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# *Penicillium expansum* lipase-catalyzed production of biodiesel in ionic liquids

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ABSTRACT

*Penicillium expansum* lipase (PEL) was used to catalyze biodiesel production from corn oil in [BMIm][$PF_6$][1] (an ionic liquid, IL) and *tert*-butanol. Both systems were optimized in terms of MeOH/oil molar ratio, reaction temperature, enzyme loading, solvent volume, and water content. The high conversion obtained in the IL (86%) as compared to that in *tert*-butanol (52%) demonstrates that the IL is a superior solvent for PEL-catalyzed biodiesel production. Poor yields were obtained in a series of hydrophilic ILs. Addition of salt hydrates affected biodiesel production predominantly through the specific ion (Hofmeister) effect. The impact of methanol on both activity and stability of PEL in the IL and in hexane was investigated, in comparison to the results obtained by two commonly used lipases, Novozym 435 and Lipozyme TLIM. The results substantiate that while different lipases show different resistance to methanol in different reaction systems, PEL is tolerant to methanol in both systems.



## 1. Introduction

A continuing dependence on petroleum fuels is widely considered environmentally unsustainable. Biodiesel, on the other hand, has been generally accepted as a promising substitute for conventional fossil diesel because it is biodegradable, nontoxic, and renewable (Ranganathan et al., 2008; Chisti, 2007). Here biodiesel is a mixture of fatty acid methyl esters (FAMEs) produced by alcoholysis of oils and fats from natural sources such as vegetables, animals, and microalgae. While the chemical approaches for biodiesel production require the use of either acid or alkali catalysts, enzymatic transesterification with methanol (MeOH) has attracted much attention, because it is more eco-friendly and holds the potential for industrial implementation (Shimada et al., 2002; Ranganathan et al., 2008). Bajaj et al. (2010) have recently published a comprehensive review about the enzymatic production of biodiesel. Su et al. (2009) have explored the use of dimethyl/diethyl carbonate as both the extraction solvent and transesterification reagent for in situ lipase-catalyzed biodiesel production. Rosa et al. (2009) have also demonstrated the potential advantages of conducting the continuous lipase-catalyzed production of biodiesel in compressed fluids such as carbon dioxide, propane, and *n*-butane.

However, the high cost of enzymes and the loss of enzyme activity caused by MeOH (a co-substrate) and glycerol (a by-product) remain an obstacle to the industrial implementation of the enzymatic process (Shimada et al., 2002; Ranganathan et al., 2008; Bajaj et al., 2010). Lipases from different sources have been investigated in the biodiesel production, but the most commonly used one is the lipase B from *Candida antarctica* (CALB), which has been commercialized by Novozymes as an immobilized form (Novozym 435) (Shimada et al., 2002; Du et al., 2004; Li et al., 2006; Modi et al., 2006; Royon et al., 2007). Insoluble MeOH is believed to be partially responsible for enzyme inhibition (Shimada et al., 2002), and the use of *tert*-butanol as a solvent has been recognized as a solution for reducing this inhibitory effect (Li et al., 2006; Royon et al., 2007), presumably due to its ability in solubilizing MeOH (Ranganathan et al., 2008).

So far, enzymatic production of biodiesel has been mainly conducted in organic media or in a solvent-free system. Ionic liquids (ILs), a promising new type of solvents for biocatalytic processes, have rarely been utilized in this application. Ionic liquids are organic salts remaining as liquids under ambient temperatures, and there has been an increasing interest in using them as a new reaction medium for biotransformations because of their unique solvent properties and their ability of presenting excellent enzyme performance (Yang and Pan, 2005; van Rantwijk and Sheldon, 2007). The following three research papers on lipase-catalyzed biodiesel production in ILs have been based on the use of the same commonly used lipase (CALB) as the catalyst: Sunitha et al. (2007) have explored the methanolysis of sunflower oil in two hydrophobic ILs, [BMIm][$PF_6$] and [EMIm][$PF_6$][2], and two hydrophilic ones, [BMIm][$BF_4$][3] and [HMIm][$BF_4$][4]; Ha et al. (2007) have compared the production yield of biodiesel from soybean oil in 23

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[1] 1-butyl-3-methylimidazolium hexafluorophosphate.

[2] 1-ethyl-3-methylimidazolium hexafluorophosphate.

[3] 1-butyl-3-methylimidazolium tetrafluoroborate.

[4] 1-hexyl-3-methylimidazolium tetrafluoroborate.

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different ILs; and Zhao et al. (2010) have developed new ether-functionalized ILs for enzymatic methanolysis of Miglyol® oil 812 and soybean oil.

Our current research is focused on the use of *Penicillium expansum* lipase (PEL) in catalyzing the biodiesel production in ionic liquids, in hope of (1) introducing a less expensive yet efficient lipase for producing biodiesel, and (2) offering a new solvent type for minimizing the enzyme inhibition problem and optimizing the production yield. Unlike the commonly used and widely investigated lipases, PEL has received only limited attention to its catalytic properties and potential applications from researchers. Previously, this enzyme has been utilized in catalyzing biodiesel production from corn oil (Li et al., 2007) and waste oil (Li et al., 2009) in organic media. Our recent study (Yang et al., 2010) investigated the catalytic activity of this enzyme in both ionic liquid and organic solvent systems and first explored its use in catalyzing the methanolysis of corn oil in the ionic liquid [BMIm][$PF_6$]. The superior conversion obtained in this IL, as compared to that obtained in the commonly used organic solvents (such as *tert*-butanol and hexane) and in the solvent-free system, has successfully demonstrated that the IL is a promising reaction medium for PEL-catalyzed biodiesel production. Our previous experimental results (Yang et al., 2010) have also suggested that direct addition of salt hydrates in the nonaqueous reaction systems affects the hydrolytic activity of PEL via a dual function: the water-buffering effect and the specific ion (Hofmeister) effect, and the latter seems to predominate in the IL system.

