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Immobilized lipase *Candida* sp. 99-125 catalyzed methanolysis of glycerol trioleate: Solvent effect

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Abstract

The immobilized lipase *Candida* sp. 99-125 catalyzed methanolysis of glycerol trioleate was studied in twelve different solvents in order to deduce the solvent effect through an attempt to correlate the highest yield with such solvent properties as hydrophobicity (log *P*), dielectric constant (ε), and Hildebrand solubility parameter (δ). The results showed that the conversion of glycerol trioleate and yield of oleic acid methyl ester were quite dependent on the solvent. The catalyst lipase in various solvents also needed different optimum amount of water to keep its maximum activity, and generally this lipase in more hydrophobic solvents required more water. The correlation between the highest yield and log *P* value was found to be reasonable except deviation of data points of certain solvents, while no obvious correlation existed between the other two parameters, dielectric constant (ε) and Hildebrand solubility parameter (δ), and the enzyme activity. The study revealed that more hydrophobic solvents such as *n*-hexane or cyclohexane were more suitable solvents for *Candida* sp. 99-125 catalyzed transesterification of glycerol trioleate to oleic acid methyl ester.

Keywords: Immobilized lipase; Methanolysis; Glycerol trioleate; Solvent

1. Introduction

Over the past two decades, there has been an increasing interest in the enzymatic-catalyzed reactions in organic media. Among these enzymes, lipases from different origins have been widely employed to catalyze hydrolysis, alcoholysis, esterification and transesterification of carboxylic esters (Kobayashi et al., 2003; Tewari et al., 2003; Ting et al., 2006; Zinni et al., 2007). The monoalkyl esters of fatty acids (also called biodiesel), which can be produced from renewable resources via lipase catalyzed reactions, have drawn attention as a non-toxic, biodegradable and renewable source of energy with quite lower exhaust emissions (Lara and Park, 2004; Royon et al., 2007). Thus these fatty acid alkyl esters are environmentally friendly and show great potential as an alternative energy.

Although lipase-catalyzed synthesis of biodiesel has many special advantages over chemical methods, the difficulty or even inability to dissolve both the hydrophobic and hydrophilic substrates in an organic solvent, which consequently influences the yield, has been taken into consideration for biochemical synthesis (Castillo et al., 2003). In general, organic solvent characteristics not only influence the mass transfer in the reaction system, but they also have great effect on the enzyme structure and optimum water content to keep its maximum activity. Thus many literatures have appeared describing the effect of different organic solvents on biocatalytic activity and much attention has been paid to the influence of the solvent physicochemical properties on enzymatic activity (Catoni et al., 1996; Vic et al., 1997; Bellot et al., 2001; Hazarika et al., 2003).

In this work, self-established immobilized lipase *Candida* sp. 99-125 was employed to catalyze the methanolysis of glycerol trioleate (TG) in twelve different organic solvents. The lipase was proved to be stable when used

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repeatedly for synthesis of biodiesel in our former studies (Nie et al., 2006; Lu et al., 2007). These solvents were selected for they have a wide range of physico-chemical properties (log *P* value ranging from −1.3 to 3.5, dielectric constant from 1.89 to 37.5 and Hildebrand solubility from 7.3 to 11.9). It is expected that there is a correlation between the oleic acid methyl ester (OAME) yield and the properties of the solvents employed. Moreover, whether optimum water amount needed for the maximum ester yield is related to the solvents is also investigated.

2. 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 and were chromatographically pure. *Candida* sp. 99-125 was prepared in our laboratory (He et al., 2002; Nie et al., 2006; Tan et al., 2006; Lu et al., 2007). Twelve different solvents dimethylsulfoxide, acetonitrile, acetone, tetrahydrofuran (THF), *t*-butanol, dichloromethane, benzene, chloroform, toluene, tetrachloride carbon, cyclohexane, *n*-hexane and all the other reagents were obtained commercially and were of analytical grade.

2.2. Lipase immobilization

The immobilized method has been established in our lab, and its procedure has been described detailedly in our former literatures (He et al., 2002; Nie et al., 2006; Tan et al., 2006; Lu et al., 2007). This lipase showed excellent activity in enzymatic synthesis of 2-ethylhexyl palmitate and fatty acid methyl esters. Firstly, 0.1 g of textile (about 9 cm^2) was pre-soaked for 1 h in 10 ml of co-immobilization solution, consisting of 5% (w/v) glutin, 2% (w/v) lecithin, 2% (w/v) polyethylene glycol-6000 and 1% (w/v) magnesium chloride. The textile was then dried at room temperature to be used as the support in the immobilization of the lipase. The support was added into 10 ml enzyme solution (5000–10,000 U/ml) and stirred for 2–3 h. Then the textile was taken out and dried at room temperature under vacuum. The activity of the immobilized lipase was determined by the olive oil emulsion method (Abramic et al., 1999). One unit of activity is equivalent to the amount of enzyme required to liberate 1 μmol fatty acid per minute from olive oil under assay conditions.

2.3. General procedure for TG methanolysis

In advance, all the solvents were dehydrated over molecular sieves 5 A. 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 solvent, 0.2 g immobilized lipase, and every 45.3 μl methanol was added to the system at 0 h, 8 h and 16 h, respectively, with a total reaction time of 24 h. Since different solvents might need varied optimum water amount to keep its maximum activity, water amount as a single parameter was optimized for these solvents in the range of 0–10% based on the TG weight. At pre-determined times, 20 μl samples were taken and centrifuged to obtain the upper layer. Then 5 μl of the upper layer was dissolved in *n*-hexane for gas chromatography analysis ($CHCl_3$ was used to solve the sample taken from the dimethylsulfoxide solvent since *n*-hexane can not dissolve the sample quite well).

2.4. Analytical procedure

The methyl ester 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 °C and 360 °C, respectively (Nie et al., 2006; Lu et al., 2007). Heptadecanoic acid methyl ester purchased from Sigma was used as an internal standard. The conversion is defined as consumed TG amount during the reaction divided by the initial TG amount (g/g). The OAME yield is defined as oleic acid methyl ester amount produced divided by the initial amount of TG (g/g).

3. Results and discussion

Twelve solvents with log *P* (the logarithm of the partition coefficient of a given compound in the standard octanol–water two-phase system) ranging from −1.3 to 3.5 were selected to investigate their effects on the methanolysis.

Table 1 illustrates the effect of different solvents with methanol added only once and Table 2 shows the conversion and OAME yield of methanolysis reaction in different solvents systems and varied water amounts. And it can be clearly seen that both the conversion and the yield of the methanolysis reaction dramatically are dependent on the solvent.

Former studies shown that methanol insoluble in the reaction system might inhibit the lipase activity, so three-step methanolysis was always employed to avoid lipase inactivation (Shimada et al., 2002). However, in this study, some amphiphilic solvents as *t*-butanol can dissolve both substrates easily. So, we first attempted to investigate whether the soluble methanol still inactivates the lipase activity, and the result is shown in Table 1. All the OAME yields did not exceed 40% except CCl_4, which showed a yield of 62.26%. In the more hydrophilic solvents systems such as acetonitrile, acetone and THF, the OAME yields were only about 4%. Meanwhile, the conversions of TG