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Towards a commercially potential process: Enzymatic recovery of phytosterols from plant oil deodoriser distillates mixture

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ABSTRACT

In order to examine the industrial potential to indirectly isolate phytosterols from deodoriser distillates (DODs), enzymatic transesterification of an industrial rapeseed and soybean oil DOD mixture with bioethanol was investigated using commercial lipases and a few newly immobilised preparations of lipases. The lipases from different sources and differing preparation forms were evaluated, in terms of thermostability, enzyme efficiency, and toleration of ethanol. Lipozyme 435 and Lipozyme NS-40044 TLL were found to be most effective biocatalysts in catalysing ethanolysis of glycerides and steryl esters from DODs. The optimum conditions are 10% enzyme load (wt% of DODs), ethanol/DODs of 3.0:1.0 (mol/mol), water content 0.125% (based on the weight of total mixture), and reaction at 30 °C for 5 h. The results demonstrated that >95% sterols can be recovered as free form (>85% sterol esters were liberated as free sterols within 4 h). With this process, the system was simplified as fatty acid ethyl esters and free sterol as major components, where free sterols can be recovered via solvent extraction or molecular distillation. Furthermore, a reuse study of enzyme in consecutive batch reactions demonstrated an excellent operation stability and reusability of Lipozyme 435 and Lipozyme NS-40044 TLL with the developed process. This work indicated that the industrially refined waste DODs can be directly subjected to an enzymatic process for high efficacy recovery of phytosterol without any pre-process, driven by robust lipase preparations.

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1. Introduction

Oil deodoriser distillates (DODs), a major by-product of deodorisation step of vegetable oil refining process, comprise many valuable bioactive compounds such as phytosterols and tocopherols. The most common DODs are from corn oil, wheat germ oil, rapeseed oil and soybean oil [1–3]. Typically, DODs contain 30–85% free fatty acids, 1–8% tocopherols, 5–30% glycerides, 0–5% sterol esters and 2–15% free sterols [4]. Therefore, DODs can be an important source to obtain phytosterols which might be used as bioactive ingredients in food production. However, the large proportion of fatty acids contained in DODs in both free and esterified forms together with other molecules including phytosterols, makes the isolation process very complicated and affects the recovery yield of phytosterols as well. Thus, developing essential processing technology is imperative.

There are two main methods with respect to DOD process and isolation of phytosterols, namely alkaline saponification and enzymatic transesterification [5]. The alkaline saponification process starts from hydrolysis of phytosterol esters where saponification of DOD is catalysed by NaOH/KOH at 90–120 °C under pressure. The separation of free phytosterol is further preceded by solvent extraction; or through molecular distillation at high vacuum based on the differences of volatility between phytosterols and other components [5]. The saponification process is generally suffering from some harsh conditions such as high reaction temperature and corrosive base; moreover, the formation of soap from free fatty acids results in another technical issue difficult to handle. To address these technical disadvantages, developing novel chemical processes is also in progress, such that Yang et al. [6] successfully recovered phytosterols from waste residue of soybean oil DODs by using a catalytic decomposition and crystallisation process. Currently, the enzymatic transesterification of DODs has become a promising alternative to traditional saponification method with the technical advantages in less environmental impact, mild reaction condition, etc. With continuous interests and investment in biofuel industry, annual production of ethanol has amounted up to 20 billion gallons, and thus ethanol has become the cheapest solvent after water [7]. Therefore, developing biocatalysis based ethanolysis of DODs for efficient recovery of phytosterols is of special interest that may lead to a totally "greener" approach. Theoretically, ethanolysis of DODs results in the release of steryl esters as free form, and the conversion of free and esterified fatty acids into fatty acid ethyl esters (FAEEs), which represents an important industrial

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product biodiesel [8,9]. The composition of resulting mixture is thus much simplified (only left FAEEs, free sterols, tocopherols, etc.), which makes further separation much easier. As a consequence, many researchers attempted to develop optimum enzymatic reaction system using the proper lipases targeted at the goal of high yield and low cost process. Shimada et al. [10] successfully converted sterol esters in soybean oil DODs to fatty acid steryl esters by lipase catalysed reactions. Watanabe et al. [11] utilised two steps of enzymatic processes, including (1) esterification of sterols with free fatty acid and hydrolysis of acylglycerols and (2) methylation of free fatty acids, to isolate phytosterols and tocopherols from soybean oil DODs. Another method for fractionation of phytosterols reported is based on the extraction with supercritical fluid carbon dioxide [12]. Despite these efforts, to develop an effective and industrially practical approach for separation and purification of phytosterols from industrial wastes is still of interests and challengeable. In this work, we used a rapeseed and soybean oil DOD mixture from a Chinese oil refinery plant as raw material and commercial lipases (including some new immobilised preparations) as biocatalysts to attempt to develop a technically efficient and industrially potential approach.

