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Production of Biodiesel by Immobilized *Candida* sp. Lipase at High Water Content

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Abstract

A new process for enzymatic synthesis of biodiesel at high water content (10–20%) with 96% conversion by lipase from *Candida* sp. 99-125 was studied. The lipase, a no-position-specific lipase, was immobilized by a cheap cotton membrane and the membrane-immobilized lipase could be used at least six times with high conversion. The immobilized lipase could be used for different oil conversion and preferred unsaturated fatty acids such as oleic acid to saturated fatty acids such as palmitic acid. The changes in concentration of fatty acids, diglycerides, and methyl esters in the reaction were studied and a mechanism of synthesis of biodiesel was suggested: the triglycerides are first enzymatically hydrolyzed into fatty acids, and then these fatty acids are further converted into methyl esters.

Index Entries: Biodiesel; lipase; *Candida* sp. lipase; membrane immobilization.

Introduction

Biodiesel, fatty acid methyl esters (FAMEs) of vegetable oil or fat, is an alternative fossil fuel for diesel engines and has attracted considerable attention in recent years because of its renewable, biodegradable, and nontoxic fuel *(1)*. Biodiesel has been industrially produced in the United States and Europe. There are two reaction systems for the synthesis of biodiesel: chemical and enzymatic processes. Although a chemical process using alkaline catalyst for biodiesel synthesis has been applied to commercial biodiesel production, there are several drawbacks including the large amount of wastewater and high energy cost. The enzymatic method can

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cover these problems. Shimada et al. *(2)* reported optimization of reaction conditions such as solvents. Kamini and Iefuji *(3)* used lipase from *Cryptococcus* spp. S-2 in a water reaction system; however, the highest conversion was only 80%. Shimada et al. *(4)* used immobilized *Rhizopus delemar* lipase to catalyze alcoholysis with long-chain fatty alcohols. Watanabe et al. *(5)* found that added methanol denatured the *Candida antarctica* lipase used and they developed a two-step methanolysis system for biodiesel production from vegetable oil using immobilized *C. antarctica* lipase, in which 95% conversion was obtained. Until now the lipase for high biodiesel conversion was only Novozym 435 (*C. antarctica* lipase). The water content must be controlled at a low level (<5%) in a biodiesel synthesis system *(4,5)*.

We have screened a new lipase-producing strain, *Candida* sp. 99-125, that produced high lipase activity (8060 IU/mL) *(6)*. This lipase has been used in many applications such as the synthesis of 2-ethyl ester *(7)*. Nie et al. *(8)* also used the lipase for biodiesel synthesis and conversion reached 93%. However, the effect of water content has not been discussed. Until now a high water content in a biodiesel reaction system was considered bad for biodiesel synthesis, and the mechanism of biodiesel fuel synthesis is also not clear. In the present study, enzymatic synthesis of biodiesel fuel by the lipase from *Candida* sp. 99-125 at high water content was investigated.

Materials and Methods

Chemicals and Vegetable Oil

Methanol and petroleum ether (boiling point 60–90°C) were from a Beijing chemical plant. Vegetable oil was from a local market.

Strain and Culture Medium

The lipase-producing strain was *Candida* sp. 99-125. Fermentation was carried out in a 30-L reactor at 28°C with stirring at 300–500 rpm and 1-vvm air input. The culture medium was composed of 4 wt% bean powder, 4 wt% oil, 0.1 wt% KH_2PO_4, and 0.1 wt% $(NH_4)_2SO_4$. The culture conditions and lipase separation are presented in ref. 6. The activity of the enzyme powder was 106,000 IU/g.

Immobilization of Lipase on Membrane

A 10-mL solution containing 0.2% (w/v) gelatin, 0.2% (v/v) Tween-80, and 0.2% (w/v) PEG6000 was added to a 50-mL beaker. A total of 0.1 g of membrane (about 9 cm^2) was added to the solution and the mixture was stirred with a glass bar for 2 h. The membrane was taken out and dried at room temperature. The dry membrane was added to 10 mL of enzyme solution (5000–10,000 IU/mL). The membrane in the solution was stirred with a glass bar for 2 to 3 h. The membrane was taken out and dried at vacuum condition. The lipase activity immobilized on the membrane was 5000 IU/g of membrane.

Reaction System

Two grams of oil and 279 μL of methanol (methanol/oil = 3:1[mol/mol]), water, and 0.3 g of immobilized lipase were added to a 50-mL flask; the methanol was added stepwise: 279 μL of methanol was added three times with each addition containing 93 μL of methanol. The reaction was carried out at 40°C in a 170-rpm shaker. The products formed were assayed by gas chromatography (GC) (GC-14A; Shimadzu).

