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Synthesis of trimethylolpropane esters with immobilized lipase from *Candida* sp. 99–125

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ABSTRACT

The lubricants of the future have to be more environmentally adapted, have a higher level of performance. Synthesis esters (SEs) which can be used as raw materials for biodegradable lubricant base oils are increasing in popularity due to superior technical properties. Direct esterification of trimethylolpropane (TMP) with fatty acid in a solvent free system, by immobilized lipase from *Candida* sp. 99–125 was studied. Investigations of important factors were carried out involving temperature, time, enzyme amount, substrates molar ratio and water content. For 2 g caprylic acid, under the optimal conditions, with 0.4 g immobilized lipase, at substrates molar ratio 1:10 (TMP to acid), temperature 40 °C and water content controlled under 0.8% (w/w), the total conversion of fatty acid with TMP reached up to 96% and the formation of trisubstituted TMP esters reached 93%. Water content controlled during esterification process was found to be critical for high yield of direct esterification.

1. Introduction

Since the late 1960s, scientists' attention for lubricants has been turned to biobased raw materials for its excellent physical properties, including biodegradability, renewablility, high lubricity and high flash points, and possibility to achieve tailor-made. Meanwhile, petroleum derived lubricants may soon no longer be available, industries have been searching for a cheap and renewable source of lubricants [1]. Furthermore, the lubricants of the future have to be more environmentally adapted [2–5]. The world annual consumption of lubricants is 40 million tonnes, and is projected to continue to rise 1.6% annually for at least the next 3 years [6]. The pollution problem is so severe that approximately 50% of all lubricants sold worldwide end up released in the environment [3,7]. Due to poor oxidative stability of natural vegetable oil and there is not enough arable land to support the widespread use of vegetable oil based lubricants [1], synthesis esters (SEs) are an attractive alternative to conventional petrobased lubricants.

Traditional methods of producing SEs are alkali-based reactions. A relatively new and promising development in the production of biodegradable lubricant oils and additives is enzymatic esterification with lipase as the catalyst. It has been suggested since the late 1990s [8–10]. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes which normally catalyze the hydrolysis of glycerol esters at lipid/water interfaces. In organic solvent systems, lipases have also been shown to catalyze ester synthesis [11]. Unlike alkali-based reactions, the products of enzyme catalyzed reaction can easily be collected and separated. The particular benefits offered by enzymes are specificity, mild conditions and reduced waste [12,13]. Studies on the synthesis of lubricant oils by chemical or enzymatic transesterification were reported [14]. Uosukainen et al. [10] studied the reaction of alcoholysis of rapeseed oil methyl ester (biodiesel) and TMP. In the chemical synthesis of triesters, the reaction required high temperature (up to 120 °C) with 0.5% alkaline. In enzymatic synthesis, rapeseed oil methyl ester was synthesized chemically. Secondly, transesterification for the synthesis of trimethylolpropane triester of rapeseed oil fatty acids was carried out. Finally, 85% conversion to triester was achieved with 90% total conversion at 60 h. Meanwhile, elimination of the by-product methanol which led to lipase denaturation was found to be the principal factors affecting the product yield. However, studies of direct esterification have not been reported.

Immobilized *Candida* sp. 99–125 lipase has been established in our laboratory and successfully applied in esterification synthesis for fatty acid esters [15–17]. This study focused on synthesis of trisubstituted TMP esters by lipase catalyzed direct esterification (Fig. 1) in a solvent free system to avoid additional transesterification step and release of toxic methanol. Caprylic acid is the common name for the eight-carbon saturated fatty acid (also known as octanoic acid), which can be found in coconut oil. Low temperature performance of caprylic acid ester is better than pamilic acid (16C) TMP ester or other long chain fatty acid TMP esters. Furthermore,

