Whole cell biocatalyst for biodiesel fuel production utilizing *Rhizopus oryzae* cells immobilized within biomass support particles

Kazuhiro Ban\(^{a}\), Masaru Kaieda\(^{b}\), Takeshi Matsumotoc\(^{c}\), Akihiko Kondob\(^{b}\), Hideki Fukuda\(^{c,\ast}\)

\(^{a}\) Fujikin Incorporated, 90 Sinke-Higashi, Higashi-Osaka 577-0026, Japan
\(^{b}\) Department of Chemical Science and Engineering, Faculty of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
\(^{c}\) Division of Molecular Science, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

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Abstract

As part of a research program aimed at producing biodiesel fuel from plant oils enzymatically cells of *Rhizopus oryzae* (*R. oryzae*) IFO4697 (with a 1,3-positional specificity lipase) immobilized within biomass support particles (BSPs) were investigated for the methanolysis of soybean oil. The *R. oryzae* cells easily became immobilized within the BSPs during batch operation. To enhance the methanolysis activity of the immobilized cells under the culture conditions used, various substrate-related compounds were added to the culture medium. Among the compounds tested, olive oil or oleic acid was significantly effective. In contrast, no glucose was necessary. Immobilized cells were treated with several organic solvents, but none gave higher activity than untreated cells. When methanolysis was carried out with stepwise additions of methanol using BSP-immobilized cells, in the presence of 15% water the methyl esters (MEs) content in the reaction mixture reached 90% — the same level as that using the extracellular lipase. The process presented here, using a whole cell biocatalyst, is considered to be promising for biodiesel fuel production in industrial applications. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In recent years, methyl esters (MEs) produced by methanolysis of plant oils, commonly referred to as biodiesel fuel, have become viewed as promising renewable sources of fuel, since they emit much lower levels of the sulfur oxides, halogens, and soot in their exhaust gas than petroleum diesel fuel. Utilization of lipase as a catalyst for biodiesel fuel production has great potential compared with chemical methods using an alkaline catalyst because no complex operations are needed, not only for the recovery of glycerol but also in eliminating of the catalyst and salt.

We have already reported that several lipases efficiently catalyzed the methanolysis of soybean oil without an organic solvent [1–4]. In a very low-water-content system (i.e. a microaqueous system) plant oil methyl esters (MEs) could be efficiently synthesized with immobilized *Candida antarctica* lipase [1–3]. In contrast, the extracellular lipase from *Rhizopus oryzae* (*R. oryzae*) catalyzed methanolysis in the presence of 4–30% water in the starting materials, whereas, the lipase was nearly inactive in the absence of water [4]. With these characteristics *R. oryzae* lipase is considered to be a potentially effective enzyme when waste oil is used as the substrate, since this inevitably contains a certain amount of water. However, the use of extracellular *R. oryzae* lipase as a catalyst requires complicated recovery, purification, and immobilization processes for the enzyme, which are disadvantages for industrial applications.

Fukuda and co-workers [5–7] have reported a promising process for the industrial interesterification of fats and oils. Unlike conventional methods using extracellular lipase obtained by recovery from the culture broth, acetone-dried cells of *R. chinensis* immobilized within polyurethane foam particles (BSPs) could be used directly as a whole cell biocatalyst.

In the present study, in order to utilize immobilized *R. oryzae* cells within BSPs as a whole cell biocatalyst, the culture conditions for lipase production were first examined, and the effects of pre-treatment of the cells and the water content in the reaction mixture on methanolysis were then investigated.
2. Materials and methods

2.1. Microorganism and media

All experiments were carried out using *R. oryzae* IFO 4697, which has a 1,3-positional specificity lipase. The basal medium used contained in 11 tap water: polypepton 70 g; NaNO3 1.0 g; KH2PO4 1.0 g, and MgSO4·7H2O 0.5 g. To examine the effect of substrate-related compounds, olive oil, oleic acid, oleyl alcohol, methyl caprate or Tween-80 was added to the basal medium. Glucose was also added when necessary.

2.2. Biomass support particles (BSPs) and shake-flask cultivation

Sakaguchi flasks (500 ml) containing 100 ml of the basal medium were inoculated by aseptically transferring spores from an agar slant, and incubated for 80–90 h at 35°C on a reciprocal shaker (150 oscillations/min; amplitude 70 mm) with BSPs. The *R. oryzae* cells became well immobilized within the BSPs as a natural consequence of their growth during shake-flask cultivation. The BSPs used for immobilization were 6-mm cubes of reticulated polyurethane foam (Bridgestone Co., Ltd., Osaka) with a particle voidage beyond 97% and a pore size of 50 pores per linear inch. Immobilization was effected by placing 150 particles inside a flask together with the medium subjected to prior sterilization.

