

UNIVERSITI PUTRA MALAYSIA

LIPASE-CATALYZED SYNTHESIS OF WAX ESTER

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Alcoholysis reaction between triolein and oleyl alcohol catalyzed by Lipozyme and Novozyme was carried out to produce oleyl oleate, a wax ester. Several reaction parameters were studied in order to get its optimal condition such as effect of time, reaction temperature, amount of enzyme, molar ratio of substrates (oleyl alcohol/triolein), various organic solvents used, initial water activity (a_w), continuously controlled water activity and addition of silica gel.

The optimal condition to produce wax ester for Lipozyme are incubation time of 5 h, temperature of 50 $^{\circ}$ C and using 30% (w/v) of Lipozyme. The molar ratio of substrate was 6:1 (oleyl alcohol/triolein) in heptane and at a_{w} 0.33 (initial water activity) or 0.32 (continuously controlled water activity) with the addition of 0.25 g of silica gel. For Novozyme, the optimum incubation time was 5 h, at reaction temperature of 60 $^{\circ}$ C using 30% (w/v) of Novozyme. The optimum molar ratio of substrates (oleyl alcohol/triolein)

was also at 6:1, in heptane at $a_w 0.33$ (initial water activity, a_w), 0.32 (continuously controlled water activity) and with the addition of 0.50 g of silica gel.

A scale up reaction was then carried out using the optimum condition catalyzed by Lipozyme. The percentage yield of wax ester produced at optimum reaction condition was 58.48% when determined by gravimetry method and 75.66% when determined using gas chromatography.



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SINTESIS ESTER LILIN MENGGUNAKAN MANGKIN LIPASE

Oleh

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Tindak balas pengesteran diantara triolein dan olil alkohol telah dijalankan untuk menghasilkan ester lilin iaitu olil oleat dengan dimangkinkan oleh Lipozyme dan Novozyme. Bagi mendapatkan hasil yang optima, beberapa keadaan yang mempengaruhi tindak balas telah dikaji. Antaranya adalah pengaruh masa, suhu, amaun enzim, nisbah olil alkohol terhadap triolein, jenis pelarut organik, aktiviti air awal, aktiviti air berterusan dan penambahan gel silika.

Berdasarkan keputusan yang diperolehi, Lipozyme memberikan hasil yang optima pada masa tindak balas selama 5 j, pada suhu 50 0 C dengan amaun enzim sebanyak 30% (w/v). Nisbah olil alkohol terhadap triolein yang diperlukan adalah 6:1 dan pelarut yang paling sesuai digunakan adalah heptana. Didapati juga aktiviti air awal yang diperlukan oleh Lipozyme adalah 0.33 dan aktiviti air berterusan adalah 0.32 dengan penambahan 0.25 g gel silika. Manakala Novozyme pula memerlukan masa tindak balas selama 5 j juga tetapi pada suhu $_{d}^{00}$ C dengan amaun enzim juga 30% (w/v).



Nisbah olil alkohol terhadap triolein yang diperlukan 6:1 didalam pelarut heptana pada aktiviti air awal 0.33 dan aktiviti air berterusan bernilai 0.32; dengan penambahan 0.50 g gel silika.

Satu ujikaji dengan skala besar telah dijalankan dengan menggunakan semua keputusan optima yang dimangkinkan oleh Lipozyme. Analisis untuk ester lilin yang diperolehi telah dijalankan dan peratusan hasil yang diperolehi ialah 58.48% dan 75.66%, masing-masing menggunakan kaedah gravimetri dan kromatografi gas.



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CHAPTER I

INTRODUCTION

Wax esters derived from natural sources such as sperm whale oil and jojoba oil have been widely used in industries such as in cosmetics and lubricant due to their excellent wetting behavior at interfaces. The use of jojoba oil has increased as it can be considered as an adequate substitute for sperm whale oil, which is depleting. However, the supply of jojoba oil was inconsistent, so a substitute for this wax ester is desired.

As an alternative, synthetic wax esters have been synthesized using chemicalcatalyzed and enzymatic-catalyzed method. Synthesis of wax esters using chemicalcatalyzed method leads to higher energy costs and degradation of esters. Enzymaticcatalyzed method offers several advantages over chemical-catalyzed method such as mild reaction conditions and environmentally friendly process. Furthermore, different types of product could be produced depending on the specificity of the lipase used.

The main reactions to produce synthetic wax esters are alcoholysis and esterification reaction. Wax esters of similar commercial values preferably obtained from alcoholysis of triglycerides of long-chain fatty acids with long-chain alcohols as it could be much cheaper and simpler than the synthetic route via esterification reaction where water must be removed to assure high yield.



