Lipase-catalyzed Production and Purification of Palm Esters Using Stirred Tank Reactors (STR)

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Abstract: Lipase-catalyzed production of palm esters was performed via alcoholysis of palm oil and oleyl alcohol in solvent and solvent-free systems using a 2 L stirred tank reactor (STR). Two immobilized lipases were tested and Lipzyme RM IM exhibited superior performance in both reaction systems. Reusability studies of the enzymes in a solvent-free system also demonstrated the high stability of Lipzyme RM IM as shown by its ability to yield more than 70% palm esters with up to 19 cycles of reusing the same enzymes. Modification of the enzyme washing process improved the stability of Lipzyme TL IM in a solvent system as demonstrated by maintaining 65% yield after 5 times of repeated enzyme use. The scale up process for both lipases was conducted in the presence of solvents by using the impeller tip speed approach. Lipzyme RM IM–catalyzed reaction in a 15 L STR produced 85.7% yield and there was a significant drop to 60.7% in the 300 L STR, whereas Lipzyme TL IM had a lower yield (65%) when the reaction volume was increased to 15 L. The low yields could be due to the accumulation of enzymes at the bottom of the vessel. Purification of palm esters via solvent-solvent extraction revealed that more than 90% of oleyl alcohol was extracted after the third extraction cycle at 150 rpm impeller speed with reduced palm esters: ethanol ratio (v/v) from 1:4 to 1:3.

Key words: Lipzyme RM IM, Lipzyme TL IM, palm esters, solvent-free system, stirred tank reactor

1 Introduction

The palm oil industry, which is the highest-yielding commodity for oils and fats, has become one of Malaysia’s most important ventures since the early 20\textsuperscript{th} century. The growth of the industry has been phenomenal with its highest ever earnings recorded in 2011. The industry has generated substantial income for the national economy by contributing RM 80.4 billion with export earnings surpassing the RM 65.2 billion mark recorded in 2008 by 23.3\%\textsuperscript{1}. In 2013, the export demand for palm oil and derived products was 4.5\% higher than the previous year and Malaysia is forecasted to supply more than 45\% of the global palm oil requirement in 2014\textsuperscript{4} and expected to continue its upward trend for 2015 and 2016. As one of the top contributors to the global vegetable oil trade, the palm oil industry has focused on identifying sustainable production measures and processes.

Palm-based specialty oleochemicals, particularly palm-based wax esters are one of the palm oil product derivatives that may have a very promising future. Wax esters, consisting of long chain fatty acids and long chain fatty alcohols exhibit excellent wetting behavior at the interface and non-irritating properties on human skin\textsuperscript{2-5}. However, the natural supply of wax esters such as jojoba oil and sperm whale oil are often expensive and scarce due to weather limitations and the global ban on whale hunting, leading to a growing interest in the production of synthetic wax esters, which possess similar characteristics as the natural wax esters but at a lower cost.

Research on these high value palm-based wax esters has developed steadily over the past decade. Few studies have been made on its up-scale production despite its wide range application especially in cosmetics, food, pharmaceutical and chemical industries. Conventionally, the produc-

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ton of wax esters consumes a lot of energy and can sometimes be very toxic not only to humans but also to the environment. In contrast, biocatalytic synthesis offers low energy consumption with high product selectivity under mild conditions\(^5\). The process is also much safer and more environmentally friendly as compared to the conventional method.

Research on this biocatalytic synthesis of palm-based wax esters utilizing enzymes as biocatalysts on a laboratory scale has been successful. Sellami et al.\(^5\) studied the synthesis of wax esters of palm stearin and cetyl alcohol using immobilized Rhizopus oryzae lipase. Meanwhile, Gunawan et al.\(^6\) performed an alcoholysis reaction between palm oil and oleyl alcohol using Lipozyme RM IM. Similar reactions were conducted by Keng et al.\(^9\) in a 0.5 L stirred tank reactor, then further scaled-up using 2 L and 75 L stirred tank reactors. Meanwhile, Basri et al.\(^4\) reported on a synthesis of palm oil esters using Lipozyme TL IM as a cheaper alternative in a 2 L stirred tank reactor. Hexane was used in all the aforementioned lipase-catalyzed reactions which due to its polarity-hydrophobicity solvent properties (log \(P\) > 4) that largely contributed to the stability of the enzyme. All reactions findings reported high yields of more than 80%.