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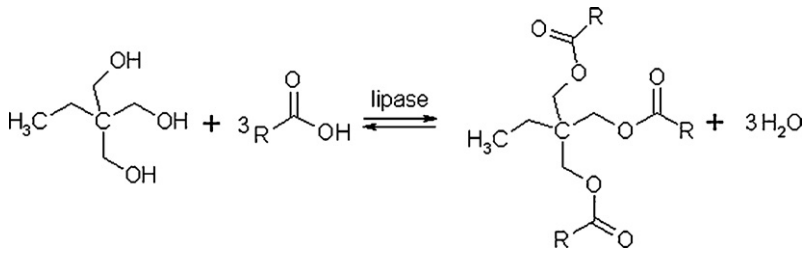

Fig. 1. The reaction scheme of esterification of TMP with fatty acid.

caprylic acid served also as solvent in this reaction because its melting point is as low as 16.8 °C. Due to three-dimensional symmetric structure of hydroxymethyl of TMP, these reactions produce unique mono-, di-, and tri-substituted TMP esters, while TMP was esterified with single fatty acid. The goal of this work was by investigating optimal reaction conditions involving effect of temperature, time, enzyme amount, substrates molar ratio and water content by different pretreatments, in order to achieve the highest trisubstituted TMP esters formation for that the hydroxyl group of mono-, disubstituted TMP esters leads to pour point reversion of lubricant.

2. Materials and methods

2.1. Chemicals

Trimethylolpropane (2-ethyl-2-(hydroxymethyl)-1, 3-propanediol, TMP) with a melting range of 56–59 °C and caprylic acid with a purity of 98% were obtained from Fuchen Chemical Co. Ltd. (Tianjin, China). The other solvents and salts of analytical grade were obtained from Beijing Chemical Factory. They were dried by molecular sieves before usage.

2.2. Lipase immobilization

Lipase (EC.3.1.1.3) was obtained from *Candida* sp. 99–125, its characterisation and catalytic properties was described in previous literatures [15]. Furthermore, the immobilized method has been established in our lab, and its procedure was expatiated in our previous literatures [16,17].

2.3. Enzymatic synthesis of TMP esters

The optimization of reaction conditions except studies of water content took place in a 50 ml shake flask, and was exposed to the air in order to remove water in case of low environmental relative humidity in Beijing. 2.019 g caprylic acid was mixed with TMP corresponding to different substrate molar ratios from 1:4 to 1:11 (TMP to fatty acid). About 25 min after addition of TMP to caprylic acid at 40 °C, a clear, homogeneous solution was observed. Heat up to 60 °C could accelerate the solution of TMP. Subsequently, different amount of lipase were added. The reaction was carried out in an orbital reciprocal shaker at 190 rpm at different temperatures from 30 °C to 50 °C with gradient of 5 °C and for different time periods from 12 h to 72 h.

To investigate effect of water content, 25.263 g acid, 6.906 g TMP, and 5 g immobilized lipase were added in a 100 ml airtight shake at 40 °C. Enzyme preparations and the organic phase of the reaction mixture were adjusted to different water content by pre-equilibration with different pretreatments at 40 °C in separate containers. Equilibration was performed overnight (at least 16 h). Thus, different initial water content of system including 0.071%, 0.105%, 0.273%, 0.335%, and 0.488% (w/w) were obtained.

2.4. Analytical procedure

Samples were obtained at schedular time, and immediately heated to ensure enzyme deactivation. The compounds including fatty acid, mono-, di-, and trisubstituted esters in the reaction mixture were quantified using a GC-2010 gas chromatography (Shimadzu, Japan) equipped with a DB-1ht capillary column (30 m × 0.25 mm × 0.1 μm; J&W Scientific, USA) and a flame ionizing detector (FID). The column temperature was held at 110 °C, then heated to 132 °C at 12 °C/min, and to 180 °C at 30 °C/min and finally to 300 °C at 20 °C/min and then maintained for 5 min. The temperatures of the injector and detector were both set at 300 °C. The retention time of caprylic acid and mono-, di-, and trisubstituted TMP esters (Fig. 2) were 2.19 min and 5.43 min, 8.13 min, and 10.25 min, respectively. The fatty acid conversion was calculated

