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Ethanolysis of rapeseed oil to produce biodiesel fuel catalyzed by *Fusarium heterosporum* lipase-expressing fungus immobilized whole-cell biocatalysts

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ABSTRACT

We demonstrated ethanolysis of rapeseed oil to produce biodiesel fuel using lipase-producing filamentous fungi immobilized on biomass support particles (BSPs) as a whole-cell biocatalyst. We prepared two types of whole-cell biocatalyst: wild-type *Rhizopus oryzae* producing triacylglycerol lipase (w-ROL) and recombinant *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase (r-FHL). Both w-ROL and r-FHL successfully catalyzed the ethanolysis of rapeseed oil, and the fatty acid ethyl ester yield was as high as 79% (w-ROL) or 94% (r-FHL). In the case of r-FHL, the residual monoglycerides (MGs) and diglycerides (DGs) were no more than 0.73 and 0.18%, respectively. In addition, r-FHL could be recycled for the ethanolysis of rapeseed oil, retaining over 85% fatty acid ethyl ester yield by the fifth cycle. r-FHL was revealed to be a promising catalyst for biodiesel production using rapeseed oil and ethanol.

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1. Introduction

Biodiesel fuel (BDF) produced by alcoholysis of vegetables oils or animal fats is viewed as a promising renewable, biodegradable, and nontoxic fuel [1]. The use of BDF is becoming increasingly important due to diminishing petroleum reserves and environmental regulations. Although an alkaline-catalyst method has been applied to BDF production, increasing environmental concerns have led to a growing interest in alternatives such as lipase catalysis, as it can avoid conventional difficulties in the recovery of glycerol, potassium, and/or sodium salt [2–4]. However, lipase-catalyzed BDF production is limited due to its higher preparation cost.

Recently, numerous studies have been published on the use of microbial lipases as whole-cell biocatalysts [2,5–12]. In particular, the direct use of lipase-producing fungal cells immobilized within porous biomass support particles (BSPs) is cost-effective, because it requires no purification or complicated immobilization processes [13,14]. *Rhizopus oryzae*, which naturally produces a 1,3-positional-specific triacylglycerol lipase, is one of the most studied fungi in lipase-catalyzed methanolysis [5–7]. Our previous studies revealed that *R. oryzae* immobilized on BSPs can efficiently catalyze methanolysis with the addition of an appropriate amount of water [10], and that a packed-bed reactor (PBR) containing BSP-immobilized *R. oryzae* can be used for long-term repeated batch methanolysis, giving a methyl ester (ME) content of around 80–90% after 10 batch cycles [9,10].

Methanol is usually used in BDF production because it is the less costly as an absolute alcohol [2,4]. However, methanol has some drawbacks such as toxicity, and it is a petroleum-derived alcohol, hence it would be preferable to use ethanol as an alcohol in BDF production because it is less toxic and can be produced from various kinds of biomass [15–19]. Although lipase-catalyzed BDF production using methanol has been extensively studied, few studies have focused on BDF production using ethanol.

Here, we report that lipase-producing fungal cells immobilized on BSPs can catalyze the alcoholysis of rapeseed oil with ethanol. In this study, as whole-cell biocatalysts, lipase-expressing wild-type fungus *R. oryzae* (w-ROL) and recombinant *Aspergillus oryzae* expressing lipase from *Fusarium heterosporum* (r-FHL) were prepared. We optimized ethyl ester productivity and demonstrated repeated batch alcoholysis reactions retaining high ethyl ester content.

Abbreviations: BDF, biodiesel fuel; BSPs, biomass support particles; w-ROL, wild-type *Rhizopus oryzae* producing triacylglycerol lipase; r-FHL, recombinant *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase; ME, methyl ester; FFA, free fatty acid; MG, monoglyceride; DG, diglyceride; TG, triglyceride.

