

## Synthesis of Oleyl Oleate, a Liquid Wax Ester, using Lipozyme

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**Abstract** : Enzymatic synthesis of a liquid wax ester, oleyl oleate from oleic acid and oleyl alcohol, was studied using a commercial immobilized lipase, Lipozyme. The effects of various reaction parameters such as reaction time, effect of temperature, amount of lipase, molar ratio of substrates (oleyl alcohol/oleic acid), organic solvent (log P) and initial water activity ( $a_w$ ) were investigated. The optimal conditions for the ester synthesis were incubation period of 2 h, temperature at 50°C, amount of lipase of 100 mg, molar ratio of substrates of 1, organic solvents of  $\log P \geq 3.5$  and initial  $a_w$  of 0.120, which resulted in a maximum yield (90%). Analysis of the products of the reaction using gas chromatography showed the presence of oleyl oleate.

Keywords: esterification, lipase, Lipozyme, oleyl oleate, synthesis,

**Abstrak** : Sintesis enzimatik satu ester lilin cecair, olil oleat daripada asid oleik dan alkohol olil, telah dikaji dengan menggunakan satu lipase terimobilisasi komersial, Lipozyme. Kesan dari beberapa parameter tindakbalas seperti masa, suhu, amaun lipase, nisbah molar substrat (olil alkohol/asid oleic), pelarut organik(log P) dan aktiviti air ( $a_w$ ) permulaan telah dikaji. Keadaan optimum untuk sintesis ester itu adalah; masa tindakbalas 2 jam, suhu, 50°C, amaun lipase, 100g; nisbah molar; 1, pelarut organik yang mempunyai  $\log P \geq 3.5$  dan  $a_w$  permulaan; 0.120 yang telah memberikan kadar hasil maksimum (90%). Analisis produk tindakbalas dengan menggunakan kromatografi gas menunjukkan kehadiran olil oleat.

Katakunci: esterifikasi, lipase, Lipozyme, olil oleat, sintesis

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### Introduction

Liquid wax esters derived from natural sources such as jojoba oil and sperm whale oil have been dominantly used in the lubricants, plasticizers and cosmetics market. This is due to their unique properties of being able to impart wetting behavior at interfaces and having a non-greasy feeling when applied on skin surfaces[1]. However, the protection of endangered sperm whale, depleted the sperm whale oil production. Meanwhile, the jojoba oil market was inconsistent due to the late initial seed production of jojoba plantation which usually takes about 5 years.

Thus, the synthesis of synthetic liquid wax esters has the potential of meeting the demand of these esters. However, chemically-catalyzed wax esters may have problems such as high production costs and the degradation of the esters when high temperature is used. Producing liquid wax ester through enzyme-catalyzed reactions have been investigated due to the possibilities of synthesizing long chain fatty esters which resemble naturally occurring wax of commercial ester [2,3]. This alternative method of using environmentally friendly enzymatic-catalyzed synthesis, offers mild reaction conditions such as atmospheric pressure, moderate temperature and pH. Enzyme-catalyzed reactions also produce relatively pure product using a simple procedure with high

efficiency. Furthermore, the enzyme used, lipase is known to be selective for long chain acids and alcohols.

In this study, the esterification reaction of oleic acid, a major fatty acid component present in palm oil and oleyl alcohol, catalyzed by Lipozyme to produce oleyl oleate was carried out. The effects of various parameters on the esterification reaction were investigated.

### Experimental Procedures

#### Materials

Lipozyme IM-20 was obtained from Novo Nordisk, Bioindustrials. Inc. (Danbury, CT). The chemicals were purchased as follows; oleic acid, BDH (England) and oleyl alcohol, Fluka (Switzerland). All other chemicals were of analytical grade.

