JOURNAL OF OLEO SCIENCE

J. Oleo Sci., Vol. **54**, No. 4, 203-209 (2005)

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Large Scale Production of Liquid Wax Ester by Immobilized Lipase

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Edited by S. Hara, Seikei Univ., and accepted December 17, 2004 (received for review October 19, 2004)

Abstract: Oleyl oleate, a liquid wax ester was synthesized by an immobilized *Candida antartica* lipase B (Novozym 435) as biocatalyst using oleic acid and oleyl alcohol. The effects of various reaction parameters were optimized to obtain a high yield of liquid wax esters. Investigation in large scale production was performed in batch mode of stirred tank reactor (STR) with one multi-bladed impeller. The optimum condition to produce liquid wax ester was, reaction time (RT); 30 min, temperature (T); 50℃, amount of enzyme (E); 90 g (900,000 PLU), agitation speed (A) of 400 rpm, number of impeller tip (N) of 2 and molar ratio of oleyl alcohol to oleic acid (M); 2:1. Analysis of the yield showed that at optimum condition, ＞90% liquid wax esters were produced. The stability of Novozym 435 showed at high percentage (＞80%) up to 4 cycles.

Key words: liquid wax ester, enzymatic, esterification, oleyl oleate

1 Introduction

Interest in lipase-catalyzed preparation of wax esters have grown in the last decade, because of the ability to obtain a wide variety of high-quality products under mild reaction conditions (1). Wax esters can be defined as a high molecular weight esters from long-chain fatty acids and alcohols (2). Interest in the study of the synthesis of wax esters arises from two important applications. First, wax esters have many industrial applications from lubricants to cosmetics and the possibility of preparing esters that resemble naturally occurring waxes of commercial interest is appealing (3). Wax esters have a wide range of application in industry especially in cosmetics due to the special characteristics such as non-toxic, novelty, good fat soluble properties and excellent wetting behaviour at interfaces.

Wax esters have been obtained in high yield by esterification of long-chain fatty acids and long-chain fatty alcohols and in moderate to good yields by alcoholysis of triacylglycerols or natural fats and oils with long-chain alcohols (5). This is due to the direct reaction between free fatty acids and fatty alcohols in the esterification reaction. However, in alcoholysis reaction, triacylglycerols or natural fats and oils must be separated first to the free fatty acids before reacting with alcohol to form an ester. Moreover, the presence of side products such as glycerol and diacylglycerol may reduce the yields compared to esterification reaction having just water molecules as byproduct. The esterification of oleic acid and fatty alcohol, catalyzed by lipases leads to a mixture of very long-chain wax ester with properties potential suitable for applications in cosmetics and lubricants (5-9).

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On the other hand, the production of wax ester in large scale by using a bioreactor system has become a commonplace recently. The bioreactor is the main part of any biochemical process in which enzyme are employed for the economic manufacture of a wide range of useful product. Many applications of enzymatic reactors use lipases, because of their action at interfaces, which makes them perfect biocatalysts for use in such reactors, which promote interfacial contact between enzymes and substrates (10). Stirred tank batch reactors shows different advantages such as enzyme can be readily separated from the reaction mixture at the end of the reaction, easy to control and the product is free of contamination from the enzyme.

This paper shows a synthesis of oleyl oleate, a liquid wax ester in large scale reaction by stirred tank batch reactor. The oleyl oleate synthesis from oleic acid and oleyl alcohol catalyzed by Novozym 435 (immobilized *Candida antartica Lipase B*) in organic solvent has been chosen as reaction model. First, the synthetic activity of a commercial immobilized Novozym 435 was analyzed as a function of the reaction time (RT), temperature (T), molar ratio of substrates (M) and amount of enzyme (E). Then, the reactor system was optimized in term of mixing such as agitation speed (A) and number of impeller tip (N). The influence of Novozym 435 as a biocatalyst in repeated use was also study as the operational stability.

2 Experimental Procedures

Materials: Novozym 435 as 10,000 PLU/g (from *Candida antartica* lipase immobilized onto macroporous acrylin resin) was kindly donated by Novo (Malaysia). Oleyl alcohol (purity, >60%) and oleic acid (purity, >85%) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Japan). All other reagents were of analytical grade and used as received.

