**Original paper**

**Highly Efficient Synthesis of Phytosterol Linolenate Catalyzed by *Candida Rugosa* Lipase through Transesterification**

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In recent years, phytosterols and their fatty acid esters have attracted much attention due to their beneficial effects on human health, while the latter showed excellent advantage in oil solubility. In this study, a novel method was developed for highly efficient synthesis of phytosterol linolenate through lipase-catalyzed transesterification of phytosterols with ethyl linolenate. The effects of lipase, and of solvents with different Log $P$ values on the transesterification of phytosterols were investigated. And the solubility of phytosterols and residual activities of lipase after exposure to different solvents were also considered. Results showed that the conversion was positive correlated with the Log $P$ values of the solvent. *Candida rugosa* lipase and iso-octane was the most suitable biocatalyst and solvent, respectively. The effects of other reaction parameters, including lipase load, reaction temperature, substrate concentration, substrate molar ratio and reaction time on the conversion of phytosterols to phytosterol linolenate were investigated. And the highest yield (95.9 ± 0.8%) of phytosterol linolenate was obtained in short reaction time (2 h) under the selected conditions: 75 mmol/L phytosterols, 1:1.75 molar ratio of phytosterols to ethyl linolenate, 120 g/L 3 Å molecular sieves and 20 g/L *Candida rugosa* lipase in iso-octane, 150 r/min and 40 °C, suggesting that *Candida rugosa* lipase–catalyzed transesterification of phytosterols was an efficient route for phytosterols esters synthesis. The solubility of phytosterols and its linolenate in vegetable oil was also compared and results showed that the oil solubility of phytosterols was significantly improved by transesterification with ethyl linolenate, facilitating the incorporation into a variety of oil- or fat-based foods.

Keywords: phytosterols, transesterification, *Candida rugosa* lipase, ethyl linolenate

**Introduction**

Phytosterols (plant sterols), mainly including beta-sitosterol, stigmasterol, campesterol and brassicasterol, are essential triterpenoid molecules that stabilize phospholipid bilayers of cell membranes in plants (Hamedi *et al.*, 2014; He *et al.* , 2016a). Phytosterols are thoroughly widespread in plants and their related products, such as vegetable oils, nuts, beans, seeds and cereals (Chien *et al.*, 2010). They are generally extracted from the deodorizer distillates produced during vegetable oil refining process or from tall oil, a by-product of the paper pulping industry (Fernandes *et al.*, 2007). Recently phytosterols have attracted much attention due to their beneficial effects on human health since they have cholesterol-lowering ability, and they can also act as anti-cancer, anti-diabetic, and anti-inflammatory agents (He *et al.*, 2014; Miras-Moreno *et al.*, 2016). Abundant studies in vivo proved that phytosterols could reduce both total cholesterol and low density lipoprotein- cholesterol (LDL-C) in animal or humans by competing with dietary and endogenous cholesterol for...
incorporation into bile salt (Cui et al., 2016; Rideout et al., 2014). A daily intake of 1.5 – 3 g phytosterols contributed to reduction in LDL-C levels up to 10% and reduced the risk of coronary heart disease by 20% (Rekha et al., 2016).

However, the unique chemical structure of phytosterols determines that they have high melting point (>130°C) and low solubility in both water and edible oils, which drastically limits the further application of phytosterols in food, medical, cosmetic and other industries. To overcome this problem, the most common and effective solution is to synthesize the corresponding phytosterol fatty acid esters since the esterified forms have much greater solubility in oils and much lower melting point and still maintain all of the excellent properties contained in the original phytosterols (Cui et al., 2016; Tan et al., 2012; Zeng et al., 2015; Hu et al., 2015). On the one hand, phytosterol fatty acid esters can be easily incorporated into a wide variety of diets and fat-based food products. On the other hand, abundant studies in recent years have confirmed that the esterified phytosterols can effectively decrease blood TC and LDL-C levels in a similar manner as the free phytosterols (Hu et al., 2015). Our previous study also found that equimolar phytosterols and phytosteryl laurate could reduce serum TC by 11.5% and 13.2%, respectively, in mice fed a high-fat-high-cholesterol diets (He et al., 2011).