#### Esterification Reaction

The reaction mixture consisted of oleic acid (2.0 mmol), oleyl alcohol (2.0 mmol), enzyme (50 mg) and n-hexane (2.0 mL). They were placed in sample vials and were incubated at 40°C, 150 rpm in a horizontal shaker waterbath for an hour. All experiments were assayed in triplicate. The control experiments were carried out with heat-treated (heated at 80°C for 1h) enzyme. The reaction was

terminated by dilution with 3.0 ml of ethanol/acetone (1:1 v/v). The remaining free oleic acids in the reaction mixture were determined by titration with 0.1 M NaOH using an automatic titrator (ABU 90, radiometer, Copenhagen) to end point of 9.5 pH. The percentage of conversion (%) for each reaction was expressed as number of moles of oleic acid consumed as a percentage of number of moles of initial oleic acid used.

#### ***Effect of Reaction Time***

The reaction mixtures were incubated at the above mentioned conditions but at different reaction times (0, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 8h). The percentage of conversion was determined as described earlier.

#### ***Effect of Temperature***

The reaction mixtures were incubated at 30°C, 40°C, 50°C, 60°C and 70°C respectively. The percentage of conversion was determined as described earlier.

#### ***Effect of Amount of Lipase***

The effect of different amounts of lipase (2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100.0, 200.0 and 300.0 mg) on the esterification reaction mixtures was investigated. The percentage of conversion was determined for each amount used as described earlier.

#### ***Effect of Molar Ratio of Substrate***

The effect of various molar ratio of oleyl alcohol ( $n$  mmol)/oleic acid (2 mmol) (1, 2, 3, 4, 5, 6, 7, 8, and 9) on the esterification reaction was studied. The percentage conversion of the product was determined as described earlier.

#### ***Effect of Organic Solvent***

The effects of various organic solvents of different polarity, which are indicated by the log P values were investigated. The solvents used were ethyl acetate (log P = 0.68), benzene (log P = 2.00), hexane (log P = 3.50), heptane (log P = 4.00), isooctane (log P = 4.20), decane (log P = 5.60), dodecane (log P = 6.60) and hexadecane (log P = 8.80)[4]. The percentage of conversion was determined as described earlier

#### ***Effect of Initial Water Activity ( $a_w$ )***

The lipase and reaction media were pre-equilibrated with the vapor of saturated salt solutions overnight (at least 16 h) at  $\approx 25^\circ\text{C}$  in separate containers. The reaction was started by mixing the substrate and enzyme preparation in the reaction vials and the mixture was then incubated at 40°C and 150 rpm in a horizontal shaker waterbath for an hour. The salts used were LiCl ( $a_w = 0.12$ ),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  ( $a_w = 0.32$ ),  $\text{MgNO}_3 \cdot 6\text{H}_2\text{O}$  ( $a_w = 0.53$ ), NaCl ( $a_w = 0.75$ ), KCl ( $a_w = 0.84$ ) and  $\text{KNO}_3$  ( $a_w = 0.94$ ) [5]. The percentage yield was determined as described earlier.

#### ***Analysis of the products***

The products of the reactions were separated and analysed by thin layer chromatography (TLC) on precoated silica gels plate (60F<sub>254</sub>, Merck, Darmstadt, Germany) and developed in the mobile phase of hexane, ether anhydrous and acetic acid (8.5:20.0:0.5, v/v). Identification was made by comparison with known standards. Analysis was also carried out on a gas chromatograph (G-3000, Hitachi, Japan) which was equipped with an RTX-35 TG polar capillary column (0.32mm  $\times$  30m) from Restek Corporation (Bellefonte, PA). Helium was used as a carrier gas with flow rate 1.0 ml/min. The injector and detector were set at 320°C. Infrared (IR) analysis was carried out using a Perkin Elmer Fourier-Transform Infrared spectrophotometer Model 1650 (Perkin-Elmer Corp., Norwalk, CT)).

