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Research article

Enzymatic synthesis of coconut oil based wax esters by immobilized lipase EQ3 and commercial lipozyme RMIM

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ABSTRACT

Background: Liquid wax esters are widely used in cosmetic as well as pharmaceutical and other industries. The demand of organic and natural products is increasing nowadays. Coconut oil contains benefit fatty acids and has been mainly used for oil-based and moisturizer products. Liquid wax esters from coconut oil and unsaturated fatty alcohol can be synthesized by enzymatic reaction; and it is interesting for using as an alternative natural ingredient in these industries.
Results: Optimal condition for coconut oil based wax ester synthesis by immobilized lipase EQ3 was 10 U of enzyme, temperature at 30°C and molar ratio of coconut oil to oleyl alcohol at 1:3 (mol/mol) (0.33X) dissolved in isooctane for 12 h, while for Lipozyme RM IM optimal condition was 10 U of enzyme, temperature at 45°C and oil/alcohol molar ratio at 1:3 (0.33X) dissolved in isooctane for 3 h. Percentage of wax esters synthesized by both lipases reached more than 88%. Both immobilized lipases catalyzed high yield of wax esters within the 2nd batch; after that, the immobilized lipases showed reduced activity and synthesized <60% of wax esters from the 3rd to 5th batch. The main composition of wax esters was ~48% oleyl laurate with 10% degradation at ~250°C.
Conclusions: The liquid wax ester synthesis by commercial Lipozyme RM IM had higher effect than immobilized lipase EQ3, but both catalysts were stable within 2 batches in the optimum condition. The characteristic properties of wax esters showed potential for use as components in cosmetics and skin care products.
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1. Introduction

Industrial applications of waxes or wax esters have increased over the last decade with continuous expansion of the cosmetics industry. Both male and female consumers are becoming more interested in healthy-looking skin and the cosmetics market has shown good growth trends with attractive adaptation strategies. Nowadays, customers are demanding organic and natural products. Coconut oil (*Cocos nucifera*) is a vegetable oil composed of natural saturated fatty acids extracted from the coconut kernel [1] which has become an important ingredient in cosmetics and skin care products. Outstanding properties of coconut oil include its ability to enhance skin barrier repair [2], anti-bacterial effect [3], anti-inflammatory effect [4], antioxidant effect [2], wound healing [2] and skin aging [4].

Wax esters or waxes are normally used as thickening agents, emollient agents, lubricants, and for viscosity and consistency boosting in skin care and cosmetics products [5]. Wax esters have hydrophobic characteristics composed of esterified long chain fatty acids and long chain alcohols with an even number of carbon atoms from 12 to 32 [6]. Wax esters are typically grouped into two forms as liquid and solid, depending on the saturation of fatty acids and alcohols, with different melting properties at room temperature. Wax esters are mainly synthesized by chemical and enzymatic reactions. Chemical reactions normally use corrosive substances and require high energy consumption that leads to dangerous handling. Wax esters can be biosynthesized by lipase catalysis through mild transesterification or esterification reactions. As the key biocatalyst, lipase from microorganisms has more selectivity and specificity to various substrates than other sources; moreover, enzyme immobilization allows it to be reusable biocatalysts in many reactions

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including wax ester synthesis. Ungcharoenwiwat and H-Kittikun [7] screened oils for wax ester synthesis through alcoholysis reactions. They found that immobilized lipase EQ3 could synthesize long chain fatty acids in oils with high yield of wax esters from oleyl alcohol and coconut oil, palm oil and jatropha oil at 60.3, 49.6 and 50.1%, respectively. Papadaki et al. [8] investigated wax ester synthesis from microbial oils to behenyl and cetyl esters using Novozyme 435. Their results showed transesterification reaction with conversion yields up to 87.3% and 69.1%, respectively. Moreover, this enzyme was efficiently reused for the synthesis of six and three cycles of palm esters and microbial esters, respectively. Ungcharoenwiwat and H-Kittikun [9] found that the immobilized lipase EQ3 had high palm oil based wax esters synthesis with >80% for 12 h, while Aguieiras et al. [10] studied SFAD-cetyl, SFAD-oleyl and PFAD-oleyl ester synthesis by fungal crude lipase; the enzyme was reused up to five repeated batch reactions.

Coconut oil based wax esters can be value-added product by applying as a potential natural additive in product of cosmetics and lubricants industries. Here, synthesis of natural liquid wax esters from coconut oil and oleyl alcohol was performed by immobilized lipase EQ3 compared with commercial immobilized lipase as Lipozyme RM IM. Various parameters affecting the alcoholysis reaction for wax ester synthesis were investigated. Moreover, characteristic properties of coconut oil based wax esters were also analyzed for potential further applications.