Phytosterol esters of fatty acids can be presently synthesized via chemical esterification and transesterification, but chemical methods often involve some problems such as excessive energy consumption, the formation of side products, browning of products, low selectivity, need for removal of catalyst from the product and so on (He et al., 2010; Hu et al., 2015). However, enzymatic catalysis allowing mild and environment friendly reaction conditions, has high selectivity, fewer side-products and is therefore attractive for the synthesis of esters (Cui et al., 2016). In recent years, several major lipases, including Novozym 435 (He et al., 2010; Wang et al., 2015), Candida rugosa lipase (Zheng et al., 2012) and others (Cui et al., 2016; Pan et al., 2012), have been used for the synthesis of phytosterol esters by direct esterification.

Compared with esterification, transesterification route displayed some advantages (Humeau et al., 1995). The side product water was produced and hardly eliminated in direct esterification, which promoted the hydrolysis reaction. The equilibrium shift towards ester synthesis was limited in spite of the presence of dehydrating reagent and an excess of acyl donor. Even if menthol or ethanol was produced during transesterification, the reaction temperature favored its elimination and then contributed to shift the reaction equilibrium towards ester synthesis. The application of alkyl esters as acyl donors for transesterification offered an effective solution to overcome equilibrium. In a previous study by Coulon et al. (1995), transesterification reaction gave better conversion than direct esterification for lipase-catalyzed synthesis of fructose olate in organic solvent when using immobilized lipase from Candida antarctica as biocatalyst. Humeau et al. (1995) investigated the effect of acyl donor on the conversion for ascorbyl palmitate synthesis using immobilized lipase from Candida antarctica as biocatalyst and found that transesterification route was superior to direct esterification. Pang et al. (2013) established a novel chemoenzymatic route for propyl caffeate synthesis via transesterification or direct esterification and found that the yield of propyl caffeate using transesterification was higher than that using esterification in the presence of Novozym 435. Therefore, the method of lipase-catalyzed transesterification synthesis might be an effective strategy to obtain better yield.

To the best of our knowledge, lipase-catalyzed transesterification synthesis of phytosterol esters of fatty acids has not yet been reported in the literature. In the present study, ethyl linolenate was firstly selected as acyl donor and used for the synthesis of phytosterol esters of fatty acids via transesterification reaction in the presence of Candida rugosa lipase. The objective of the present study was to develop a novel enzymatic route for highly efficient synthesis of phytosterol linolenate via transesterification of phytosterols with ethyl linolenate. The effects of various parameters, including reaction media, lipase load, reaction temperature, substrate concentration, substrate molar ratio and reaction time on the conversion of phytosterols to phytosterol linolenate were investigated and the solubility of the free phytosterols and esterified forms in vegetable oil were also compared.

Materials and Methods

Materials Phytosterols (purity>95%) were generous gifts from Jiangsu Spring Fruit Biological Products Co., Ltd. (Taixing, China). Ethyl linolenate (purity>80%) was purchased from Henan Linuo Biochemical Co., Ltd. (Anyang, China). Candida rugosa lipase (lyophilized powder, Type VII, 700 U/mg) was provided by Sigma-Aldrich China Co., Ltd. (Shanghai, China). Novozym 435 (lipase B from Candida antarctica, immobilized on a macroporous acrylic resin, 10,000 PLU/g) and Lipozyme RM IM (lipase from Rhizomucor miehei, immobilized on an anionic exchange resin, 275 IUN/g) were obtained from Novo Nordisk Co., Ltd. (Shanghai, China). Methanol, iso-propanol and n-hexane used for HPLC analysis were of spectral grade and provided from Tedia Company Inc. (Shanghai, China). Acetone, dimethyl sulfoxide (DMSO), tert-pentanol, tert-butanol, n-hexane, cyclohexane, n-heptane, n-octane, isooctane, ethyl acetate, petroleum ether (bp 60–90°C) and other common reagents used were of analytic grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Lipase-catalyzed transesterification reaction Phytosterols (0.025~1.0 mmol), ethyl linolenate (0.03~1.25 mmol), lipase (25~200 mg), 4 Å molecular sieves (120 mg) and organic solvent (5 mL) were added into a 10 mL screw-capped vial. The vial was placed in a water-bath controlled at 30–60°C and the reaction mixtures were shaken at 150 r/min for 0.5–36 h (Fig. 1). Over the time course of the reactions, a portion of the reaction mixture...
(100 μL) was periodically removed from the reaction for thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). All reactions were performed in duplicate unless otherwise specified. The results were expressed as mean ± standard deviation (SD).

