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Production of biodiesel fuel from soybean oil catalyzed by fungus whole-cell biocatalysts in ionic liquids

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ABSTRACT

The methanolysis of soybean oil to produce a fatty acid methyl ester (ME, i.e., biodiesel fuel) was catalyzed by lipase-producing filamentous fungi immobilized on biomass support particles (BSPs) as a whole-cell biocatalyst in the presence of ionic liquids. We used four types of whole-cell biocatalysts: wild-type *Rhizopus oryzae* producing triacylglycerol lipase (w-ROL), recombinant *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase (r-FHL), *Candida antarctica* lipase B (r-CALB), and mono- and diacylglycerol lipase from *A. oryzae* (r-mdlB). w-ROL gave the high yield of fatty acid methyl ester (ME) in ionic liquid [Emim][BF_4] or [Bmim][BF_4] biphasic systems following a 24 h reaction. While lipases are known to be severely deactivated by an excess amount of methanol (e.g. 1.5 Mequiv. of methanol against oil) in a conventional system, methanolysis successfully proceeded even with a methanol/oil ratio of 4 in the ionic liquid biphasic system, where the ionic liquids would work as a reservoir of methanol to suppress the enzyme deactivation. When only w-ROL was used as a biocatalyst for methanolysis, unreacted monoglyceride remained due to the 1,3-positional specificity of *R. oryzae* lipase. High ME conversion was attained by the combined use of two types of whole-cell biocatalysts, w-ROL and r-mdlB. In a stability test, the activity of w-ROL was reduced to one-third of its original value after incubation in [Bmim][BF_4] for 72 h. The stability of w-ROL in [Bmim][BF_4] was greatly enhanced by cross-linking the biocatalyst with glutaraldehyde. The present study demonstrated that ionic liquids are promising candidates for use as the second solvent in biodiesel fuel production by whole-cell biocatalysts.

1. Introduction

Biodiesel fuel, i.e., fatty acid methyl esters (MEs), is a renewable and environmentally friendly fuel produced by transesterification of a triglyceride with methanol (methanolysis) [1,2]. Although alkali-catalyzed methanolysis of a triglyceride is one of the most powerful methods for the production of commercial biodiesel, there are some drawbacks in this system: high energy consumption, difficulty in recovering by-product glycerol, and the removal of the alkaline catalyst from reaction mixtures. Alternatively, enzymatic methanolysis using lipase has attracted considerable attention in biodiesel production because biocatalysis offers many advantages that help to overcome the afore-mentioned problems [3–7]. Immobilized lipases such as Novozyme 435 have mainly been used as biocatalysts for biodiesel production [5,6]. Also, utilization of a lipase-producing microorganism such as filamentous fungi (e.g. *Rhizopus oryzae* and *Aspergillus oryzae*) as a whole-cell biocatalyst is a promising approach [4]. The preparation of a biocatalyst is a facile procedure requiring no complex purification process, and the prepared whole-cells can be directly used as lipase containers. We have developed an efficient biodiesel production system using microorganisms immobilized on biomass support particles (BSPs) as whole-cell biocatalysts [8–13].

However, in lipase-catalyzed biodiesel production, methanol that will not dissolve in oil has traditionally caused a critical problem of enzyme deactivation. To avoid lipase deactivation by excessive methanol, several approaches have been examined including a stepwise addition of methanol [5–9], and use of organic solvents [14] or a salt solution [15]. Although these approaches seemed effective, laborious procedures—the periodic addition of methanol or the removal of organic solvents from the biodiesel product—were required and not suitable for large-scale production.

Many researchers have recently become interested in using ionic liquids as reaction media for biotransformation [16–18]. Ionic liquids are functional solvents with unique properties such as negligible volatility, thermal stability, and relatively high polarity. Diverse types of biocatalysts including lipases [19], proteases [20,21], and whole-cell biocatalysts [22,23] have so far been used

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for biotransformation in ionic liquids. Recently, Koo and coworkers reported biodiesel fuel production by immobilized *Candida antarctica* lipase B (Novozyme 435) in ionic liquids [24]. These reports prompted us to employ ionic liquids as reaction media in biodiesel production by whole-cell biocatalysts.