In this current study, we extend our interest to the following three aspects: (1) optimizing the IL system for PEL-catalyzed biodiesel production, (2) examining the effect of salt hydrates on this catalytic process, and (3) investigating the tolerance of PEL against MeOH. The latter was carried out through assessing the effect of MeOH on both activity and stability of PEL in the IL and in organic media, in comparison to the results obtained by two other commercial lipases commonly used for biodiesel production, namely, Novozym 435 and Lipozyme TLIM. Discussion was then made regarding the enzyme inhibition induced by MeOH during the biodiesel production.

## 2. Methods

### 2.1. Materials

Lipase from *P. expansum* (PEL, 10,000 U/g of hydrolytic activity) was kindly donated by Shenzhen Leveking Bioengineering Co. Ltd., China. This enzyme was produced by spraying the concentrated supernatant from fermentation with addition of a certain amount of starch as a thickening agent. Two other immobilized lipases, Novozym 435 (from *C. antarctica*) and Lipozyme TLIM (from *Thermomyces lanuginosa*) were purchased from Novozymes (China) Investment Co. Ltd. *p*-Nitrophenyl palmitate (pNPP) and *p*-nitrophenol (pNP) were purchased from Sigma–Aldrich China Inc. All the fatty acid methyl esters used for GC characterization were of chromatographic purity also from Sigma–Aldrich China Inc. Refined corn oil was obtained from a local supermarket in Shenzhen, China. The ionic liquid [BMIm][$PF_6$] (99%, HPLC) was purchased from ShangHai Cheng Jie Chemical Co. Ltd. All other reagents used were of analytical grade from local manufacturers in China.

### 2.2. PEL-catalyzed biodiesel production

The general procedures of the enzymatic reactions, both in the ionic liquid [BMIm][$PF_6$] and in the organic solvent *tert*-butanol, and GC analysis of the biodiesel products have been described in (Yang et al., 2010). In both reaction systems, each of the following parameters was optimized independently: MeOH/oil molar ratio, reaction temperature, enzyme loading, solvent volume, and water content. Unless otherwise stated, the reaction condition for converting 1.0 g of corn oil was: two molar equivalent of MeOH, 2 ml of a given solvent, 200 mg of the PEL, conducted at 40 °C with agitation of 220 rpm. For the experiments on investigating the effect of water content and salt hydrates, both solvents were dried over molecular sieves prior to use.

### 2.3. Effect of methanol on the activity of three lipases

Activity assays of PEL in hexane and in [BMIm][$PF_6$] have been described in (Yang et al., 2010), following the hydrolysis of pNPP to pNP. The effects of MeOH were assessed by adding different amounts of MeOH into the reaction systems and comparing the amount of pNP that was produced. For the assays in hexane, a certain amount of each enzyme (160 mg of PEL, 60 mg of Novozym 435, and 10 mg of Lipozyme TLIM) was added to 2.0 ml hexane containing 20 mM pNPP in the presence of 0–2.5% v/v MeOH and the reaction was carried out at 37 °C with agitation of 220 rpm. Periodically, 0.1 ml sample was taken and mixed thoroughly with 2.0 ml of 0.1 M NaOH solution. After centrifugation, the bottom phase was taken for absorbance reading at 400 nm with a LAMBDA 25 UV/Vis spectrophotometer from Perkin–Elmer. Blank reactions (without addition of the enzyme) were also conducted in parallel, and their rates were small enough to be neglected. The molar extinction coefficient for pNP in 0.1 M NaOH was determined accordingly, as detailed in (Yang et al., 2010). For the activity assays in [BMIm][$PF_6$] where pNPP has a low solubility, the substrate solution was prepared by dissolving pNPP in isopropanol in a concentration of 20 mM. The reaction was then started by adding the same enzyme amount (as stated above in the hexane system) to a 5 ml capped bottle containing 0.2 ml of the substrate solution and 2.0 ml of [BMIm][$PF_6$]/MeOH mixture (MeOH 0–20%, v/v).

### 2.4. Effect of methanol on the stability of three lipases

For testing the MeOH effect on lipase stability in hexane, a certain amount of each lipase (80 mg of PEL, 60 mg of Novozym 435, and 10 mg of Lipozyme TLIM) was incubated in 4.0 ml hexane containing different amounts of MeOH (0–5%, v/v) at 50 °C for 12 h. After vacuum drying to remove the solvent (which is volatile), the enzyme was subjected to activity assay in hexane (in the absence of MeOH) as described above. For the stability tests in [BMIm][$PF_6$], each enzyme (80 mg of PEL, 60 mg of Novozym 435, and 10 mg of Lipozyme TLIM) was incubated in the IL containing 0–100% (v/v) MeOH (the IL and MeOH are miscible with each other within the whole range) at 50 °C for 12 h. After centrifugation and subsequent removal of the supernatant, the remaining enzyme was assayed for its activity in the IL (in the absence of MeOH) as described above. It needs to be mentioned that although the IL is viscous and non-volatile and can hardly be removed completely, efforts have been made to ensure that a minimal amount of the IL remained with the treated enzyme.

## 3. Results and discussion

### 3.1. Optimization of the PEL-catalyzed biodiesel production

In view of our recent finding that the ionic liquid [BMIm][$PF_6$] and the organic solvent *tert*-butanol offer a higher conversion of PEL-catalyzed methanolysis from corn oil than the solvent-free milieu does (Yang et al., 2010), we focused on the optimization of the first two systems in this study. The following parameters were examined (Fig. 1): MeOH/oil molar ratio, reaction