2. Materials and methods

2.1. Enzymes and chemicals

Coconut oil was purchased from a local market in Songkhla, while oleyl alcohol was obtained from Sigma Chemicals (St. Louis, MO). Commercial immobilized lipase: Lipozyme RM IM (*Rhizomucor miehei*) was obtained from Novozymes (Bagsvaerd, Denmark) which was immobilized on Duolite A568, a weak anionic resin by phenol-formaldehyde copolymers [11]. Immobilized lipase EQ3 (*Burkholderia* sp. EQ3 isolated from wastewater of fish canning factory (Songkhla, Thailand)) was prepared with Accurel EP 100 (macroporous polypropylene-based hydrophobic porous support, Akzo Nobel Membrana) as carrier with particle size 200–400 μm [7,9]. The immobilized lipases were stored at 4°C until required for use. All reagents were of analytical grade and used as received.

2.2. Analysis

2.2.1. Lipase activity determination

Hydrolysis activity of lipase was measured in a two-phase system with palm oil as substrate using cupric acetate-*pyridine* reagent [12]. Lipase activity was determined by measuring the amount of fatty acids liberated as palmitic acid. One unit of enzyme activity was defined as the quantity of enzyme that released 1 mol of palmitic acid per min at the specified conditions [13,7].

2.2.2. Determination of coconut oil based wax esters

All samples from alcoholysis reaction were analyzed in triplicate. Compositions in the reaction mixture in terms of wax esters, triglyceride, free fatty acid and oleyl alcohol were analyzed by a thin-layer chromatography-flame ionization detector (TLC-FID) (Iatroscan MK5, Iatron Laboratories Inc., Tokyo, Japan). Solvent condition for TLC was performed in a mixture of hexane/diethyl ether/formic acid (64:6.4:0.4, v/v/v) for 30 min [14]. Chromarods were scanned and each compound was qualitatively detected in peak areas by FID using ChromStar 6.3 software. The percentage of the peak area was calculated to the percentage of conversion of the corresponding compounds that was assumed to be the percentage content of the corresponding compound [7].

Determination of coconut oil based wax esters composition was carried out by a Trace GC Ultra Gas Chromatograph coupled to an ISQ Mass Spectrometry detector (Thermo Scientific, MA, USA). For the gas chromatography system, a TR-5MS capillary column was used. The column temperature program was applied as follows: 150°C held for 1 min, ramped to 225°C (20°C/min) held for 1 min, heated to 300°C (5°C/min) held for 40 min and final temperature of 340°C (10°C/min) held for 10 min. The mass spectrometer was set at 290°C for transfer line and scanned from 35 amu to 500 amu. Electron impact ionization was employed with an ion source temperature of 250°C, with concentration based on the percentage of peak areas [7].

2.2.3. Statistical analysis

Results of wax ester synthesis were expressed as mean ± standard deviation (SD). All data were analyzed utilizing one-way ANOVA. Tukey's post-hoc test (SPSS software) was used to determine statistical significance which was set at $p < 0.05$.

2.3. Wax esters synthesized by immobilized lipase EQ3 and commercial Lipozyme RM IM

The alcoholysis reaction was carried out using molar ratio of coconut oil at 110 mg and oleyl alcohol at 120 mg (150 μmol:450 μmol, 1:3 mol/mol) dissolved in hexane 1 ml in a 10 ml screwed cap tube. 2 U of Immobilized lipase (hydrolysis activity of immobilized lipase EQ3: 0.14 U/mg carrier, Lipozyme RM IM: 0.60 U/mg carrier) was added in the reaction, and the mixture was incubated at 37°C under shaking with a horizontal shaker at 150 rpm for 72 h. The reaction was sampled at an adequate amount of 10 μl, diluted with chloroform 20 μl and analyzed for percentage of wax esters by TLC-FID [7].

2.3.1. Amount of immobilized lipase

The effect of enzyme dosage on alcoholysis reaction was investigated. The different amounts of immobilized lipases were accurately weighted for 2, 5 and 10 U in the reaction mixture. Other substances and conditions were carried out as the above mentioned conditions.

2.3.2. Incubation temperature

The wax ester synthesis reaction was incubated at different temperatures namely 30°C, 37°C and 45°C with using the optimal enzyme concentration in the reaction from previous studies.

2.3.3. Molar ratio of substrate

Molar ratios of coconut oil and oleyl alcohol (mol/mol) were varied at 1:1 (150 μmol:150 μmol), 1:2 (150 μmol:300 μmol), 1:3 (150 μmol:450 μmol) and 1:4 (150 μmol:600 μmol) for the reaction mixture at the optimal condition from previous studies.

2.3.4. Substrate concentration

The wax ester synthesis was studied by taking different substrate concentrations in the reaction based on optimal molar ratio of coconut oil and oleyl alcohol (mol/mol) from previous studies (1:3 mol/mol, 150 μmol:450 μmol). Substrate concentrations were varied at 0.33X, 0.66X and 1.0X.

2.3.5. Various organic solvents

Various organic solvents were used instead of hexane (log P = 3.50) in the reaction mixture as toluene (log P = 2.50), heptane (log P = 4.00) and isooctane (log P = 4.50).

2.3.6. Reusability

Both immobilized lipases were repeatedly used at 12 h per batch for five batches of reaction. After each batch of wax ester synthesis, the immobilized lipases were washed with excess solvent in the same optimal reaction to remove the substrate and product, and then

filtered and dried at room temperature. The immobilized lipase was weighed and used in the next batch.

