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# Enzymatic synthesis of palm-based ascorbyl esters

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ABSTRACT

The synthesis of palm-based ascorbyl esters through transesterification of ascorbic acid and palm oil in tert-amyl alcohol catalyzed by immobilized lipase is described. Highest conversion (70–75%) was determined after 16 h reaction at 40 °C using lipase (Novozyme 435 from *Candida antartica*) with an ascorbic acid to palm oil mole ratio of 1:8. The purified product was further characterized by $^{13}$C NMR and GC–MS and the mixture of ascorbyl monoesters obtained were identified as ascorbyl monooleate (61%), ascorbyl monopalmitate (30%) and ascorbyl monostearate (9%). The antioxidant activity of palm-based ascorbyl esters was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) test. The results showed that pure palm-based ascorbyl esters have an antioxidant activity with an $IC_{50}$ value of 0.1 mg/mL.



## 1. Introduction

L-Ascorbic acid (Vitamin C) is a water-soluble vitamin with a high antioxidant activity that is used as an additive in foods and cosmetics [1]. The solubility of this compound in water is good; however, this hydrophilic character reduces its effectiveness in stabilizing fats and oils [2]. To overcome this problem, ascorbic acid was esterified with fatty acid to produce ascorbyl fatty acid ester [3]. The fatty acid ester of ascorbic acid is a potential antioxidant and surfactant in food and cosmetics with high fat content [2]. The esterification process can be either chemical or enzymatic; however, the latter was preferred because of its advantages – high catalytic efficiency, mild reaction condition, and inherent selectivity of the natural catalyst [4].

In the enzymatic catalytic reaction, lipase was widely used as a biocatalyst [2]. Research on synthesis of ascorbyl fatty acid ester using unsaturated and saturated fatty acid as an acyl donor in organic solvent catalyzed by lipase has been reported [1,2,3,5]. However, the use of palm oil in the synthesis of ascorbyl fatty acid ester has not been explored. Palm oil is a major commodity and is abundantly available in Malaysia. It contains a mixture of monounsaturated, polyunsaturated, and saturated fatty acid. The major fatty acids are palmitic acid (43.9%) and oleic acid (38.4%) [6].

In this study palm oil will be used as a source of fatty acids in the transesterification with ascorbic acid using commercial immobilized lipase. Optimal conditions for the enzymatic conversion were determined and the product obtained was further characterized.

## 2. Materials and methods

### 2.1. Enzymes and chemicals

A commercially immobilized lipase was used throughout this work. This enzyme, Novozyme 435, is a preparation of lipase B from *Candida antartica* immobilized on a macrosporous acrylic resin; Lipozyme TLIM was from *Thermomyces lanuginosa* and RMIM was from *Rhizomucor miehi* (Novo Nordisk Industry, Bagsvaerd, Denmark). L-Ascorbic acid (purity >99%), tert-amyl alcohol (TAA), molecular sieve 5 Å, and Kieselgel G 60 were from Merck (Darmstadt, Germany). Palm oil was obtained locally.

### 2.2. Lipase-catalyzed synthesis of palm-based ascorbyl esters

A constant amount of ascorbic acid was mixed with palm oil in a scintillation vial. Molecular sieve type 5 Å (treated by heating at 100 °C for 8 h prior to use) and TAA as an organic medium were added into the reaction system and agitated in a shaker (B. Braun) at 200 rpm and 40 °C for 24 h. The reaction was started by the addition of enzyme. The experiment was prepared in triplicates for each sample and standard deviations were calculated.

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### 2.3. Analysis

#### 2.3.1. Thin-layer chromatography (TLC) analysis

Quantitative analysis of palm-based ascorbyl esters was made using TLC on 60 silica gel plates using a solvent mixture of chloroform:methanol:acetic acid:water at the ratio of 81:9:8:2 (v/v/v/v) as a mobile phase. The product was visualized by spraying with 5% sulfuric acid in ethanol and heating at 110 °C for 30 min, followed by quantitative analysis using a photodensitometer (Shimadzu CS-9301 PC) at 500 nm. Ascorbyl palmitate was used as standard. Product concentration was expressed as gram of product in total volume of the reaction mixture (g/L).