This present work is an extension of what was published\(^4\), in which palm oil wax esters or palm esters were produced by the alcoholysis of palm oil and oleyl alcohol using Lipozyme RM IM and Lipozyme TL IM in a 2 L stirred tank reactor. The study was performed using solvent-free reactions under optimum conditions as an attempt to reduce production costs without jeopardizing the palm ester yield. Reusability studies of both enzymes and downstream processing of palm esters via liquid-liquid extraction were also taken into consideration. Subsequently, a scale-up of the process to a larger STR volume (15 L) was performed using a constant impeller tip speed prior to proceeding to 300 L STR.

2 Materials and Methods

2.1 Materials

Immobilized enzyme, Lipozyme TL IM (commercial lipase from Thermomyces lanuginosus immobilized on silica gel) and Lipozyme RM IM (commercial lipase from Rhizomucor miehei immobilized on microporous anionic exchange resin) were purchased from Novo-Nordisk, Denmark. Refined bleached deodorized (RBD) palm oil was obtained from Golden Jamalina Sime Darby, Malaysia. Fatty acids in Malaysian palm oil are 0.1–0.3% of lauric acid, 0.9–1.5% of myristic acid, 39.2–45.2% of palmitic acid, 3.7–5.1% of stearic acid, 37.5–44.1% of oleic acid and 8.7–12.5% of linoleic acid. Oleyl alcohol, \(n\)-hexane and ethanol were purchased from Merck, Germany. All other chemicals were used without further purification and were of analytical grade.

2.2 Experimental set up

2.2.1 2 L stirred-tank reactor (STR)

The 2 L STR reactor with a working volume of 1.5 L was made of borosilicate glass (13.0 cm i.d.) with a stainless steel top-plate and baffle insert. The temperature in the vessel was controlled using an open thermostat system with a circulation pump. The agitation system consisted of a rotary shaft located at the center of the top-plate and equipped with a six-bladed Rushton turbine (RT) impeller as shown in Fig. 1. The stainless steel top-plate consisted of various openings for sampling and electrodes (2 L, Biostat MD, B. Braun, Germany).

2.2.2 Synthesis of palm esters in solvent and solvent-free systems using Lipozyme RM IM

Palm oil (600 mmol) and oleyl alcohol (1800 mmol) were placed in the reaction vessel followed by the addition of \(n\)-hexane to a working volume of 1.5 L. Lipozyme RM IM (16.8 wt%) was subsequently added and was continuously reacted at an impeller speed of 250 rpm and temperature of 50°C for 5 hours. The optimum conditions were based on previous work\(^4\). At the end of the reaction, a 10 ml sample was collected for gas chromatography. The hexane was then removed and recovered by a rotary evaporator. In the solvent-free system, the same reaction conditions were performed but without \(n\)-hexane.

2.2.3 Synthesis of palm esters in solvent and solvent-free systems using Lipozyme TL IM

Palm oil (600 mmol) and oleyl alcohol (1800 mmol) were placed in the reaction vessel, followed by the addition of \(n\)-hexane to a working volume of 1.5 L. Lipozyme TL IM (24.7
wt %) was subsequently added and the mixture was continuously reacted at an impeller speed of 275 rpm and temperature of 60°C for 3 hours. The optimum conditions were based on previous work.

2.2.4 Gas chromatography analysis

The reaction mixture was quantified by injecting a 1 µL sample into an Agilent 68900 gas chromatograph equipped with a RTX-65 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, Restek Corporation, USA) and a flame ionization detector. The temperature of injector and detector were set at 250°C and 300°C, respectively. Meanwhile, the oven temperature was maintained at 150°C for 2 min, and increased by 20°C per min to 290°C and held at that temperature for 10 min. Nitrogen was used as a carrier gas at a flow rate of 1.4 mL/min. The product composition was quantitated by an internal standard method using methyl laurate.

2.2.5 Reusability studies of Lipozyme RM IM and Lipozyme TL IM in a solvent-free system

The reusability of enzymes in 2 L STR was based on the optimized reaction conditions using Lipozyme RM IM and Lipozyme TL IM in both the solvent and solvent-free systems. At the end of the reaction, the enzyme was filtered from the reaction mixture using a stainless steel test sieve with 20 µm mesh. The enzyme was washed thrice with hexane followed by distilled water and dried in open air at room temperature for 4 h before being used again.

2.2.5.1 Reusability studies - modification on the enzyme washing process

The previous procedure in 2.2.5 was adopted for the reaction in solvent system except the enzyme was washed thrice with only distilled water and dried in an oven at room temperature 4 h before being used again.

