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Study on the effect of cultivation parameters and pretreatment on *Rhizopus oryzae* cell-catalyzed transesterification of vegetable oils for biodiesel production

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Abstract

Enzymatic methanolysis of vegetable oils for biodiesel production has become a hot point recently, in which study on whole cell as catalyst is an important field. In this paper, whole cell (*Rhizopus oryzae* IFO 4697) was adopted directly as biocatalyst for biodiesel production. Effects of carbon source on cell growth and whole cell-catalyzed methanolysis of vegetable oils for biodiesel production were studied. The results showed that different oils contained in the cultivation medium had varied effects on the whole cell-catalyzed methanolysis of oils; with some specified oil as the carbon source for cell cultivation, those cells expressed higher catalytic activity in catalyzing the transesterification of the same oil for biodiesel production. The initial reaction rate was increased notably (204%) with oil pretreatment on the cells before catalyzing the reaction, which was possibly due to the improved mass transferring of substrates. Under the optimized conditions, the maximum methyl ester yield could reach 86%.

Keywords: Whole-cell biocatalyst; Pretreatment; Lipase; Methanolysis; Biodiesel

1. Introduction

Biodiesel (fatty acid methyl esters), which is produced by transesterification of triglycerides with methanol, has become more attractive recently because of its environmental benefits and the fact that it is made from renewable resources [1]. Biodiesel fuels have many attractive features, such as it is domestically produced, offering the possibility of reducing petroleum imports; it is biodegradable and the combustion products have reduced levels of particulates, carbon monoxide, sulfur oxides, hydrocarbons, soot, and nitrogen oxides [2–4].

Biodiesel can be produced by chemical methanolysis using an alkali or acid-catalysis or by enzymatic methanolysis using lipases. At present, the chemical methanolysis using an alkali-catalysis has been applied for biodiesel production in the industry. However, requirement of removal of catalyst and excessive energy requirements are the major drawbacks for such chemical process, and free fatty acids and water would interfere with the reaction seriously. Enzymatic methanolysis utilizing lipase has become more attractive for biodiesel fuel production, since the glycerol produced as a by-product can easily be recovered and the purification of fatty methyl esters is simple to accomplish [5,6]. Several extracellular lipases, such as *C. antarctica*, *Lipozyme* TL IM and *Rhizopus oryzae* (F-AP15), have been used in the transesterification for biodiesel production [4,6–8]. However, the main hurdle to biodiesel production using extracellular lipases for industrial application is the cost of the lipase. Since extracellular lipase production requires complicated recovery, purification, and immobilization processes which account for a large part in the lipase cost [9,10]. As a means of reducing the cost, utilization of whole-cell biocatalysts is significantly advantageous since no purification is necessary. It has been demonstrated that the whole cells can efficiently catalyze the methanolysis of vegetable oils for biodiesel production [9,11]. In this paper, whole cell (*R. oryzae* IFO 4697) is used directly as the biocatalyst for biodiesel production (Fig. 1). Effect of different cultivation parameters on cell growth and whole cell-catalyzed methanolysis of vegetable oils for biodiesel production were examined systematically.

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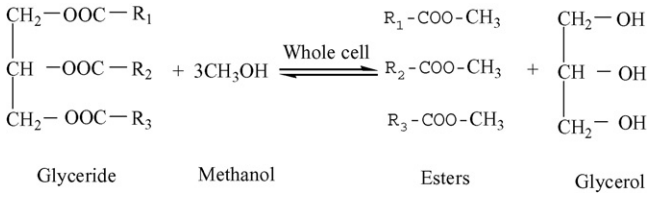

Fig. 1. Whole cell-catalyzed methanolysis of vegetable oils for biodiesel production.

2. Materials and methods

2.1. Materials

The whole cell *R. oryzae* IFO 4697 was purchased from ATCC. Palmitic acid methyl ester, stearic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester, linolenic acid methyl ester, and heptadecanoic acid methyl ester (served as the internal standard) were purchased from Sigma and were chromatographically pure. All other chemicals were obtained commercially and were of analytical grade.

2.2. Cells cultivation

The culture medium contains 30 g/l oil, 70 g/l peptone, 1.2 g/l $NaNO_3$, 1.2 g/l KH_2PO_4 and 0.5 g/l $MgSO_4.7H_2O$.

One hundred milliliters of basal medium containing about 10^6 spores was cultivated for approximately 40 h at 35 °C on a shaker (130 rpm).

The cells were separated from the culture broth by filtration. After being washed with tap water, the cells were frozen in a −70 °C refrigerator, and dried under a vacuum for about 12 h.