The purposes of this project are:

- 1. To produce oleyl oleate, a wax ester which is known to be as a principal component of sperm whale and jojoba oil from *Simmondsia chinensis*.
- To screen commercially available immobilized lipases for best catalyst to synthesis oleyl oleate.
- 3. To study the reaction parameters to find the optimal conditions such as reaction time, amount of enzyme, reaction temperature, molar ratio of substrate, various organic solvents, initial water activity, continuously controlled water activity and effect of adding silica gel.
- To do a scale up reaction at the optimal conditions using the best immobilized lipase tested.
- To analyze the product using thin layer chromatography, column chromatography, gas chromatography and Fourier Transform Infrared Spectroscopy.



CHAPTER II

LITERATURE REVIEW

Lipases

Lipases catalyzed the reversible hydrolysis of glycerol ester bonds and also the synthesis of glycerol esters. Under certain circumstances, lipases also catalyze a number of transesterification reactions. These reactions can be illustrated by Equations 1-5 as follows (Gandhi, 1997):

i. Hydrolysis

$$RCOOR' + H_2 O \rightleftharpoons RCOOH + R'OH$$
(1)

ii. Synthesis

•	Esterification	
	RCOOH + R'OH \rightleftharpoons RCOOR' +H ₂ O	(2)

• Interesterification

 $RCOOR' + R''COOR* \rightleftharpoons RCOOR* + R''COOR'$ (3)

Alcoholysis

 $RCOOR' + R'OH \rightleftharpoons RCOOR'' + R'OH$ (4)

Acidolysis

 $RCOOR' + R''COOH \rightleftharpoons R''COOR' + RCOOH$ (5)

The last three reactions are often grouped together into a single term called transesterification.



The last three reactions are often grouped together into a single term called transesterification.

Hydrolysis refers to the splitting of a fat/ester into its acid and glycerol/alcohol in the presence of water. Both fatty acid and glycerol/alcohol have important industrial applications for example, the usage of fatty acids to produce soap (Hoq *et al.*, 1985). However, the hydrolysis of fats and oils is an equilibrium reaction, and therefore it is necessary to change the direction of the reaction to ester synthesis by modifying the reaction conditions (Jaeger *et al.*, 1994; Lazar and Eirich, 1989). The reversible reaction in this case is controlled by the water content of the reaction mixture so that, in nonaqueous environment lipases catalyze ester synthesis. Another potent role is to deprotect (by hydrolysis) the ester-protecting groups in synthetic intermediates so as to exploit the ability of lipases to catalyze hydrolysis under mild conditions (Kazlauskas, 1994).

Esterification mixtures generally contain only the substrates and enzyme, and the products are ester and water. On the other hand, transesterification processes, such as alcoholysis, acidolysis and interesterification, give rise to alcohol, acid, or ester instead of water. Hence, transesterification becomes more lucrative when any of these are the desired products (Gandhi, 1997). In interesterification, a controlled quantity of water is required, in addition to the amount needed for the enzyme to maintain the active hydrated state. As the presence of too much water will decrease the amount of ester synthesis products, the water content should be carefully adjusted (whereupon interesterification)

predominates over hydrolysis), to achieve the accumulation of the desired reaction products (Benjamin and Pandey, 1998; MacRae and Staines, 1994).

Characteristic and Properties of Lipases

Enzymes work to modify specific chemical bonds usually at specific sites on a molecule, in contrast to ordinary chemical reactions that occur at random in response to the laws of thermodynamics. Enzymes permit control of products produced and also increase yield by reducing side products; these advantages are coupled with mild reaction conditions and low waste treatment costs (Posorske, 1984).

Lipase has a wide spectrum of specificities. The specificity of lipase is controlled by the molecular properties of the enzyme, structure of the substrate and factors affecting binding of the enzyme to the substrate (Jensen, 1983). Types of specificity are:

- i. Substrate specificity
- ii. Positional specificity
- iii. Fatty acid specificity
- iv. Stereospecificity



Substrate Specificity

Rua and Ballesteros (1994) studied the behavior of two forms of *Candida rugosa* lipases (A and B) on the rate of hydrolysis using *p*-nitrophenyl butyrate as substrate and observed that both lipases showed striking variations in their *K*m values. The behavior of Lipase B could be consistent with an enzyme, which still obeys Michaelis-Menten kinetics but with a *K*m value for *p*-nitrophenyl butyrate much higher than the ester solubility under the experimental conditions. With triolein, both lipases showed relatively similar and slow activity up to 20mM substrate, but higher substrate concentrations showed much enhancement of hydrolysis by Lipase B. Clapes and Adlercreutz (1991) studied the catalytic parameters of α -chymotrypsin-catalyzed esterification of N-acetyl armino acids in acetonitrile and ethyl acetate. The specificity of the enzyme for the amino acid side chain in both solvents was approximately the same as in water, the specificity towards N-protecting groups was reversed i.e. a much higher rate was observed with Nacetyl than with N-benzyloxycarbonyl derivatives. More favorable hydrophobic interactions were responsible for the higher rate of hydrolysis of the substrates with the large non-polar protecting groups in water.