2. Materials and methods

2.1. Materials

Rapeseed and soybean oil deodoriser distillate (DOD) mixture was obtained from Kerry Oleochemical Industry Co., Ltd. (Shanghai, China). Bioethanol (99.8% purity) was purchased from VERBIO Ethanol Zörbig GmbH & Co. KG (Leipzig, Germany). Campesterol, β-sitosterol, cholesterol, stigmasterol and tocopherol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Oleic acid, ethyl oleate, glycerol monooleate, 1,3-dioleate, triolein and cholesteryl oleate used for determination of standard curves are from Nu-Chek Prep, Inc. (Elysian, MN, USA) and with a minimum purity of 95%. Lipozyme RM IM, Lipozyme TL IM, Lipozyme NS-4004 TLL, and Lipozyme 435 were procured from Novozymes A/S (Bagsvaerd, Denmark). *Pseudomonas cepacia* and *Psuedomonas fluorescen* lipases were obtained from Amano Pharmaceutical Co. Ltd. (Tokyo, Japan). *Candida rugosa* and *Candida cylindraceae* lipases were purchased from Sigma–Aldrich (St. Louis, MO, USA). Immobilised lipase *Candida antarctica* Lipase A, NZL-101 was purchased from Codexis, Inc. (Pasadena, CA, USA). All solvents such as methanol, *n*-propanol, *n*-hexane and acetone were of HPLC grade and obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Chemical composition and physicochemical characteristics of DODs

The composition of DODs including free sterol, steryl ester, tocopherols, free fatty acid, triacylglycerol, diacylglycerol and monoacylglycerol was determined by high performance liquid chromatography (HPLC) analysis according to the method described by Torres et al. [13]. The HPLC analysis was performed on a Hitachi-Merck HPLC system (Darmstadt, Germany) equipped with a silica 60-column conjugating to an Alltech evaporative light scattering detector (ELSD, Deerfield, IL). The ELSD conditions were 2.2 bar, 35 °C and gain 3. The flow rate was 2 ml/min. The column temperature was maintained at 35 °C. The elusion programme of mobile phases follows the previous report of Torres et al. [13]. The concentration of individual component was calculated based on the comparison of corresponding HPLC peak area with its standard curves, and the composition percentage of all components was thus estimated by summarising individual components.

The determination of total unsaponifiable matter of DODs was performed according to the AOCS official method Ca 6b-53 [14]. The DODs were saponified to liberate esterified phytosterols as free forms and hydrolyse glycerides. The saponification was performed for 1 h using 5 ml of 50% KOH, 25 ml of 95% ethanol with about 2.5 g DODs under reflux. The total unsaponified extract was saponified again with 5 ml of 50% KOH for 1 h. The procedure was repeated once more to ensure complete hydrolysis of the ester bonds. The water content of DODs was measured by a Karl–Fischer moisture meter (MKS-1, Kyoto Electronics Co. Ltd., Kyoto, Japan). Colour of the DODs was measured by using a Lovibond tintometer (Model F, The Tintometer Ltd., Salisbury, Wilts, UK) in a 1 in. cell on the Lovibond scale in transmittance mode and expressed as (5R + Y) units.

Fatty acid composition of the DODs was analysed by gas chromatography (GC) after methylation according to the AOAC method (1997) as described elsewhere [15]. The analysis was carried on a Thermofisher Trace GC system, equipped with a flame ionisation detector. Supelco capillary column (Omegawax, 30 m × 0.25 mm) was used. Temperature was programmed from 140 to 240 °C at a rate of 4.0 °C/min. The temperature of both injector and detector was set at 260 °C. Helium was used as carrier gas with a flow rate of 1 ml/min throughout the analysis. Identification of fatty acid methyl esters was based on comparison of retention time of tested samples to methyl ester standards. The determination was done in duplicate.

Table 1
Selected commercial lipases obtained from different sources for the alcoholysis reaction.

Code	Lipase
A	Lipozyme RM IM (immobilised *Rhizomucor miehei* lipase; particle diameter 0.2–0.6 mm)
B	Lipozyme TL IM (immobilised *Thermomyces lanuginosus* lipase; particle diameter 0.3–1.0 mm)
C	CAL-A NZL-101 (immobilised *Candida antarctica* lipase A)
D	Lipozyme NS-40044 TLL (*Thermomyces lanuginosus* lipase)
E	*Pseudomonas cepacia* lipase
F	*Pseudomonas fluorescen* lipase
G	*Candida rugosa* lipase
H	*Candida cylindraceae* lipase
I	Lipozyme 435 (immobilised *Candida antarctica* lipase B; particle diameter 0.3–0.9 mm)

2.3. Ethanolysis reaction

Ethanol other than methanol was selected for enzymatic alcoholysis of DODs in this work, because ethanol is less toxic (permissible for food process) than methanol and it does not deactivate lipases to the extent as methanol [8]. Initially, the effects of different types of commercial lipases on the production of fatty acid ethyl esters were conducted. Then, the effect of temperature, enzyme content, mole ratio of ethanol/DODs, water content and reaction time were intensively investigated, respectively.

Effects of biocatalysts on ethanolysis of the DODs were performed using the lipases from different sources and different immobilisations forms (Table 1.) DODs (5 g) were alcoholysed in a mixture containing with 5% (wt% of DODs) of various lipases at a mole ratio of ethanol to DODs of 1.0:1.0 in the presence of 0.15% water (wt% of total reaction mixture). The mixture was agitated at 200 rpm and 50 °C for 24 h. Thereafter, the reaction was monitored by periodical sample withdrawing and HPLC analysis.