2.3. Whole cell-catalyzed methanolysis of vegetable oils

The methanolysis reactions were carried out in a 50 ml screw-cap bottle at 35 °C on a shaker (130 rpm). The compositions of the reaction mixtures were as follows: soybean oil 9.65 g, methanol 0.35 g, and 0.1 M acetate buffer (pH 5.6) 0.5 ml. One molar equivalent of methanol was 0.35 g against 9.65 g soybean oil. Fifty microliters of samples were taken from the reaction mixture at specified time, centrifuged to obtain the upper layer and analyzed by capillary gas chromatography.

2.4. Cells pretreatment

Oil pretreatment on cells was carried out as follows: the cells were preincubated in soybean oils for some time before catalyzing the methanolysis of oils for biodiesel production. While for methyl esters pretreatment, the cells were first preincubated in methyl esters for some time, and then filtrated, washed and dried for further use.

2.5. Gas chromatography (GC) analysis

The methyl esters contained in the reaction mixture was analyzed using a GC-14A gas chromatography connected to a wax TM-10 capillary column supplied by Agilent. Five microliters of the aforementioned mixture and 300 μl of 1.4 mmol/l heptadecanoic acid methyl ester (hexane as the solvent) which is served as the internal standard were precisely measured and mixed thoroughly. The column temperature was kept at 180 °C for 0.5 min, heated to 250 °C at 15 °C/min, and then maintained for 6 min. The temperatures of the injector and detector were set at 245 and 250 °C, respectively.

Table 1
Effect of carbon sources on cell growth

Carbon sources	Cells weights (g/l)
Refined soybean oil	15.4
Refined olive oil	15.6
Refined cottonseed oil	16.0
Crude rapeseed oil	13.9
Crude soybean oil	14.8
Glucose	6.0

3. Results and discussion

3.1. Effect of carbon sources on whole cell-catalyzed methanolysis of soybean oil for biodiesel production

To examine the effect of carbon source on cells growth and the methanolysis activity, glucose and different oils were used as carbon sources respectively in cells cultivation. Table 1 shows the weights of dry cells with different carbon sources and Fig. 2 shows the time courses of methanolysis catalyzed by cells cultivated with different carbon sources. From Fig. 2 it can be seen that cells expressed methanolysis activity with oils as the carbon source cells, while no activity with glucose as the carbon source. Therefore it can be concluded that oils acted as carbon sources as well as inducers for lipase production [9].

During the whole cell-catalyzed methanolysis of oils for biodiesel production, it has been found that cells expressed varied methanolysis activities with different oils as the carbon source in cells cultivation (Fig. 2). The cells cultivated with

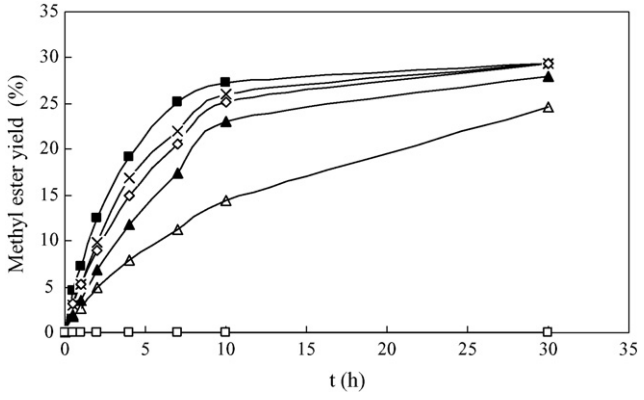

Fig. 2. Effect of carbon sources on whole cell-catalyzed methanolysis of soybean oil. Reaction conditions: 35 °C, 150 rpm, pH 5.6, 5% buffer (w/w, based on oil weight), 5% cells (w/w, based on oil weight), one molar equivalent of methanol, refined soybean oil (■), refined olive oil (×), refined cottonseed oil (◯), crude rapeseed oil (△), crude soybean oil (▲), and glucose (□).

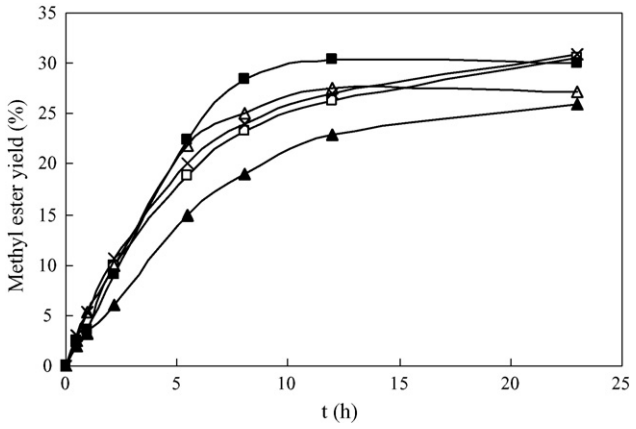

Fig. 3. Effect of oil carbon sources on biodiesel production. Reaction conditions: 35 °C, 150 rpm, pH 5.6, 5% buffer (w/w, based on oil weight), 5% cells (w/w, based on oil weight), one molar equivalent of methanol, the methanolysis of soybean oil catalyzed by the cells with olive oil as carbon source (□, ■), the cells with cottonseed oil as carbon source catalyzed the methanolysis of soybean oil (▲), and the cells with cottonseed oil as carbon source catalyzed the methanolysis of cottonseed oil (△). The cells with soybean oil as carbon source catalyzed the methanolysis of soybean oil (×).