**TLC analysis** The sample (200 μL) removed periodically from the reaction system was dissolved in 2 mL absolute ethanol / n-hexane (1:1, v/v) and then used for TLC analysis. A small quantity of sample solution (10 μL) was evenly pointed on a preliminarily activated TLC plate. Development was then carried out using petroleum ether (60–90°C) / ethyl acetate (19:1, v/v) as developing agent and the TLC plate was located by iodine vapor staining for 10 min. Rf values of different substrates and products were as follows: 0.04–0.13 (phytosterols), 0.65–0.82 (ethyl linolenate) and 0.86–0.93 (phytosterol linolenate).

**Product purification by column chromatography** At the end of the lipase-catalyzed transesterification reaction, the solvent used was removed by rotary evaporation. The solid reaction mixtures were obtained and used for silica-gel column chromatographic purification. The samples were eluted with petroleum ether (60–90°C) / ethyl acetate (19:1, v/v) at the flow rate of 3 mL/min. The eluent was preliminarily detected by TLC analysis. The eluent containing desired product was further analyzed by HPLC analysis. The fractions only containing phytosterol linolenate were collected by rotary evaporation under vacuum.

**Quantitative analysis by HPLC** Aliquot fractions (100 μL) removed periodically from the reaction mixtures were diluted in 2 mL of absolute ethanol / n-hexane (1:1, v/v) for quantitative analysis. The sample (10 μL) was analyzed by Agilent 1100 HPLC using a symmetry-C18 column (5 μm, 4.6 mm × 150 mm, Waters) controlled at 35°C. The sample was eluted with methanol/isopropanol/n-hexane (8:1:1, v/v/v) as mobile phase at the flow rate of 1.0 mL/min. The eluate was monitored with a Schambeck ZAM 4000 evaporative light scattering detector (ELSD) at 50°C and nitrogen as carrier gas at pressure of 0.5 bar. The conversion of phytosterols to phytosterol linolenate were defined as follows:

The conversion of phytosterols to phytosterol linolenate (%) = \[ \frac{\text{peak area of phytosterol linolenate in reaction mixtures} + \text{peak area of phytosterols in reaction mixtures}}{\text{peak area of phytosterol linolenate in reaction mixtures}} \times 100 \]

**Determination of the solubility of phytosterols and its linolenate** The solubility of phytosterol linolenate in soybean oil was studied at 25°C according to previous literature with minor modification (He et al., 2016b). In detail, 0.1 g phytosterols or 2.5 g phytosterol linolenate was added into a 20 mL flask, respectively. The flask was heated with a oil bath equipped with a magnetic stirring apparatus at 25°C. Subsequently the soybean oil was added dropwise until the sample was completely dissolved. Thereafter, the flask was allowed to stand for 2 h under magnetic stirring (200 r/min). The oil volume were promptly adjusted and recorded on the basis of the dissolution. The oil solubility was calculated by the amount of soybean oil to be added and expressed as g/100 mL, 25°C.

**Determination of the residual enzyme activity** To investigate the effect of different solvents on the lipase activity, 0.1 g Candida rugosa lipase were added into vials and treated with 2 mL different solvents in a water-bath controlled at 50°C and shaken at 150 r/min for 24 h. After pretreatment, the solvent were removed by vacuum drying at 50°C. The residual enzyme activity was measured in a reaction system including phytosterols (0.25 mmol), ethyl linolenate (0.31 mmol), Candida rugosa lipase (200 mg), 4 Å molecular sieves (600 mg) and iso-octane (5 mL). The other samples (except lipase) were added into the corresponding vials and then the vials were placed in a water-bath controlled at 50°C and shaken at 150 r/min for 24 h. The residual lipase activity was expressed as the molar conversion of phytosterols to phytosterol linolenate using HPLC.

