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Effect of water on methanolysis of glycerol trioleate catalyzed by immobilized lipase *Candida* sp. 99–125 in organic solvent system

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ABSTRACT

In this study, hydrolysis and methanolysis of glycerol trioleate (TG) by lipase *Candida* sp. 99–125 were investigated under different water conditions. Both the reaction rates were relatively low without water, while increasing water content to 5 wt.% (or more, from 10–20%) based on the TG amount caused remarkable higher TG conversion for both reactions. Moreover, comparing the time course curves of the hydrolysis and methanolysis, it could be concluded that the methanolysis reaction catalyzed by this *Candida* sp. 99–125 appeared to accord with the successive reaction mechanism. TG was first hydrolyzed to partial glycerides and oleic acid (OA), then oleic acid methyl ester (OAME) was produced by esterification of the OA with methanol. This water effect was also confirmed by the experiments that water substitutions such as *t*-butanol and some surfactants added into the system did not get such high yields as that of the water included system. So these results showed that water took part in the methanolysis reaction, and successive hydrolysis–esterification process might be the catalytic mechanism of this lipase.

1. Introduction

Lipases are extensively selected as mild and environment-friendly biocatalysts in hydrolysis, alcoholysis, esterification and transesterification process in organic solvent media [1–4]. To enhance the stability of the lipase, immobilized methods are widely studied for its advantage to reduce the high cost of the catalyst by repeated utilization. Lipase-catalyzed methanolysis of vegetable oils for fatty acid methyl esters (FAMEs, also called biodiesel) synthesis shows great potential as an alternative energy for the reserves shortage and thus many studies are focused on this subject [5,6].

For the lipase-catalyzed biodiesel production in non-aqueous media, water plays multiple roles and it has strong influence on the catalytic activity and stability of the lipase [7,8]. First, water is essential for the integrity of the three-dimensional structure of the protein molecule, and therefore some essential water is needed to keep the enzyme active in organic solvents. Besides, it is well known that water participates in the esterification or hydrolysis thermodynamically reversible reactions and thus water content affects the equilibrium conversion. Moreover, excess water amount might make the lipase more flexible and lead to some unintended side-reactions such as hydrolysis especially in the transesterification process. Consequently optimum water content required to keep the maximum enzymatic activity exists for most lipases, and the amount for a certain reaction depends on the lipase, the immobilized support and the organic solvent employed.

In this study, with self-established immobilized lipase *Candida* sp. 99–125 employed as catalyst, both hydrolysis and methanolysis of glycerol trioleate (TG) under different water contents in *n*-hexane solvent were investigated in detail. Reactions under varied water amounts were performed to obtain the optimum condition and some explanations about the reason why this lipase needed so much water were also discussed.

2. Materials and methods

2.1. Materials

TG was obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Oleic acid methyl ester (OAME) and heptadecanoic acid methyl ester were purchased from Sigma (St. Louis, USA) and were chromatographically pure. Chemical pure Span 60 and Tween 80 were obtained from Shantou Xilong Chemical Factory, Guangzhou, China. Dioctyl sulfosuccinate sodium salt (Aerosol OT or AOT) with 96% content was purchased from Acros Organics, New Jersey, USA. *Candida* sp. 99–125 immobilized on textile membrane was prepared in our laboratory, and the procedure has been described in detail in our earlier literature [9–12]. All the other reagents were obtained commercially and were of analytical grade.

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2.2. Hydrolysis reaction

In advance, the solvent *n*-hexane was dehydrated over molecular sieves 5A. Before initiating the reaction, the immobilized lipase, TO, water and *n*-hexane were preheated in a 40 °C incubator for 30 min. Unless otherwise stated, typical hydrolysis was carried out in a 50 ml stoppered flask, incubated in a reciprocal shaker at 40 °C and 180 rpm for a total reaction time of 12 h. The reaction system contained 1 g TG, 2 ml *n*-hexane, 0.2 g immobilized lipase. Water amount varied from 0% to 20% based on the TG weight and an extra experiment with excess water for equal weight amount to TG was also conducted for comparative study. At pre-determined time, 20 μl sample was taken and centrifuged to obtain the upper layer. Then 5 μl of the upper layer was dissolved in *n*-hexane for gas chromatography analysis.