#### 2.3.2. Conversion of ascorbic acid

A series of experiments containing several mixtures of substrates, solvents, molecular sieves, and immobilized lipase using established optimum conditions was conducted to determine ascorbic acid conversion. At the end of the reaction, ascorbic acid content was determined with the use of ascorbic acid sensors (Merck, Darmdstat, Germany). Ascorbic acid conversion was calculated from the decrease in ascorbic acid after completion of reaction compared with that in the control (without enzyme).

#### 2.3.3. High-performance liquid chromatography (HPLC)

The HPLC analysis of palm-based ascorbyl esters was done based on the method reported by Kuwabara et al. [1] with slight modification. The equipment used was Shidmadzu 10A-VP, on an Ascentic C18 column (5 μm, 150 mm × 4.6 mm) with methanol/water/phosphoric acid (95/5/0.1, v/v/v) as a mobile phase at a flow rate of 1 mL/min. Detection was achieved at a wavelength of 245 nm using ascorbyl palmitate as standard.

#### 2.3.4. Structural analysis

For structural analysis, the product was isolated from TLC silica gel plates ($R_f$ value = 0.5–0.6). Silica gel containing the product was eluted using TAA, filtered, and vacuum-dried. The isolated product was finally identified by spectral studies. IR spectra were recorded using PerkinElmer Spectrum 2000. The mass spectrometry data were obtained on Agilent Tech 6890N GC with Agilent test 5973 innert MSD (USA). The $^{13}C$ NMR spectra were recorded on a Variant Unity Inova (USA) at 500 MHz.

#### 2.3.5. Antioxidant effect (DPPH radical)

The antioxidant effect of the synthesized palm-based ascorbyl esters was evaluated using the method reported by Hou et al. [7] with slight modification. The assay mixture contained 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical solution, ethanol, and the test sample. Scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at wavelength of 517 nm. The antioxidant activity of the test compounds was expressed as $IC_{50,}$ which was defined as the concentration of test compounds required to inhibit the formation of DPPH radicals by 50%.

## 3. Results and discussion

### 3.1. Mechanism for palm-based ascorbyl esters synthesis

The mechanism for the synthesis of palm-based ascorbyl esters in these studies is illustrated in Fig. 1. Triglyceride molecules in palm oil were first hydrolyzed to free fatty acids by the immobilized lipase Novozyme 435 followed by transesterification of the liberated free fatty acids with ascorbic acid in the presence of organic solvent and molecular sieve to produce palm-based ascorbyl esters.

### 3.2. Selection of the best biocatalyst

Three types of immobilized lipase were screened for their ability to synthesize palm-based ascorbyl esters. The activities of the enzyme were evaluated on the basis of product intensity as determined by TLC analysis. The order of activities was as follows:

Novozyme435 > Lipozyme TLIM > Lipozyme RMIM

Novozyme 435 was the most effective enzyme due to its thermostable character; it was particularly useful in the synthesis of esters and amides. It also has a broad substrate specificity to promote a reaction between a wide range of primary and secondary alcohols and carboxylic acids [8]. Therefore, it was used in the subsequent experiments.

### 3.3. Influence of molecular sieve

In the transesterification reaction, the water content of the medium not only affects the rate of reaction but also the equilibrium position [2]. An excess amount of water may slow down the reaction rate [9] and favor hydrolysis [10]. It can be controlled by continuously adding a water adsorbent such as diatomaceous earth, silica, and molecular sieve [11]. The use of molecular sieve in the transesterification reaction not only dried the reaction mixture but also shifted the equilibrium condition of the synthesis