**Determination of substrate solubility** To determine the effect of the substrate solubility on the conversion, the solubility of phytosterols in various solvents was investigated based on the previous literature with some modification (He et al., 2016a). In brief, an amount of 0.41 g of phytosterols was added into screw-capped vial with 2 mL DMSO, or 5 mL acetone, tert-pentanol, tert-butanol, cyclohexane, n-hexane, n-heptane, n-octane or iso-octane. These solutions were incubated in a water-bath shaker (50°C) at 150 r/min for 2 h. Subsequently, 1 mL of the upper phase was accurately taken out, weighed, recorded and then dried under vacuum to remove the solvent. At the end of drying, the remaining solid sample was weighed and recorded. The substrate solubility was calculated according to the following formula:
The solubility of phytosterols (mmol/L) = \frac{The sample weight at the end of drying under vacuum (g)}{414 (g/mol)} \times 1000

Results and Discussion

Screening of lipases  In this study, three lipases in either powdered or immobilized forms were selected as the potential biocatalyst due to their excellent catalytic activity (Li et al., 2008; Miao et al., 2014; Torres et al., 2008). Novozym 435 and Lipzyme RM IM are both immobilized lipases from Candida antarctica and Rhizomucor miehei, respectively, while Candida rugosa lipase was powdered lipase. The influence of different lipases on the conversion of phytosterols to phytosterol linolenate via transesterification is presented in Fig. 2. As shown in Fig. 2, different lipases displayed different catalytic activities for the transesterification of phytosterols with ethyl linolenate. The free lipase from Candida rugosa displayed high catalytic activity for the transesterification of phytosterols with ethyl linolenate, while the two immobilized lipases were found to have substantially poor efficiency. In detail, the conversion below 20% was obtained when using Novozym 435 (12.3 ± 0.9%) and Lipzyme RM IM (11.4 ± 0.2%) as biocatalyst. Candida rugosa lipase exhibited the highest catalytic activity and the conversion approached 52.1 ± 1.9%. Miao et al. compared the catalytic activity of Candida rugosa lipase, Novozym 435, Lipzyme RM IM and Lipzyme TL IM when used for the synthesis of phytosterol laurate by direct esterification and the results showed that Candida rugosa lipase displayed the highest catalytic activity (Miao et al., 2014). According to Miao et al., the esterification rate can achieve 82.0% using the lipase from Candida rugosa as biocatalyst in n-hexane, whereas the conversion only reach 52.1% using the same lipase. The discrepancy may be attributed to the difference of acyl donor and reaction conditions. Based on the above results, Candida rugosa lipase was selected as the biocatalyst for the transesterification of phytosterols with ethyl linolenate in subsequent experiments.

Effect of solvent  Reaction solvent is one of the most important factors for lipase-catalyzed esterification or transesterification reaction in non-aqueous media. On the one hand, reaction solvent can influence the mass transfer in the reaction system by changing the solubility of the substrate (He et al., 2012). On the other hand, organic solvents have great effects on the enzyme structure, thus affecting the lipase activity and stability (He et al., 2016a; Pan et al., 2012). The Log P value was defined as the logarithm of the partition coefficient of a given compound in the standard two-phase system of octanol/water and mainly used for describing the solvent hydrophobicity (He et al., 2016a; Jia et al., 2010). In general, the higher the Log P was, the stronger the hydrophobicity of solvents.