In the present study, we have investigated biodiesel production in ionic liquids catalyzed by lipase-producing *R. oryzae* and *A. oryzae* as whole-cell biocatalysts. In this system, the whole-cells can be used as a lipase-immobilized matrix, and ionic liquids would work as the reservoir phase of methanol to avoid deactivation of the biocatalysts. Ionic liquids, which are totally composed of salt, do not dissolve ME and unreacted oil, and form biphasic systems, where the recovery of ME would be more facile. Moreover, ionic liquids can also be expected to act as an extracting phase for the by-product glycerol [25], which often has a negative effect on lipase activity in biodiesel production [26]. In the present study, we demonstrated the availability of ionic liquids in biodiesel production catalyzed by whole-cell biocatalysts.

2. Materials and methods

2.1. Materials

1-Ethyl-3-methylimidazolium tetrafluoroborate ([Emin][BF_4]), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmin][BF_4]) were purchased from Kanto Chemical Co. (Tokyo, Japan), and 1-ethyl-3-methylimidazolium trifluoromethylsulfonate ([Emin][TfO]) was obtained from Merck. Soybean oil and methanol were purchased from Nacalai Tesque (Kyoto, Japan) and 25% glutaraldehyde solution was obtained from Wako Pure Chemical Ind. (Osaka, Japan). Fungal strains, *R. oryzae* IFO 4697 and *A. oryzae* niaD300, were obtained from the NITE Biological Resource Center (NBRC, Chiba, Japan) and the National Research Institute of Brewing (Hiroshima, Japan), respectively. Biomass support particles (BSPs: Bridgestone Co. Ltd., Osaka, Japan) used for cell immobilization were 6 mm × 6 mm × 3 mm cuboidal solids of reticulated polyurethane foam with a particle voiding of more than 97% and a pore size of 50 pores per linear inch. All other chemicals used were of analytical reagent grade.

2.2. Whole-cell biocatalysts

Two types of fungal strains, *R. oryzae* IFO 4697 and *A. oryzae* niaD300, were used as a host strain. Since wild-type *R. oryzae* (w-ROL) originally produces 1,3-position-specific lipases, this strain was used without genetic manipulation. Alternatively, *A. oryzae* was genetically engineered to produce *Fusarium heterosporum* lipase (r-FHL) [12], *C. antarctica* lipase B (r-CALB) [11], and *A. oryzae* mono- and diglyceride lipase (r-mdlB) [13], according to previous reports. Table 1 summarizes the whole-cell biocatalysts used in the present study.

The BSPs (150 pieces) and the fungal spores were incubated in Sakaguchi flasks containing basal medium (100 ml, pH 5.6) composed of 70 g/l polypeptone, 1.0 g/l $NaNO_3$, 1.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, and 30 g/l olive oil at 30 °C for 96 h using a reciprocal shaker (150 oscillations per min, amplitude 70 mm). The fungal cells were spontaneously immobilized on the BSPs during natural growth in shake-flask cultivation. The cells immobilized on BSPs were separated from culture broth by filtration, washed twice with distilled water, and dried for more than 48 h before use in the experiments.

Table 1
Whole-cell biocatalysts used in the present study.

Abbreviation of whole-cell biocatalysts[a]	Host fungi	Lipase (specificity)
w-ROL	*R. oryzae*	Endogenous lipase (1,3-specific)
r-FHL	*A. oryzae*	*F. heterosporum* lipase (1,3-specific)
r-CALB	*A. oryzae*	*C. antractica* lipase B (nonspecific)
r-mdlB	*A. oryzae*	*A. oryzae* mono-diglyceride lipase (mono-diglyceride specific)

[a] Prefix w- and r- means wild-type or recombinant fungi, respectively.