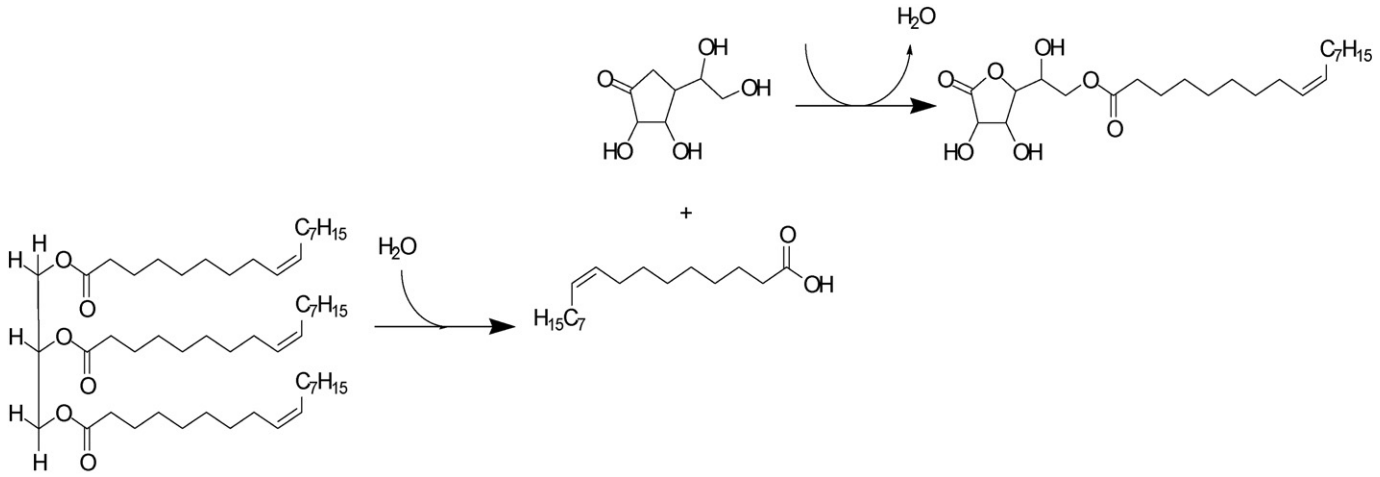


**Fig. 1.** Lipase-catalyzed transesterification of ascorbic acid and palm oil using immobilized Novozyme 435.

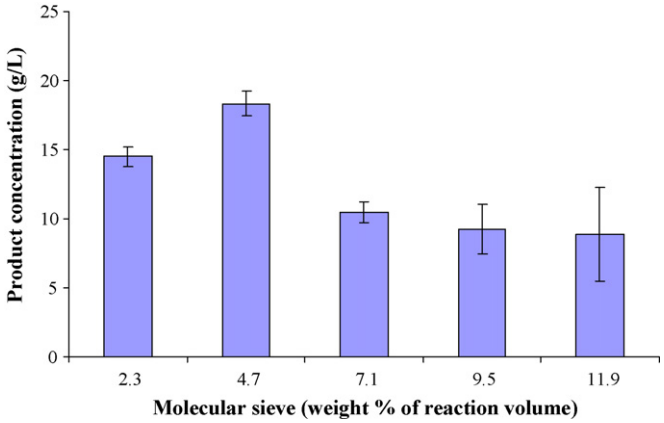


**Fig. 2.** Effect of molecular sieve on the synthesis of palm-based ascorbyl esters. The reaction was carried out with 0.15 mmol L-ascorbic acid and 1.5 mmol palm oil in 4 mL tert-amyl alcohol and 40 mg immobilized lipase at 40 °C for 24 h. Vertical bars represent standard deviation.

