

High performance enzymatic synthesis of oleyl oleate using immobilised lipase from *Candida antarctica*

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

Keywords: Oleyl oleate, immobilised lipase, esterification, liquid wax ester.

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High performance enzymatic synthesis of oleyl oleate, a liquid wax ester was carried out by lipase-catalysed esterification of oleic acid and oleyl alcohol. Various reaction parameters were optimised to obtain high yield of oleyl oleate. The optimum condition to produce oleyl oleate was reaction time; 5 min, organic solvents of log P \geq 3.5, temperature; 40-50°C, amount of enzyme; 0.2-0.4 g and molar ratio of oleyl alcohol to oleic acid; 2:1. The operational stability of enzyme was maintained at >90% yield up to 9 cycles. Analysis of the yield of the product showed that at optimum conditions, >95% liquid wax esters were produced.

Wax esters are an important class of fine organics that are widely used in cosmetic, pharmaceutical and lubricant industries (Wiling, 1996; Decagny et al. 1998; Goma-Doncescu and Legoy, 1997; Mukerjee and Kiewitt, 1988; De et al. 1999). Special characteristics of non-toxicity, good fat soluble properties and excellent wetting behaviour at interfaces make them very special to be used in cosmetic formulations (cleansers, conditioners and moisturizers) (Peter and Robert, 2001). Natural wax ester can be extracted from animals and plant materials such as beeswax, sperm whale and jojoba oil. However, they are often in short supply and at present too expensive for commercial exploitation (Prapulla et al. 1992; Langrand et al. 1998).

Currently, most of the wax esters used commercially are of synthetic origin. They are generally produced by chemical reaction of an alcohol with an organic acid in the presence of an acid catalyst. The chemically synthesized product is cheap but not natural. The high-temperature process can lead to degradation of the esters and undesired side reactions; the resulting energy cost is high (Awang et al. 2003). To overcome this problem, enzymatic synthesis can be attractive as it is typically very selective and is performed at moderate temperatures and pressures. Esters produced through biocatalysis can be considered close to 'natural' and can potentially satisfy recent consumer demand (Gillies et al. 1987).

Biotechnology production of esters with lipases is important and lipase-catalysed reactions have recently received greater consideration over the traditional chemical synthetic methods. The use of lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) to catalyse reactions in organic solvent is well documented (Akoh et al. 1992; Hari Krishna et al. 1999; Hari Krishna et al. 2000). One of the driving force for this research is the possibility of preparing a synthetic wax esters which resemble naturally occurring waxes of commercial interest. The specificity of the lipase to form an ester bond permits control of specific reactions which will also increase yield. There are many published papers on the catalytic performance of wax ester synthesis. However there are no reported work on high productivity (product/time) on the synthesis of wax ester.

A better understanding of various reaction parameters affecting the esterification with long-chain substrates is essential for possible large scale synthesis. This paper shows a high performance synthesis of oleyl oleate, by enzymatic synthesis route using an immobilised lipase from *Candida antarctica* (Novozym 435) in organic solvents.

MATERIALS AND METHODS

Materials

Novozym 435 as 10,000 PLU (from *Candida antarctica* lipase immobilised onto macroporous acrylin resin) was kindly donated from Novo (Malaysia). Oleyl alcohol (purity, >60%), oleic acid (purity, >85%) and authentic standard of oleyl oleate (purity, >99%) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Japan). All other reagents were of analytical grade and used as received.

Oleyl oleate synthesis

The reaction system consisted of 2 mmol of oleic acid, 4 mmol of oleyl alcohol, 2.0 ml of hexane and 0.3 g of Novozym 435. The mixture was incubated at 37°C using a horizontal waterbath shaker. The agitation speed was set at 150 rpm and the reaction mixture was continuously reacted for 180 min. The reaction was then terminated by dilution with 7.0 ml of ethanol/acetone (1:1 v/v).