2.2.6 Liquid-liquid extraction of palm esters

2.2.6.1 The effect of impeller speed

The purification of palm esters was conducted via liquid-liquid extraction using ethanol with phase ratio of palm esters to ethanol 1:4 in a 2 L STR. Continuous mixing occurred for 30 min at room temperature and the impeller speed varied from 150, 200, 250 and 300 rpm. The mixture was allowed to separate into two layers. The lower layer was collected for GC analysis.

2.2.6.2 The effect of extraction

A 1:4 of palm esters and ethanol was mixed at impeller speed of 150 rpm for 30 min in a 2 L STR at room tempera-

| Dimension                      | 2 L   | 15 L  | 300 L |
|--------------------------------|-------|-------|-------|
| Diameter of the tank, D_t (cm) | 13.0  | 18.5  | 50.0  |
| Height of the tank, H (cm)     | 13.0  | 36.0  | 160.0 |
| Diameter of the impeller, D_i (cm) | 5.5   | 7.5   | 20.0  |
| Ratio D_i/D_t                   | 0.408 | 0.408 | 0.400 |

Fig. 2  Schematic diagram of the a) 15 L and b) 300 L STR.
schematic diagrams of the reactors are shown in Fig. 2(a) and Fig. 2(b). Reaction conditions were based on optimum conditions\(^4\)\(^5\) in a 2 L STR for Lipozyme RM IM and Lipozyme TL IM, respectively and were upscaled proportionally to the volume of the STRs. All reactions were performed in a solvent system (Tables 2 and 3). The percentage yield of the collected samples was determined as previously mentioned.

### Results and Discussion

#### 3.1 Production of palm esters in solvent and solvent-free systems for Lipozyme RM IM and Lipozyme TL IM

Figure 3 represents the comparative studies between Lipozyme RM IM and Lipozyme TL IM on the percentage yield of palm esters in solvent (hexane) and solvent-free reaction systems. Lipozyme RM IM and Lipozyme TL IM are immobilized lipases obtained from *Rhizomucor miehei* and *Thermomyces lanuginosus*, respectively and the catalytic activity (10790 U) was maintained with optimum amounts for both reactions involving Lipozyme RM IM and Lipozyme TL IM. The reaction between palm oil and oleyl alcohol was based on the assumption that one mole of palm oil produces three moles of palm esters as shown by the reaction scheme in Fig. 4. Meanwhile, the composition of palm esters produced was observed to coincide with the composition of fatty acids in palm oil. They mainly consisted of oleyl palmitate, oleyl oleate, oleyl stearate, oleyl myristate and oleyl laurate as depicted in Fig. 5. At the time when the reactions were conducted, it was observed that presence of solvents did not have significant effect on the distribution of oleyl esters. However, further optimization need to be carried out for both enzymes in solvent-free system by taking into account factors such as temperature, substrate loading and enzyme amount which may contribute to the distribution of esters in palm esters. Overall, reactions with Lipozyme RM IM produced a higher yield than Lipozyme TL IM. Reactions catalyzed by Lipozyme RM IM have been extensively researched for higher yields especially in catalytic reactions involving longer alcohol/fatty acid (C8 - C18) chain\(^1\)\(^2\)\(^3\)\(^4\). Similar findings were also reported by previous researchers\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\), on the preference of Lipozyme RM IM. Conversely, Lipozyme TL IM was observed to produce lowest esterification activity and highest hydrolytic activity in comparison to Lipozyme RM IM\(^11\)\(^12\). Furthermore, solvents provide protection from extreme conditions, such as increase in viscosity, which usually limit mass
transfers. This was seconded by Ghamgui et al. and Karra-Châabouni et al., who highlighted that immobilized lipase was more effective in organic solvents than in the absence of organic solvent. However, reactions in solvent-free systems for both Lipozyme RM IM and Lipozyme TL IM have shown good comparable results with reactions in solvent systems, which could provide alternative approaches to a greener production process with reduced production costs. Therefore, further investigation to optimize the production of palm esters in a solvent-free system is necessary for both lipases.