2.3.7. Characteristics of coconut oil based wax esters

Characteristics of coconut oil based wax esters were analyzed in duplicate. Wax ester samples were detected by GC–MS to determine their composition. Moisture content, saponification and acid values were determined according to the AOAC method [15]. Surface tension was measured using a Model 20 Tensiometer (Fisher Science Instrument Co., PA, USA) at 25°C. Thermostability properties of liquid wax esters were determined using a Differential Scanning Calorimeter, DSC7 and Thermogravimetric Analyzer, TGA 7 (PerkinElmer, MA, USA) to scan the decomposition step of liquid wax esters. All measurements were performed under nitrogen gas condition. The samples were weighted at 5 mg and placed in an aluminum crucible with a cap. For DSC, the samples were heated from −30°C to 600°C, while for TGA temperature was set at 50–1000°C [7].

3. Results and discussion

3.1. Amount of immobilized lipase

Coconut oil based wax ester synthesis was investigated by two types of immobilized lipase. The amount of biocatalyst in the reaction had a major influence on reaction rate. The results are shown in Fig. 1. Wax ester reaction of immobilized lipase EQ3 and Lipozyme RM IM dramatically increased with increasing reaction time from 6–12 h. For immobilized lipase EQ3, wax esters were obtained with the highest amount at 88.13%, while Lipozyme RM IM synthesized the wax esters at 83.93% for 24 h. Then, the wax ester yield slightly increased and maintained a constant rate until 72 h.

To determine the effect of enzyme loading, the reaction was performed with 2, 5 and 10 U of immobilized lipase for 72 h. The percentage of wax esters rose with increasing the enzyme dosage and the highest yield of wax esters was obtained at 10 U of enzyme. For the reaction of immobilized lipase EQ3, enzyme loading at 2, 5 and 10 U showed rapid synthesis for 24 h with the value being 52.88, 68.88 and 88.13%, respectively. After that, the synthesis slightly increased to 83.39, 87.76 and 91.13% at 72 h, respectively. For the reaction of Lipozyme RM IM, the percentage of wax esters also increased with reaction mixture containing 2–10 U of enzyme. The synthesis reaction at 10 U of Lipozyme RM IM significantly increased to 83.93% while the reaction of 5 U (61.77%) and 2 U (47.32%) of enzyme gave lower yields than 10 U at 24 h. Moreover, the synthesis reactions at 10 U of both immobilized lipase EQ3 and Lipozyme RM IM were not significantly different at 24 h. Coconut oil based wax esters using immobilized lipase EQ3 and Lipozyme RM IM at 10 U showed the highest catalytic efficiency.

In case of very low percentage of wax ester synthesis at low concentration of Lipozyme RM IM (2 U), this phenomenon might be resulted from the low amount of lipase molecule on carrier surrounding in large excess substrate that was not sufficient for efficient interaction of catalyst and substrate [16], and it might be lower dispersion of immobilized lipase leading the reduction of substrate diffusion to the active site [17]. While, the increasing of immobilized lipase EQ3, the reaction was rapid and obtained the high yield of wax esters, but the initial rate was slower than Lipozyme RM IM. It might involve the low amount of enzyme on carrier resulting to having high amount of solid particle in the reaction. Although, the increasing of contact surface may help in raising mass transfer, but the high amount of carrier might increase mass transfer resistance [18] or diffusion limitation of substrate [19]. For the equilibrium point of reaction, Zhong et al. [20] found that high enzyme concentration enhanced the effective contact area with substrate molecules by increasing the probability of substrate enzyme collision. In addition, surface contact area of enzyme and substrate might reach saturation when an excessive amount of enzyme was added. Ungcharoenwiwat et al. [7] reported that increasing the amount of enzyme led to an increased initial rate but did not influence reaction equilibrium.

3.2. Incubation temperature

Alcoholysis reaction of coconut oil and oleyl alcohol was carried out using 10 U of both immobilized lipases. The reactions were incubated at various temperatures of 30, 37 and 45°C for 24 h. Temperature is one of the most important variables affecting transesterification. Results are shown in Fig. 2. Wax ester synthesis using immobilized lipase EQ3 was significantly different at 30–45°C. The reaction at 30°C of incubation gave the highest yield of wax esters at 84.74% for 18 h and then slightly increased to 89.55% at 24 h. Furthermore, the synthesis reaction at 45°C obtained 86.12% wax esters for 18 h but gave a significantly different amount of 87.29% wax esters for 24 h when compared with 30°C. This result was similar to Ungcharoenwiwat and H-Kittikun [13] who reported that lipase from *Burkholderia* sp. EQ3 had an optimal temperature of around 25–37°C. For wax esters synthesized by Lipozyme RM IM, optimal temperature was 45°C. The