Identification of reaction product

The product was periodically tested using thin layer chromatography, TLC (Merck type DC-plastic foilein Keisel gel 60 F₂₅₄); Fourier Transform Infrared Spectroscopy, FTIR (Perkin Elmer, model 1650) and gas chromatography, GC (Hitachi, model G 3000). Preliminary detection and identification of reaction product were facilitated by TLC. The developing solvent system used was ethyl acetate:hexane:acetic acid (20:8.5:0.5, v/v). Further identification was carried out by FTIR. Final identification was performed by GC 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°C and the final temperature was set at 300°C. The zone temperature for injector and detector were set at 330°C and 350°C. The temperature was increased at 10°C per minute to 280 °C, and then it was increased at 5°C per minute to the final temperature.

Analysis of reaction product

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 with 0.1 M NaOH in an automatic titrator (Radiometer, ABU 90). All the samples were assayed in triplicate and the experiment was repeated twice.

$$\text{Conversion of wax ester (\%)} = \frac{\text{Volume of NaOH used (without enzyme)} - \text{Volume of NaOH used (with enzyme)} \times 100}{\text{Volume of NaOH used (without enzyme)}}$$

Effect of reaction time. The effect of time in the wax ester synthesis was investigated by varying reaction periods (1, 2, 3, 4, 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 min) while fixing the other conditions. The percentage conversion was determined as described above.

Effect of various organic solvents. The reactions were studied using various organic solvents (benzene, log P=2.0; toluene, log P=2.5; hexane, log P=3.5; n-heptane, log P=4.0; nonane, log P=5.1; n-hexadecane, log P=8.8) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

Effect of reaction temperature. The reaction mixtures were incubated at various reaction temperatures (30, 40, 50, 60 and 70°C) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

Effect of amount of enzyme. The reactions were studied using various amount of enzyme (0.1, 0.2, 0.3, 0.4 and 0.5 g) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

Effect of molar ratio. The reaction mixtures were reacted with different molar ratio of substrates, mmol oleyl alcohol/mmol oleic acid (molar ratio= 1, 2, 3, 4 and 5) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

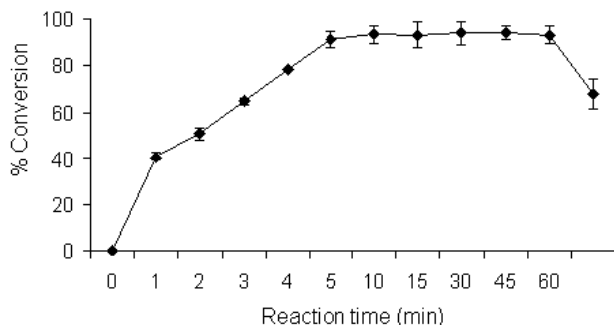


Figure 1. Effect of reaction time on the synthesis of oleyl oleate. Reaction condition: temperature, 37°C; molar ratio of substrate (mmol oleyl alcohol/mmol oleic acid), 2:1; amount of enzyme, 0.3 g; and agitation speed of 150 rpm.

Effect of heat on enzyme stability. Three types of Novozym 435 preparation were used in the reaction. They were fresh Novozym 435, Novozym 435 heated at 50°C for 60 min and Novozym 435 incubated in hexane for 60 min at 50°C before being used for esterification reaction. Reaction conditions for the other parameters were followed

as described above. The activity of Novozym 435 was assayed in term of percentage of conversion of oleyl oleate as described above.

Effect of enzyme reusability. After each cycle, the reaction mixture was removed and the Novozym 435 was rinsed with excess hexane, filtered and the solvent was evaporated under a stream of nitrogen before being used with fresh substrates. Reaction conditions for the other parameters were followed as described above. The activity of Novozym 435 was determined in term of percentage of conversion of oleyl oleate as described above.

RESULTS AND DISCUSSION

Identification of reaction product

Products from esterification reaction between oleic acid and oleyl alcohol catalysed by Novozym 435 were monitored by TLC. The presence of the oleyl oleate, oleic acid and oleyl alcohol were detected as brown spots when visualized by an iodine reagent. Further identification was carried out by FTIR showed a characteristic absorption of ester bond at 1742 cm⁻¹. Final identification of reaction mixture was performed by GC by comparing the ester with a known authentic standard. The profile of GC chromatogram showed major peak of oleyl oleate presence at retention time of 14.14 min, oleyl alcohol at 3.54 min and oleic acid at 4.29 min.