Oleyl oleate synthesis: Oleyl oleate synthesis catalyzed by Novozym 435 was carried out in a stirred tank reactor (Biostat MD, by B. Braun, Germany, 2 L overall volume) being equipped with agitator system, a temperature control, sampling ports and sump and dump line for emptying of the bioreactor (see **Fig. 1**). First, the bioreactor was set up to the selected temperature condition. The reaction was started by pouring 600 mmol of oleic acid, 1200 mmol of oleyl alcohol, 90 g (900,000 PLU) of Novozym 435 and 600 ml of hexane into the vessel. The reaction was maintained during 1 h at 150 rpm agitation rate. At regular interval of time, samples were taken through a sampling port. After each sample collection, the reaction was terminated by dilution of ethanol:acetone (1:1, v/v).

Determination of the percentage conversion of oleyl oleate (%): The percentage conversion (%) of oleyl oleate was measured by determining the remaining unreacted fatty acids in the reaction mixture by titration

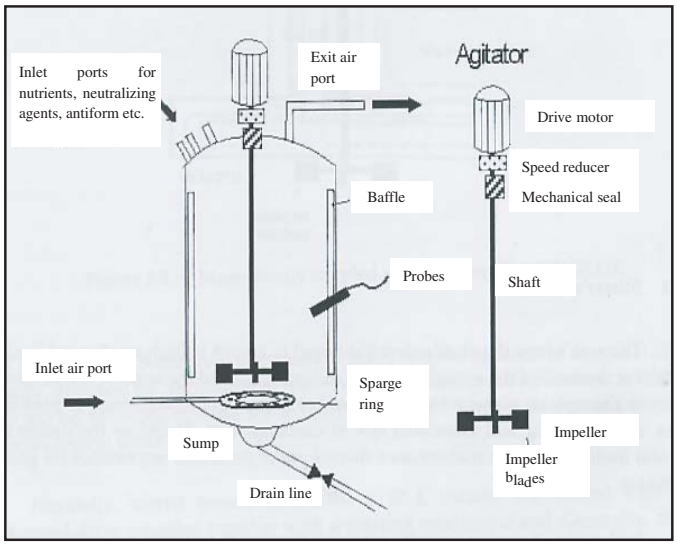

Fig. 1 Stirred Tank Reactor with One Multi-Bladed Impeller.

with 0.1 M NaOH in an automatic titrator.

Conversion of wax ester (%) =

$$\frac{\text{Volume of NaOH used (without enzyme)-Volume of NaOH used (with enzyme)}}{\text{Volume of NaOH used (without enzyme)}} \times 100$$

Identification of product:

Fourier Transform Infrared Spectroscopy (*FTIR*): The reaction mixture in hexane before incubation and after incubation were analyzed by using Fourier Transform Infrared Spectroscopy (FTIR) (Perkin Elmer, model 1650). A KBr pellet was used with the purified samples to record the spectrum. The presence of oleyl oleate was identified by comparing the ester with the known standard.

Gas Chromatography (*GC*): GC analysis was performed with a Hitachi (G 3000) instrument equipped with a medium polar capillary column RTX-65-TG (Restek Corporation, USA). Helium was used as carrier gas at 1.0 ml/min. The initial column temperature was 150℃ and the final temperature was set at 300℃. The zone temperature for injector and detector were set at 330℃ and 350℃. The temperature was increased at 10℃ per min to 280℃, and then it was increased at 5℃ per min to final temperature. The presence of oleyl oleate was identified by comparing the ester with the known standard.

Effect of reaction time (*RT*): The effect of time in the wax ester synthesis was investigated by varying reaction periods (5, 10, 15, 30, 45 and 60 min). The percentage of conversion was determined as described above.

Effect of temperature (*T*): The reaction mixtures were incubated at various reaction temperatures (30, 37, 40, 45 and 50℃). Samples were collected at varying reaction periods for 60 min. The percentage conversion was determined as described above.

Effect of amount of enzyme (*E*): The reactions were studied using various amount of enzyme (50, 60 and 90 g). Samples were collected at varying reaction periods for 60 min. The percentage conversion was determined as described above.

Effect of molar ratio (*M*): The reaction mixtures were reacted with different molar ratio of substrates, *n*mmol oleyl alcohol/2mmol oleic acid (molar ratio = 1, 2, 3 and 4). The percentage conversion of the product was determined as described above.

Effect of agitation speed (*A*): The effect of agitation speed of STR in the reaction mixtures were studied. They are 100, 200, 300 and 400 rpm. The percentage conversion was determined as described above.