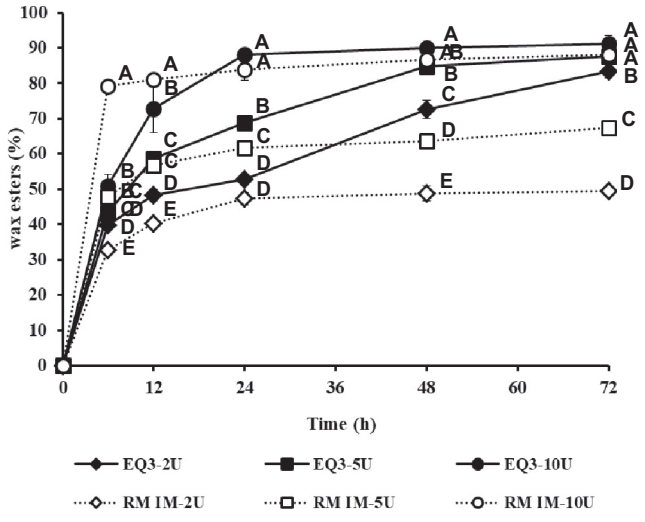

Fig. 1. Effect of time and enzyme concentration on the synthesis of coconut oil based wax esters by the immobilized lipase EQ3 and Lipozyme RM IM (reaction mixture: coconut oil 110 mg and oleyl alcohol 120 mg (1:3 mol/mol), hexane 1 mL at 37°C and 150 rpm for 72 h).

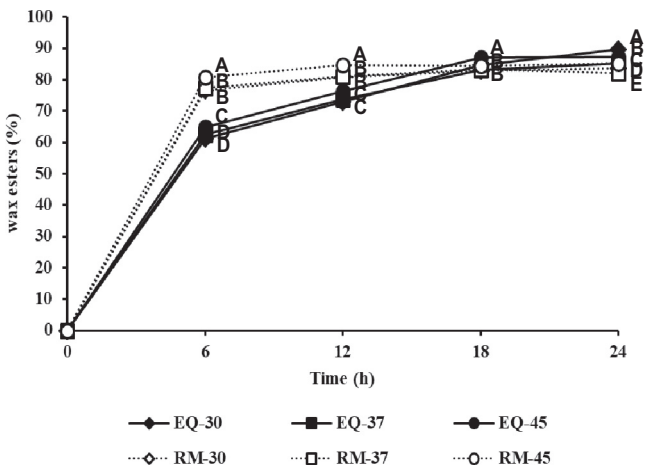

Fig. 2. Effect of temperature on the synthesis of coconut oil based wax esters by the immobilized lipase EQ3 and Lipozyme RM IM (reaction mixture: immobilized enzyme 10 U, coconut oil 110 mg and oleyl alcohol 120 mg (1:3 mol/mol), hexane 1 mL and 150 rpm for 24 h).

synthesis dramatically increased to 80.88% wax esters at 6 h and then slightly changed to 85.32% wax esters at 24 h. The reaction at 37°C obtained 77.41 and 82.30% wax esters for 6 h and 24 h, while the lowest synthesis was recorded for the reaction mixture incubated at 30°C. Higher temperature can increase enzyme activity and reaction rate; however, lipases are proteins that denature at high temperatures due to conformational changes in their three-dimensional structures that lead to low enzyme activity. Different enzymes can catalyze at different optimal temperatures. Optimal temperatures for coconut oil based wax ester synthesis were 30°C for immobilized lipase EQ3 and 45°C for Lipozyme RM IM.

3.3. Molar ratio of oil and alcohol

Molar ratio of substrate as oil to alcohol for transesterification influences the yield of synthesis. Moreover, the appropriate ratio of substrate can produce products with high purity and low remaining substrate. An alcoholysis reaction of both immobilized lipases was performed at 1:1 (150 μmol: 150 μmol), 1:2 (150 μmol: 300 μmol), 1:3 (150 μmol: 450 μmol) and 1:4 (150 μmol: 600 μmol) of coconut oil and oleyl alcohol in hexane and 10 U of enzyme for 24 h. Results are shown in Fig. 3. Coconut oil based wax esters showed considerable synthesis according to increasing molar ratio. The appropriate molar ratio of substrate for immobilized lipase EQ3 and Lipozyme RM IM was 1:3 (mol/mol). The trend of synthesis by immobilized lipase EQ3 at higher oil to alcohol molar ratios from 1:1 to 1:3 (mol/mol) gave greater wax ester conversion in a shorter time with significant difference. At 1:3 (mol/mol) of substrate, immobilized lipase EQ3 catalyzed the reaction faster than Lipozyme RM IM and produced wax esters with highest yield up to 85.86% for 18 h; after that, catalysis reaction slightly increased to 86.32% at 24 h. This result was similar to Lipozyme RM IM. Lipase showed rapid synthesis with a high amount of wax esters at 79.73% within 6 h. Percentage of wax esters slightly increased and remained constant at 85.14% for 24 h. The catalysis reaction of both immobilized lipases reduced when molar ratio of coconut oil and oleyl alcohol was 1:4 (mol/mol). Many previous reports also demonstrated good yield of wax esters at an appropriate molar ratio of 1:3 (mol/mol) by enzymatic transesterification [8,9,21]. An excess alcohol may lead to imbalance of enzyme loading and substrate in the reaction. Moreover, high amounts of alcohol can destroy the water layer around the enzyme molecules and may inhibit the reaction phase between enzyme and substrate. At high concentration of alcohol, oleyl alcohol cannot totally dissolve in the oil and become replaced the organic solvent and affects the protein structure unstable [5,22,23].

