical methods: The amount of enzyme (protein) is determined according to Bradford's method[12] by using the Bovine Serum Albumin (BSA) as the standard. The amount of bound protein (AP*)* is determined indirectly from the difference between the Amount of Protein Introduced (API) and the Amounts of Protein Remain (APR) in the prepared solution as shown in equation below:

$$AP = API–APR \quad (1)$$

Lipase activity assay is determined based on the amount of fatty acid methyl ester produced by using Thin Layer Chromatography (TLC) method. The solvent system which is mixed of hexane and chloroform (1:1 molar ratio) has been prepared. The sample is plotted on TLC plate by using capillary tube. Then, the TLC plate is placed into the solvent system in vertically form. We need to wait for while to make sure the solvent system absorb to the TLC plate. The TLC place is dried and put onto iodine pallets in the beaker for colorized the sport on the TLC plate. The sport present on the TLC plate is identified to calculate percent yield of ester. The percent yield is calculated as stated below:

$$\%\text{Yield} = \frac{\text{Highest peak of sample TLC plate X 100\%}}{\text{High of TLC plate}}$$

One unit of enzyme activity is defined as the amount of the enzyme catalyzed the liberation of one percent conversion of cooking oil.

RESULTS

Immobilization of lipase: Chitosan has been selected to be a support for immobilize lipase, which is a biodegradable polymer obtained abundantly from chitin. The chosen method of immobilization was simple adsorption, whereby the enzyme adheres to the surface of the support particles by weak physical forces, such as van der Waals forces of attraction or dispersion forces.

Lipase assay: Lipase concentration was determined by comparing the absorbance of lipase at 595 nm wavelength with BSA standard curve. The amount of bound enzyme was determined indirectly from the difference between the amount of enzyme introduces and the amount of enzyme remain in the solution, which is shown in Table 1.

Immobilized lipase prepared in previous had been used as a catalyst for transesterification reaction that to study their activity on particular reaction. Figure 1 shows the ester conversion of transesterification reaction using immobilized enzyme.

Conventional method of transesterification reaction was used free lipase as a catalyst in order to drive forward the reaction to form ester and glycerol. Figure 2 is show the ester conversion of transesterification reaction using free lipase as catalyst. The diamond shape in the figure is indicate the 1:4 oil methanol molar ratio system and the triangle in shape is for 1:6 oil methanol molar ratios system.

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	2.0002	2131.000	-
	Lipase remain	1.645	1736.333	-
	Lipase immobilization	-	394.667	21.925
Free lipase	-	1.856	1970.777	-

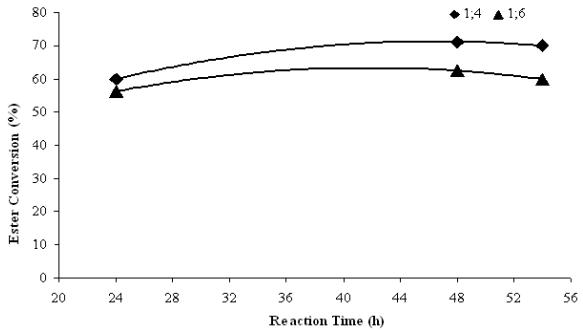

Fig. 1: Ester conversion for immobilized lipase on transesterification reaction

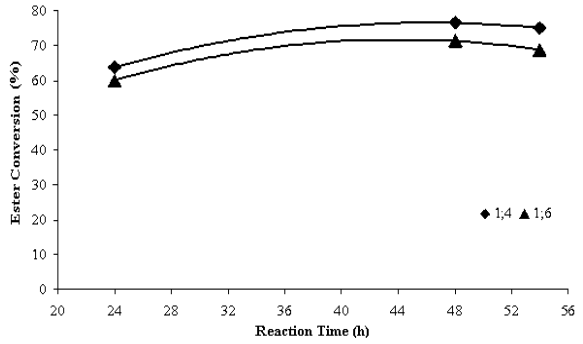

Fig. 2: Ester conversion for free lipase on transesterification reaction

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

Result shows in Table 2 indicate the ester conversion of each parameter that has been studied on transesterification reaction for both immobilized lipase and free lipase.