Effect of temperature change was examined over the range from 20 to 80 °C. The mixture of DODs (5 g) was mixed with 5% (wt% of DODs) of the selected lipase (Lipozyme TL IM, Lipozyme NS-40044 TLL or Lipozyme 435) at a mole ratio of ethanol/DODs, 1.0:1.0 with 0.15% water (wt% of total mixture). The mixtures were stirred at 200 rpm at 20, 30, 40, 50, 60, 70 or 80 °C for 24 h. The reaction was monitored by periodical sampling and HPLC analysis.

To obtain optimum enzyme load of selected lipases, 3, 5, 7, 10, 15 and 20% lipases (wt% of DODs) with other identical conditions were examined, respectively. The reaction was conducted with agitation at 200 rpm and 30 °C (optimal temperature selected from the aforementioned experiments) for 24 h. The reaction was monitored by periodical sampling and HPLC analysis.

Effects of mole ratios of ethanol to DODs on transesterification of DODs were examined at the mole ratios of ethanol to DODs (0.5:1.0, 1.0:1.0, 2.0:1.0, 3.0:1.0, 4.0:1.0 and 5.0:1.0). The mole amount of the DODs was estimated by the average molecular weight of the DODs with oleic acid as a representative fatty acid, where the components FFAs, monoglycerides, diglycerides, triglycerides and steryl fatty acid esters containing reactive acyl groups of alcoholysis were taken into consideration for calculation. The mixture was incubated with 10% (wt% of DODs) of lipase in the presence of 0.15% water (wt% of total mixture), with agitation at 200 rpm and temperature 30 °C for 24 h. The reaction was monitored by periodical sampling and HPLC analysis.

The effects of water contents (0, 0.125, 0.15, 0.5, 1, 1.5 and 2%, wt% of total mixture) on enzymatic transesterification of DODs were studied by mixing the DODs (5 g) and ethanol (ethanol to DODs of 3.0:1.0 (mol/mol)) with 10% (wt% of DODs)) enzyme. The reaction was conducted at 30 °C for 24 h with agitation at 200 rpm.

The time course of lipase catalysed transesterification of DODs was conducted at 200 rpm at 30 °C with 5 g of DODs mixed with ethanol (ethanol/DODs, 3 mol:1 mol) in the presence of 0.125% water (wt% of total mixture). The reaction was monitored by sampling at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h for HPLC analysis. The HPLC analysis follows the same method as for the analysis of original DOD sample. The sample (20 μl) withdrawn from the reaction mixture was dissolved in 1 ml solvent mixture of chloroform:methanol (2:1, v/v) and subjected to centrifugation at 12,000 rpm for 5 min prior to injection. The concentration of individual component was calculated based on the comparison of corresponding HPLC peak area with its standard curves, and the composition percentage of all components was thus estimated by summarising individual components.

2.4. Reusability of enzyme

The reuse test of Lipozyme 435 and Lipozyme NS-40044 TLL was performed with consecutive batch reactions with 10% enzyme load (wt% of DODs), ethanol/DODs of 3.0:1.0 (mol/mol), water content 0.125% (wt% of total mixture). The reaction was

Table 2
Chemical composition and some physicochemical characteristics of mixed rapeseed and soybean oil DODs.

Characteristics/component	Value/content[a]
Colour (Lovibond scale)	
Red	23.06 ± 0.30
Yellow	12.00 ± 0.12
Blue	2.50 ± 0.08
Moisture (wt%)	0.06 ± 0.01
Unsaponification matter (wt%)	26.94 ± 0.05
Sterols (wt%)	11.15 ± 0.30
Sterol esters (wt%)	5.35 ± 0.14
Free fatty acid (wt%)	47.64 ± 1.4
Tocopherols (wt%)	1.81 ± 0.06
Triacylglycerol (wt%)	5.32 ± 0.20
Diacylglycerol (wt%)	1.91 ± 0.11
Monoacylglycerol (wt%)	22.75 ± 2.92
Hydrocarbons and unknown unsaponifiable compounds	10.33 ± 0.07

[a] Values are given as means ± SD of three separate determinations.

conducted with agitation at 200 rpm and 30 °C for 5 h. At the end of the reaction, the resulting mixture was immediately sampled for HPLC analysis. The immobilised lipase was recovered by a sequential filtering and washing with *t*-butanol for 2 times; and the resulting enzyme was reused for the next batch reaction under the same conditions. This process was repeated nine times. The remaining activity of enzyme was calculated based on a relative value (%) of the yield of FAEEs in individual cycle to the yield of FAEEs in the first cycle.