Effect of reaction time

Time course study gives an insight into the performance of an enzyme as the reaction progresses. Such progress curves help determine the shortest time necessary for obtaining good yield and so enhance cost-effectiveness of the process (Yee et al. 1997). The profile of wax ester produced at various time intervals is presented in Figure 1. The conversion of oleyl oleate was increased with increasing reaction time. Novozym 435 gave highest percentage yield within a reaction period of 5 min. After 5 min (91.3%), the percentage yield was relatively constant. This may be due to the reactions having achieved the equilibrium. Similar finding was reported by Chen et al. 1995, although the esterification reaction used immobilised *Rhizopus niveus* fungal cells. The initial reaction rate was rather insensitive to the water produced, since little water was formed during the initial stage (Chen et al. 1995). The yields remained constant after 5 min of reaction may be due to the production of water molecule, which had increased in an adequate value to accommodate the hydrolysis process (Virto and Adlercreuz, 2000).

Effect of various organic solvents

During the last decade, the tremendous potential of enzymes as practical catalyst for chemical processes in non-aqueous environments has been well recognized (Lozano et al. 2004). The use of biocatalyst in organic solvents offers many advantages over using pure water, such as the increase in solubility of poorly water-soluble organic substrates, avoiding unwanted side reactions and degradation of common organic reagents, as well as the ability to shift the thermodynamic equilibrium of many processes to the synthetic way, thus favouring product recovery (Klibanov, 1997). The polarity of the organic solvents employed for the esterification reaction is known to affect the enzyme activity (Hari Krishna et al. 2001). The log P value of the solvents is widely used parameter to describe solvent polarity and their possible effect on enzyme activity where P is the partition coefficient of a given solvent between water and octanol in a two-phase system. It is generally recommended that the use of solvents with log P >4.0 (nonpolar) result in better esterification. Figure 2 shows the effect of various organic solvents on the Novozym 435 catalysed synthesis of oleyl oleate. Generally all the solvents showed high percentage yield especially with n-hexadecane (93.2%) with log P 8.8, followed by nonane (92.1%) and hexane (91.8%) with log P both 5.1 and 3.5 respectively. Other solvents with log P <3.5 tend to give relatively lower esterification. This was probably due to the following reasons, (i) they enhanced dissociation of weak organic acid and built up the net proton concentration in the homogeneous phase, which led to the reverse reaction (hydrolysis), and (ii) they might also strip off the essential water around the enzyme present as microaqueous layer thereby, affecting the active conformation of the enzyme. Our observation are in agreement with the literature reports showing relatively lower ester conversions in solvents with log P <2.5 (Hari Krishna et al. 2001).

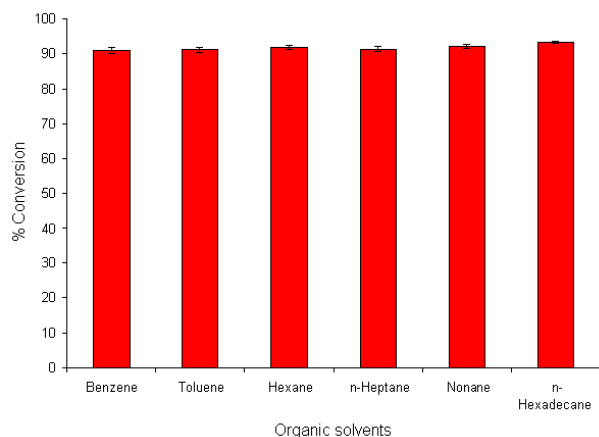


Figure 2. Effect of various organic solvents on the synthesis of oleyl oleate. Reaction condition: reaction time, 60 min; temperature, 37°C; molar ratio of substrate (mmol oleyl alcohol/mmol oleic acid), 2:1; amount of enzyme, 0.3 g; and agitation speed of 150 rpm.