Effect of number of impeller blade (*N*): The reactions were studied at varying number of impeller blade (number of impelle = 1, 2 and 3) in the STR. The percentage conversion was determined as described above.

Effect of enzyme reusability: The effect of enzyme reusability on the esterification reaction was studied using STR. The stability of enzyme was determined as percentage conversion of oleyl oleate.

3 Results and Discussion

Effect of reaction time (*RT*): The time course is a good indicator of enzyme performance and reaction progress. Enzyme having good performance will need a shortest time to obtain good yields. Therefore it can minimize the process expenses. **Figure 2** shows the reaction time profile for the esterification reaction of oleic acid and oleyl alcohol catalyzed by Novozym 435. The data suggest that a period of 30 min (93.22%) was optimum point for the reaction at 37-50℃, while prolonged periods up to 60 min the yield was unchanged. With time, the velocity of reaction was seen to remain constant until equilibrium was reached. They are several factors that have contributed to this reason as follows. As the reaction progressed, the substrates concentration decreased which led to a fall in the degree of saturation

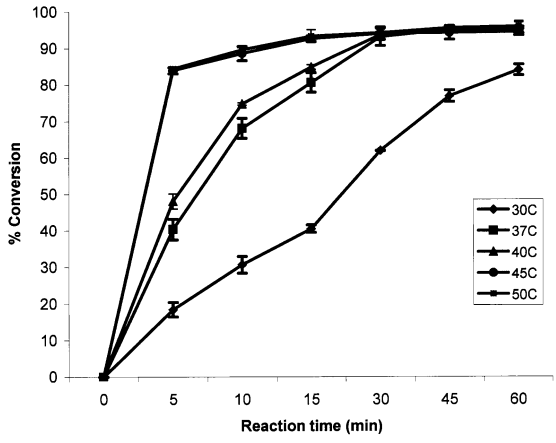

Fig. 2 Effect of Reaction Time and Temperature Studies. Reaction condition: agitation speed; 150 rpm, molar ratio of oleyl alcohol to oleic acid; 2:1 and enzyme amount; 90 g.

of the enzyme with substrate (11). On the other hand, the concentration of products and synthesis water had gradually increased and may have so promoted the reverse hydrolytic reaction (11).

Effect of temperature (*T*): Changes in the reaction temperature can effect the activity and stability of the enzymes and thus the rate of reaction (11). **Figure 2** also shows the influence of temperature on the esterification reaction within temperature range of 30-50℃ at various time intervals. The percentage yield was increased with increasing temperature from 30-50℃. This is due to the fact that, energy received from heat of higher temperature was used to increase the frequency of collision between the molecules (13). The percentage yield was at optimum at 45-50℃ (94.54%-96.02%). High percentage yield is also attributed to the enzyme immobilization, which has the advantage of conferring stability to the lipase (14). Novozym 435 is a lipase from *Candida antartica* immobilized on a microporous acrylic resin is a very tolerance product with maximum activity in the high temperature (15). Generally, an increase in temperature improves solubility of a compound and reduce viscosity resulting in enhancement of the reaction rate. However, high temperatures may also cause enzyme denaturation and so compromise the final yield (16,17). At low temperature on the other hand, solubility is reduced with the subsequence high viscosity causing mass transfer limitations, retarding reaction rate and lowering final yields (18).

Effect of amount of enzyme (*E*): The influence of varying the amount of enzyme at various time intervals is shown in **Fig. 3**. Amount of enzyme plays a crucial role in any biocatalytic process especially in large scale production. Its influence on the reaction was therefore assessed to facilitate determination of the minimal amount necessary for achieving good yields. Enzyme quantities corresponding to 50-90 g were added and assayed in various time intervals. The yield increased rapidly from 50 g (33.20%) to 90 g (95.18%) of Novozym 435 after 60 min reaction time. However, at 90 g of enzyme amount, after 30 min reaction the yield is remain constant due to the reaction achieving a saturation point. In this case, limitation of the substrate available. In esterification reaction, the amount of enzyme would influence the total reaction time, which is required to achieve a desired conversion. According to Aracil *et al*. (1992) (19) the most significant effect in enzymatic esterification reaction is the initial catalyst concentration. This effect has positive influence on ester yield. Increase in the initial concentration of immobilized lipase correspond to an increase of an ester yield.