2.5. Statistical analysis

The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests [16]. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Chemical composition and physicochemical characteristics of the DODs

Chemical composition and some physicochemical characteristics of rapeseed and soybean oil DOD mixture are shown in Table 2. The DODs are brown in colour, in which the red, yellow and blue values appeared in Lovibond scale were 23.06, 12.00 and 2.50, respectively. The DODs are composed of high content of unsaponified matters (26.94%) but negligible water (0.06%). Khatoon et al. [17] reported that Indian soybean oil DODs from two industry sources were dark in colour and had a high amount of unsaponifiable matter (11.8 and 21.9%). From the analysis results of the DOD mixture, the free sterols and steryl ester were found to be 11.15 and 5.35%, respectively. The total sterol content was higher than that of the previous report of Hirota et al. [18] who found 10.3% of total sterols in soybean oil DODs. Khatoon et al. [17] reported an Indian soybean oil DOD contains 6–10% of phytosterols. The content of tocopherols in this DOD mixture is 1.81%, which is much lower than that of two previous reports for 2 soybean oil DODs that the tocopherol contents are up to 10.4% [18] and 20.1% [13], respectively. Among 26.94% unsaponified matters, apart from phytosterols and tocopherols, up to 10.33% is hydrocarbons and other unknown compounds [17,18] (Table 2). The analysis results also showed that the DOD mixture from rapeseed and soybean oils evaluated in this work contains 47.64% free fatty acids, 5.32% triacylglycerols, 1.91% diacylglycerols and 22.75% monoglycerols. The content of free fatty acids is close to the report of Khatoon et al. [17] who found 22.7 and 49.9% of free fatty acids in two soybean oil DODs. Hirota et al. [18] reported their observations that the free fatty acid, triacylglycerol, diacylglycerol and monoglycerol contents in soybean oil DOD were 30.1, 9.5, 3.5 and 4.1%, respectively.

The analysis of fatty acid composition in the DODs showed that oleic acid is the major unsaturated fatty acid (30.14%), followed by linoleic acid (25.16%) and linolenic acid (4.60%). Palmitic acid represents the most abundant saturated fatty acid (11.44%). Arachidic acid and erucic acid are also detected as minor components (data not shown), indicating their rapeseed oil source. The results suggested the fatty acid profile of rapeseed and soybean oil DOD mixture somehow reflected the fatty acid compositions in original soybean and rapeseed oils.

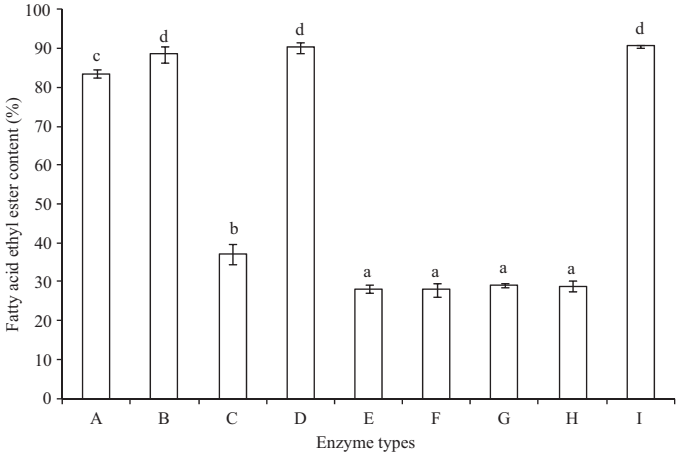

Fig. 1. The contents of fatty acid ethyl esters derived from DODs catalysed by different lipases (A–I). Bars represent the standard deviations from triplicate determinations. Different letters indicate significant differences ($P < 0.05$). DODs (5 g) were mixed with 5% (wt% of DODs) of various lipases at a mole ratio of ethanol to DODs of 1.0:1.0 in the presence of 0.15% water (wt% of total mixture). The reaction was conducted at 200 rpm at 50 °C for 24 h.

3.2. Lipase screening for transesterification of DODs

The performance of lipases in catalysing transesterification of DODs is shown in Fig. 1, which was expressed as the formation of fatty acid ethyl esters in 24 h. In general, all immobilised lipases, namely Lipozyme TL IM, Lipozyme NS-40044 TLL, Lipozyme 435 and Lipozyme RM IM achieved relatively higher yield of FAEEs (Fig. 1). The FAEE yield of Lipozyme RM IM catalysed transesterification is 83.6%. Lipozyme TL IM and NS-40044 are from the same source (*Thermomyces lanuginosus*) but with different carriers, both of which yield good conversion of DODs (>85% FAEEs). Lipozyme 435 obtained a maximum value of FAEE content up to 90.6%; while all free lipases gave considerably lower yield of FAEEs (<30%). The results indicated that immobilised lipases are much more effective in catalysing ethanolysis of DODs, which might be ascribed to efficient interaction of substrates and the enzyme molecules bound on the surface of carrier, as well as preventive effect from carrier for ethanol-induced deactivation of enzyme. Statistical analysis also depicts that Lipozyme TL IM, Lipozyme NS-40044 TLL and Lipozyme 435 are top lipases in catalysing transesterification of DODs, and their efficiencies are significantly higher than the others ($P < 0.05$). Therefore, these three immobilised lipases were selected for further study.