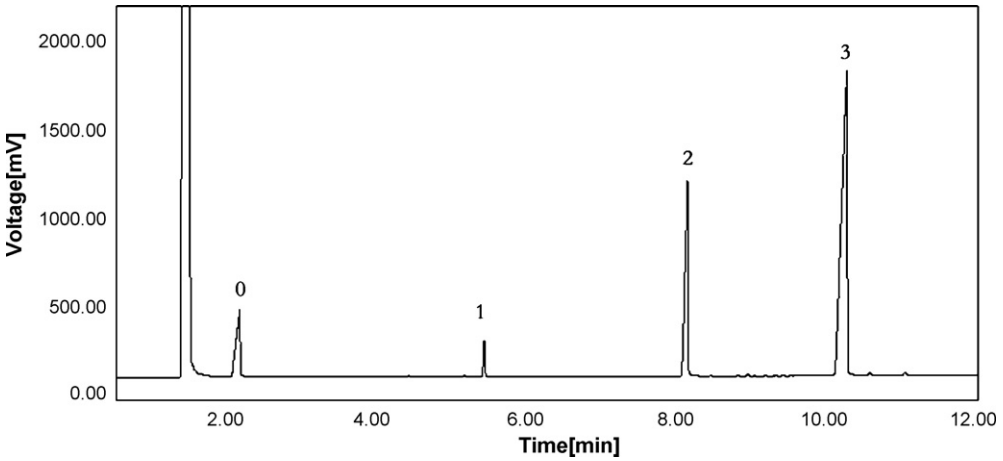

Fig. 2. GC chromatograms of direct esterification enzymatic products. 0 – caprylic acid (2.19 min); 1 – monosubstituted TMP esters (5.43); 2 – disubstituted TMP esters (8.13 min); 3 – trisubstituted TMP esters (10.25 min).

as molar percentage of the maximum theoretic conversion (TMP was completely converted to trisubstituted TMP esters), and the formation of trisubstituted TMP esters was molar percentage of trisubstituted TMP esters to all esters formed.

The compounds were identified by GC–MS. The mass spectrometer (GC-QP2010, Shimadzu, Japan) was operated under electron impact ionization conditions (electron energy 70 eV, source temperature 200 °C, interface temperature 200 °C). Full scan mass spectra were acquired ranging from 50 to and 600 amu, using a scan rate of 2 scans/s from 2 min to 15 min. Trisubstituted TMP esters *m*/*z*: 369, 297, 281, 191, 127, 98, and 57.

Karl-Fischer moisture titrator equipped with a double platinum electrode from Bejing Xianqu Weifeng Technology Development Co. Ltd. was applied to quantify the water content of sample. About 100 mg of sample was analyzed at room temperature with one-component system using monopropellant Karl-Fischer reagent as titrating solution and methanol-dry as the solvent.

3. Results and discussion

3.1. Effect of temperature

In enzymatic catalysis, reaction temperature plays one of the most important roles. Higher temperature enhances mass transfer, and the solubility of TMP, and then accelerates the release of water from the system. However, too high temperature leads to enzyme denaturation. The highest total conversion was observed at 40 °C (Fig. 3), which was consistent with previous literature [15,18]. As the temperature increased from 30 °C to 40 °C, the total conversion increased 14%, and the formation of trisubstituted TMP esters rose about 15%. However, the conversion did not show significant difference when the temperature further increased. The temperature reported [10] was as high as 65–70 °C using Novozym 435 in order to enhance the solubility of TMP. However, the higher conversion was obtained in this study at lower temperature. This result suggested that the impact of temperature on enzyme activity was more important than others such as TMP solubility.

3.2. Effect of enzyme amount

The effect of enzyme amount on synthesis TMP ester was investigated from 0.1 g to 0.5 g (4–20% based on substrates weight). Fig. 4 shows that about 60% of total conversion and 30% of the formation