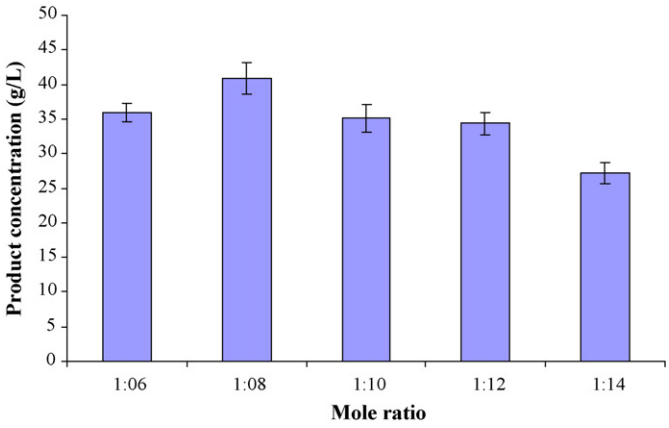


**Fig. 4.** Effect of the initial mole ratio of ascorbic acid to palm oil on the synthesis of palm-based ascorbyl esters. The reaction was carried out at 40 °C, 40 mg immobilized lipase, 4.7% (weight % of reaction volume) molecular sieve in 0.5 mL tert-amyl alcohol. Vertical bars represent standard deviation.

by adsorbing the water formed [12]. In this study, molecular sieve 5 Å was selected as recommended by the manufacturer to remove excess amount of water produced in the reaction [13]. To estimate the load of molecular sieve required for the reaction, a series of experiments containing various amounts of molecular sieve ranging from 20 to 100 mg (approximately 2–12% weight % of reaction volume) were conducted. At the end of the reaction, the product was determined by TLC analysis using ascorbyl palmitate as standard. As shown in Fig. 2, the maximum product concentration (20 g/L) was obtained at 40 mg molecular sieve, approximately 5% (weight % of reaction volume). A similar finding was reported by Song and Wei [2] when using 4 Å molecular sieve in the synthesis of vitamin C ester using immobilized lipase from *Candida* sp. More than 5% (weight % of reaction volume) molecular sieve may strip essential water from enzyme molecules that cause enzyme deactivation.

### 3.4. Amount of organic solvent

Organic solvent strongly influenced transesterification, which is in accordance with literature [3]. As reported earlier, TAA is the most suitable solvent for synthesis of ascorbyl esters because it is able to dissolve the reactants at high concentrations and because it does not deactivate the enzyme [14]. In this experiment, the amount of TAA used was varied from 25 to 95% (weight % of reaction volume). Fig. 3 shows that at 63% (weight % of reaction volume) TAA,

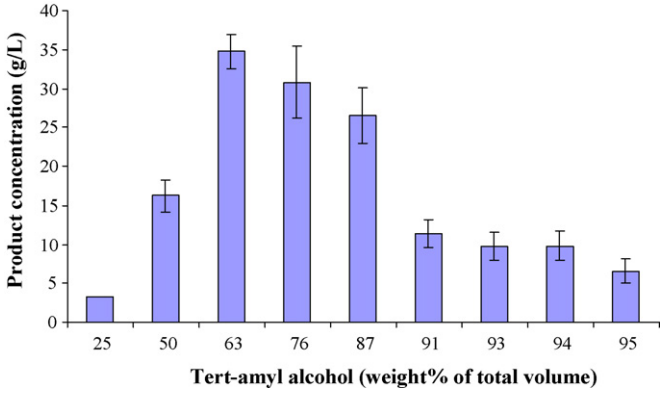


**Fig. 3.** Effect of tert-amyl alcohol (TAA) on the synthesis of palm-based ascorbyl esters. The reaction mixture contained 1.5 mmol ascorbic acid, 15 mmol palm oil, 40 mg immobilized lipase and 4 mL tert-amyl alcohol. Vertical bars represent standard deviation.

product formation was increased by approximately 75% (from 20 to 35 g/L). The higher solubility of the substrates at these conditions may contribute to a high rate of synthesis of palm-based ascorbyl esters. At more than 63% (weight % of reaction volume) solvent, a decrease in production of palm-based ascorbyl esters was observed.