3.3. Effect of temperature on ethanolysis of DODs catalysed by selected lipases

As depicted in Fig. 2, three immobilised lipases display similar profile of activity dependency on temperature, that is, a steep increase of activity from 20 to 30 °C; a declining tendency of enzyme activity from 30 to 60 °C; and a diving drop of enzyme activity when temperature is higher than 70 °C. All three types of lipases achieved optimum yield at 30 °C, however, they also differed in varying enzyme stability range: Lipozyme 435 produced the fatty acid ethyl

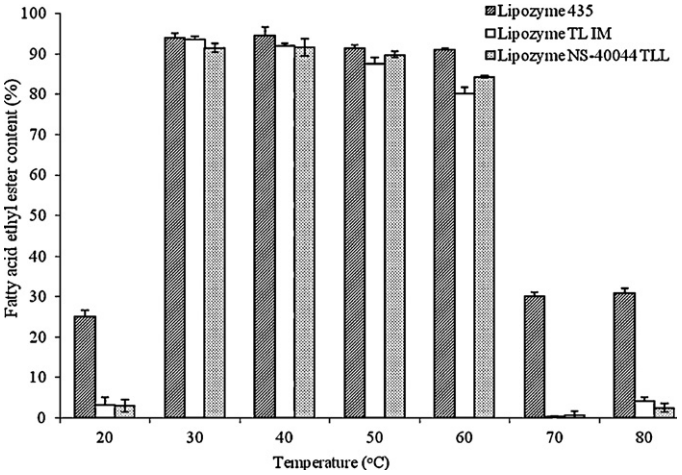

Fig. 2. Effect of temperature on synthesis of fatty acid ethyl ester from DODs catalysed by selected lipases. Bars represent the standard deviations from triplicate determinations. DODs (5 g) were mixed with 5% (wt% of DODs) of selected lipases including Lipozyme TL IM, Lipozyme NS-40044 TLL and Lipozyme 435 at a mole ratio of ethanol to DODs of 1.0:1.0 with 0.15% water (wt% of total mixture). The reactions were conducted at 200 rpm at 20, 30, 40, 50, 60, 70 or 80 °C for 24 h, respectively.

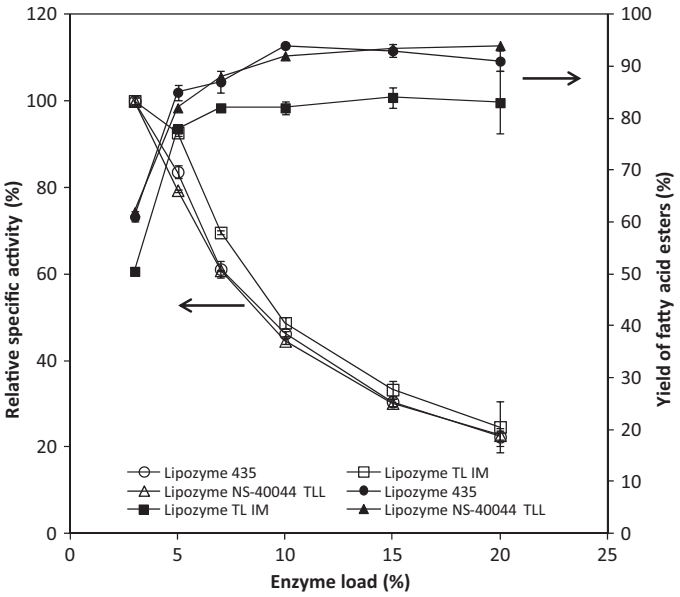

Fig. 3. Effect of enzyme load on the yield of fatty acid ethyl esters (filled symbols) and relative specific activity (empty symbols). The relative specific activity was a relative value of specific activity (yield of fatty acid esters per unit of enzyme load) to the specific activity at 3% enzyme load (as 100%). Bars represent the standard deviations from triplicate determinations. DODs (5 g) were mixed with different contents (3, 5, 7, 10, 15 and 20% (wt% of DODs)) of selected lipases including Lipozyme TL IM, Lipozyme NS-40044 TLL and Lipozyme 435 at a mole ratio of ethanol to DODs of 1.0:1.0 with 0.15% water (wt% of total mixture). The mixtures were stirred at 200 rpm at 30 °C for 24 h.

ester at higher yield at the widest range of temperature from 30 to 60 °C ($P < 0.05$); while Lipozyme NS-40044 TLL and Lipozyme TL IM showed relatively high activity in the ranges of 30–50 °C. Lipozyme 435 and Lipozyme NS-40044 TLL are new preparations from Novozymes; the carriers of Lipozyme 435 (*C. antarctica* Lipase B) and Lipozyme TL IM (lipase from *T. lanuginosus*) are hydrophilic silica, while the carrier of Lipozyme NS-40044 TLL is hydrophobic zeolites. It is not surprising that Lipozyme 435 has broader activity range than Lipozyme TL IM, likely because *C. antarctica* Lipase B could retain high activity at lower water activity than *T. lanuginosus* lipase [19,20], while Lipozyme NS-40044 TLL and Lipozyme TL IM are from the same source, and their activity difference is marginal even though the properties of their carriers are different [21].

Kinetically the increase of temperature leads to an increasing reaction rate by enhancing effective interactions between substrates and biocatalyst intrinsically and mass transfer, which could explain a steep increase of enzyme activity from 20 to 30 °C (indicating a big energy barrier at this range that controls reaction rate). Inspection of the composition of the DODs reveals the major components are FFAs (oleic acid as representative FA), monoglycerides, and sterols (Table 2). Considering that oleic acid has a melting point at 13–14 °C and monooleate 35 °C, it is reasonable to suspect that from 20 to 30 °C the reaction mixture undergoes a phase transition improving mutual dissolution with ethanol, as experimentally observed significant change of the cloudiness of the system from 20 to 30 °C. Thus, it is logical to postulate that a rapid increase of reaction rate in the temperature range of 20–30 °C might be ascribed not only to intrinsic enhancement of kinetic as a function of temperature, but also to increasing mass transfer due to decreasing viscosity of the reaction mixture [22]. However, unlike the optimum temperature at 30 °C in this system, in many other systems of *C. antarctica* Lipase B and *T. lanuginosus* lipase mediated reactions, the optimum temperature is in the range of 50–60 °C [23], which suggested a different kinetic characteristic pertaining to enzymatic ethanolysis of DODs. As observed in other ethanol-mediated reaction systems [8,19], ethanol-induced deactivation of enzyme represents the leading reason why higher temperature is not preferable due to a faster denaturation of lipase protein at enhanced temperature.