## **Results and Discussion**

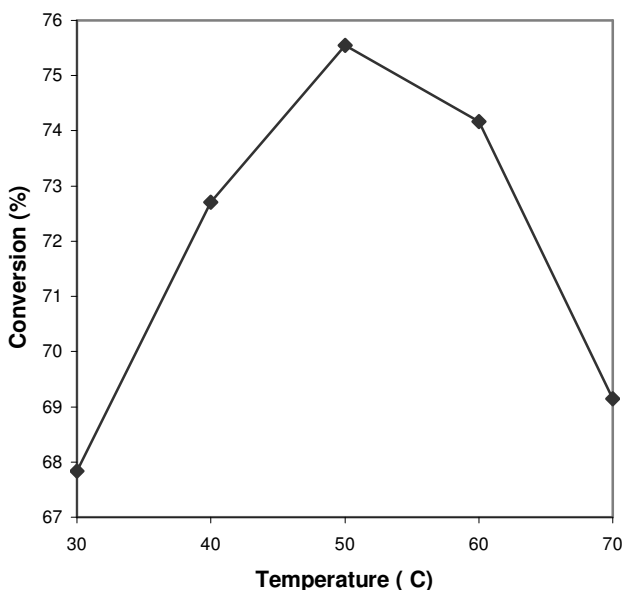
### ***Time***

The effect of time on the esterification reaction is shown in Fig.1. The percentage conversion of liquid wax ester increased with increasing reaction time and proceeded rapidly within the first 2 h. The reaction rate remained constant thereafter. This may be due to the reaction has achieved the equilibrium state where the rate of forward reaction is equal to the rate of backward reaction, hence the concentration of the product was unchanged [6]. Furthermore, water can also produce water-organic solvent miscibility and decreased the solubility of substrate in organic solvent.

**Fig. 1. Effect of time course on the esterification of oleic acid and oleyl alcohol. The percentage of conversion (%) for each investigated time was expressed as no. of mole of oleic acid consumed in the reaction as a percentage of no. of mole of initial oleic acid used.**

**Effect of Temperature**

The effect of reaction temperature on the percentage conversion of ester is described in Fig. 2. The percentage of conversion increased with increasing temperature and reached the maximum percentage conversion at 50°C as the energy from the heat was used to increase the frequency of interaction of lipase to substrates. The percentage of conversion started to decrease slightly with temperature between 50-60°C and decreased further from 60-70°C. Similar result was reported by Aracil *et al.* [7]. This phenomenon is due to the denaturation of the enzyme by heat at high temperature.



**Fig. 2.** Effect of temperature on the esterification of oleic acid and oleyl alcohol. The percentage of conversion (%) for each investigated temperature was expressed as no. of mole of oleic acid consumed in the reaction as a percentage of no. of mole of initial oleic acid used.

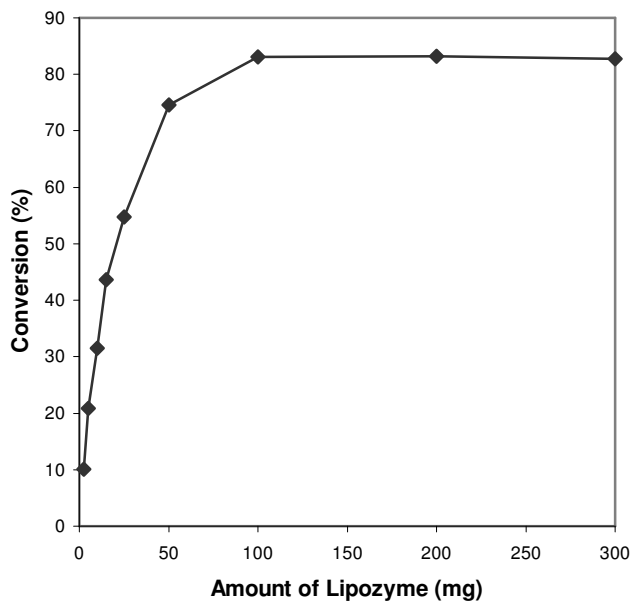
**Effect of Amount of Lipase**

Fig. 3 shows the effect of amount of lipase on the esterification reaction. The percentage conversion increased rapidly up to 50.0 mg and reached maximum conversion of 82% at 100.00 mg of lipase. However, the conversion showed saturation on the addition of lipase up to 300 mg. This may be due to the limiting effect of the substrates.