### 3.5. Mole ratio

In the esterification reaction, a further tool to increase production is the use of one substrate in excess [3]. Mole ratios of 1:1.5 ascorbic acid:oleic acid [2]; 1:4 ascorbic acid:oleic acid [3]; 1:2 ascorbic acid:palmitic acid, [14]; and 1:6 ascorbic acid:palmitic acid) [15] were reported for synthesis of ascorbyl esters. In our study, the mole ratio of the substrates varied from 1:6 to 1:14. No product was detected when a mole ratio less than 1:6 was used (results not shown). The relationship between the product concentration obtained during transesterification and the mole ratio of substrates used is shown in Fig. 4. The highest product formation (40 g/L) was determined at a mole ratio of 1:8. However, for mole ratio value of palm oil more than 8, reduction in production of palm-based ascorbyl esters was observed. Excess palm oil concentration in the organic phase may induce conformational changes that limit the access of the hydrophilic ascorbic acid to the catalytic site of the enzyme. Humeau et al. [5] reported reduction in ascorbyl palmitate production in transesterification of ascorbic acid with methyl palmitate using lipase B from *C. antartica* at a mole ratio of methyl palmitate more than 7.

### 3.6. Enzyme loading

Higher enzyme loading can improve the lipase catalyzed, esterification, acidolysis, as well as acyl group migration [16,17]. In this experiment, enzyme load was varied from 3 to 15% (weight % of substrates). Maximum production (45 g/L) was achieved at 12% (weight % of substrate) of enzyme loading (Fig. 5). Zhao et al. [18] reported maximum acidolysis of lard with capric acid in organic solvent at 15% (weight % of substrates) enzyme loading using immobilized lipase TLIM from *Thermomyces lanuginose.*

### 3.7. Reaction time

To determine the shortest time possible to achieve the highest production, the time course of reaction was monitored and the results are shown in Fig. 6. Free fatty acids content in the reaction

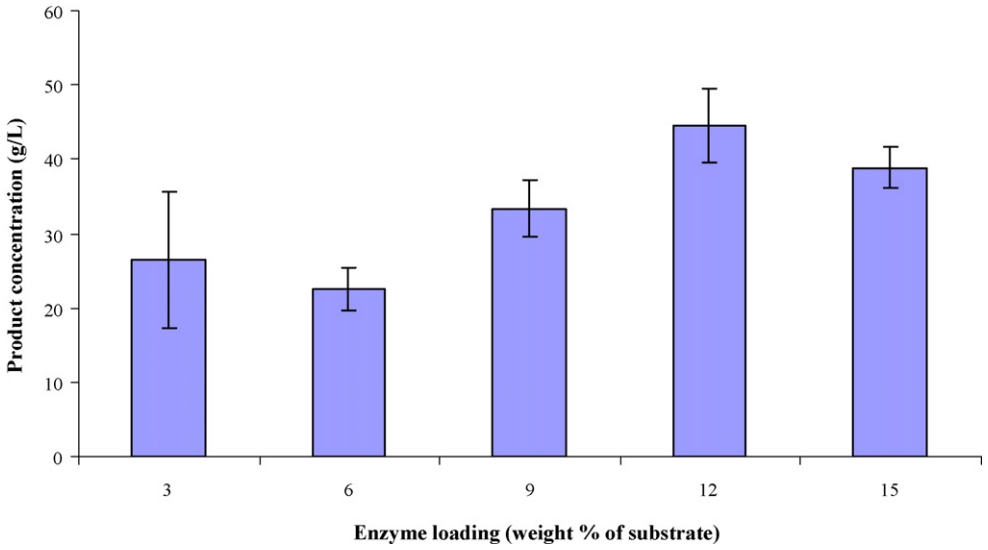


**Fig. 5.** Effect of enzyme load on the synthesis of palm-based ascorbyl esters. (The reaction mixture composed of 1.5 mmol ascorbic acid, 12 mmol palm oil, 40 mg molecular sieve and 0.5 mL tert-amyl alcohol. The experiment was conducted at 40 °C for 18 h.)