The effect of reaction solvents on the transesterification of phytosterols with ethyl linolenate was investigated and the Log P values, phytosterols solubility, product conversion and residual activities of Candida rugosa lipase after exposure to different solvents are presented in Table 1. In this study, nine organic solvents with Log P from -1.3 to 4.7 were used for the candidate solvent. DMSO, with Log P value of -1.30, had the highest solubility of phytosterols and the lowest hydrophobicity among all solvents used, whereas no phytosterol linolenate was synthesized in DMSO. As shown in Table 1 the Candida rugosa lipase subjected DMSO showed no residual activity in iso-octane. This may be attributed to the strong polarity of DMSO, which rapidly deprived the necessary water from enzyme molecules thus making the lipase completely deactivated. In one of our previous studies, no goal product was obtained in DMSO using Lipzyme RM IM as biocatalyst for the synthesis of water-soluble plant stanol derivatives (He et al., 2012) Similarly, the conversion below 5% was obtained in acetone. However, Candida rugosa lipase subjected acetone displayed certain residual activity in iso-octane, indicating that the lipase was partially deactivated in acetone. With the increase of Log P value from 0.3 to 4.7, the conversion of phytosterols to phytosterol linolenate gradually increased from 20.6 ± 1.4% to 81.7 ± 6.7%. Although the solubility of phytosterols was the lowest in iso-octane with Log P of 4.7, the highest conversion was obtained. This trend can be explained by the following two reasons. On the one hand, iso-octane had the strongest hydrophobicity, so it couldn’t reduce the enzyme activity and affect the transesterification, which could be supported by the highest residual activity in iso-octane. One the other hand, the product (phytosterol linolenate) had weak polarity, the solvent (iso-octane) had strong hydrophobicity. The goal product showed stronger solubility in hydrophobic solvent, which contributed to shift the reaction equilibrium towards transesterification. As reported in a previous study (Pan et al., 2012), iso-octane was found to be the most suitable solvent for the synthesis of phytosterol esters from oleic acid and phytosterol in the presence of immobilized lipase Candida sp. 99 – 125. Base on the above
analyses, iso-octane was selected as the optimal solvent and used for subsequent experiments.

**Effect of temperature**  Reaction temperature was crucial to lipase-catalyzed reaction in non-aqueous media. On the one hand, the substrate solubility in solvent was influenced by reaction temperature. In general, the higher the temperature, the greater the solubility. On the other hand, the activity, stability and reusability of the lipase were strongly associated with reaction temperature (He et al., 2016a). Too high temperature was unfavorable for the stability and reusability of the lipase. Furthermore, reaction solvent was also easily volatilized at high temperature. The effect of temperature on the conversion of phytosterols to phytosterol linolenate in the lipase-catalyzed reaction was investigated at temperature ranging from 30 to 60 °C, and the results are shown in Fig. 3. As the reaction temperature increased, the conversion of phytosterols to phytosterol linolenate increased. When the temperature reached 40 °C and higher, the conversion began to decrease. Similar trend was found in a recent study by Miao et al., in which Candida rugosa lipase had optimal activity at 45 °C in n-hexane for lipase-catalyzed esterification of phytosterols with lauric acid (Miao et al., 2014). In the present study, the maximum conversion of 94.1 ± 2.1% was achieved at 40 °C in iso-octane for lipase-catalyzed transesterification reaction for 24 h.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Log P</th>
<th>Solubility (mmol/L)</th>
<th>Conversion (% to Phytosterols)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>-1.3</td>
<td>&gt;500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetone</td>
<td>-0.23</td>
<td>&gt;200</td>
<td>3.4 ± 0.5</td>
<td>63.9 ± 2.9</td>
</tr>
<tr>
<td>tert-butanol</td>
<td>0.3</td>
<td>&gt;200</td>
<td>20.6 ± 1.4</td>
<td>73.2 ± 4.9</td>
</tr>
<tr>
<td>tert-pentanol</td>
<td>1.3</td>
<td>&gt;200</td>
<td>25.8 ± 2.6</td>
<td>76.3 ± 7.3</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>3.2</td>
<td>&gt;200</td>
<td>57.4 ± 1.5</td>
<td>81.5 ± 5.1</td>
</tr>
<tr>
<td>n-hexane</td>
<td>3.5</td>
<td>108.2 ± 3.8</td>
<td>54.9 ± 2.0</td>
<td>79.7 ± 1.5</td>
</tr>
<tr>
<td>n-heptane</td>
<td>4</td>
<td>139.8 ± 4.4</td>
<td>54.6 ± 3.6</td>
<td>81.5 ± 6.4</td>
</tr>
<tr>
<td>n-octane</td>
<td>4.5</td>
<td>121.3 ± 7.1</td>
<td>65.8 ± 1.1</td>
<td>83.3 ± 4.7</td>
</tr>
<tr>
<td>Iso-octane</td>
<td>4.7</td>
<td>102.4 ± 1.4</td>
<td>81.7 ± 6.7</td>
<td>85.1 ± 0.3</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effect of temperature on conversion of phytosterols to phytosterol linolenate in lipase-catalyzed reaction. Reaction conditions: 50 mmol/L phytosterols, 1:1.25 molar ratio of phytosterols to ethyl linoleate, 20 g/L Candida rugosa lipase, 0.6 g 4 Å molecular sieves in 5 mL iso-octane, 24 h and 150 r/min.