3.4. Substrate concentration

Substrate concentration for coconut oil based wax ester synthesis was investigated to determine the optimal concentration to give a product with the lowest remaining substrate and improve reaction rate of the enzyme as well as the conversion of equilibrium limited reactions to produce the high purity product. Substrate concentrations for wax ester synthesis by immobilized lipase EQ3 and Lipozyme RM IM were studied based on the optimal substrate ratio from previous experiment and varied by dilution to 0.33, 0.66 and 1.0X of optimal substrate ratio (1:3 mol/mol). Results of wax ester synthesis reaction for 24 h are shown in Fig. 4. Percentage of wax esters showed direct variation on increasing substrate concentration, but the reaction rate gave reversed variation. For synthesis by immobilized lipase EQ3, 0.33X of substrate concentration exhibited rapid synthesis with the highest percentage of wax esters at 18 h of 91.54%, followed by 0.66X and 1.0X at 85.86 and 84.57%, respectively. After that, the synthesis showed equilibrium point at the end of the reaction. Similarly, wax ester synthesis by Lipozyme RM IM gave faster reaction rate at 0.33X of substrate with a short time than immobilized lipase EQ3 (~72%) at 6 h for 90.62% with significant difference. Substrate concentrations at 0.66 and 1.0X were 84.00 and 80.91% at 6 h and then synthesis slightly increased until 24 h. These findings were similar to Kumar et al. [24] who noted that lipase from *Aspergillus terreus* NCFT 4269.10 showed high catalysis at substrate concentrations of 0.25–2.0% but it was inhibited at higher concentrations than 2.0%.

Steep increasing was shown in the initial rate of reaction with increasing substrate concentration because the enzymes were saturated with substrate leading to effective binding and high conversion rate until reaching the maximum rate. It has remarked that the reaction rate of enzyme to product formation depends on enzyme activity [25]. Moreover, high percentage of wax esters at low substrate concentration was observed because wax ester analysis by TLC-FID measured as the relative percentage of compounds in the reaction. The low percentage of wax esters at high substrate concentration had more impurity of remaining substrate. Moreover, high saturation of substrate affected enzyme structure and the binding between substrate and enzyme because of unavailable catalytic sites and the

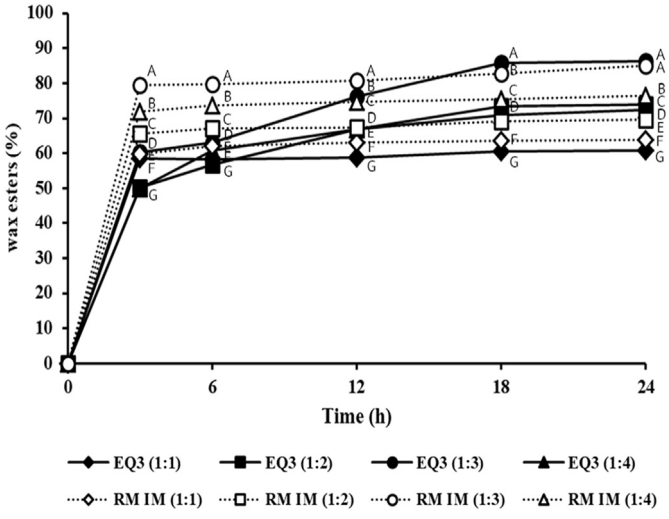

Fig. 3. Effect of molar ratio of coconut oil and oleyl alcohol on coconut oil based wax esters by the immobilized lipase EQ3 (reaction mixture: immobilized enzyme 10 U, hexane 1 mL, 30°C and 150 rpm for 24 h) and Lipozyme RM IM (reaction mixture: immobilized enzyme 10 U, hexane 1 mL, 45°C and 150 rpm for 24 h).

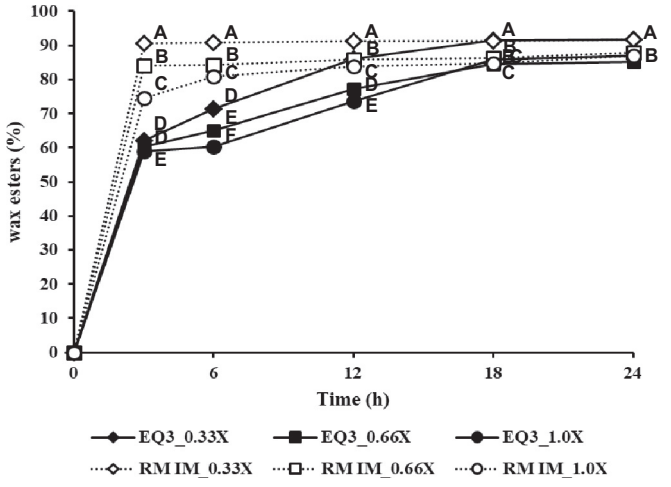

Fig. 4. Effect of substrate concentration on coconut oil based wax esters by the immobilized lipase EQ3 (reaction mixture: immobilized enzyme 10 U, coconut oil and oleyl alcohol 1:3 mol/mol, hexane 1 mL, 30°C and 150 rpm for 24 h) and Lipozyme RM IM (reaction mixture: immobilized enzyme 10 U, coconut oil and oleyl alcohol 1:3 mol/mol, hexane 1 mL, 45°C and 150 rpm for 24 h).

viscosity of oleyl alcohol [5]. Although, at low substrate concentration had affected the yield of wax esters, but it had more conversion of wax esters with high purity that might be increasing the enzyme reusability because of short time immersion in the system.