medium was determined by titration with 0.5 mM NaOH using phenolphthalein as indicator. The result shows that the initial free fatty acid content (FFA) in palm oil was 15%. Hydrolysis of palm oil by lipase increased FFA in the reaction system to approximately 45% and reached equilibrium after 2 h. Formation of palm-based ascorbyl esters was initiated by lipase catalyzed transesterification of the liberated free fatty acids with ascorbic acid. Maximum production (61 g/L) was obtained after 16 h and decreased gradually after 18 h which may attribute to enzyme inhibition by the excess free fatty acids. Ascorbic acid content before and after reaction was determined as described in Section 2. The conversion of up to 70–75% was achieved under these conditions. In comparison, low product formation was reported in the synthesis of ascorbyl ester using fatty acids such as oleic acid (20 g/L) [2] or palmitic acid (15–19 g/L) [5] may be due to hydrolysis reaction promoted by the excess water in the reaction system.

### 3.8. Structural analysis

At the end of the reaction, after filtration, the reaction mixture was evaporated under reduced pressure. The product was analyzed by TLC as described in Section 2 except using mobile phase consisting of petroleum ether:diethyl ether:acetic acid at a ratio of 105:45:0.2 (v/v/v) and it was found that it contains triglycerides (10.5%), free fatty acid (41%), diglycerides (37%), monoglycerides (7%), palm-based ascorbyl esters (4.5%). The presence of palm-based ascorbyl esters in the reaction system was detected by HPLC using ascorbyl palmitate as standard. Characterization of purified product isolated from silica gel chromatography was carried out employing FTIR, $^{13}$C NMR, and GC–MS. From GC–MS and $^{13}$C NMR analyses, the pure product obtained was a mixture of monoester of ascorbic acid consisting of oleic (61%), palmitic (30%), and stearic acids (9%) with molecular ion at $m/z$ = 256, 265, and 284, respectively. The ester bond was detected at IR (CO–O ester) = 1731.84 cm$^{-1}$. $^{13}$C NMR (500 MHz, CDCL3) δ 179.54 (C-2, C-1′, C-1‴), 130.11 (C-4,C-5), 130.06 (C-9″,C-10″), 72.29 (C-3), 72.19 (C-6), 61.54 (C-7), 34.8 (C-8″, C-2‴), 34.34 (C-2′, C-14′, C-3″), 32.34 (C-7″, C-12″), 27.42 (C-6″), 27.38 (C-6′, C-12′), 25.08 (C-5′, C-4″), 24.02 (C-4′, C-4‴), 23.61(C-3′, C-3‴), 22.78 (C-15′, C- 17″), 14.2 (C-16′, C-18″).

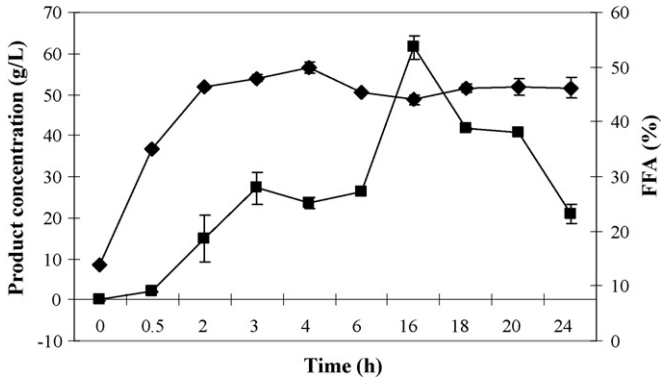


**Fig. 6.** Effect of reaction time on production of palm-based ascorbyl esters and free fatty acids content. (The reaction mixture contained 1.5 mmol ascorbic acid, 12 mmol palm oil, 40 mg immobilized lipase and 0.5 mL tert-amyl alcohol.) Vertical bars represent standard deviation.