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2. Materials and methods

2.1. Strains, media, and culture conditions

The filamentous fungus *R. oryzae* IFO 4697, which produces a 1,3-position specific lipase, was used without genetic manipulation. *R. oryzae* was grown in a basal medium consisting of 70 g/l polypeptone, 1.0 g/l $NaNO_3$, 1.0 g/l KH_2PO_4, and 0.5 g/l $MgSO_4 \cdot 7H_2O$. Glucose or olive oil as the sole carbon source was added to the medium at a concentration of 10 or 30 g/l, respectively. Recombinant *A. oryzae*, which expresses lipases from *F. heterosporum* [11,20,21], was grown in dextrin–peptone (DP) medium consisting of 20 g/l glucose, 20 g/l polypeptone, 5 g/l KH_2PO_4, and 0.5 g/l $MgSO_4 \cdot 7H_2O$. The pHs of the basal and DP media were initially adjusted to 5.6 and 6.8, respectively.

2.2. Preparation of BSP-immobilized cells

Sakaguchi flasks (500 ml) containing 100 ml DP medium, fungal spores, and 150 BSPs were incubated at 30 °C for 96 h on a reciprocal shaker (150 opm). The BSPs used for cell immobilization were 6 mm × 6 mm × 3 mm cuboids of reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage of more than 97% and a pore size of 50 pores per linear inch (ppi). Fungal cells were spontaneously immobilized within BSPs as a natural consequence of their growth during shake-flask cultivation. The resulting BSP-immobilized cells were separated from the culture broth by filtration and washed with distilled water. Immobilized *R. oryzae* cells were dried at room temperature for 48 h, and immobilized *A. oryzae* cells were lyophilized for 24 h before use in subsequent experiments. The prepared whole-cell biocatalysts, wild-type *R. oryzae* and recombinant *A. oryzae* expressing *F. heterosporum* lipase, are abbreviated as w-ROL and r-FHL, respectively.

2.3. Cross-linking of immobilized w-ROL with glutaraldehyde

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

2.4. Cell weight on BSPs and lipase activity assay

The cell weight immobilized on one BSP was measured according to the previous work [12]. Ten pieces of BSPs immobilizing fungi cells were taken, washed with acetone for 10 min to remove adherent olive oil, and dried for 24 h at 80 °C. The BSPs plus dried cells were then weighed and treated with sodium hypochlorite solution (approximately 10 vol.%) to remove the immobilized cells. The weight of immobilized cell was estimated from the difference between the weights.

Lipase hydrolysis activity of the whole-cell biocatalysts was measured using hydrolysis of *p*-nitrophenyl butyrate (pNPB) as a chromogenic substrate [22]. The reaction was conducted using 0.01% pNPB solution and two pieces of the whole-cell biocatalysts (BSPs) at 30 °C. After the reaction for 10 min, trichloroacetate was added to the reaction mixture to stop the reaction, followed by measurement of absorption of product *p*-nitrophenol at 400 nm. As a control, the same reaction was performed without the whole-cell biocatalysts. Units are defined as the micromole of the product generated per minute.

Table 1
Characteristic evaluation of the whole-cell biocatalysts.

	Immobilized cell weight on BSPs (mg/BSP)	Lipase activity of the cell (U/g-dry cell)
w-ROL	2.11	8.12
r-FHL	2.48	2.67

2.5. Alcoholysis reaction

The alcoholysis reaction was carried out in a screw-capped bottle at 30 °C with incubation in a reciprocal shaker (150 opm). The composition of the reaction mixtures in the batch operations were as follows: for methanolysis: rapeseed oil 9.65 g, methanol 0.35 g; for ethanolysis: rapeseed oil 9.5 g, ethanol 0.5 g; for propanolysis: rapeseed oil 9.37 g, 1-propanol or 2-propanol 0.63 g; for butanolysis: rapeseed oil 9.23 g, 1-butanol 0.77 g. These were mixed in 50 ml screw-capped bottles with 100 pieces of BSPs and 0.5 ml distilled water. To fully convert the oil to its corresponding alkyl esters, each one molar equivalent of alcohol was added stepwise at 24, 48, and 72 h. For the repeated use of the enzyme, BSP-immobilized cells were dried at room temperature after washing with tap water, and alcoholysis was carried out five times with fresh reaction substrates.