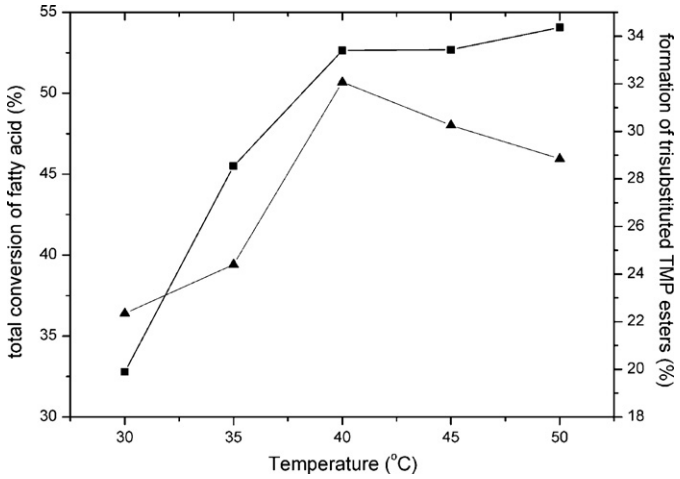

Fig. 3. Effect of temperature on esterification. -▲- the total conversion, -■- the formation of trisubstituted TMP esters, respectively. Reaction conditions: TMP 0.2684 g, fatty acid 2.0189 g, substrates molar ratio 1:7 (TMP to acid), enzyme amount 0.4 g, at 190 rpm for 24 h.

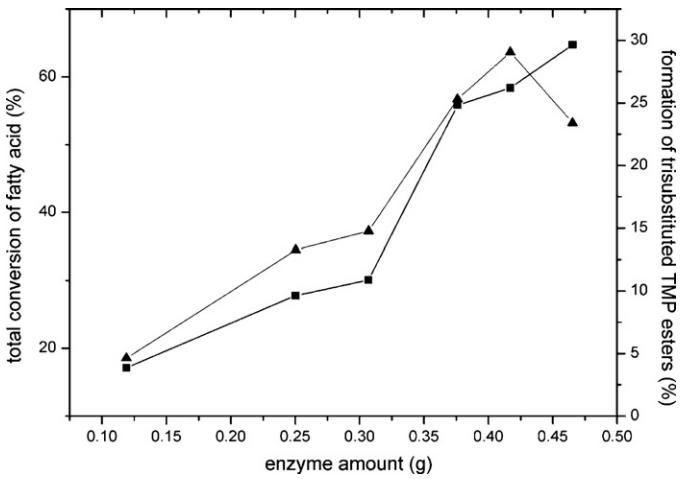

Fig. 4. Effect of enzyme amount on esterification. -▲- the total conversion, -■- the formation of trisubstituted TMP esters, respectively. Reaction conditions: TMP 0.2684 g, fatty acid 2.0189 g, substrates molar ratio 1:7 (TMP to acid), at 40 °C and 190 rpm for 24 h.

of trisubstituted were achieved after 24 h of reaction at enzyme amount of 0.4 g (16% based on substrates weight). The lipase used in this study was immobilized on textile, which had a large surface of about 91 cm^2/g. After immobilization the carrier surface was hydrophobically modified, to avoid adsorption of water. At the enzyme amount greater than 0.4 g, there is a reduction in the total conversion but the formation of trisubstituted TMP esters is still increasing. No side reaction was observed at the enzyme amount greater than 0.4 g. It was assumed that the immobilized enzyme showed surface effect due to the large and hydrophobic area. At the enzyme amount greater than 0.4 g, the surface area of the solution was little smaller than that of the immobilized enzyme. The mono- and disubstituted TMP esters may accumulate on the surface due to their hydrophobicity, which shifted the reaction to trisubstituted TMP ester, and even the total conversion was slightly lower than that at the enzyme amount 0.4 g. Upon increasing the enzyme amount further, reaction conversion did not increase significantly but even decrease slightly. This phenomenon may be explained that the form of immobilized lipase led to the limit of mass transfer and the lack of substrate to access the active site of enzyme.

3.3. Effect of substrates molar ratio

To obtain the highest formation of trisubstituted TMP esters, substrates molar ratio (TMP to acid) from 1:4 to 1:11 was investigated. Fig. 5 shows that there was no significant difference of total conversion. The highest yield of trisubstituted TMP esters was observed with molar ratio (TMP to acid) 1:10. However, when molar ratio (TMP to acid) increased to 1:11, the formation of trisubstituted TMP esters decreased slightly, possibly due to substrate inhibition by excess fatty acid. The fatty acid amount was constant in this study, and the TMP amount varied with substrates molar ratio. Thus, with the lower substrates molar ratio, the relative higher TMP concentration led to the generation of mono- and di-substituted TMP esters (data not shown). Further experiments on the reuse of fatty acid and mono- and di-esters are considered.