2.3. Preparation of cells immobilized within BSPs

BSP-immobilized cells were separated from the culture broth by filtration. After washing them with tap water, the BSPs were treated with an organic solvent (methanol, ethanol, isopropanol or acetone) by immersion for 0–30 min, or left untreated, and then, dried under a vacuum for about 48 h. Immobilized cells with a water content of approximately 5% were obtained and used as a methanolysis catalyst. The filtrate from the culture broth was used for analysis of the hydrolysis activity.

2.4. Enzyme reaction

The methanolysis and hydrolysis reactions were carried out at 35°C in a 50-ml screw-cap bottle with incubation on a reciprocal shaker (150 oscillations/min; amplitude 70 mm). The compositions of the reaction mixtures were as follows: for the methanolysis, soybean oil 9.65 g, 0.1 M acetate buffer (pH 5.6) 0–3.0 ml, and methanol 0.35 g, were added with 50 BSPs to a screw-cap bottle; for the hydrolysis reaction, 0.1 M acetate buffer (pH 5.6) 9.0 ml, 0.1 M CaCl2 1.0 ml, olive oil 2.0 g, and culture broth 0.5 ml were added to a screw-cap bottle. These two mixtures were incubated for 2.5 and 1 h, respectively. One molar equivalent of methanol was 0.35 g against 9.65 g soybean oil.

To fully convert the oil to its corresponding MEs, when the ME content in the reaction mixture reached approximately 30 and 60%, 0.35 g of methanol was added twice. In this case, the reaction mixture was incubated for 72 h. The methanolysis products were analyzed by capillary gas chromatography (cGC) as described below.

2.5. Analysis

The biomass concentration within a BSP was measured as follows. Twenty particles were taken and washed with acetone several times to remove substrate-related compounds and then dried for 24 h at 100°C. The particles plus dried cells were weighed and treated with an aqueous solution of sodium hypochlorite (approximately 10% v/v) to remove biomass. The cleaned particles were rinsed, dried, and reweighed. The biomass was estimated from the difference between the weights. Water content in the BSPs was determined by Karl Fischer titration (Kyoto Electronics Mfg. Co., Ltd., Japan).

The ME and free fatty acid (FFA) contents in the reaction mixture were quantified using a GC-18A gas chromatograph (Shimadzu Corp., Kyoto) connected to a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Folsom, CA). Samples (150 μl) were taken from the reaction mixture at specified times and centrifuged to obtain the upper layer.

For cGC analysis, 100 μl of the upper layer and 20 μl tricaprylin were precisely measured into a 10-ml bottle, to which a specified amount of anhydrous sodium sulfate as a dehydrating agent and 3.0 ml hexane were added. Tricaprylin served as the internal standard for cGC. A 1.0-μl aliquot of the treated sample was injected into a GC-18A gas chromatograph connected to a DB-5 capillary column (0.25 mm × 10 m) to determine the ME and FFA contents in the reaction mixture. The ME and FFA contents were, respectively, determined as the ratio of MEs and FFAs converted to the reaction mixture without water and glycerol. The column temperature was held at 150°C for 0.5 min, raised to 300°C at 10°C/min, and maintained at this temperature for 3 min. The temperatures of the injector and detector were set at 245 and 250°C, respectively.

The detailed hydrolysis procedure of Fukumoto et al. [8] was followed, with 1 mmol of fatty acids liberated per min being defined as 1 unit (U).