3.5. Various organic solvents

Optimization of organic solvents for coconut oil based wax ester synthesis by immobilized lipase EQ3 and Lipozyme RM IM was studied by preparing reaction mixtures with optimal condition in toluene (log P = 2.50), hexane (log P = 3.50), heptane (log P = 4.0) and isooctane (log P = 4.50) for 24 h. Results are shown in Fig. 5. Various organic solvents greatly affect enzymatic reaction. The log P value of the solvent can evaluate its polarity; a more positive value indicates higher lipophilicity or hydrophobicity. Wax ester synthesis by both immobilized lipases increased when the reactions were dissolved in organic solvents with log P > 3.0. Thus, organic solvents with high hydrophobicity can increase the solubility of non-polar substrates. Wax ester synthesis by immobilized lipase EQ3 dramatically increased with hydrophobic solvent. High percentage of wax esters was synthesized in isooctane at 88.35% followed by hexane and heptane with 85.97 and 83.50% at 12 h; after that, the wax esters reached 91.30, 90.80 and 90.25% at 24 h, respectively. Reaction in toluene showed the lowest wax ester yield at 43.70% for 24 h. This result was similar to synthesis by Lipozyme RM IM. Wax ester synthesis was faster than by immobilized lipase EQ3, and obtained the highest yield in isooctane followed by hexane and heptane at 88.85, 86.30 and 84.17% at 3 h, respectively. Then, percentage of wax esters insignificantly increased to 90% at 24 h. Moreover, the enzyme showed the lowest catalysis in toluene as percentage of wax esters with 87% for 6 h and 87.83% for 24 h. This result suggested that the appropriate organic solvent for coconut oil based wax esters by immobilized lipase EQ3 and Lipozyme RM IM was isooctane. Lipase is generally stable and active in hydrophobic solvents because it has a hydration layer around the enzyme structure to preserve its activity. Hydrophobic solvents can improve substrate solubility and promote viscosity reduction which supports the accessibility of reactant molecules in a biocatalyst microenvironment [25]. Moreover, it has been reported that organic solvents with log P < 3.0 could prevent essential water moieties for enzymatic reactions around the immobilized particles or enzyme molecules which affect enzyme structure and reduce its activity [5,7].

In addition, the productivity of wax esters by immobilized lipase EQ3 at the optimum condition for 12 h was calculated as 7.36% per hour and the productivity of Lipozyme RM IM for 3 h was 29.62% per hour. The commercial Lipozyme RM IM exhibited high potential synthesis than immobilized lipase EQ3 for 4 times. The Lipozyme RM IM was more suitable for commercial industry because it had improved yield of enzyme production and its purity through using *Aspergillus oryzae* as expression system [26] that lead to have high immobilization yield and use low amount of immobilized enzyme. Although, the immobilized lipase EQ3 which was wild-type lipase had lower productivity of wax esters, its synthesis had insignificantly production compared with Lipozyme RM IM at 12 h and it was valuable to study for its improvement.

3.6. Immobilized enzyme reusability

Coconut oil based wax ester synthesis by immobilized lipase EQ3 was carried out in substrate molar ratio of 1:3 with 0.33X dissolved in isooctane and 10 U of immobilized lipase at 30°C for 12 h, whereas the reaction was incubated at 45°C for Lipozyme RM IM. After synthesis, both immobilized lipases were filtrated and washed five times with the same organic solvent used in the synthesis. Then, the immobilized lipases were dried in a desiccator under vacuum condition for 12 h before use in the next batch of synthesis. Reusable immobilized lipases were recycled repeatedly for five batches. Results are shown in Fig. 6. For wax esters synthesized by immobilized lipase EQ3, enzyme efficiency tended to decrease in each batch. Percentage of wax esters in the first to fifth batches were 88.64, 84.34, 69.37, 41.53 and 32.64%, respectively. Lipase activity decreased drastically for the last three cycles with retaining more than 50% at the third batch of reaction. This result was consistent with synthesis by Lipozyme RM IM as 86.64, 85.40, 60.66, 48.62 and 32.06% in the first to fifth batch, respectively. Percentages of wax esters synthesized by both enzymes in many batches showed no significant differences. Decreasing catalysis of lipases might cause the formation of glycerol molecules as a by-product from transesterification of wax ester synthesis. These residual polarity molecules, attaching around solid supports and enzyme molecules, could not be washed out from the immobilized enzyme by hydrophobic organic solvents and impacted on substrate entering the enzyme molecules. Another study into the effect of