3.4. Effect of water content

Water content in the reaction medium is a key parameter in nonaqueous enzymology both for the maintenance of three-dimensional structural integrity and also for optimal catalytic activity of the enzyme. Thus, water content of system was

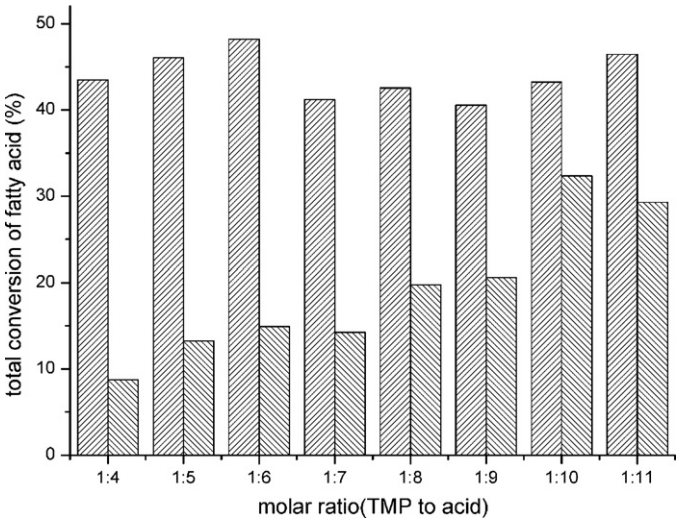

Fig. 5. Effect of substrates molar ratio (TMP to acid) on esterification. The left column represents the total conversion; the right column represents the formation of trisubstituted TMP esters, respectively. Reaction conditions: fatty acid 2.0189 g, enzyme amount 0.4 g, at 40 °C and 190 rpm for 24 h.

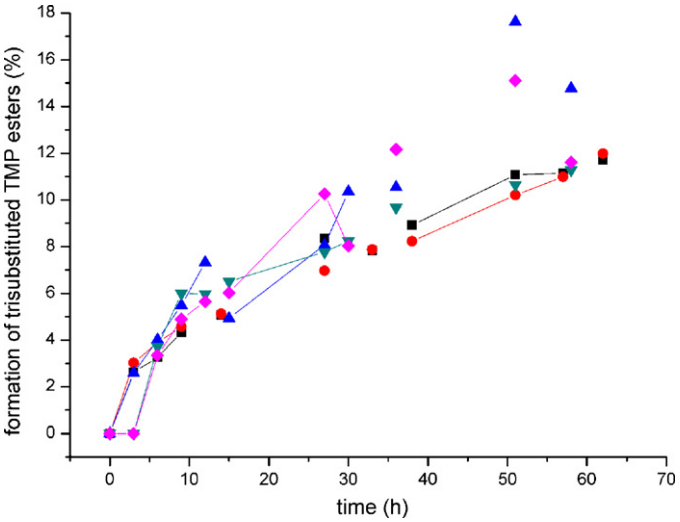

Fig. 7. The formation of trisubstituted TMP esters during esterification process. Reaction conditions: TMP 6.906 g, fatty acid 25.263 g, enzyme amount 5 g, at 40 °C and 190 rpm. Initial water content, -■- 0.071%, -●- 0.105%, -▲- 0.273%, -▼- 0.335%, -♦- 0.488%.

analyzed to evaluate the effect of water in this work. Figs. 6 and 7 show the final total conversion with the initial water content of 0.105%, achieved 67.85%, and was almost 20% higher than the lowest. However, the initial conversion rate showed no significant difference, which is only 5% between the highest and the lowest after 10 h. The final formation of trisubstituted TMP esters was only about 15%. Worthy of noticing that, while the initial water content was above 0.3% (Fig. 9), there was almost no trisubstituted TMP esters generated in the first 4 h. As the water content reached over 0.8% (Fig. 9) after 10 h, the total conversion rate and the formation rate to trisubstituted TMP esters slowed down due to the increased water content. Possibly, the water tolerance limit of this immobilized lipase to form trisubstituted TMP esters was 0.8% (w/w) which needs to be confirmed by more sophisticated experiment design.