DISCUSSION

Based on the result in Table 1, the concentration of lipase introduce was 2131 µg mL^{-1} and the lipase remain in solution after immobilized process was 1736.3333 µg mL^{-1}. The lipase concentration on the surface of chitosan was defined as the different between lipase concentrations introduce and lipase remain in the solution after immobilized process. Therefore, the lipase concentration has been bound on the chitosan was 394.6667 µg mL^{-1}. Based on the method described at the earlier part, each parameter will use 2 gram of immobilized 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 21.9259 µg lipase g-chitosan^{-1}.

The enzymatic transesterification reaction between immobilized and free lipase was discussed below.

Immobilized lipase on enzymatic transesterification reaction: Based on Fig. 1, the highest conversion of ester was achieved at 48 hour reaction time and oil to methanol 1:4 molar ratio with the value of 72.25%. At 24 h reaction time, the conversion of ester is 60% and at 54 h reaction time the ester conversion is 70%. Meanwhile, 62.5% was the highest conversion achieved at 48 hours reaction time for 1:6 molar ratios system. The ester conversion at 24 and 54 h reaction time for 1:6 oil methanol molar ratio systems were 56.25 and 60%.

As the reaction time was increased the percentage of ester conversion also increase until it reach 48 h of reaction time 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 long operation time. Further increase in the reaction time (more than 54 h) does not increase the production of ester. Therefore, the optimum reaction time for transesterification reaction using immobilized lipase is 48 h.

Another important parameter affecting the yield of ester in transesterification process is the molar ratio of oil to alcohol. In order to shift the transesterification reaction in forward direction, it is necessary to use either excess amount of alcohol or to remove one of the products from the reaction mixture. 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.

Free lipase on transesterification reaction: Based on Fig. 2, in 1:4 molar ratio systems, the highest conversion of 76.5% was achieved at 48 h reaction time and the lowest conversion at 24 h reaction time which is 63.75%. The ester conversion was decreased to 75% as the reaction time was increased to 54 h. For the time being, the ester conversion was found highest at 48 h reaction time for 1:6 molar ratio systems, which was 71.25%. At 24 and 54 h reaction time, the ester conversions are 60 and 68.75%.

Free lipase activity was increased due to the increase of the reaction time until 48 hours, but decreased when the reaction time is further increase to 54 h and beyond. The increased of reaction time for this reaction was reduced the lipase activity due to the low survival of free lipase operating in long period of reaction. Further increased the operating time (more than 54 h) do not increased the production of ester. Thus, optimum reaction time for transesterification reaction using free lipase was 48 h.

Comparison between immobilized lipase to free lipase on transesterification reaction: Based on the result in Table 2, oil methanol molar ratio of 1:4 gives a better conversion of ester than 1:6 oil methanol molar ratios system on transesterification reaction. The ester conversion for three reaction time (24, 48 and 54 h) in 1:4 molar ratio systems was higher than ester conversion in 1:6 oil methanol molar ratios systems with the values were 3.75, 5.25 and 6.25%. This may be due to the low survival of free lipase on excess methanol that inhibits their activity during transesterification reaction. Hence, excess methanol (more than 1:4 molar ratio) is seems give a bad effect to the ester production on transesterification reaction using free lipase as catalyst.

Based on the results, the highest conversion of ester was obtained by free lipase at 48 h reaction time and 1:4 molar ratio systems with the value of 76.50%. However, the lowest conversion of ester was 56.25 % achieved by immobilized lipase at 24 h reaction time and 1:6 molar ratio systems. Generally, the conversion of ester for free lipase is higher than immobilized lipase on transesterification reaction for all parameters studied.

The activity of free lipase is seemed higher than activity of immobilized lipase on transesterification reaction. The interaction between the enzyme and its substrate is usually by weak forces. In most cases, van der Waals forces and hydrogen bonding are 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.

CONCLUSION

The successful adsorption of lipase on chitosan beads was achieved by previously soaked the support with hexane and then soaked with lipase solution. The experiment results show that immobilized lipase has an optimum reaction time of 48 hours and the oil methanol ratio is 1:4. Meanwhile, the optimum reaction time for free lipase also same as immobilized lipase which was 48 hours by using 1:4 oil methanol molar ratio systems. However, the conversion of ester for free lipase is higher than ester conversion of immobilized lipase. The chitosan beads were appear as appropriate support for immobilized lipase on transesterification reaction even though the ester conversion was lower than free lipase. On the other hand, immobilized lipase was provided an important advantage such as easy separation from the product and has a high potential to reuse. Further studies on reusability of immobilized lipase may see the potential of this support in order to drive forwards the ester production in transesterification reaction.

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