3.4. Effect of enzyme load on transesterification of DODs with ethanol

Proper enzyme load is an important operation variable to achieve a faster reaction and better enzyme efficiency. The dependency of FAEE yields on the dosage of three selected lipases is presented in Fig. 3. For all 3 enzymes, an increase in enzyme concentration up to 10% (wt% of DODs) resulted in the increase in fatty acid ethyl ester content. However, with a further increase in enzyme concentration from 10 to 20%, the content of fatty acid ethyl esters remained almost unchanged. This was probably due to the limitation of available substrate in the reaction mixture to saturate catalytic sites of lipases, and a dynamic balance between increasing substrate–biocatalyst interaction and decreasing mass transfer with the increase of enzyme concentration. In terms of efficiency, at 10% of enzyme load, Lipozyme 435 is slightly better than Lipozyme NS-40044 > Lipozyme TL IM; while at 20% enzyme load Lipozyme NS-40044 achieved highest yield of fatty acid ethyl esters.

However, the relative specific activities of all three enzyme preparations decrease significantly as enzyme load increases. Thus, taken an integral consideration of enzyme activity (denoted as fatty acid ester yield) and specific activity, a preferable enzyme load is in the range of 7–10%, and 10% was used for further test.

3.5. Effect of substrate ratio on ethanolysis of DODs

The effects of mole ratios of ethanol to DODs on the production of fatty acid ethyl esters are presented in Fig. 4. As indicated, regardless of enzyme types, the increase in mole ratio of ethanol/DODs from 0.5:1.0 up to 3.0:1.0 led to an increase in fatty acid ethyl ester content. However, further increase in mole ratio (4.0:1.0 and 5.0:1.0) negatively affected the formation of fatty acid ethyl ester (Fig. 4), and the deactivation effect from enhancing concentration of ethanol might be the main reason. This has been intensively discussed elsewhere [9,19]. This result indicated that an appropriate

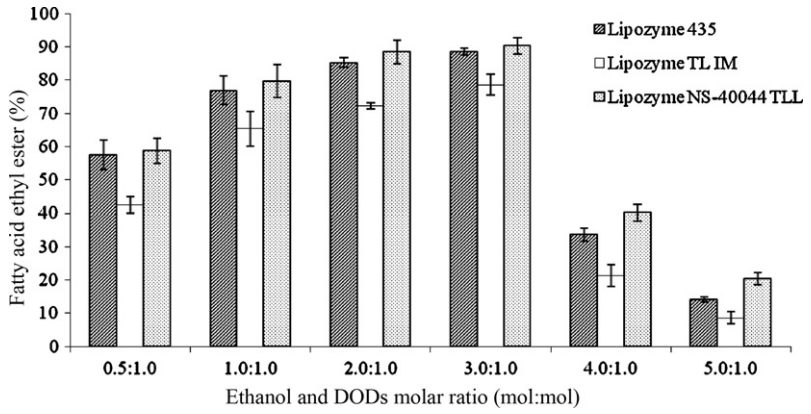

Fig. 4. Effect of mole ratio of ethanol to DODs on synthesis of fatty acid ethyl esters from DODs catalysed by selected lipases. Bars represent the standard deviations from triplicate determinations. DODs (5 g) were mixed with different mole ratios of ethanol to DODs (0.5:1.0, 1.0:1.0, 2.0:1.0, 3.0:1.0, 4.0:1.0 and 5.0:1.0). The mixture was incubated with 10% (wt% of DODs) of Lipozyme TL IM, Lipozyme NS-40044 TLL or Lipozyme 435 in the presence of 0.15% water (wt% of total mixture). The mixtures were stirred at 200 rpm at 30 °C for 24 h.

mole ratio of ethanol to DODs for enzymatic transesterification of DODs catalysed by Lipozyme TL IM, Lipozyme 435 or Lipozyme NS-40044 TLL was 3.0:1.0 (mol/mol). In terms of enzyme efficacy, at the same mole ratio of ethanol to DODs Lipozyme TL IM exhibited the lowest conversion, and Lipozyme NS-40044 TLL achieved a slightly better FAEE yield than Lipozyme 435 but the difference is marginal. Taking all reactive substrates into consideration, the composition of DODs is very complicated and thus it is difficult to reason this in a stoichiometric way.