refined oils expressed higher activity than those with crude oils as the carbon sources, which may due to the negative effect caused by some minor components such as phospholipids contained in the crude oils [12].

3.2. Effect of oil carbon sources on whole cell-catalyzed methanolysis of different vegetable oils for biodiesel production

The cells cultivated with soybean oil, olive oil and cottonseed oil as carbon sources were collected and adopted further to catalyze the methanolysis of different oil for biodiesel production. It has been noticed interestingly that with some specified oil as the carbon source, those cells expressed higher catalytic activity in catalyzing the same oil for biodiesel production. As shown in Fig. 3, the cells with olive oil as carbon source expressed higher catalytic activity in catalyzing the methanolysis of olive oil than catalyzing the methanolysis of soybean oil. For the cottonseed oil, the similar result was obtained.

The effect of soybean oil concentration on cells growth and methanolysis activity was also investigated. It has been found that when the concentration of soybean oil was between 20 and 30 g/l, the cells exhibited higher methanolysis activities. The methyl ester (ME) yield was up to 30% at 8 h just as shown in Fig. 4.

3.3. Effect of oil pretreatment on whole cell-catalyzed methanolysis for biodiesel production

Since the lipase produced by *R. oryzae* IFO 4697 is intracellular, mass transferring of substrates could affect reaction rate of the methanolysis. Effect of soybean oil pretreatment on whole cell before catalyzing the methanolysis was therefore investigated. The cells were preincubated in soybean oil for 24, 48 or 72 h. With oil pretreatment on the cells, the initial reaction rates

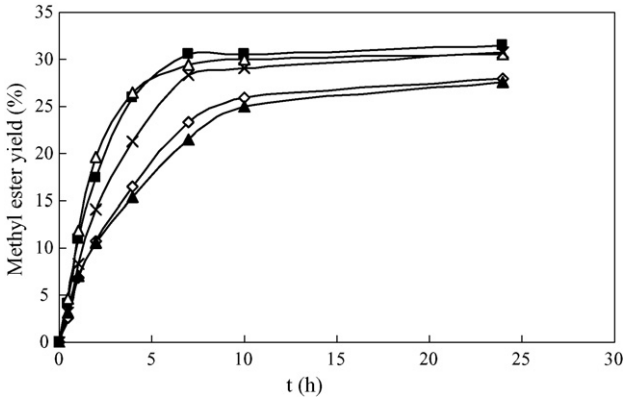

Fig. 4. Effect of soybean oil concentration on the methanolysis. Reaction conditions: 35 °C, 150 rpm, pH 5.6, 5% buffer (w/w, based on oil weight), 5% cells (w/w, based on oil weight), one molar equivalent of methanol, 10 g/l (◊), 20 g/l (■), 30 g/l (△), 40 g/l (×), and 50 g/l (▲).

of the methanolysis were increased notably (Fig. 5). The longer time the cells preincubated in the oil, the higher the initial reaction rates were. After 72 h oil pretreatment, the initial reaction rate was 24.78 mmol/(l min), 204% of the control, which might be due to the improved mass transferring.

3.4. Effect of methyl ester pretreatment on whole cell-catalyzed methanolysis

Whole cells were immersed in methyl ester for some time and the effect of methyl ester pretreatment on whole cell-catalyzed methanolysis of soybean oils was also investigated (after the pretreatment, methyl esters were removed totally). It was found interestingly that the initial reaction rate of the methanolysis decreased when the cells were subject to methyl ester immersing pretreatment before catalyzing the reaction (Fig. 6). The longer time the cells were preincubated in the methyl esters, the lower the initial reaction rates were. After 72 h methyl esters pretreatment, the initial reaction rate was 0.72 mmol/(l min), which was much lower than the control. The results were quite different from the pretreatment results of the immobilized lipase-