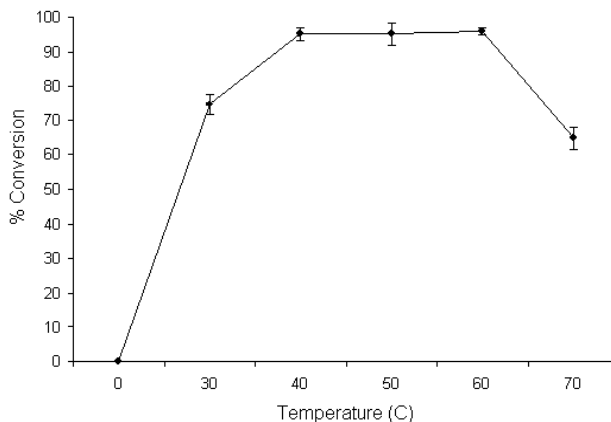


Figure 3. Effect of reaction temperature on the synthesis of oleyl oleate. Reaction condition: reaction time, 60 min; molar ratio of substrate (mmol oleyl alcohol/mmol oleic acid), 2.1; amount of enzyme, 0.3 g; and agitation speed of 150 rpm.

Effect of reaction temperature

The effects of reaction temperature can be apportioned to its effect on substrate solubility as well as its direct influences on the reaction and the enzyme (Facioli and Barrera-Arellano, 2001). Effect of reaction temperature on esterification reaction is shown in Figure 3. Initially, the percentage conversion of wax ester was increased with increasing temperature from 0°C to 40°C. The conversion was slightly constant at maximum range of 40-60°C (95.1-95.9%) before dropping at 70°C (94.8%). This is probably because beyond a critical temperature, the lipase may have been deactivated. The results is similar in the findings by most reviewed papers that Novozym 435 was optimally used at 40°C to 60°C (Bourg-Garros et al. 1998; Lozano et al. 2003). The conversion decreased slightly after 60°C probably caused by the vibration and movement of the enzyme molecule, which would affect the hydrogen bonds and other bonds in the lipase structure. Hence, the enzyme molecule will unfold and alter its tertiary and quaternary structure or globular structure (three-dimensional conformation). Consequently the catalytic power of lipase will be reduced, because denaturation process has occurred. At high temperature (50-60°C), a higher water evaporation rate may shift the position of the equilibrium to the product side and increases the yield (Chen et al. 1995). It also promotes collisions between enzyme and substrate molecules to result in accelerated rates of reaction. According to Carta et al. (1991) immobilisation on lipase will alter its sensitivity to temperature. Change in the reaction temperature can affect the activity and stability of the enzymes and thus the rates of reaction (McGilvery and Goldstein, 1983).

Effect of amount of enzyme

From an applied point of reaction, the substrate concentration should be as high as possible to obtain a

higher degree of esterification. Simultaneously, the amount of immobilised enzyme used should be as low as necessary to obtain the desired product (Kuan et al. 2001). The influence of varying amount of enzyme on the esterification reaction of oleic acid and oleyl alcohol was shown in Figure 4. The percentage conversion had increased from 0.1 g (93.5%) to 0.4 g (95.4%) and kept constant when the amount of enzyme used was about 0.5 g (95.1%). Amount of enzyme at 0.2 g (95.2%) to 0.4 g (95.4%) was sufficient to catalyse this esterification reaction. The excess enzyme did not contribute to the increase in the percentage conversion. This is also similar with what was reported by Torres and Otero, (2001) that an excess of enzyme did not increase in percentage conversion and sometime it would decrease the yield of the product. At saturation point, all the substrates are bound to the enzyme and added enzyme molecule could not find any substrate to serve as a reactant. In esterification reaction, the amount of enzyme will influence the total reaction time, which is required to achieve desired conversion (Bloomer et al. 1992). According to Aracil et al. (1993) the most significant main effect in enzymatic esterification reaction is the initial catalyst concentration.