2.3. Methanolysis reaction

Methanolysis had the similar procedure with hydrolysis reactions except that stepwise methanol was added into the system. The solvent *n*-hexane was dehydrated over molecular sieves 5A in advance, and the immobilized lipase, TO, water and *n*-hexane were preheated in a 40 °C incubator for 30 min before initiating the reaction. Unless otherwise stated, typical methanolysis was carried out in a 50 ml stoppered flask, incubated in a reciprocal shaker at 40 °C and 180 rpm. The reaction system contained 1 g TG, 2 ml *n*-hexane, 0.2 g immobilized lipase, and every 45.3 μl methanol was added to the system at 0, 4 and 8 h, respectively, with a total reaction time of 12 h. Water amount varied from 0% to 20% based on the TG weight and an extra experiment with excess water for equal weight amount to TG was also conducted for comparative study. At pre-determined time, 20 μl sample was taken and centrifuged to obtain the upper layer. Then 5 μl of the upper layer was dissolved in *n*-hexane for gas chromatography analysis. All the experiments were replicated at least three times and the results presented were the mean values for the replicated data.

2.4. Different strategy for water substitution

The optimum water amount required for *Candida* sp. 99–125 lipase to maintain the highest transesterification activity was 20 wt.%, which was quite higher compared with other lipases. However, higher water amount might cause difficulties in the downstream process especially for industrialized scale. Therefore various water substitutes or surfactants were added into the methanolysis system to investigate whether they have the similar effect as water. All these procedures were the same with the methanolysis process above except that water (200 μl) was substituted by 200 μl *t*-butanol, Span 60, Tween 80 or 200 mg AOT, respectively.

2.5. Analytical procedure

The methyl ester and fatty acid contents 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; J&W Scientific, USA) and a flame ionizing detector (FID). The column temperature was held at 100 °C, heated to 180 °C at 15 °C/min, to 230 °C at 10 °C/min and finally to 330 °C at 20 °C/min and then maintained for 5 min. The temperatures of the injector and detector were set at 350 and 360 °C, respectively [11,12]. Heptadecanoic acid methyl ester purchased from Sigma was used as an internal standard.

3. Results and discussion

3.1. Hydrolysis of TG under different water contents

It is well known that the hydrolyzing activity of a lipase is not consistent with its esterification or transesterification activity. So it was worthwhile to determine the hydrolyzing activity of this lipase under different water contents first. And the results of TG hydrolysis at varied water contents were shown in Fig. 1.

It could be concluded from Fig. 1 that oleic acid (OA) content curves vary with different amounts of water. A flat OA content curve was obtained in absence of water, showing that water was an essential substrate in the hydrolysis reaction. Increasing water content to 5 wt.% (or more) based on the TG amount caused remarkable OA enhancement correspondingly and the initial hydrolysis rates in the first 30 min were similar except the system without water. However, time course curves of TG hydrolysis were different with time lasted, illustrating that dissimilar equilibrium conversions were affected by the water content [8]. Higher OA content after 12 h was obtained as the water percentage increased and about 80.3% OA was yielded when 20 wt.% water added into the hydrolysis system. And OA yield for the system in absence of water was no more than 20%. Possible explanation might be that the amount of available water as a substrate of the reaction, could be too low to allow good reaction rates for no water added system [13].

3.2. Methanolysis of TG under different water contents

Methanol was completely consumed in methanolysis of triacylglyceride with less than 1/3 molar equivalent of methanol for the stoichiometric amount, but the methanolysis was decreased significantly by adding more than 1/2 molar equivalent of methanol. And former studies showed that excessive methanol insoluble in oil might inactivate the lipase [12,14], so every 1/3 molar equivalent of methanol for the stoichiometric account to TG was added to the mixture under different water contents at 0, 4 and 8 h, respectively. The results were shown in Fig. 2a and b.

It was visible in Fig. 2a that OAME content was more than 75% after 12 h reaction when 5–20 wt.% water amount based on TG was added, and the maximum OAME yield 80.6% existed at 20% water amount. Similar results had been obtained in our previous studies

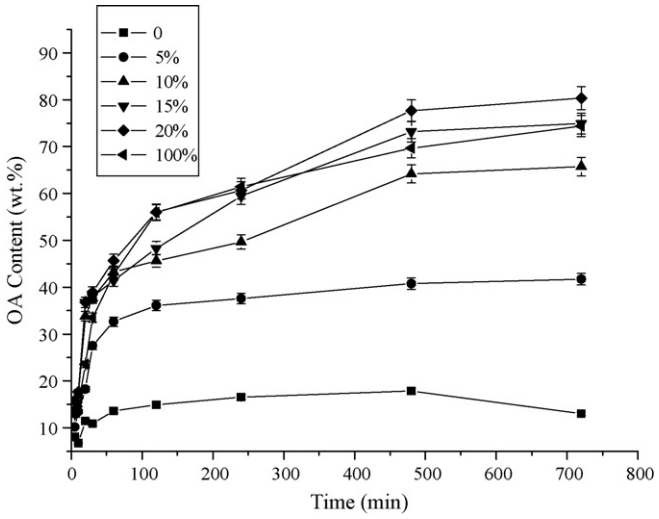

Fig. 1. Time course curves of hydrolysis of TG under different water contents. Reaction conditions: TG 1 g, 0.2 g immobilized lipase, varied water amount based on TG weight, 2 ml *n*-hexane, temperature 40 °C, 180 rpm, and total reaction time 12 h.