3.2 Enzyme reusability in a solvent-free reaction system

Immobilized lipases are more beneficial than free lipases due to their ability to be reused and recovered multiple times with considerable high catalytic activity. The operational stability of Lipozyme RM IM and Lipozyme TL IM was evaluated for their repeated use in the solvent-free alcoholysis of palm oil and oleyl alcohol in Fig. 6(a). For better understanding, the result was compared with that of the reusability study performed in the solvent system as shown in Fig. 6(b). Both lipases performed better in terms of enzyme reutilization in the solvent system reaction than in the absence of solvent. Lipozyme RM IM was able to retain beyond 70% of yield in 19 consecutive batches and decreased to 66.8% in the 20th batch for the solvent-free system. Meanwhile, the yield of palm esters using Lipozyme TL IM was comparable for both systems of above 60% after the third batch but reduced significantly to 39% in the fifth batch in the solvent-free system. Further decrease in yield was observed to be below 35% until the 11th batch, after which the run was discontinued to prevent substrate waste. The reusability and stability of immobilized enzymes can be explained by the diverse enzyme properties such as superiority of Lipozyme RM IM over Lipozyme TL IM in solvent-free conditions. This may be attributed by the reaction conditions and the substrate used as mentioned earlier. Another hypothesis is the difference in nature of the immobilization supports that can alter the enzyme conformation as well as its operational stability. Lipozyme TL IM that was immobilized on silica had a high affinity towards glycerol, a by-product of the reaction. The partition of substrates and products from the enzyme environment might cause the active site to be inaccessible to the substrate. Although enzyme washing can help to minimize the negative effects of glycerol but the selection of solvents and number of steps required have significant impact on the operational stability of the enzymes. In this study, both enzymes were washed thrice with hexane and water consecutively. Hexane was reported to result in the lowest amount of glycerol removal and lower enzyme decay activity due to its hydrophobic properties. However the use of polar solvent such as ethanol can lead to the change of support morphology by dissolving the lipase supports. Furthermore, the combined effects of desorption or leakage of enzyme from the support as well as mechanical loss during repeated filtration, washing, and drying activities could reduce the yield of palm esters.
3.3 Enzyme reusability in solvent system reaction – Modification of enzyme washing process

As part of the effort to minimize the production cost and negative impact on the environment, modification on the enzyme reusability washing process was performed with Lipozyme RM IM and Lipozyme TL IM in a solvent mediated reaction. At the end of every cycle, the enzymes were filtered and washed three times with only distilled water prior to drying. No hexane was used. Figure 7 shows that the percentage yield of palm esters was maintained above 75% and 65% until the 5th cycle for Lipozyme RM IM and Lipozyme TL IM, respectively. There was a significant increase in palm esters yield for Lipozyme TL IM in comparison with the results illustrated in Fig. 6b, which shows gradual yield drop to 53% on the 4th cycle when hexane was used for enzyme washing. Based on the results, water may be used without any assistance from a co-solvent due to its excellent ability to remove glycerol adsorbed onto the enzyme without denaturing the enzyme into unfolded state. This is advantageous from the viewpoints of cost of production and cost to the environment to not require the use of hexane. Furthermore, removing hexane helped reducing the number of steps required for enzyme washing process which prevents the loss of a significant amount of enzyme during repeated enzyme recovery activities, such as filtering, washing and drying when hexane and water were used consecutively.

3.4 Liquid-liquid extraction

3.4.1 Effect of impeller speed

The purification of palm esters through the removal of residual oleyl alcohol was conducted using liquid-liquid extraction technique in a 2 L STR. Figure 8 shows the effect of impeller speed on the percentage of oleyl alcohol extracted from palm esters. The percentage extraction of oleyl alcohol showed no significant difference as the impeller speed was further increased from 150 to 300 rpm, which indicates that the extraction of oleyl alcohol may not be largely affected by the mass transfer limitation between the reaction mixture and ethanol. Increases in impeller speed may have led to increases in surface contact area between oleyl alcohol and ethanol due to reduced droplet size in the dispersion of silicone oil in water-surfactant solution. However, it did not increase the uptake of oleyl alcohol by ethanol, which was probably caused by the saturation of the solute in the extraction phase. On another note, impeller speeds under 150 rpm was not applicable in this study due to non-homogeneous mixing. Therefore, the optimum impeller speed of 150 rpm was adopted in subsequent studies.

3.4.2 Effect of extraction cycles

A purification study of palm esters was further investigated by studying the effect of extraction cycles of oleyl alcohol from palm esters. This study was performed to ensure optimum efficiency of the extraction process to obtain a high purity of palm esters. Figure 9 depicts the percentage of oleyl alcohol extracted at different extraction cycles. The ratio of palm esters to ethanol and impeller speed was used in every cycle, based on previous experiments. 65% of oleyl alcohol was extracted in the first cycle and increased to 89% after the second cycle. A multicycle extraction process could result in a higher degree of extraction than that of a single stage extraction due to increased uptake of oleyl alcohol by ethanol and reduced solute saturation in the extraction phase. This agrees well with published data in the desulfurization of oxidized sulfur-containing compounds. The removal of sulfur was reported to increase from 93% to 93.7% after the second cycle. Similar extraction trends were also observed for the extraction of acrylamide.