Positional Specificity

Marangoni and Rosseau (1995) reported that some lipases display position specificity (regiospecificity) towards fatty acids in triacylglycerols. For instance, lipases from *Mucor miehei*, *Rhizopus delemar*, and porcine pancreas attack at the primary



hydroxyl positions (1,3) of glycerol preferentially and are said to be 1,3-specific. On the other hand, lipases from *C. rugosa, Chromobacterium viscosum* and castor bean are nonspecific with respect to position. Thus, the regiospecificity and substrate selectivity of lipases can be advantageously exploited for their use in structural determination (Alford and Smith, 1965) of triglycerides, and for the synthesis of a specific and defined set of mono- and/or diglycerides.

Fatty Acid Specificity

The lipase from Geotrichum candidum is selective toward cis-unsaturated ($\Delta 9$) fatty acids, such as oleic acid (Sonnet, 1988). Leblanc *et al.* (1998) reported that Pancreatic lipase 250 (Solvay Enzyme Inc. Elkhart, IN) was generally active with both long chain and short chain fatty acids, whereas Pancrealipase (Biozymes Inc., Quebec) was more active with long chain fatty acids (C₄-C₉ fatty acids). Using either enzyme, yields were higher for linear acids than for branched acids.

Stereospecificity

The lipase from *C. rugosa* catalyzed the stereospecific esterification of racemic naproxen with trimethylsilyl methanol in isooctane, and the improvements in (S) naproxen ester productivity and enzyme solubility were demonstrated by adding bis-(2ethylhexyl)sodium sufosuccinate as the best surfactant (Tsai *et al.*, 1996). Tawaki and Klibanov (1992) demonstrated the enantiomeric preference of lipase from *Aspergillus*



oryzae by conducting transesterification reaction of N-acetyl-phenyalanine chloroethyl ester with propanol in hydrophilic solvents such as dimethylformamide and pyridine, and hydrophobic solvents such as toluene and octane. In hydrophobic solvents, R-enantiomer can be obtained and S-enantiomer in hydrophilic solvents.

Immobilized Lipases

Many lipases from microbial sources have been found to be promising catalysts for the hydrolysis and synthesis of fats and oils (Ruckenstein and Wang, 1993). As an example, lipase from *C. rugosa* gives high activity in hydrolytic and synthesis reactions. However, the lipase particles (especially in powder forms) sometimes tend to aggregate and attach to the walls of the reactor, especially when the enzyme is hydrated to obtain catalytic activity. These problems can be reduced, by immobilizing the enzyme on a solid support (Koskinen and Klibanov, 1996).

Immobilization refers to the localization or confinement of a lipase (Malcata *et al.*, 1990). Immobilization can increase the lipase stability. Immobilizing a lipase by attaching it to a support or entrapping it in a matrix, will make it possible to recover and reuse the lipase, or use it in a continuous process. At the same time, by attaching or entrapping the lipase to a support, we are able to remove it from the reaction mixture thereby simplifying downstream processing. Two good examples of immobilized lipases are Lipozyme and Novozyme. Lipozyme is a M. miehei lipase immobilized onto



macroporous anionic resin. On the other hand, Novozyme is a *Candida antartica* lipase immobilized onto macroporous acrylic resin.

Parameters Influencing the Catalytic Activity of Lipases

Temperature

Known relationships derived by van't Hoff and Arrhenius hold for dependences of the equilibrium constant, rate and chemical reactions on the temperature. An empirical rule suggests that the reaction rate is approximately doubled, by elevating the temperature by 10 0 C. These general laws can also be used for enzymatic reactions in certain temperature intervals. Most enzymes are denatured mainly in aqueous solutions for temperatures exceeding 40-50 0 C. As mentioned above, an increased thermostability is observed for an enzyme in an organic solvent containing a small amount of water. A number of enzymes resistant to higher temperatures can be isolated from thermophilic bacteria that occur in hot natural springs. Samad *et al.* (1990) reported that thermophilic *Rhizopus rhizopodiformis* are stable at temperature above 60 0 C.

Organic Solvent

Enzymatic reactions performed in nearly anhydrous organic solvents are particularly interesting and intriguing field of research. In nearly anhydrous organic solvents, the absence of a continuous aqueous phase, around the enzymes make it possible for them to interact directly with the solvents. However, the influence of organic 24