Meanwhile, esterification process (Fig. 8) was studied in a shake flask exposed to air in order to control water content at a low level. The bell-type curve of mono- and di-substituted TMP esters showed that trisubstituted TMP esters were generated step by step.

The process of direct esterification is similar to transesterification using sodium methoxide as catalyst [19]. Before 6 h, due to the high TMP concentration, the system mainly generated monosubstituted TMP ester. However, the formation of di- and tri-substituted TMP esters increased slightly. While the concentration of monosubstituted reached higher level, the production rate of disubstituted TMP esters increased, and the highest yield was observed at 20 h. Meanwhile, the formation of trisubstituted TMP esters rose. Finally, the total conversion and the formation of trisubstituted TMP esters was 96% and 93%, respectively. The results of direct esterification were comparable to transesterification [14], however, without release of toxic methanol. Uosukainen et al. [10] screened several lipases to catalyze transesterification of TMP with methanol esters, including Lipozyme IM (sn-1, 3), and Novozym 435 (not specific) etc. Lipozyme IM was able to catalyze completely substituted TMP esters, which could be a result of spontaneous acylmigration [20] catalyzed by the carrier resin or Lipozyme is not 1,3-specific in the

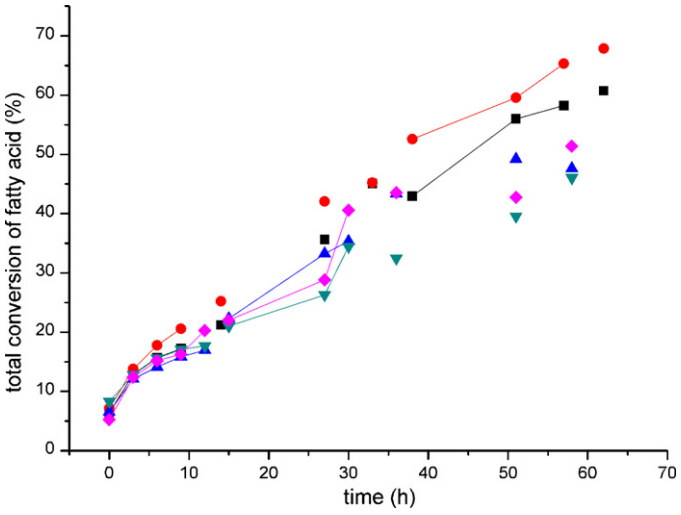

Fig. 6. The tendency of total conversion of fatty acid during esterification process. Reaction conditions: TMP 6.906 g, fatty acid 25.263 g, enzyme amount 5 g, at 40 °C and 190 rpm. Initial water content, -■- 0.071%, -●- 0.105%, -▲- 0.273%, -▼- 0.335%, -♦- 0.488%.

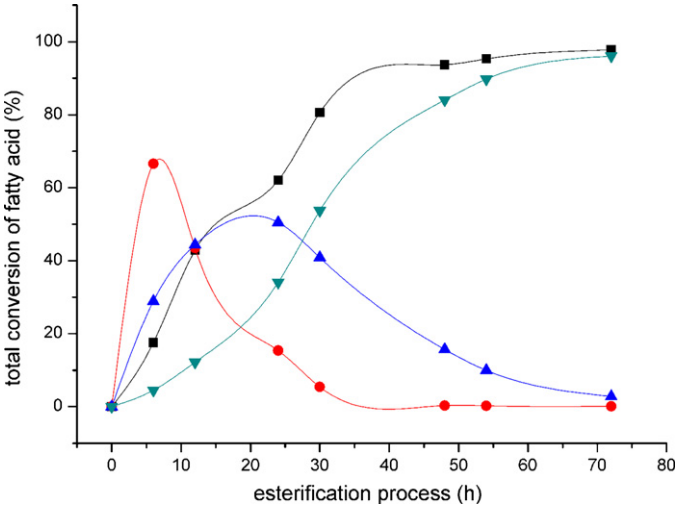

Fig. 8. Esterification process of TMP to acid. -■- the total conversion; -●- the formation of monosubstituted TMP ester, -▲- the formation of disubstituted TMP esters, -▼- the formation of trisubstituted TMP esters, respectively. Reaction conditions: TMP 0.2684 g, fatty acid 2.0189 g, enzyme amount 0.4 g, at 40 °C and 190 rpm for 24 h.