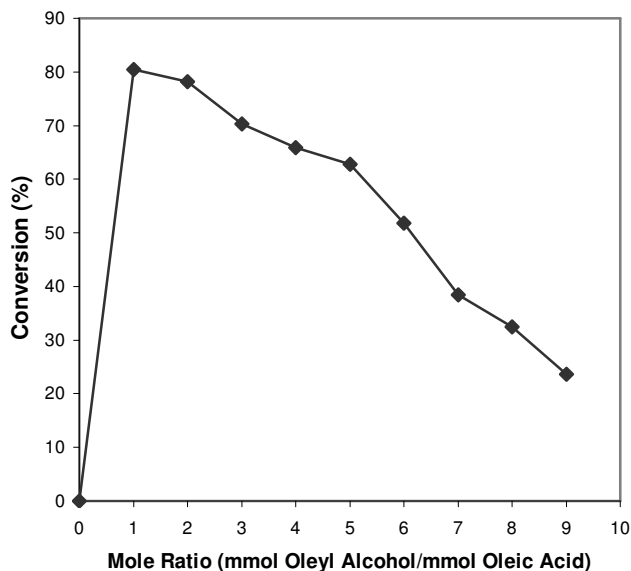
**Effect of Molar Ratio**

The effect of the molar ratio on the esterification reaction is illustrated in Fig. 4. The molar ratio of 1 (oleyl alcohol/oleic acid) produced highest percentage conversion compared to the other molar ratios. Increasing the molar ratio further decreased the percentage of conversion. This may be due to the excess of oleyl alcohol which distorted the essential

water layer from the Lipozyme and hindered the interaction between substrate and lipase [8,9].



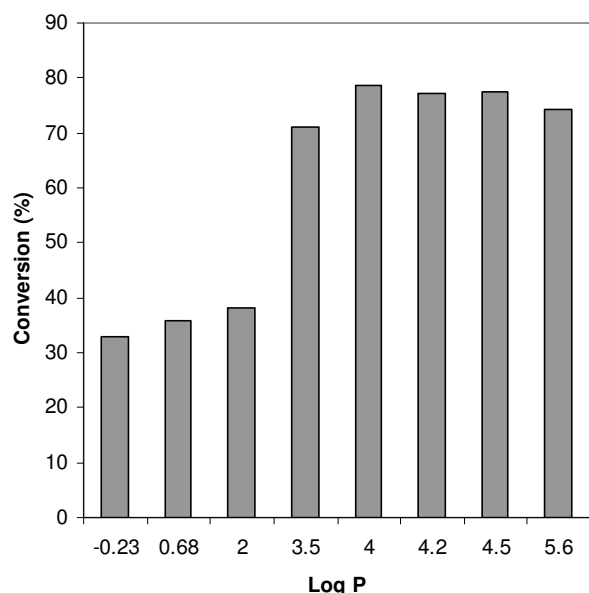
**Fig. 3.** Effect of amount of Lipozyme on the esterification of oleic acid and oleyl alcohol. The percentage of conversion (%) for each investigated amount of enzyme was expressed as no. of mole of oleic acid consumed in the reaction as a percentage of no. of mole of initial oleic acid used.



**Fig. 4.** Effect of molar ratio on the esterification of oleic acid and oleyl alcohol. The percentage of conversion (%) for each investigated molar ratio was expressed as no. of mole of oleic acid consumed in the reaction as a percentage of no. of mole of initial oleic acid used.

### Effect of Organic Solvent (Log P)

The effect of using various organic solvents on the esterification reaction is shown in Fig. 5. The lipase exhibited high percentage of conversion in non-polar solvents with  $\log P \geq 3.5$  where  $\log P$  is the partition coefficient between water and 1-octanol. The low percentage of conversion observed in polar solvents with  $\log P < 3.5$  was probably due to the ability of the polar solvent to distort the essential water, which may strip off the water layer from the enzyme molecules [10].