### 3.9. Antioxidant effect

The antioxidant activities of ascorbic acid, its derivatives, and palm oil by using the DPPH method were summarized in Table 1. Palm-based ascorbyl esters exhibited an $IC_{50}$ value of 0.1 mg/mL. This value is 10 times higher than ascorbyl palmitate and may be due to the presence of mixture of ascorbyl monoesters. Nevertheless, the results show that the transesterification of ascorbic acid with palm oil using immobilized lipase produce ascorbic acid derivatives with good free radical scavenging activity.

**Table 1**
Antioxidant activities of ascorbic acid, its derivatives and palm oil.

| Sample | $IC_{50}$ (mg/mL) |
|---|---|
| Ascorbic acid | 0.0022 |
| Ascorbyl palmitate | 0.012 |
| Palm-based ascorbyl esters | 0.1 |
| Palm oil | 22.6 |

The effect of DPPH radical will be evaluated by the method by Hou et al. [7] with a slight modification. The assay mixture contained DPPH radical solution, 99% ethanol, and sample. The solution was rapidly mixed and scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. $IC_{50}$ = concentration of compound that required for inhibition of formation of DPPH radicals by 50%.

## 4. Conclusions

Synthesis of palm-based ascorbyl esters was strongly influenced by type of immobilized lipase used, percentage of organic solvent, mole ratio, reaction time, enzyme, and molecular sieve loading. Transesterification of ascorbic acid with palm oil using immobilized lipase produced a mixture of ascorbyl monoesters consisting of ascorbyl monooleate (61%), ascorbyl monopalmitate (30%) and ascorbyl monostearate (9%) with potential antioxidant activities.

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## References

[1] K. Kuwabara, Y. Watanabe, S. Adachi, K. Nakanishi, R. Matsuno, J. Biochem. Eng. 16 (2003) 17–22.
[2] Q.X. Song, D.Z. Wei, J. Mol. Catal. B: Enzym. 18 (2002) 261–266.
[3] M. Adamczak, U.T. Bornscheuer, W. Bednarski, Process Biochem. 40 (2005) 3177–3180.
[4] F.X. Malcata, H.R. Reyes, H.S. Garcia, C.G. Hill Jr., C.H. Amudson, J. Am. Oil Chem. Soc. 67 (1990) 890–910.
[5] C. Humeau, M. Girardin, B. Rovel, A. Miclo, J. Mol. Catal. B: Enzym. 5 (1998) 19–23.
[6] Tomarelli RM. Randomized palm oil fat composition for infant formula, US Patent No. 5000975 (1991) 1–18.
[7] W.C. Hou, R.D. Lin, K.T. Cheng, Y.T. Hung, C.H. Cho, C.H. Chen, S.Y. Hwang, M.H. Lee, Phytomedicine 10 (2003) 170–175.
[8] Novozyme 435, Product Sheet Oils and Fats, 2001, pp. 1–3.
[9] N. Fontes, J. Partridge, P.J. Halling, S. Barrieiros, Biotechnol. Bioeng. 77 (2002) 296–305.
[10] F. Cho, J.M. Deman, O.B. Allen, ELAEIS 6 (1993) 25–38.
[11] L.H. Posorke, G.K. Lefebvre, C.A. Miller, T.T. Hansen, B.L. Glenvig, J. Am. Oil Chem. Soc. 65 (1988) 922–926.
[12] T. Maugard, J. Tudella, M.D. Legoy, Biotechnol. Prog. 16 (2000) 358–362.
[13] Drying Agent, Merck KGaA, Damstadt, Germany.
[14] F. Viklund, J. Alander, K. Hult, J. Am. Oil Chem. Soc. 80 (2003) 20–25.
[15] H.J. Hsieh, G.R. Nair, W.T. Wu, J. Agric. Food Chem. 54 (2006) 5777–5781.
[16] X. Xuebing, Int. News Fats Oils Related Mater. 11 (2000) 1121–1129.
[17] X. Xuebing, Eur. J. Lipid Sci. Technol. 102 (2000) 287–303.
[18] H. Zhao, Z. Lu, X. Bie, F. Lu, Z. Liu, J. Food Eng. 78 (2007) 41–46.