2.6. Gas chromatography analysis

Samples were obtained from the reaction mixture at intervals and centrifuged at 12,000 rpm for 5 min for phase separation. The upper oil layer was analyzed using a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) connected to a ZB-5HT capillary column (0.25 mm × 15 m; Phenomenex, USA). The temperatures of the injector and detector were set at 320 and 370 °C, respectively. The column temperature was initially maintained at 130 °C for 2 min, increased to 350 °C at 10 °C/min, then to 370 °C at 7 °C/min, and finally maintained at this temperature for 10 min. Chromatographic peaks were identified by comparing their retention times with those of a standard solution. Tricaprylin served as the internal standard for the quantification of the alkyl esters, free fatty acids (FFAs), monoglycerides (MGs), diglycerides (DGs), and triglycerides (TGs) in the reaction mixture. The contents were calculated as the ratio of each material present in the reaction mixture without water and glycerol. The detailed procedure for the determination of alkyl ester content is described in a previous paper [12].

3. Results and discussion

3.1. Alkyl ester production with various kinds of alcohols using w-ROL and r-FHL

In the present study, we prepared two types of whole-cell biocatalysts, w-ROL and r-FHL. Lipase-producing fungal cells were spontaneously immobilized on BSPs during cultivation. We could obtain immobilized lipase as a whole-cell biocatalyst without any complex and laborious procedures. We examined the amount of cell immobilized on BSPs and the lipase activity of each whole-cell biocatalyst (Table 1). It was revealed that the amount of the cells on BSPs in w-ROL and r-FHL was 2.11, 2.48 mg per BSP, respectively. Adhesion of the fungal cells was appeared to be similar in these biocatalysts. On the other hand, the hydrolysis activity of w-ROL and r-FHL was shown to be 8.12, 2.67 U/g-dry cell, respectively. Although it is very difficult to estimate the amount of lipase which takes part in the reaction in the whole-cells, the amount of active lipase is likely to be higher in w-ROL than that in r-FHL.

Then, we studied the alcoholysis of oil with various kinds of alcohols using lipase-expressing fungi immobilized on BSPs. A mix-

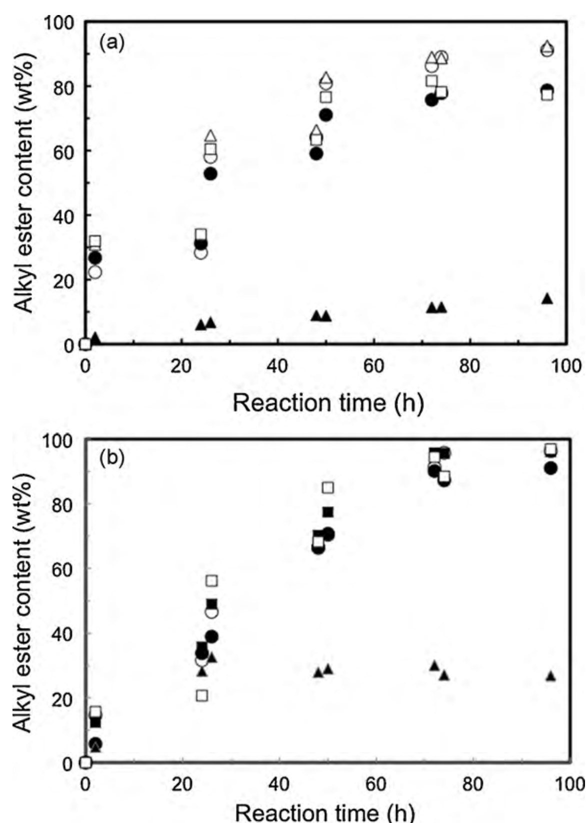

Fig. 1. Time-course of alkyl ester content by alcoholysis of rapeseed oil with various alcohols using w-ROL (a) or r-FHL (b). Symbols: (○) methanol; (●) ethanol; (△) 1-propanol; (▲) 2-propanol; (□) 1-butanol.