Effect of molar ratio (*M*): The effect of molar ratio of substrate on the esterification reaction is shown in **Fig. 4**. The optimal molar ratio of oleyl alcohol/ oleic acid was 2 (90.32%). Increasing the molar ratio of oleyl alcohol to oleic acid beyond this (molar ratio = 2) would decrease the esterification activity. This observa-

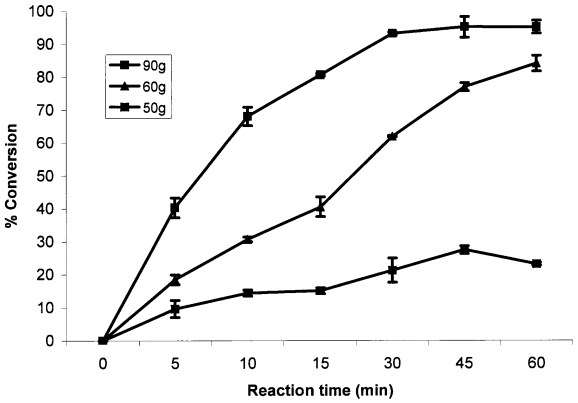

Fig. 3 Effect of Amount of Enzyme. Reaction condition: reaction time; 60 min, agitation speed; 150 rpm, molar ratio of oleyl alcohol to oleic acid; (2:1) and temperature; 37℃.

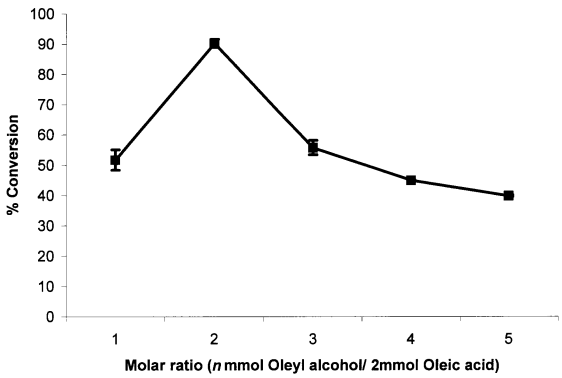

Fig. 4 Effect of Molar Ratio of Substrates. Reaction condition: reaction time; 60 min, agitation speed; 150 rpm, amount of enzyme; 90 g and temperature; 37℃.

tion may reflect the ability of the excess oleyl alcohol to distort the essential water layer from enzyme. At the same time, the excess oleyl alcohol would be a hindrance to interaction between acid substrate and lipases (20,21). This is different with what was reported by Chen and Wang, (1997) (21) In their studies, the optimal molar ratio to produce oleyl oleate was 1. The percentage yield of wax ester at higher molar ratio was relatively low. This may be due to the presence of high amount of oleyl alcohol instead of hexane. Oleyl alcohol has a higher density than hexane, thus the interaction of the substrates and the enzyme may be inhibited.

Effect of agitation rate (*A*): The effect of agitation speed on the synthesis of wax esters by esterification using Novozyme 435 is shown in **Fig. 5**. The percentage conversion was increased gradually with increasing agitation speed from 100 rpm (29.74%) to 400 rpm (93.76%). Agitator play a major role in a molecule movement. Higher agitation speed can increase the movement of molecules, thus resulting the high combination of enzyme to substrates. Moreover, it will also increase external mass transfer rates between the bulk phase of the reaction mixture and the surface of enzyme. In this study, the use of multi-bladed impeller primarily produce radial flow and a high energy dissipation density in the proximity of the agitator (23).

Effect of number of impeller tip (*N*): The effect of the number of impeller tip was shown in **Fig. 6**. The purpose of this study is to find the most appropriate number of impeller tip for a simple 2L stirred tank reactor.

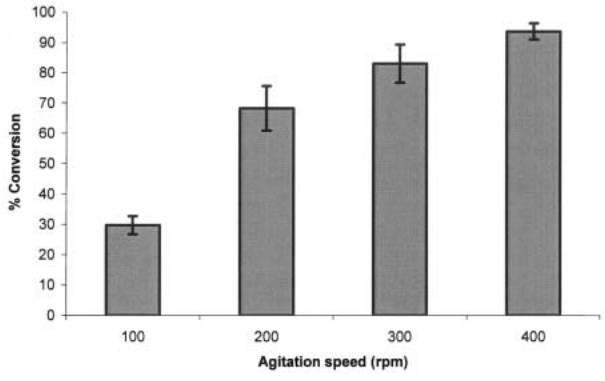

Fig. 5 Effect of Agitation Speed. Reaction condition: reaction time; 60 min, enzyme amount; 90 g, molar ratio of oleyl alcohol to oleic acid; (2:1) and temperature; 37℃.