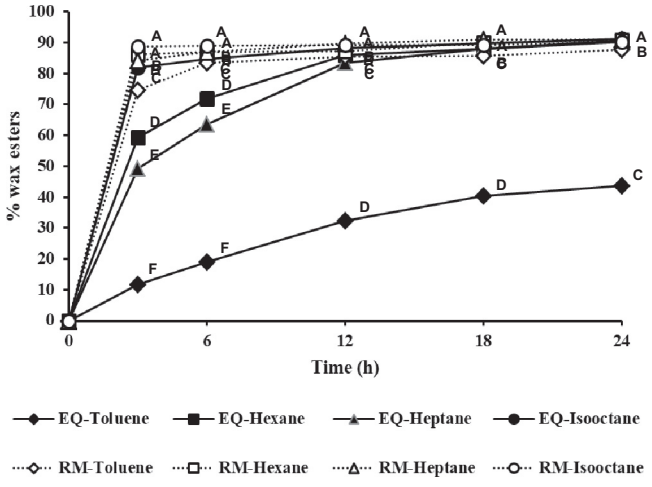

Fig. 5. Effect of organic solvents on coconut oil based wax esters by the immobilized lipase EQ3 (reaction mixture: immobilized enzyme 10 U, coconut oil and oleyl alcohol 1:3 mol/mol (0.33X), 30°C and 150 rpm for 24 h) and Lipozyme RM IM (reaction mixture: immobilized enzyme 10 U, coconut oil and oleyl alcohol 1:3 mol/mol (0.33X), 45°C and 150 rpm for 24 h).

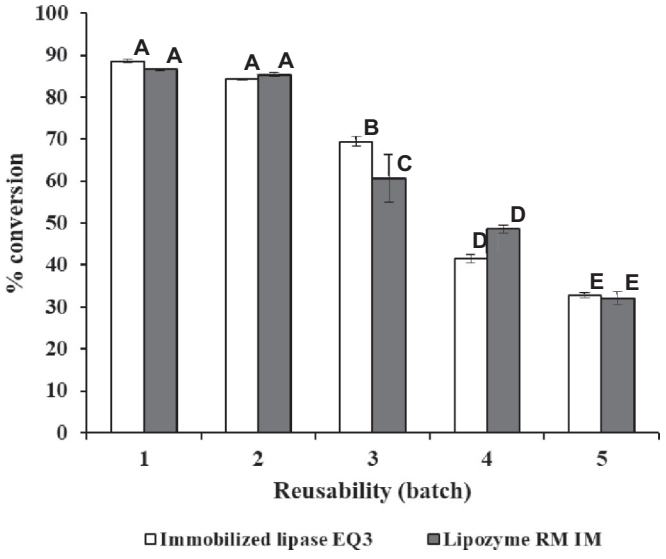

Fig. 6. Enzyme reusability on coconut oil based wax esters by the immobilized lipase EQ3 (reaction mixture: immobilized enzyme 10 U, coconut oil and oleyl alcohol 1:3 mol/mol (0.33X) in isooctane, 30°C and 150 rpm for 12 h) and Lipozyme RM IM (reaction mixture: immobilized enzyme 10 U, coconut oil and oleyl alcohol 1:3 mol/mol (0.33X) in isooctane, 45°C and 150 rpm for 12 h).

organic solvent on washing the immobilized lipase for enzyme reusability found that organic solvents like methanol and butanol could discharge glycerol molecules absorbing enzymes at more than 85%, whereas hexane could wash glycerol to 40% [27]. However, the higher polarity of solvent was better at discharging glycerol from immobilized enzymes but might destroy the water layer surrounding the enzyme and result in desorption. This result concurred with Marín-Suárez et al. [28] who studied the reusability of Lipozyme RM IM for transesterification of waste fish oil for biodiesel production. They found that the enzyme lost activity more than 16% after 10 cycles, probably due to solvent inhibition and glycerol adsorption by enzyme support. Furthermore, glycerol is insoluble in hexane or high hydrophobic solvent; therefore, it cannot remove the glycerol layer in the case of more hydrophilic supports (Lipozyme RM IM) [29]. For immobilized lipase EQ3, prepared by absorption technique on Accurel as polypropylene-based hydrophobic porous support, the enzyme might be easily desorbed or inactivated after the washing step. Washing with hydrophobic solvent like isooctane was not sufficient to efficient elimination of the oil layer or wax esters formed around the enzyme. The washing solution is important in studies of enzyme reusability.

3.7. Characteristics of coconut oil based wax esters

Liquid wax esters from coconut oil and oleyl alcohol synthesized by immobilized lipase EQ3 at optimal condition were collected and analyzed for wax ester composition by gas chromatography-mass spectrophotometry (GC–MS) [7], saponification value, acid value, moisture content (AOAC 2005) and surface tension property. Results are shown in Table 1. Liquid wax esters comprise different carbon chain lengths of C_{28}-C_{36}. More unsaturated wax esters result in liquid form at room temperature. Total wax esters had 85% with the major component as oleyl laurate at 47.90% followed by oleyl myristate, palmityl laurate and oleyl palmitate at 15.98, 9.79 and 6.56%, respectively. The percentage of the area of wax esters was based on fatty acid composition in coconut oil which had lauric acid (~47%) as a major component followed by myristic acid (~19%) and oleic acid (~6.2%), respectively [30]. Moreover, the oleyl alcohol used in reaction was industrial grade with 85% purity (mixed with oleyl and palmityl alcohol) as the reason why it had both oleyl and palmityl esters. From a previous report, immobilized lipase EQ3 was used for jatropha oil and palm oil based wax ester synthesis at similar amounts of wax esters to this experiment. Thus, lipase preferred transesterified catalysis with long chain fatty acids [7,9].