ture of one molar alcohol and rapeseed oil was incubated with w-ROL or r-FHL expressing fungi immobilized on BSPs at 30 °C on a reciprocal shaker. After starting the reaction, one equimolar alcohol was added stepwise at 24, 48, and 96 h. The time-course of rapeseed oil catalyzed by w-ROL and r-FHL, respectively, is shown in Fig. 1. The final fatty acid alkyl ester (FAAE) yields after 96 h are summarized in Table 2. When r-FHL was used as a catalyst, the final FAAE yields with methanol, ethanol, 1-propanol, and 1-butanol were 96, 94, 96, and 97%, respectively. In the case of w-ROL, the final the FAAE yields of methanol, ethanol, 1-propanol, and 1-butanol were 83, 79, 93, and 69%, respectively. These results clearly show that these short and linear chain alcohols are suitable for BDF production using whole-cell biocatalysts to the same degree as for purified lipase-catalyzed BDF production [3]. In contrast, the yields of 2-propylester catalyzed by w-ROL or r-FHL were significantly lower (w-ROL: 14%; r-FHL: 27%). After the alcoholysis reaction, the lipase activities of w-ROL and r-FHL were retained (data not shown), indicating that denaturation of lipase by 2-propanol would be negligible. Therefore, it can be concluded that the low yield of 2-propylester was ascribed to low reactivity of secondary alcohol, 2-propanol. However, there should be several factors which affect the transesterification efficiency, including the denaturation of lipase by alcohol, alcohol accessibility to active site of lipase, and nucleophilicity of alcohol, etc. Therefore, we could not readily conclude the reason for the effect of alcohol species.

Table 2
Fatty acid alkyl ester yields after the alcoholysis reaction using w-ROL or r-FHL. Alcohols were added at 0, 24, 48, and 78 h. Fatty acid alkyl ester yields after 96 h are shown.

	Alkyl ester content (wt%)				
	Methanol	Ethanol	1-Propanol	2-Propanol	1-Butanol
w-ROL	83	79	93	14	69
r-FHL	96	94	96	27	97

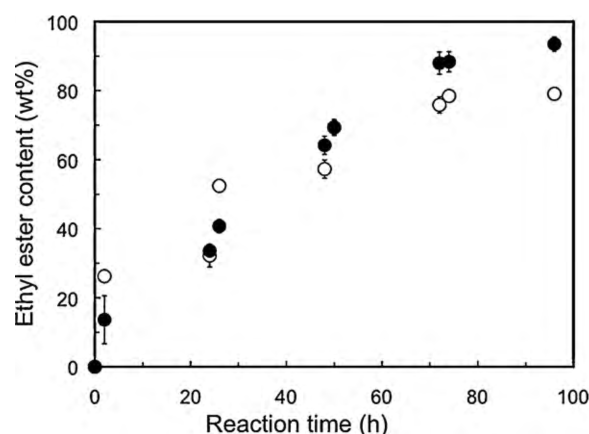

Fig. 2. Time-course of ethanolysis of rapeseed oil with w-ROL or r-FHL. Symbols: (○) w-ROL; (●) r-FHL. Reaction conditions: 5 wt% water concentration based on oil and alcohol; stepwise addition of one molar equivalent alcohol to oil at 0, 24, 48, and 72 h; temperature: 30 °C.

3.2. Time-course and remaining compounds of the ethanolysis reaction using w-ROL and r-FHL

The time-courses of ethanolysis of rapeseed oil catalyzed by w-ROL and r-FHL are shown in Fig. 2. One equimolar amount of alcohol to oil was added stepwise at 24, 48, and 72 h. Although r-FHL showed a relatively lower initial reaction rate, the final fatty acid ethyl ester (FAEE) yield reached a high value of over 94% after 96 h. In contrast, w-ROL showed a higher initial reaction rate compared to r-FHL, but the final FAEE yield by w-ROL resulted in a relatively lower conversion of 79% after 96 h. As can be seen in Table 1, the amounts of the cell on the BSPs were almost same in w-ROL and r-FHL while the lipase activities were considerably different. The higher ethanolysis rate by w-ROL would be due to the higher lipase activity. On the other hand, as for the final FAEE yield after 96 h, higher conversion was obtained in r-FHL system. Both *R. oryzae* lipase and *F. heterosporum* lipase are characterized by 1,3-positional specificity, hence fatty acid alkyl esters would be formed by spontaneous migration of the acyl groups from the second position to the outer positions. Higher FAEE yield achieved by r-FHL was probably due to the higher acyl migration ability of r-FHL [11]. To confirm the acyl migration in the esterification, the composition of the reaction mixture in ethanolysis reaction catalyzed by the two whole-cells were checked by thin-layer chromatography (TLC). When r-FHL was used, the concentration of 2-MGs, 1,2(2,3)-DGs, which has the acyl group in 2-position, was appeared to be low compared with that of w-ROL (data not shown). These results would suggest that r-FHL has excellent acyl migration ability and accelerate the transesterification.