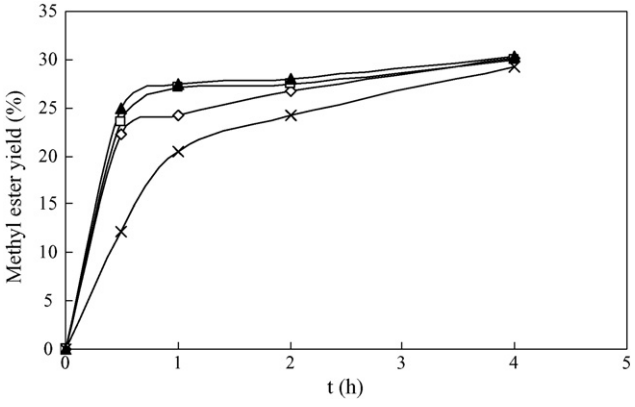

Fig. 5. Effect of soybean oil pretreatment on the methanolysis of soybean oil. Reaction conditions: 35 °C, 150 rpm, pH 5.6, 5% buffer (w/w, based on oil weight), 5% cells (w/w, based on oil weight), one molar equivalent of methanol, 24 h (◊), 48 h (□), 72 h (▲), and control (×).

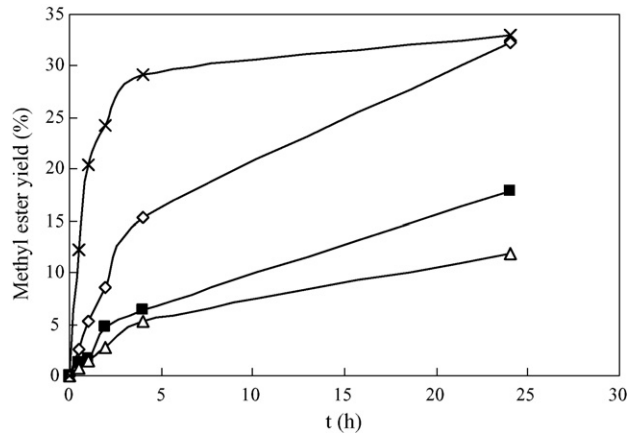

Fig. 6. Effect of methyl ester pretreatment on whole cell-catalyzed methanolysis of soybean oil. Reaction conditions: 35 °C, 150 rpm, pH 5.6, 5% buffer (w/w, based on oil weight), 5% cells (w/w, based on oil weight), one molar equivalent of methanol, 24 h (◊), 48 h (■), 72 h (△), and control (×).

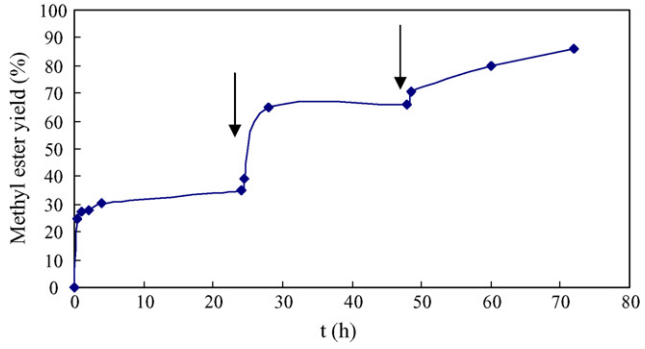

Fig. 7. Methanolysis of soybean oils with three stepwise addition of methanol for biodiesel production. Reaction conditions: 35 °C, 150 rpm, pH 5.6, 5% buffer (w/w, based on oil weight), 5% cells (w/w, based on oil weight), arrows indicate the addition of one molar equivalent of methanol.

catalyzed methanolysis of oils for biodiesel production, which progressed much faster when the immobilized lipase was pretreated by methyl oleate immersing [8].

3.5. Whole cell-catalyzed methanolysis with three stepwise addition of methanol

It has been reported that too much methanol would lead to serious inactivation of the lipase [2,11,13]. So three stepwise addition of methanol was adopted in whole cell-catalyzed methanolysis of oils for biodiesel production. Under the optimum conditions, the ME yield of the whole cell-catalyzed methanolysis was about 86% with three stepwise addition of methanol (Fig. 7).

4. Conclusion

The whole cell *R. oryzae* IFO 4697 can be used directly as biocatalyst in biodiesel production. The cells had no methanolysis activity when only glucose was used as the carbon source. Different oils contained in the cultivation medium had varied effects on the whole cell-catalyzed methanolysis of soybean oils for biodiesel production. With some specified oil as the carbon source for cell cultivation, those cells would express higher catalytic activity in catalyzing the methanolysis of the same oil for biodiesel production. The initial reaction rate of the whole cell-catalyzed methanolysis increased notably with oil pretreatment, which was possibly due to the improved mass transferring of substrates. While the initial reaction rate of the whole cell-catalyzed methanolysis decreased significantly after cells being pretreated by methyl ester, which might result from the changes of the secondary structures of the lipase.

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