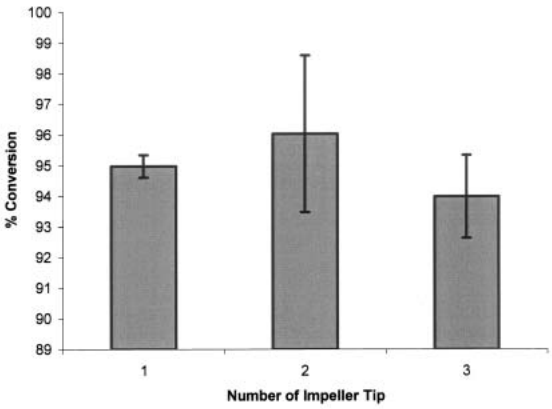

Fig. 6 Effect of Number of Impeller Tip. Reaction condition: reaction time; 60 min, enzyme amount; 90 g, molar ratio of oleyl alcohol to oleic acid; (2:1) agitation speed; 400 rpm and temperature; 37℃.

Figure 6 showed the changes of different number of impeller tip in wax ester yield with time. It indicates that the percentage conversion of 2 impeller tips reached a maximum conversion followed by number of impeller 1 and 3. Optimum number of impeller tip of 2 provide a good mixing of the whole tank because no stagnant areas are appeared between impellers whereas impeller tip of 3 is too close together clearly act as one wide impeller. Flow to the middle is restricted, power input is reduced and the tank is inadequately agitated (23).

Effect of enzyme reusability: Although there are a number of industrially useful reaction that can be catalyzed by enzymes, the biocatalyst are still too expensive for application to be economical. The use of enzymes from alternative sources, such as recombinant enzymes or immobilized enzymes (for repeated use) might help to drive down the product cost and make the enzymatic process economically viable (23). **Figure 7** shows the profile of enzyme reusability for oleyl oleate synthesis. As shown in **Fig. 7** the percentage conversion of oleyl oleate was maintained at more than 80% from cycle 1 to 4. However, the yield started to decreased drastically thereafter from cycle 5 to 7. This is maybe due to high agitation speed of the reactor causes an affect to the immobilized enzyme. The half life of Novozym 435 in this reaction is up to 6 cycles.

Esterification reaction at optimum conditions: Six parameters were chosen to optimize the synthesis of

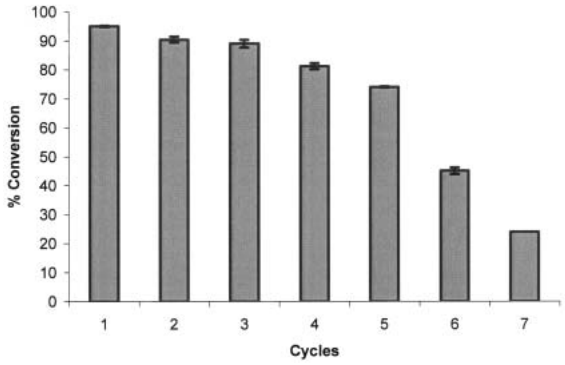

Fig. 7 Effect of Enzyme Reusability. Each operational cycles corresponds to 60 min of reaction.

oleyl oleate, namely reaction time, temperature, impeller speed, number of impeller tip, molar ratio of substrates and amount of enzyme. The study on the esterification reaction indicated that the highest conversion of >90% could be obtained in reaction time (RT); 30 min, when conducting the reaction at temperature 37℃. The optimum molar ratio of oleyl alcohol/oleic acid (M), in the esterification reaction was 2:1. Novozym 435 can tolerate high temperature (T); 50℃, high agitation speed (A); 400rpm and number of impeller tip (N); 2. The activity of Novozyme 345 was maintained more than 80% up to fourth cycles and its half-life was at 6 cycles.

4 Conclusion

This work suggests that wax esters can be produced at high percentage of yield by esterification of oleic acid and oleyl alcohol, using Novozyme 435 in large scale production.

Acknowledgments

This project was financed by a grant from IRPA Project, No.01-02-04-0000-PR, Malaysia.

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