**Fig. 4.** Effect of enzyme load on conversion of phytosterols to phytosterol linolenate in lipase-catalyzed reaction. Reaction conditions: 50 mmol/L phytosterols, 1:1.25 molar ratio of phytosterols to ethyl linoleate, Candida rugosa lipase, 0.6 g 4 Å molecular sieves in 5 mL iso-octane, 24 h, 150 r/min and 40 °C.

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**Table 1.** Log P values, phytosterols solubility, product conversion and residual activities of Candida rugosa lipase after exposure to different solvents.

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<tr>
<th>Solvent</th>
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<td>&gt;500</td>
<td>0</td>
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</tr>
<tr>
<td>Acetone</td>
<td>-0.23</td>
<td>&gt;200</td>
<td>3.4 ± 0.5</td>
<td>63.9 ± 2.9</td>
</tr>
<tr>
<td>tert-butanol</td>
<td>0.3</td>
<td>&gt;200</td>
<td>20.6 ± 1.4</td>
<td>73.2 ± 4.9</td>
</tr>
<tr>
<td>tert-pentanol</td>
<td>1.3</td>
<td>&gt;200</td>
<td>25.8 ± 2.6</td>
<td>76.3 ± 7.3</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>3.2</td>
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<tr>
<td>Iso-octane</td>
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<td>81.7 ± 6.7</td>
<td>85.1 ± 0.3</td>
</tr>
</tbody>
</table>
Enzyme dosage from 5 to 20 g/L resulted in a significant conversion enhancement, but the conversion slightly increased with a further increase in lipase load. An excellent synthesis method should consider both the conversion of substrate to the desired product and economical interest of the reaction, in other words, using less amount of *Candida rugosa* lipase to obtain satisfactory production of phytosterol linolenate. Using minimal amount of *Candida rugosa* lipase such as 5 g/L would be economically attractive, but the conversion of phytosterols to phytosterol linolenate only reached 40.6 ± 6.4% after 24 h. Increasing of *Candida rugosa* lipase dose lead to higher conversion of phytosterols to phytosterol linolenate. The formation of phytosterol linolenate was much higher with 20 g/L lipase dose and resulted in a conversion of 90.4 ± 5.2%. As the further increase of lipase dose, the conversion of phytosterols to phytosterol linolenate slightly but not significantly increased with a further rise from 20 g/L to 40 g/L, indicating that 20 g/L *Candida rugosa* lipase was enough to make substrate fully transesterified. This trend is similar to the results from a previous study by Zheng *et al.*, in which the highest production of phytosterol esters was obtained when using 15 g/L immobilized *Candida rugosa* lipase as biocatalysts via direct esterification (Zheng *et al.*, 2012). Based on these results, *Candida rugosa* lipase was used for all further experiments at an enzyme load of 20 g/L.