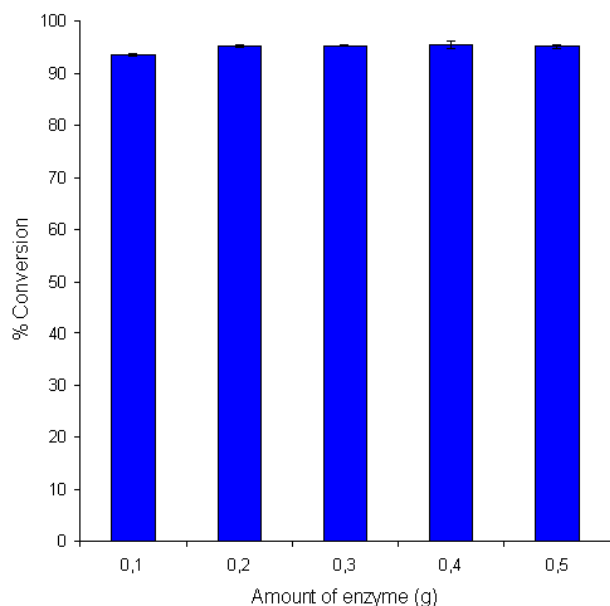


Figure 4. Effect of amount of enzyme on the synthesis of oleyl oleate. Reaction condition: reaction time, 60 min; temperature, 37°C; molar ratio of substrate (mmol oleyl alcohol/mmol oleic acid), 2:1; and agitation speed of 150 rpm.

Effect of molar ratio of substrates

Relative proportions of the various substrates in a reaction mixture define the physical and chemical properties of a reaction system. High acylation yields can be achieved with high substrate concentrations in the reaction media (Bloomer et al. 1992). The effect of molar ratio of substrates on the esterification reaction is shown in Figure

5. The optimal molar ratio (mmol oleyl alcohol/ mmol oleic acid) was 2:1 (97.2%). Increasing the mole ratio of oleyl alcohol to oleic acid beyond this (molar ratio = 2:1) would decrease the esterification activity. This observation may reflect the ability of the excess oleyl alcohol to distort the essential water layer from enzyme. At the same time, the excess of oleyl alcohol will hinder the interaction frequency between substrate and lipases (Kanasawud et al. 1992; Claon and Akoh, 1994). This is may be due to the presence of high substrates concentration, the viscosity of the reaction mixture surrounding the enzyme molecule may be increased due to the increase in the alcohol leading to ineffective mixing of reactants and subsequent reduction in reaction rate (Erbeldinger et al. 1998). This is contrary to what was reported by Chen et al. 1995. The optimal molar ratio to produce oleyl oleate was 1:1. The percentage yield of wax ester at higher molar ratio was relatively lower which was due to the presence of high amount of oleyl alcohol instead of the solvent. Oleyl alcohol has a higher density than hexane, thus the interaction of the substrates and the enzyme may be inhibited.

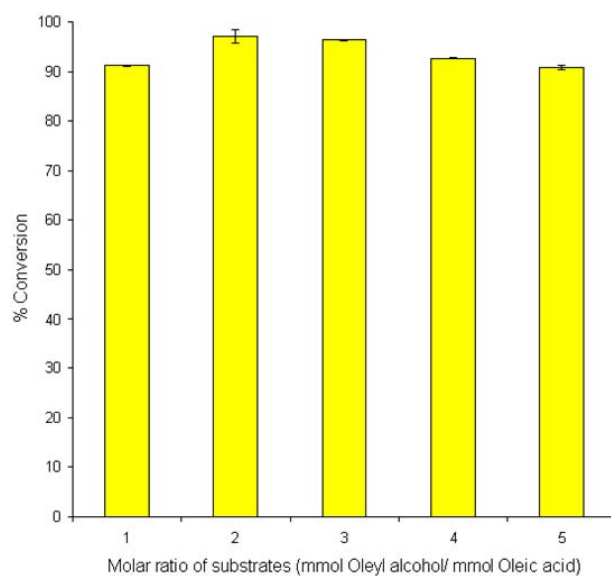


Figure 5. Effect of molar ratio of substrate on the synthesis of oleyl oleate. Reaction condition: reaction time, 60 min; temperature, 37°C; amount of enzyme, 0.3 g; and agitation speed of 150 rpm.

Effect of heat and organic on enzyme stability

As shown in Figure 6, percentage yield of more than 90% was achieved although the enzyme was heated at 50°C for 60 min in the oven before it was used for esterification reaction. Incubation in hexane at 50°C for 60 min, which was the solvent of choice for the esterification reaction, also did not have any deleterious effect on the enzyme activity. This is mainly attributed to enzyme immobilisation, where enzyme acquires greater resistance toward thermal inactivation (Yee et al. 1997). A similar finding was reported by Ramamurthy and McCurdy, (1994) for their

work on lipase-catalysed esterification of oleic acid and methanol in hexane.