Determination of Lipase Activity

Lipase activity was determined according to an olive oil emulsion method *(9)*. The fatty acids released were determined by titration with 0.5 mol/L of NaOH. One unit of lipase was defined as the amount of enzyme that released 1 μmol of fatty acid/min at 37°C.

Product Analytical Methods

The products including triglycerides, diglycerides, monoglycerides, and FAMEs were assayed by GC (GC-14A; Shimadzu) with a capillary column (DB1-ht, 0.25 mm × 15 m; Agilent). The detector was a flame ionization detector and the column temperature was increased from 100 to 300°C at a rate of 10°C/min and from 300 to 350°C at rate of 5°C/min.

The fatty acid composition in oil was assayed by chemical methanolysis by BF_3-methanol at 60°C for 30 min and then the FAMEs were determined by a GC according to ref. *10*. Each value was measured three times and an average value was taken as the final value. For each experiment, two replications were made and the maximum standard deviation was <12%.

The conversion used in all figures is the molar conversion, defined as the ratio of moles of FAMEs formed to moles of triglycerides in oil added in the reaction system.

Results and Discussion

Methanolysis of Different Oils

Lipase from *Candida* sp. has no position specificity, which means that it will show the same catalytic property for all oils. However, the lipase shows a different degree of methanolysis for different oils (Table 1).

Safflower and soybean oils have higher conversion, whereas palm oil has the lowest conversion. In fact, these oils differ in fatty acid composition, and we compared the methyl ester composition of these oils, as shown in Table 2. It can be seen that the lipase from *Candida* sp. 99-125 prefers unsaturated fatty acids such as oleic acid and linoleic acid and that these fatty acids were converted into methyl esters easily, whereas the palmitic acid in oils was converted only 85–90% into methyl ester. A chemical methanolysis method such as BF_3-methanol alcoholysis to determine the composition of fatty acid often denatured these unsaturated fatty acids, resulting in a low content value of these fatty acids in oils. This result is also confirmed by

Table 1
Conversion of Different Oils by Lipase From *Candida* sp. 99-125

	Soybean oil	Safflower oil	Linseed oil	Corn oil	Palm oil
Methyl ester conversion (%)	91	91.8	90.8	91.2	88.5

Reaction conditions: 2 g of oil, oil:methanol = 1:3 (mol/mol); immobilized lipase:oil = 15:100 (w/w); temperature of 40°C; total reaction time of 30 h; water content of 15% (w/w).

Table 2
Comparison of Different Fatty Acids in Oils by Enzymatic Conversion

	Soy bean oil		Palm oil	
	Content in oil (%)[a]	Part converted into methyl ester (%)	Content in oil (%)[a]	Part converted into methyl ester (%)
Palmitic acid	17.6	90	50.5	88
Oleic acid	42.3	95	46.0	96
Linoleic acid	38.4	96		
Linolenic acid			2.4	98

[a]Fatty acid content was determined by chemical methanolysis with B F_3-methanol.

direct esterification by the *Candida* sp. 99-125 lipase *(11)*. In direct esterification of fatty acids by this lipase, linoeic acid also had the highest conversion (90%).

Addition Model of Methanol on Conversion to Biodiesel Fuel

Methanol denatures lipase and, therefore, the methanol must be added stepwise in reducing the denaturing effect, as shown in Fig. 1. When the final total volume of methanol used was the same, increasing methanol feeding number enhanced the conversion of FAMEs (biodiesel). However, after methanol was added more than three times (in each step one-third of the total methanol was added) the influence on conversion was not as notable. This result is similar to that of Watanabe et al. *(5)*, who used three-step methanolysis of oil for biodiesel synthesis.

Effect of Water Content on Conversion of Biodiesel

In most lipase catalytic ester syntheses, a low water content (<5%) should be kept owing to hydrolysis of triglycerides *(4,12)*. However, the synthesis of biodiesel by *Candida* sp. 99-125 lipase can be carried out at a high water content, as indicated in Fig. 2. Ninety-five percent conversion was obtained at 10–20% (v/v) water content. The mechanism of methanolysis of oil is that the oil (triglycerides) is first hydrolyzed into fatty acids, and then these free fatty acids (FFAs) formed are converted into methyl ester. Hydrolysis needs a high water content, and this is shown in Fig. 3.