A high saponification value (SV) gave a higher proportion of low molecular weight fatty acids in the oil or vice versa [31,32]. The SV is used for determination of the average molecular weight of oil. Here, the wax ester samples exhibited 124.77 mgKOH/g that indicating the transesterification reaction between oleyl alcohol and fatty acids to produce long chain wax esters with increasing molecular weight. For acid value and water content, coconut oil based wax esters showed high acid value with 7.41 mgKOH/g, whereas coconut oil was less than 4.0 mgKOH/g. High acid value might cause high contents of free fatty acids from triglyceride hydrolysis in the reaction related to water content [33]. Water content in wax ester samples was 0.10% while coconut oil had 0.01%. Kusdiana and Saka [34] found that water content at 0.1% could decrease transesterification for methyl ester synthesis caused by hydrolysis to free fatty acids. For surface tension reduction, wax esters exhibited lower surface tension than coconut oil and could be applied in the cosmetics industry.

Table 1
Characteristics of coconut oil based wax esters.

Characteristics	Coconut oil based wax esters
1. Fatty acid composition (area%)	
Palmityl luarate (28:0)	9.79
Oleyl luarate (30:1)	47.90
Oleyl myristate (32:1)	15.98
Oleyl palmitate (34:1)	6.56
Oleyl oleate (36:2)	3.24
Oleyl stearate (36:1)	1.62
Total	85.09
2. Saponification value (mgKOH/g)	124.77 ± 1.98
3. Acid value (mgKOH/g)	7.41 ± 0.19
4. Moisture content (%)	0.10 ± 0.01
5. Surface tension	30.17 ± 0.29

Melting and degradation points were measured by DSC and TGA curves. Generally, the DSC technique is used to measure chemical or physical changes in oil properties that occur as temperature varies with time. TGA is a widely used technique for identifying the decomposition steps of different solid and liquid materials by monitoring the sample weight. The melting process resulted in an endothermic peak in the DSC curve. Results are shown in Fig. 7. The first peak of endothermic phenomenon was at −0.67°C and other points were closely connected with lower temperature than the first peak (beginning at ~ −16.42°C). Moreover, the endothermic process increased in temperature range of nearly 80–393.7°C and the weight of wax esters in the TGA graph decreased as temperature increased. There was only one main decomposition step at which wax esters degraded with weight loss of 97.16% at 337.02°C and 99.78% at 423.61°C, indicating vapor-phase sample decomposition. Coconut based wax esters should, therefore, be applied at temperatures lower than 250°C (remaining 90% wax esters) for highest stability.

4. Conclusions

Immobilized lipase EQ3 was used for wax ester synthesis from coconut oil and oleyl alcohol and compared with Lipozyme RM IM. Immobilized lipase EQ3 obtained the highest percentage of wax esters as 88.35% for 12 h with a slower synthesis than Lipozyme RM IM at 88.85% for 3 h under optimal conditions. Reusability of both immobilized lipases was two cycles with high percentage of wax ester synthesis. Characteristics of coconut oil based wax esters showed that more than 85% were mainly composed of oleyl laurate (47.90%). Wax esters had a melting point at ~ −0.67°C and degradation to 90% remaining at ~250°C. Moreover, the washing step of immobilized lipase should be improved to reduce the cost of production. Wax esters

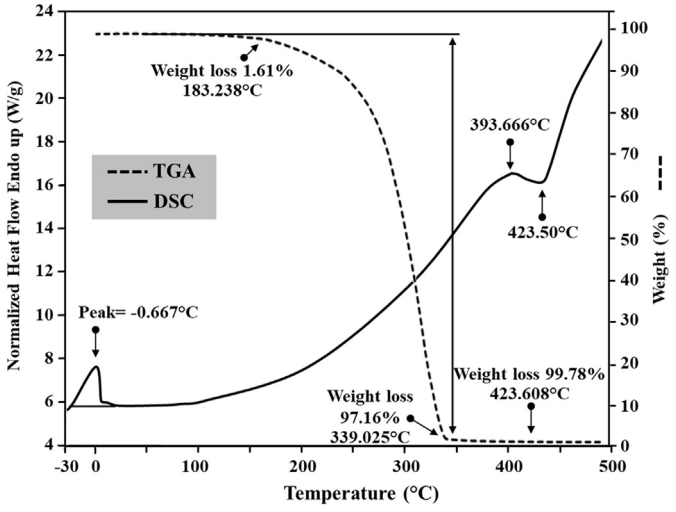

Fig. 7. DSC and TGA analysis of coconut oil based wax esters.

synthesized by enzymatic methods show potential for large scale production to replace chemical catalysis methods for wax ester synthesis.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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