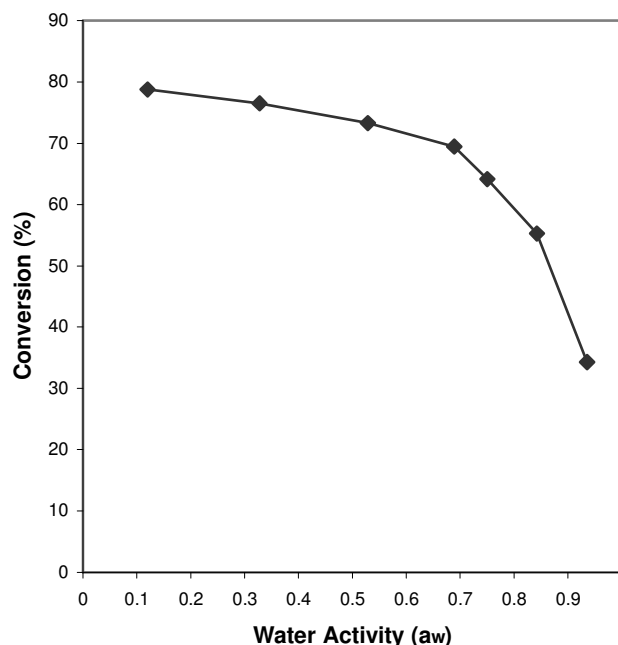
**Fig. 5.** Effect of organic solvent on the esterification of oleic acid and oleyl alcohol. The percentage of conversion (%) for each investigated organic solvent was expressed as no. of mole of oleic acid consumed in the reaction as a percentage of no. of mole of initial oleic acid used.

### Effect of Water Activity ( $a_w$ )

The effect of initial  $a_w$  on the esterification reaction was investigated and the results are shown in Fig. 6. This technique is based on the equilibrium concept, whereby, at equilibrium,  $a_w$  of any component is equal in any phase at equilibrium [11]. In these studies, Lipozyme required a lower optimum  $a_w$  of 0.12 (LiCl) to give highest conversion. Lower percentage yield at higher  $a_w$  was due to the enzyme becoming less active when the extent of hydration increases. It may reflect trivial effects such as catalyst aggregation and enzyme inactivation during  $a_w$  equilibrium.

### Esterification Reaction at Optimum Conditions

The esterification reaction was carried out at optimum conditions (incubation period of 2 h, temperature at 50°C, amount of lipase of 100 mg, molar ratio of substrates of 1, hexane as organic solvent and initial  $a_w$  of 0.120). A high yield of 90% was obtained.



**Fig. 6.** Effect of water activity,  $a_w$ , on the esterification of oleic acid and oleyl alcohol. The percentage of conversion (%) for each investigated  $a_w$  was expressed as no. of mole of oleic acid consumed in the reaction as a percentage of no. of mole of initial oleic acid used.

### Analysis of Products

The products of the reaction after incubation were ascertained by TLC and the ester spot showed a retention time of 10.586 min when detected with gas

**Fig. 7.** Gas chromatogram of the esterification of oleic acid and oleyl oleate. Symbols: hexane (A), oleic acid (B), oleyl alcohol (C), oleyl oleate (D)

chromatography. Comparison with the known standard showed that the ester was oleyl oleate. A typical GC chromatogram of the products of the esterification reaction is shown in Fig. 7. Peak 1 represents the solvent (hexane). Peaks 2, 3 and 4 are oleyl alcohol, oleic acid and oleyl oleate at 3.503 min, 4.366 min and 10.586 min, respectively.

The infrared spectral band at  $1177.0\text{ cm}^{-1}$  and the strong carbonyl stretching frequency at  $1740.0\text{ cm}^{-1}$  confirmed that the ester bond is formed in the reaction mixture during the incubation period.

### Conclusion

The observations suggest that oleyl oleate can be synthesized from oleic acid and oleyl alcohol, by Lipozyme at mild condition with high percentage of conversion.

### Acknowledgement

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