3.6. Effect of water content on enzymatic ethanolysis of DODs

The water concentration is one of the important parameters that influence the catalytic activity of lipase [24]. The effects of water concentrations on enzymatic ethanolysis of trioleoylglycerols have been studied by Piyatheerawong et al. [19]. In this work, the effect of water content (as percentage of total mixture weight) on the formation of fatty acid ethyl ester from DODs catalysed by 3 different lipases is depicted in Fig. 5. Generally, Lipozyme TL IM showed the inferior efficiency to Lipozyme 435 and Lipozyme NS-40044 TLL at all water contents tested, and similar observations have been obtained in the examinations of the effects of other parameters (Figs. 2–4). Piyatheerawong et al. [19] proposed that most enzymes require a certain amount of water to maintain the conformation of their active sites, which depends greatly on the specific structure of individual enzymes. While according to our results, for these 3 immobilised lipases the yield of FAEE at 0.125% water content is higher compared to the system negligible water content; no significant change of FAEE yield was observed when water content increased to 0.15%. Further increase in water content to 0.5%, the content of fatty acid ethyl ester dramatically decreased (approximately >50%), which is believed the transesterification was reversed by increasing hydrolysis as observed relatively higher concentration of free fatty acids (data not shown). Hence it can be concluded that 0.125–0.15% is an optimal range of water content; and 0.125% water was determined as an appropriate content for ethanolysis of DODs catalysed by Lipozyme IL IM, Lipozyme 435 or Lipozyme NS-40044 TLL.

3.7. Time course of lipase catalysed ethanolysis of DODs

Fig. 6 shows a typical time course of transesterification of DODs catalysed by 3 immobilised lipases. A general trend with the reaction evolution is that the contents of FAEEs and free sterols continuously increase corresponding to the decreases of free fatty acids and steryl esters. All the reactions reach equilibrium at around 4 h; the composition of the reaction mixture remains almost constant after 5 h (Fig. 6). As aforementioned, Lipozyme 435 displays highest activity (FAEE content about 90% at 5 h) (Fig. 6A); while the difference of FAEE yields between Lipozyme TL IM and Lipozyme

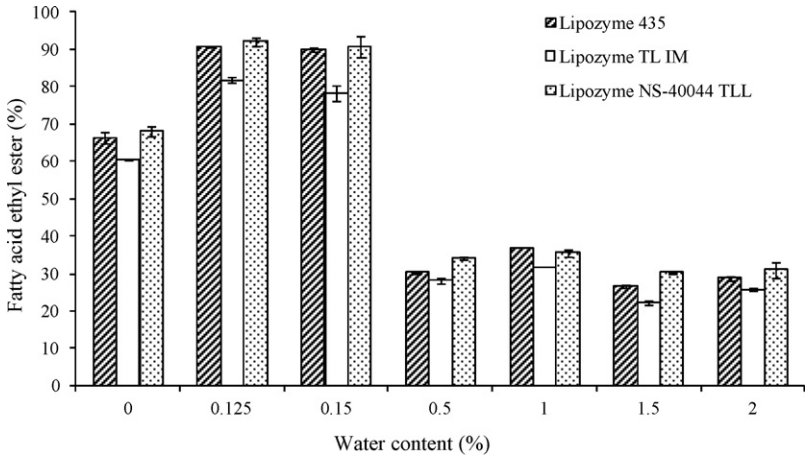

Fig. 5. Effect of water content on synthesis of fatty acid ethyl esters from DODs catalysed by selected lipases. Bars represent the standard deviations from triplicate determinations. DODs (5 g) were mixed with 10% (wt% of DODs)) Lipozyme TL IM, Lipozyme NS-40044 TLL or Lipozyme 435 at a mole ratio of ethanol to DODs of 3.0:1.0 with different water contents ranging from 0, 0.125, 0.15, 0.5, 1, 1.5 or 2% (wt% of total mixture). The mixtures were stirred at 200 rpm at 30 °C for 24 h.