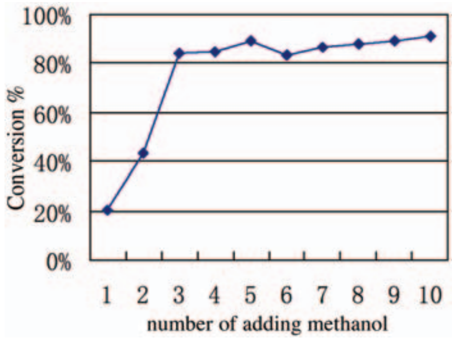

Fig. 1. Effect of methanol addition number on conversion. The conditions were as follows: Total methanol:oil = 1:3 (mol/mol); immobilized lipase:oil = 15:100 (w/w); temperature = 40°C; total reaction time = 30 h.

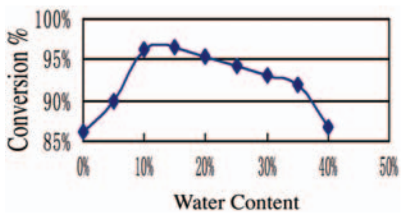

Fig. 2. Effect of water content on conversion of biodiesel. The conditions were as follows: 2 g of oil, oil:methanol = 1:3 (mol/mol); immobilized lipase:oil = 15:100 (w/w); temperature = 40°C; total reaction time = 30 h.

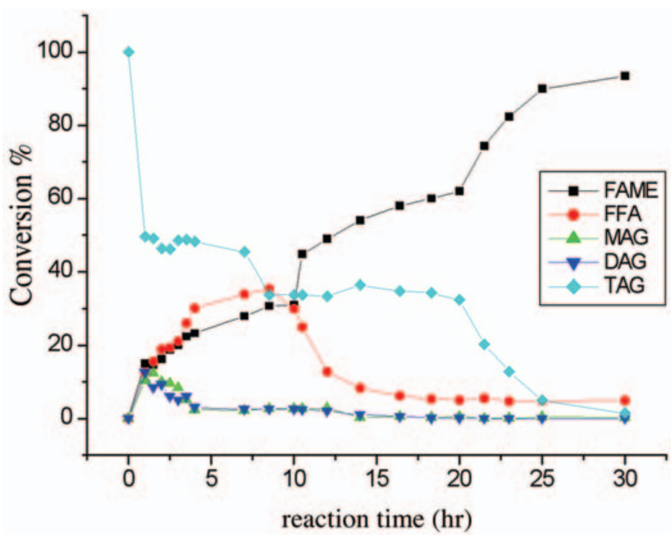

Fig. 3. Component change in reaction. The conditions were as follows: 2 g of oil, oil:methanol = 1:3 (mol/mol); immobilized lipase:oil = 15:100 (w/w); temperature = 40°C; total reaction time = 30 h; water content = 15% (w/w). DAG, diglyceride; MAG, monoglyceride; TAG, triglyceride.

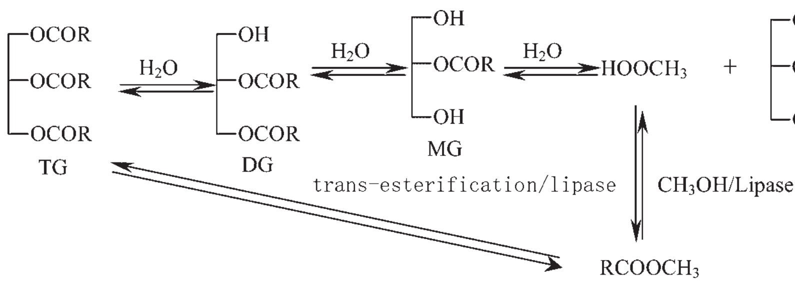

Fig. 4. Mechanism of synthesis of methyl ester by lipase catalysis. TG, triglyceride; DG, diglyceride; MG, monoglyceride.

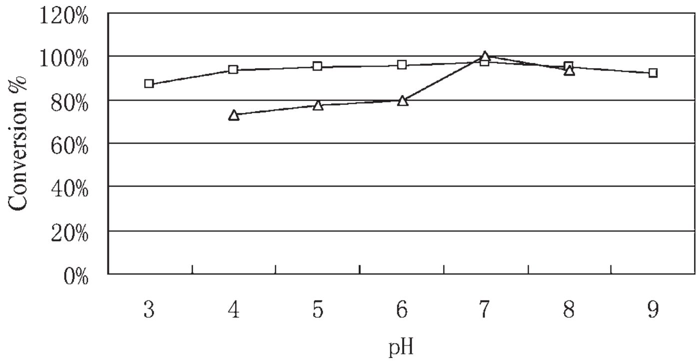

Fig. 5. Effect of pH on conversion of biodiesel. The conditions were as follows: 2 g of oil, oil:methanol = 1:3 (mol/mol); immobilized lipase:oil = 15:100 (w/w); temperature = 40°C; total reaction time = 30 h; water content = 15%. (□) Content of FAME; (△) enzyme activity.