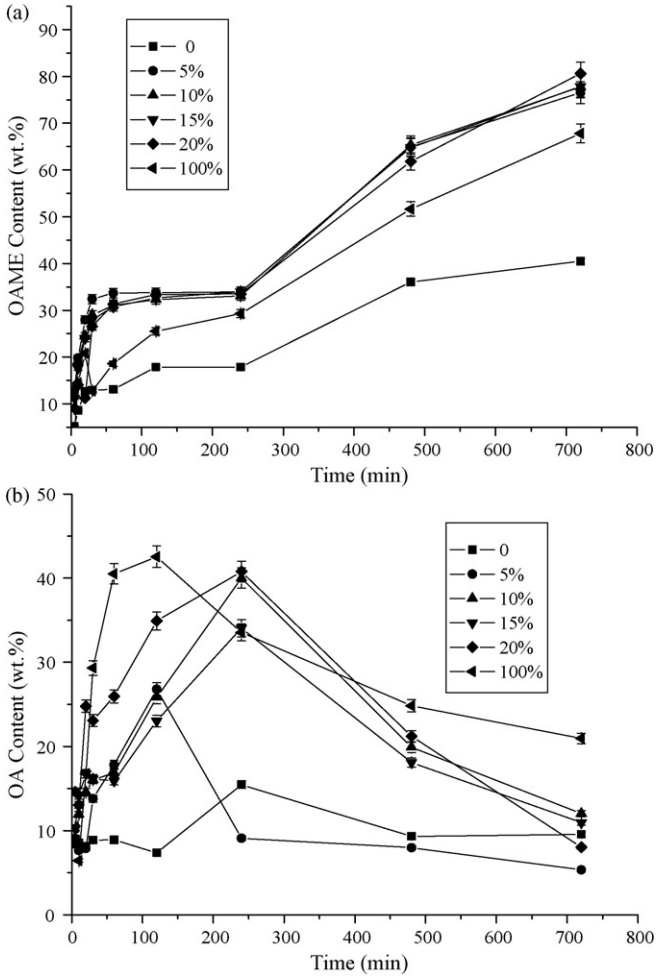

Fig. 2. Time course curves of methanolysis of TG under different water contents. (a) OAME content curves; (b) OA content curves. Reaction conditions: TG 1 g, 0.2 g immobilized lipase, varied water amount based on TG weight, 2 ml *n*-hexane, temperature 40 °C, 180 rpm, every 1/3 molar equivalent of methanol (45.3 μl) were added to the system at 0, 4 and 8 h, respectively, and total reaction time 12 h.

with lard as substrate [12]. Although 100 wt.% water had similar initial trend during the first 2 h, its equilibrium yield (67.8%) was a little lower, suggesting that more water in the system might not favor the transesterification reaction. In contrast, reaction rate was much lower without any water added, and the OAME content reached a plateau at only about 40.5%.

As shown in Fig. 2b, bell-shaped OA content curve illustrated that OA content declined after first increment to maximum. In the 5–20 wt.% range, more water amount had faster hydrolyzing rates, while 100 wt.% produced the most OA (20.9%) after the completion of the methanolysis.

Similar course curves in Fig. 2a and b in the presence of 5–20 wt.% water indicated that similar reaction mechanism might exist in that water amount range, which would be discussed below.

3.3. Comparison of hydrolysis and methanolysis

Since highest OAME yield was obtained under 20 wt.% water amount in *n*-hexane system, which was consistent with our earlier studies for the methanolysis of animal fats and TG, this maximum value was selected for comparison studies of TG hydrolysis and methanolysis. Fig. 3 shows the time curves of TG methanolysis and hydrolysis under 20 wt.% water amount.