Effect of enzyme reusability

The ability of immobilised lipase to retain its synthetic activity during recycling was examined by several workers (Liu and Shaw, 1995; Sellapan and Akoh, 2000; Zhang et al. 2000). In our work, the retention of the Novozym 435 activity after repeated use was assessed in term of % conversion of oleyl oleate at the end of each cycle. Figure 7 demonstrates the reusability of Novozym 435 on a small-scale by using screw-capped vials. Novozym 435 retained high activity even after 9 uses (91.9%). The stability may be due to immobilisation and low water content. Water content plays an important role in enzyme stability, as water is responsible for the internal structural flexibility and for heat-induced inactivation (Sellapan and Akoh, 2000). However, the percentage conversion was decreased from 90% to 83% at 13 cycles.

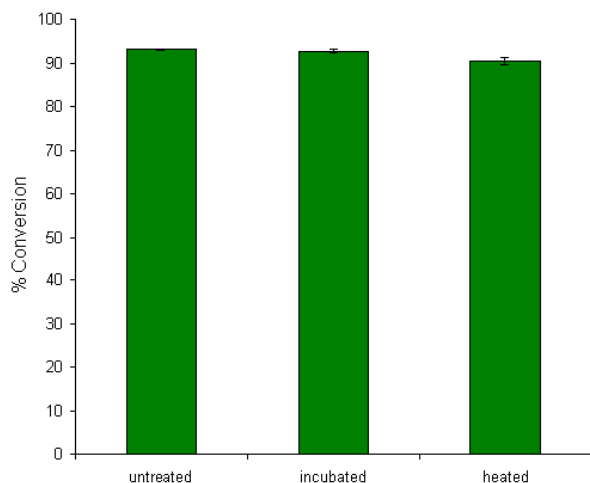


Figure 6. Effect of heat of enzyme stability on the synthesis of oleyl oleate. Reaction condition: reaction time, 60 min; temperature, 37°C, molar ratio of substrate (mmol oleyl alcohol/mmol oleic acid), 2:1; amount of enzyme, 0.3 g; and agitation speed of 150 rpm.

CONCLUDING REMARKS

Five parameters were chosen to optimise the synthesis of oleyl oleate, namely reaction time, temperature, various organic solvents, molar ratio of substrates and amount of enzyme. The reusability and stability of Novozym 435 on heat were also studied in term of % conversion. The optimum yield of products (>90%) was obtained within 5 min, temperature between 40°C to 50°C, organic solvents with log P \geq 3.5, molar ratio of 2:1 and amount of enzyme of 0.2-0.4 g. Novozym 435 was also stable in the presence of heat and organic solvent and the activity was maintained at >90% up to 9 cycles. This work suggests that oleyl oleate, a liquid wax ester can be produced at a very high yield and in a short period by esterification of oleic acid

and oleyl alcohol, using immobilised lipase from *Candida antartica* (Novozym 435). The high percentage conversion is also essential for possible large scale synthesis.

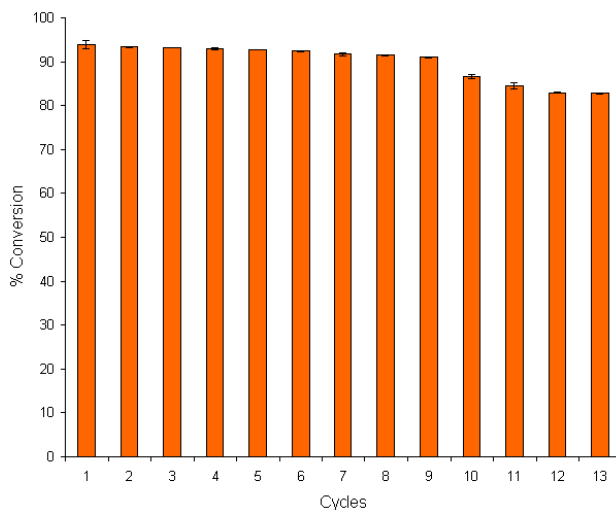


Figure 7. Effect of enzyme reusability on the synthesis of oleyl oleate. Reaction condition: reaction time, 60 min; temperature, 37°C; molar ratio of substrate (mmol oleyl alcohol/mmol oleic acid), 2:1; amount of enzyme, 0.3 g; and agitation speed of 150 rpm.

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