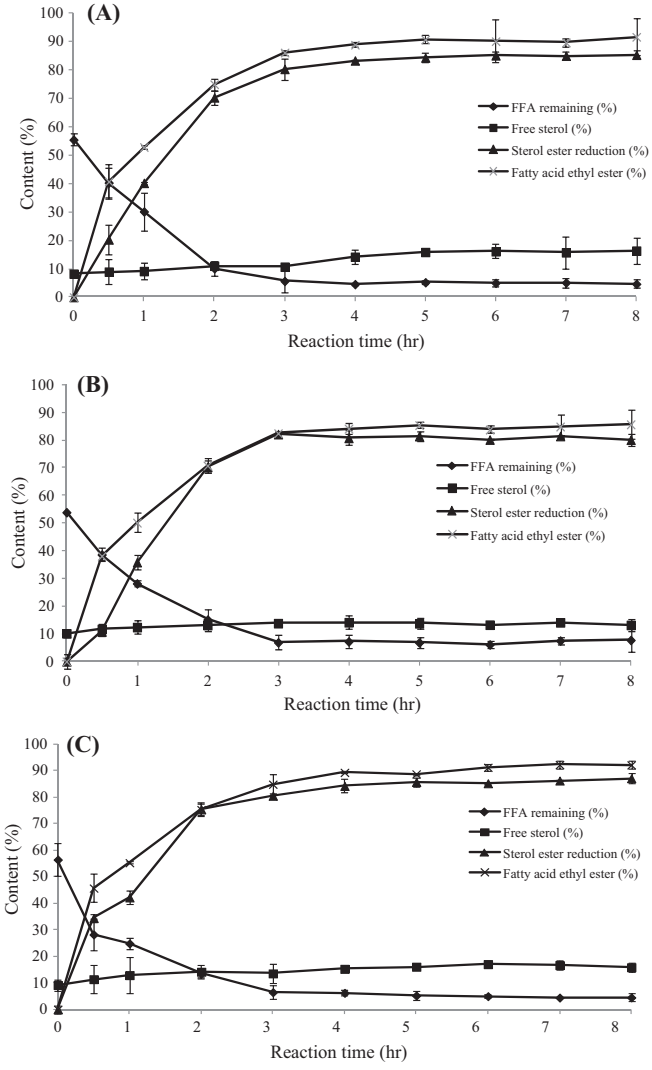

Fig. 6. A typical time course of fatty acid ethyl ester content, free fatty acid remaining content, sterol ester reduction (a decrease in sterol ester content at each interval time compared with the initial content of sterol ester in the reaction mixture) and free sterol content from DODs ethanolised by Lipozyme 435 (A), Lipozyme TL IM (B) and Lipozyme NS-40044 TLL (C). Bars represent the standard deviations from triplicate determinations. DODs (5 g) were mixed with 10% (wt% of DODs) Lipozyme TL IM, Lipozyme NS-40044 TLL or Lipozyme 435 at a molar ratio of ethanol to DODs of 3.0:1.0 with 0.125% water (wt% of total mixture). The mixtures were stirred at 200 rpm at 30 °C for 8 h.

NS 40044 mediated systems is unremarkable (83–88%) (Fig. 6B and C); even though the 2 enzyme preparations differ in carrier property and immobilisation technique. To confirm the function of time on the production of fatty acid ethyl esters, the reactions have been further progressed up to 48 h. It was noted that the reaction time prolonged to 48 h had no significant effect on the formation of fatty acid ethyl esters (data not shown).

As aforementioned, in the DODs sterols occurred as free form at 11.15% (wt%) and as ester form at 5.35% (wt%), we are thus able to estimate the possible recovery of total sterols based on the conversion of sterol esters in Fig. 6. As depicted in Fig. 6A and C, >85% conversion of sterol esters could be achieved, which means the sterol ester will be decreased to <15% of 5.35% (0.8%), that is to say, only <0.8%/(11.15% + 5.35%) = 4.86% of total phytosterols still existed as sterol ester form; or >95% sterol can be recovered as free form through essential separation process, such as short path distillation.

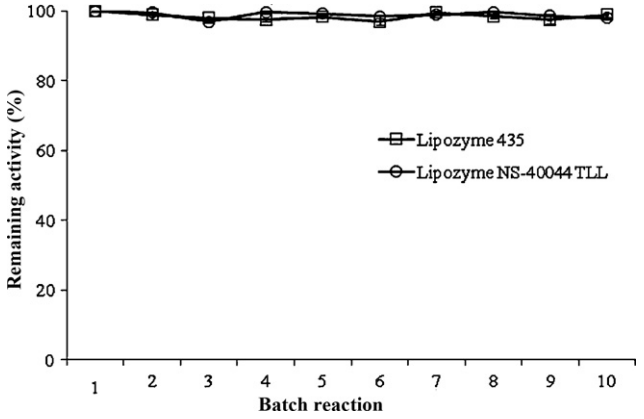

Fig. 7. Reusability of Lipozyme 435 and Lipozyme NS-40044 TLL in consecutive batch reactions. The reaction was conducted 30 °C for 5 h with agitation at 200 rpm; where the enzyme load is 10% (wt% of DODs), ethanol/DODs of 3.0:1.0 (mol/mol), water content 0.125% (wt% of total mixture). At the end of each batch reaction, the immobilised lipase was recovered by sequential operations: filtering and washing with *t*-butanol for two times, prior to being reused for the next batch under the same conditions. This process was repeated nine times.

3.8. Reusability of enzyme

An excellent reusability of enzyme is of special interest from commercial point of view. In this work, Lipozyme 435 and Lipozyme NS-40044 TLL were regenerated by washing with *t*-butanol after each reaction cycle. Insignificant loss of enzymatic activity of both lipases was observed after consecutive reuse for 10 cycles (Fig. 7), which indicated their operation stability in this reaction. Most likely it is because the relatively low operation temperature and protective effect from FFAs and other glycerides [25]; and the regeneration process by *t*-butanol washing may also help in recovery of enzyme activity [25]. Good operation stability and reusability of Lipozyme 435 and Lipozyme NS-40044 TLL in this system indicate the commercial value of the process developed in this work.

4. Conclusion

Enzymatic recovery of phytosterols from rapeseed and soybean oil DOD mixture can be achieved via the ethanolysis of DODs catalysed by Lipozyme 435 or Lipozyme NS-40044 TLL with 10% enzyme load (wt% of DODs) at the mole ratio of ethanol to DODs of 3.0:1.0 in the presence of 0.125% water (wt% of total reaction mixture) at 30 °C for 4 h. After this enzymatic ethanolysis process, >95% phytosterols are expected to be recovered as free form through further essential process, such as short path distillations. A valuable by-product biodiesel can also be produced simultaneously. Furthermore, good operation stability and reusability of Lipozyme 435 and Lipozyme NS-40044 TLL under optimised reaction conditions indicate the commercial value of the process developed in this work.

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