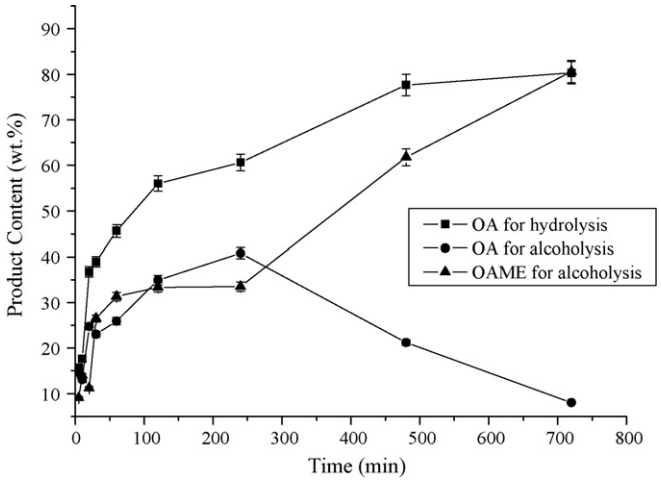

Fig. 3. Comparison of hydrolysis and methanolysis under 20% water content. The reaction conditions were the same as that in Figs. 1 and 2.

Hydrolysis reaction proceeded faster than the methanolysis in the first 8 h, and the equilibrium contents for these two reactions were similar after 12 h reaction. That phenomenon and the bell-shaped OA curve for methanolysis indicated that the methanolysis reaction catalyzed by this *Candida* sp. 99–125 appeared to accord with the successive reaction mechanism [15]. That was to say, TG was first hydrolyzed to partial glycerides and OA, then OAME was produced by esterification of the OA with methanol. This mechanism was also confirmed by the result that a maximum OA yield existed for the methanolysis process. The mechanism was similar with that of the lipase *C. rugosa* and *P. Cepacia* in Kaieda et al.'s prior studies [16,17].

3.4. Related study: effect of water substitutes or surfactants

One of the distinct characteristics of *Candida* sp. 99–125 was that this lipase needed much more water to maintain high transesterification activity, while the activity of Novozym 435 could be still high in absence of water [14]. One reason might be that water could dilute methanol, which was a strong lipase inhibitor during the process. Another explanation might be that water in organic solvent media offered sufficient interfacial area where the transesterification reaction occurred [18]. To deduce whether water had similar functions above, various water substitutes or surfactants were added into the methanolysis system and the results were shown in Table 1.

It could be concluded from Table 1 that *t*-butanol as water substitute did enhance the OAME content to some extent but not too much (less than 5%), compared with the system without water. Span 60 and Tween 80 did not have any positive effect on the yield while

Table 1
OAME content of TG methanolysis with water substitutes or surfactants

Different water substitutes	OAME content (%)
No water added	40.5
t-Butanol (200 μl) added	46.9
AOT (200 mg) added	61
Span 60 (200 μl) added	35.5
Tween 80 (200 μl) added	33.7
200 μl water added	80.6

Reaction conditions: TG 1 g, 0.2 g immobilized lipase, 200 μl *t*-butanol, Span 60, Tween 80 or 200 mg AOT added into the system, respectively, 2 ml *n*-hexane, temperature 40 °C, 180 rpm, every 1/3 molar equivalent of methanol (45.3 μl) were added to the system at 0, 4 and 8 h, respectively, and total reaction time 12 h.

AOT substitution favored the transesterification equilibrium but still not as high as that of water. These results illustrated that water analogs might have property diluting methanol, and some surfactant did provide interfacial area for lipase-catalyzed methanolysis. However, all these water substitutes did not have better effect as that of water.

Another effect of water might be that the binding of glycerol to lipase surface might be relieved by the high amount of water thus shifting the equilibrium to the OAME synthesis direction. However, results from esterification reaction showed that water did not have that glycerol relieving effect (data not shown). And many other lipases like Novozym 435 did not need so much water for methyl ester synthesis. This also confirmed the fact that main effect of water was not partitioning glycerol from the protein surface.

Considering the hydrolysis and methanolysis curves above, successive reaction mechanism showed that water took part in the methanolysis. So although water might have some similar effects as *t*-butanol or AOT, the main effect was that a hydrolysis–esterification process occurred during the whole methanolysis process.

4. Conclusion

The initial water content in *n*-hexane solvent strongly affects both reaction rates and equilibrium yields of TG hydrolysis and methanolysis catalyzed by immobilized lipase *Candida* sp. 99–125. 20 wt.% amount water based on the TG weight added into the reaction system leads to the highest OAME yield for methanolysis and OA yield for hydrolysis, respectively. The increase of methanolysis yield can be explained by the successive reaction mechanism. Although water analogs might have property diluting methanol (*t*-butanol), and some surfactants (AOT) did provide interfacial area for lipase-catalyzed methanolysis, but all these water substitutes did not have the same effect as water. Therefore main effect of water might be that it participated in the methanolysis reaction, that is to say, successive hydrolysis–esterification mechanism proceeded during the whole process. More detailed microcosmic investigations on the explanations why this lipase needs so much water to keep its high transesterification activity are still necessary at molecular level in future.

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