3.4.3 Reduction of ethanol ratio in a solvent-solvent extraction

The amount of ethanol used for palm esters purification
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Fig. 9 Effect of extraction cycle on the extraction of oleyl alcohol. Reaction conditions: contact time: 30 min, temperature: 25°C and impeller speed: 150 rpm.

Fig. 10 Effect of ethanol ratio on extraction of oleyl alcohol. Reaction conditions: contact time: 30 min, temperature: 25°C, number of cycles: 3 and agitation speed: 150 rpm.

Fig. 11 Scale-up of palm esters production in solvent system reaction.

is a key factor in determining the purity and the cost of purification of palm esters. In previous experiments (3.4.1 and 3.4.2), the phase ratio of 1:4(v/v) of palm esters to ethanol was using a 500 mL separating funnel. Further study aimed to purify the palm esters at a reduced cost by varying the volume ratio of palm esters to ethanol from 1:1 to 1:4 using a 2 L STR. Figure 10 shows that the removal percentage of oleyl alcohol increased when the ratio for ethanol increased. An abrupt change was observed from ratio 1:1 (5.29%) to 1:2 (89.96%) which could be due to insufficient amounts of ethanol needed to bind with oleyl alcohol at a similar phase ratio of palm esters and ethanol. This was followed by a gradual increase of oleyl alcohol removal with the highest percentage removal of 94.69% at ratio of 1 to 4. More than 85% of oleyl alcohol was success-

fully removed from the palm esters at a volume ratio of 1:2 after the third cycle of extraction, which indicates a reduced amount of ethanol required in achieving more than 85% purity of palm esters. Hence, the ratio of 1:3 has shown to be adequate to obtain palm esters with greater than 90% purity.

3.5 Scale-up of palm esters production catalyzed by Lipzyme RM IM and Lipzyme TL IM in a solvent system

The study of palm esters production catalyzed by Lipzyme RM IM and Lipzyme TL IM was further extended into scaling up the reactor volume to 15 L and 300 L by using the impeller tip speed approach. The results are depicted in Fig. 11. The scale up for Lipzyme RM IM from 2 L to 15 L STR showed a good translation with almost similar yield of 86.6% to 85.7%. This was probably due to multiple (3 RT) impeller systems used in the 15 L reactor in comparison to a single impeller system used in the 2 L scale reaction. However, the yield decreased to 60.7% when the volume was scaled up to 300 L. In comparison, the yield for Lipzyme TL IM shows a significant reduction from 78.0% to 65.0% when the volume was increased to 15 L the study was not extended to 300 L to avoid substrate and enzyme waste. The reduction in yield could be attributed to the loss of enzyme suspension caused by inadequate power consumption during the scale up process. As a result, the immobilized enzyme particles accumulated at the bottom of the vessel, which resulted in a non-well mixed reaction and therefore limited the enzyme-substrate interaction. Many researchers have reported the application of constant power consumption rule as a scale up approach for reaction involving suspension of solid particle. However, scaling up with constant power consumption could result in increased impeller tip speed that could damage the immobilized lipase. Additionally, it was also
mentioned that the stirred vessel may have rather complex spatial solid distribution and can be dependent on the geometry of the mixer, impeller speed, flow pattern and the characteristics of the suspension, all of which can be further explored.

4 Conclusion

There is a higher yield of palm esters by using Lipzyme RM IM than Lipzyme TL IM in reactions with and without solvents in a 2 L STR but the difference is small. However, the operational stability of Lipzyme RM IM in the solvent-free system greatly outperformed Lipzyme TL IM in up to 19 cycles of same enzymes being reused with no significant activity loss. The purification of palm esters via solvent-solvent extraction using a 2 L STR were optimized and high amounts of oleyl alcohol were successfully extracted (> 90%). Furthermore, the scale up production of palm esters at a constant impeller tip speed to 15 L for Lipzyme TL IM and 300 L for Lipzyme RM IM in solvent systems have a negative effect on the yield of palm esters. The results suggest that scaling up production using the STR needs to be further studied using different approaches such as constant power consumption with different impeller types for process efficiency and cost effectiveness.

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