From a practical point of view, produced BDF should meet extremely high standards; the concentrations of residual TGs, DGs, and MGs in FAEE should be negligible. Components of the reaction mixture after ethanolysis of rapeseed oil by w-ROL and r-FHL are shown in Table 3. When r-FHL was used, residual MGs and DGs were 0.73 and 0.18%, which were 100-fold lower compared to those of w-ROL. In addition, TGs were not detected in the r-FHL system, which suggests that r-FHL could completely convert rapeseed oil to ethyl ester without producing MGs and DGs. In the w-ROL system,

Table 3
Components of the reaction mixture after ethanolysis of rapeseed oil by w-ROL and r-FHL for 96 h.

Components (wt%)	w-ROL	r-FHL
FAEE	79	94
MGs	13	0.73
DGs	10	0.18
TGs	0.17	n.d.
FFAs	6.6	1.4

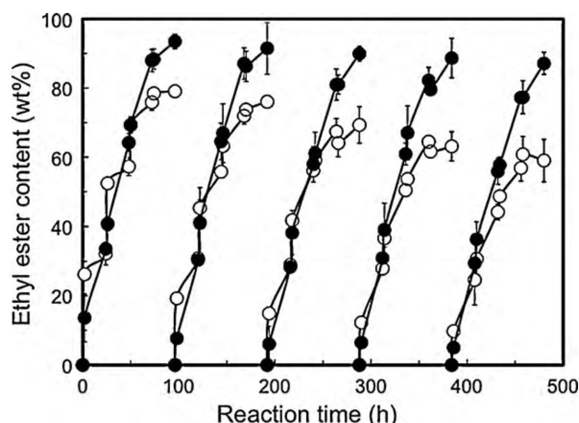

Fig. 3. Time-course of ethyl ester content during 5 repeated batch cycles of ethanolysis of rapeseed oil with w-ROL or r-FHL. Symbols: (○) w-ROL; (●) r-FHL. Reaction conditions: 5 wt% water concentration based on oil and alcohol; stepwise addition of one molar equivalent alcohol to oil at 0, 24, 48, and 72 h; temperature: 30 °C.

MGs and DGs remained at 13 and 10% probably due to the lower acyl migration ability of w-ROL.

3.3. *Recycling of whole-cell biocatalysts in repeated batch ethanolysis*

To reduce the cost of BDF production and simplify the production process, repeated batch ethanolysis is a useful approach. After one cycle of ethanolysis, whole-cell biocatalysts were washed to remove glycerol, and then mixed with fresh rapeseed oil and ethanol for the next ethanolysis reaction. The time-course of FAEE content in five repeated cycles of ethanolysis using w-ROL or r-FHL is shown in Fig. 3. In the w-ROL system, the FAEE production rate gradually decreased with repeated cycles, resulting in an FAEE content of about 60% on the fifth cycle. In our previous study, the detachment of fungi cells from BSPs could not be observed [9], suggesting that the decrease of the FAEE yield during repeated reactions could be caused by deactivation of w-ROL. On the other hand, when r-FHL was used as the catalyst, the FAEE content reached a high value of over 90% in the first cycle and retained its original productivity after five cycles. Lipase from *F. heterosporum* has been reported to show high organic solvent tolerance [11,20], which should contribute to higher FAEE production by r-FHL even in harsh ethanolysis conditions. It has been also reported that a glycerol layer formed on the surface of the BSPs could cause the loss of lipase activity during repeated use of whole-cell biocatalysts [23]. However, in the r-FHL system, no significant negative effect of residual glycerol was observed.

4. Conclusion

We successfully demonstrated biodiesel fuel production from rapeseed oil and ethanol using whole-cell biocatalysts. Although both w-ROL and r-FHL catalyzed the ethanolysis reaction, r-FHL was revealed to be more suitable for repeated ethanolysis reactions since it retained higher activity. Our results also showed that the r-FHL-catalyzed ethanolysis reaction achieved higher FAEE productivity, to a degree almost the same as with methanolysis, which demonstrates the feasibility of BDF production using bioethanol in place of methanol. Our whole-cell biocatalyst needs a small amount of water (5–15%), suggesting that water-containing bioethanol could be directly utilized. Our results should expand the utility of whole-cell biocatalysts for BDF production by ethanolysis.

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