2.3. Methanolysis of soybean oil catalyzed by whole-cell biocatalysts in ionic liquids

Methanolysis of soybean oil was carried out in the presence of ionic liquids. Typically, soybean oil (1 ml), ionic liquids (1 ml), methanol (molar ratio of methanol/soybean oil is 1–8), and distilled water (5 wt.% for soybean oil) were mixed in a 10 ml screw-capped vial, where a biphasic system was formed. Subsequently, whole-cell biocatalysts immobilized on BSPs were added to the reaction mixture to initiate methanolysis in a reciprocal shaker at 250 rpm and 30 °C. Dioleoylglycerol was used as a substrate for r-mdlB. In the ionic liquid-free system, the same reaction mixtures as above were prepared without adding ionic liquids.

Each sample (100 μl) was taken from the reaction mixture periodically and centrifuged at 12,000 rpm for 5 min to complete phase separation (upper phases: ME and unreacted soybean oil, lower phases: ionic liquids, methanol, and water). For ME analysis, the upper phase (20 μl) was mixed with tricaprylin (10 μl, internal standard) in a 2 ml tube, followed by the addition of hexane (1.5 ml) and an appropriate amount of anhydrous sodium sulfate. Then, 1.0 μl of the treated sample was injected into the GC-2010 gas chromatograph (Shimadzu Co., Kyoto, Japan), which was equipped with a ZB-5HT capillary column (0.25 mm × 15 m; phenomenex, USA). The column temperature was kept at 130 °C for 2 min, raised to 350 °C at a rate of 10 °C/min, then to 370 °C at a rate of 7 °C/min, and finally maintained at this temperature for 10 min. The ME content was determined to be the ratio of ME in total components in the reaction mixtures without water and glycerol. All experiments were conducted in triplicate: data represent the average of three experiments and error bars indicate the standard deviation.

2.4. Stability test of the biocatalysts in ionic liquids

Whole-cell biocatalysts immobilized in BSPs (20 particles) were incubated in ionic liquids (5 ml) at 30 °C for 72 h, followed by washing twice with deionized water to remove the ionic liquids. Subsequently, residual activities of the biocatalysts exposed to ionic liquids were measured in methanolysis without adding ionic liquids. The same reaction was conducted with the biocatalysts that were not exposed to ionic liquid but incubated in air at 30 °C as a control to evaluate the effect of the ionic liquid.

2.5. Cross-linking of whole-cell biocatalyst on BSPs with glutaraldehyde

Cross-linking of whole-cell biocatalysts with glutaraldehyde was carried out by adding 0.1 vol% of glutaraldehyde solution (200 ml) to BSP-immobilized w-ROL (200 particles) and incubating them in a bioshaker (25 °C, 250 rpm) for 1 h. The cross-linked cells were separated from the solution by filtration, followed by washing with deionized water and drying for 24 h at room temperature to obtain glutaraldehyde-treated whole-cell biocatalysts.

In recycling tests, the whole-cell biocatalysts were directly used for the next methanolysis with no treatment.

3. Results and discussion

3.1. Activity of whole-cell biocatalysts in ionic liquids

While much research has been intensively investigated in biocatalytic reactions in ionic liquids, few studies have focused on biotransformation using whole-cell biocatalysts. In such a system, the catalytic performance of whole-cell biocatalysts are known to depend on ionic liquids and fungus species [22,23,27]. In the present study, wild-type *R. oryzae* producing original triacylglycerol lipase (w-ROL) and recombinant *A. oryzae* genetically engineered to produce *F. heterosporum* lipase (r-FHL) [12], *C. antarctica* lipase B (r-CALB) [11], and *A. oryzae* mono- and diglyceride lipase (r-mdlB) [13] were used as whole-cell biocatalysts (Table 1). These fungus cells were successfully immobilized on BSPs during cultivation. As reaction media for biodiesel production, four kinds of imidazolium-based ionic liquids were examined. In our preliminary test, these ionic liquids did not form an emulsion in the reaction mixture and thus seemed to be suitable for reaction media. First, we examined the catalytic activity of these four types of whole-cell biocatalysts in ionic liquid- and ionic liquid-free system (Fig. 1). ME production was performed using soybean oil with 4-molar methanol catalyzed by the biocatalysts. In conventional ionic liquid-free system, r-mdlB exhibited an activity in ME production whereas the activities of the other biocatalysts were negligible. It appeared that r-mdlB showed a little higher tolerance to methanol compared with other biocatalysts. On the other hand,