FFA content increased at the beginning of the reaction (before 10 h of reaction), and then the FFA contents decreased, whereas the methyl ester contents continuously increased. The monoglycerides and diglycerides were only formed at the first 2 h of reaction and then they were further hydrolyzed into fatty acids. After 17 h of reaction, the final one-third of methanol was added to the three-step methanolysis system, the methyl ester continued to increase while the FFA, monoglyceride, and diglyceride contents were low, but the triglyceride content continued to decrease, meaning that the triglycerides were directly converted into methyl eaters in this stage.

Figure 4 shows the mechanism of biodiesel. In the early stage of the reaction, methanolysis mainly contributed to the hydrolysis of oil, and in the late stage of the reaction, interesterification played an important role.

Influence of pH in Water Phase

The pH for the stable lipase was in the range of 7.0–9.0 (Fig. 5), and the pH in the water phase influenced the synthesis of biodiesel fuel, as shown in Fig. 5. The optimal pH for biodiesel synthesis was in the range of 5.0–8.0, and the highest conversion was obtained at 7.0.

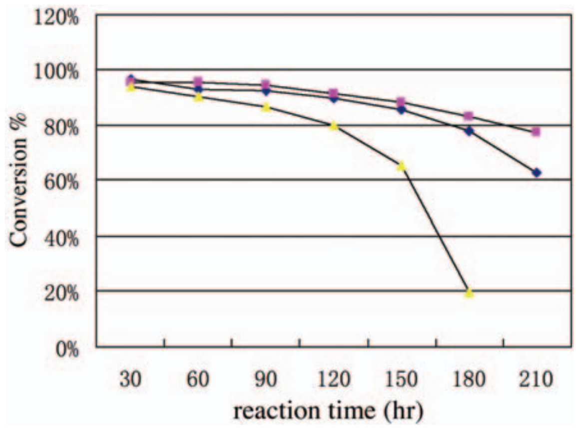

Fig. 6. Reuse of immobilized lipase at different water content. The conditions were as follows: 2 g of oil, oil:methanol = 1:3 (mol/mol); immobilized lipase:oil = 15:100 (w/w); temperature = 40°C; each batch reaction time = 30 h. (◆) 15% water content; (■) 20% water content; (△) 25% water content.

Table 3
Comparison of Enzymatic Synthesis of Biodiesel

Solvent	Lipase and substrate	Conversion (%)	Reference
Organic solvent			
Dimethylsulfoxide	Lipase from *Cryptococcus* spp. S-2, vegetable oil	85	*3*
Diethyl ether	Lipase from *Cryptococcus* spp. S-2, vegetable oil	70.4	*3*
Hexane	Lipase from *Cryptococcus* spp. S-2, vegetable oil	86.1	*3*
Petroleum ether and water	Lipase from *Candida* sp.	92–96	This work
Solvent free	Lipozyme, sunflower oil, and ethanol	60–80	*13*
	Lipase from *C. antarctica*	90–92	*5*
	Lipase from *Rhizopus oryzae*, plant oil	30–60	*14*
	Novozym 435, cottonseed oil		*15*

Reuse of Immobilized Lipase

The ability to reuse immobilized lipase is important for large-scale production of biodiesel. The water content influenced reuse, as shown in Fig. 6. When the water content was in the range of 15–20%, the immobilized lipase on the membrane could be reused six times with conversion up to 90%, and the half-life of the immobilized lipase was more than 200 h.

Table 3 provides a comparison of biodiesel synthesis. The results show that the synthesis of biodiesel by lipase from *Candida* sp. 99-125 is an efficient process at a high water content.

Conclusion

Lipase from *Candida* sp. 99-125 is an effective lipase for biodiesel synthesis and prefers unsaturated fatty acids such as oleic acid and linoleic acid. The lipase could catalyze biodiesel synthesis at a high water content (10–20%), and the optimal water content was 15% (w/w). The mechanism of synthesis is that triglycerides in oils are first enzymatically hydrolyzed to fatty acids, and then these fatty acids are further converted into methyl esters by the immobilized lipase. The lipase could be immobilized on a cotton membrane and the immobilized lipase could be reused at least six times with high conversion (>90%). The lipase is a potential lipase for biodiesel synthesis.

Acknowledgments

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