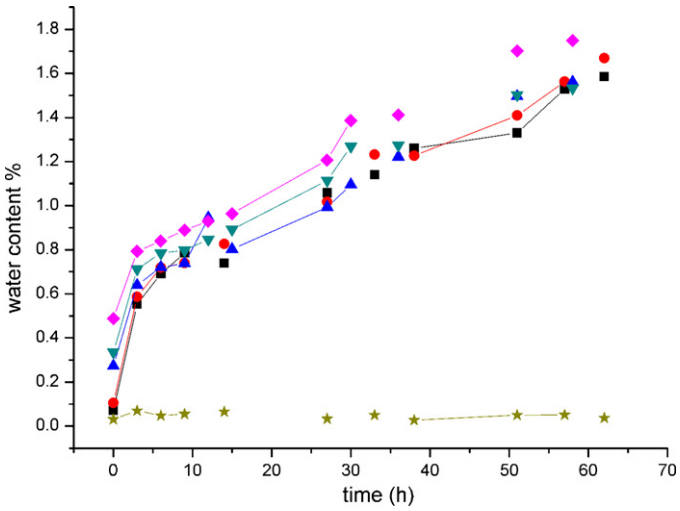

Fig. 9. The water content in the reactant during esterification process. Reaction conditions: TMP 6.906 g, fatty acid 25.263 g, enzyme amount 5 g, at 40 °C and 190 rpm. Initial water content, -■- 0.071%, -●- 0.105%, -▲- 0.273%, -▼- 0.335%, -♦- 0.488%, -★- the process water content of reaction in shake flask exposed to air.

conditions used. However, the same result was observed in this work, which indicated equality of hydroxymethyl of TMP.

The initial water content of investigations in airtight shake flask was in range of about 0.1–0.5% (w/w, Fig. 9). Without any pretreatment, the initial water content was 0.335% due to the hygroscopic capacity of TMP with three hydroxyl groups. Fig. 9 shows the tendency of water content of system during esterification process. With higher initial water content, water content of process kept in a higher level. After about 10 h, water content with different pretreatments all reached 0.6%. After then, the increasing rate slowed down, and finally was in range of 1.4–1.8%. However, compared to airtight shake flask reaction, the process water content of esterification in a shake flask exposed to air kept in lower range of 0.03–0.06%. Figs. 8 and 9 show that the best method to synthesize TMP esters is working in an open air flask, where the water contents is kept under 0.1%. Therefore, water content of system is a key parameter to high yield of trisubstituted TMP esters. In addition, controlled water content during esterification process at a certain level is required. Meanwhile, controlled initial water content is not necessary for direct esterification that is different to transesterification.

4. Conclusion

The lipase from *Candida* sp. 99–125 immobilized on a textile membrane was able to catalyze esterification of TMP with fatty acid. In this study, the immobilized lipase had an optimal temperature at 40 °C. Water content should be controlled under 0.8% (w/w) during esterification process. The optimum of enzyme amount was 16% (w/w) based on the weight of substrates. Substrates molar ratio showed significant impact on formation of trisubstituted TMP esters. Under the optimal reaction condition, the total conversion of fatty acid with TMP reached up to 96% and the formation of trisubstituted TMP esters reached 93%. Water content controlled during esterification process is found to be more important and effective than at the beginning for direct esterification. Direct esterification achieved comparable results to transesterification, however, without the methanol toxicity. The purity of trisubstituted TMP esters was 98% after one-step purification of molecular distillation. Further experiments are on the way to study the interaction between factors using response surface modeling and to control water under a certain level during esterification process.

Acknowledgements

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