**Effect of substrate concentration** The effect of phytosterol concentration from 5 mmol/L to 200 mmol/L on the conversion of phytosterols to phytosterol linolenate in lipase-catalyzed transesterification reaction was investigated under the same substrate molar ratio. The results are shown in Fig. 5. At fixing substrate molar ratio, the concentration of another substrate, ethyl linolenate also increased with the rise of phytosterol concentration. As shown in Fig. 5, the conversion firstly increased when phytosterol concentration from 5 mmol/L to 25 mmol/L, and attained 92.7 ± 4.0% at 25 mmol/L phytosterols. The conversion of phytosterols to phytosterol linolenate then gradually decreased when phytosterol concentration from 25 mmol/L to 75 mmol/L, and still attained 87.2 ± 1.1% at 75 mmol/L phytosterols. Meanwhile, the product (phytosterol linolenate) concentration increased in a straight line with the increasing of phytosterol concentration from 5 mmol/L to 75 mmol/L. The product concentration still increased with the increase of phytosterol concentration from 75 mmol/L to 125 mmol/L, but the conversion evidently decreased with a further increase in phytosterol concentration. According to our previous study, the lower conversion was observed in lipase-mediated synthesis of water-soluble plant stanol derivatives at higher substrate concentrations (He *et al.*, 2016a), which was in agreement with the present result. This may be attributed to that most of phytosterols were really undissolved in organic solvent, especially at higher concentration. In other words, a large number of phytosterols were added in reaction media, but only the dissolved phytosterols directly participated in transesterification. Excess substrates were strongly absorbed on the enzyme active site and inhibited the lipase activity, which may also account for this phenomenon (Yadav and Dhoot, 2009). Based on these results, phytosterols was used in all further experiments at the concentration of 75 mmol/L.

**Effect of phytosterols to ethyl linolenate molar ratio** The influence of molar ratio of phytosterols to ethyl linolenate on conversion of phytosterols to phytosterol linolenate in lipase-catalyzed reaction was evaluated and the results are presented in Fig. 6. As expected, equimolar ratio of both substrates can appear as ideal in terms of economical cost and further separation for the final products, but such ratio was not advantageous for phytosterol linolenate synthesis. As shown in Fig. 6, the conversion of phytosterols to phytosterol linolenate reached 80.0 ± 2.3% for 24 h under the equimolar of phytosterols to ethyl linolenate. In actual, almost no displacement of the equilibrium...
Lipase-Catalyzed Transesterification Synthesis of Phytosterol Linolenate

Candida rugosa lipase could be effectively used for the synthesis of phytosterol esters in water-in-[Bmim]PF$_6$ microemulsion. And the highest conversion rate could reach 87.9% and 95.1% under the optimized conditions: 305 mmol/L Tween 20, 5.4:1 molar ratio of water to Tween 20 for 24 h and 48 h, respectively. This discrepancy may be attributed to the difference of reaction type, acyl donor and other reaction parameters. In this study, the highest conversion of phytosterols to phytosterol linolenate (>95%) was obtained in 2 h, suggesting that the free Candida rugosa lipase exhibited highly efficient catalytic activity for the transesterification of phytosterols with ethyl linolenate.

**Comparison of the oil solubility** The solubility of phytosterols and its linolenate at 25°C was studied. The solubility of phytosterols in soybean oil was 1.41 ± 0.03 g/100 mL, while the solubility of phytostreols linolenate reached above 25 g/100 mL under the same conditions, which was above 18 times of the solubility of free phytosterols in soybean oil. In a previous study by Deng et al. (2011), the solubility of phytosterols in vegetable oil was increased by 22 times by esterifying with polyunsaturated fatty acids via chemical method, which was in agreement with our results. Our results showed that the coupling of phytosterols and ethyl linolenate by transesterification in the presence of Candida rugosa lipase significantly improved the solubility of phytosterols in vegetable oil, thereby facilitating the incorporation into a variety of oil-based systems.

In the present study, we presented a highly efficient method for phytosterol linolenate synthesis via transesterification. Candida rugosa lipase performed significantly better than Novozym 435 and Lipozyme RM IM in carrying out the transterification of phytosterols and ethyl linolenate. The conversion was positive correlated with the Log $P$ values of the solvent. The highest yield (95.9 ± 0.8%) of phytosterol linolenate was obtained in short reaction time (2 h) under the selected conditions: 75 mmol/L phytosterols, 1:1.75 molar ratio of phytosterols to ethyl linolenate, 120 g/L 3 Å molecular sieves and 20 g/L Candida rugosa lipase in iso-octane, 150 r/min and 40°C. The solubility of phytosterols in vegetable oil was significantly improved by transesterification with ethyl linolenate, thus facilitating the incorporation into a variety of oil- or fat-based foods.

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**References**


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