3. Results and discussion

3.1. Effects of substrate-related compounds added in the culture medium on methanolysis activity

To examine the effects of substrate-related compounds on methanolysis activity, cells were cultivated with BSPs in the presence of various substrate-related compounds. The concentration of each compound was 30 g/l, and glucose was added at 3 g/l, to the basal medium. The BSPs were
Table 1
Effects of substrate-related compounds on lipase production by immobilized *R. oryzae* cells

<table>
<thead>
<tr>
<th>Substrate-related compound</th>
<th>Cells immobilized within BSPs</th>
<th>Extracellular hydrolysis activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell concentration (mg/BSP)</td>
<td>Methyl ester content (wt.%)</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>7.09</td>
<td>10.4</td>
</tr>
<tr>
<td>Olive oil</td>
<td>8.52</td>
<td>12.8</td>
</tr>
<tr>
<td>Oleyl alcohol</td>
<td>0.99</td>
<td>1.4</td>
</tr>
<tr>
<td>Methyl caprate</td>
<td>2.21</td>
<td>0.2</td>
</tr>
<tr>
<td>Tween 80</td>
<td>3.34</td>
<td>5.0</td>
</tr>
<tr>
<td>None</td>
<td>3.12</td>
<td>1.2</td>
</tr>
</tbody>
</table>

not treated with an organic solvent. Table 1 shows the ME contents after 2.5-h methanolysis with 15% water content in the reaction mixture.

Except for methyl caprate, the ME contents in the presence of each substrate-related compound was greater than that without them. Two of the compounds, olive oil and oleic acid, greatly enhanced the ME content. However, the extracellular hydrolysis activity was higher without the addition of a compound than with any of them. The reason may be that these compounds act to maintain lipase within the cells to some degree. On the basis of the results, olive oil was selected as the most suitable compound to enhance intracellular methanolysis activity using cells immobilized within BSPs.

Fig. 1 shows surface and cross-sectional micrographs of a 6-mm cubic particle with immobilized cells cultivated in the presence of olive oil. The cells can be seen to have formed a dense film near the particle surface, which would severely limit the amount of oxygen in the center. Cellular adhesion to the support matrix appears strong, which means the cells would not be easily released from the particles even with vigorous agitation of the reaction mixture.

To determine the optimal olive oil concentration, its initial concentration (with 3 g/l of glucose) was varied between 0 and 50 g/l (Fig. 2). The ME content increased with the olive oil concentration up to 30 g/l, but beyond this concentration, no change in ME content was observed. Consequently, 30 g/l olive oil was used in the subsequent experiments.

3.2. Effect of glucose concentration added in the culture medium on methanolysis activity

Fig. 3 shows the effect of the initial glucose concentration on the methanolysis activity. Olive oil and glucose were added to the culture medium at 30 and 3 g/l, respectively. The ME content was high an initial glucose range from 0 to 5 g/l, but beyond this it decreased gradually. A similar trend was also slightly apparent for the numbers of cells immobilized within the BSPs. It is thought that the presence of a large amount of glucose causes a reduction in lipase production,
and it was clear that no glucose was necessary to enhance the methanolysis activity of the immobilized \textit{R. oryzae} cells.

This trend is almost identical to the case of intracellular lipase production from \textit{R. chinensis} for the interesterification of fats and oils [5].

3.3. Treatments of BSPs-immobilized cells with organic solvents

To investigate the effects of organic solvents on cell permeabilization, BSP-immobilized cells were treated with three various alcohols (methanol, ethanol, or isopropyl alcohol) or acetone (all \(\geq 99\%\) pure). Fig. 4 shows the effects of the treatment time on the ME content after incubation for 2.5 h. In the cases of isopropyl alcohol and acetone, there was almost no decrease in the ME content even with 30 min treatment. In contrast, with ethanol or methanol resulted in a rapid decrease in the ME content; with methanol almost all catalytic activity was lost. These findings indicate that the use of non-treated cells is preferable to obtain a highly active, whole cell biocatalyst, and also that the concentration of methanol in the reaction mixture should be kept at a low level since methanol directly attacks the enzyme [1].

We previously reported that permeabilization of recombinant \textit{Saccharomyces cerevisiae} cells with alcohol solutions resulted in significantly effective whole cell biocatalysts for the conversion of methylglyoxal to S-lactoylglutathione [9]. However, unlike the production of S-lactoylglutathione, in which a non-permeable substrate was used, in the methanolysis of oil a certain amount of methanol is itself utilized as a substrate, resulting in a high degree of substrate permeation into \textit{R. oryzae} cells without any treatment for the cell membrane.

3.4. Effect of water content in the reaction mixture on methanolysis activity

To complete the bioconversion of soybean oil to the corresponding ME, at least three molar equivalents of methanol are necessary. Since a high methanol concentration causes irreversible denaturation [1], a molar amount of methanol equal to that of oil was added twice at the times indicated by the arrows in Fig. 5, which shows the time courses of methanolysis with stepwise additions of methanol under an increasing water content. When methanolysis was carried out with 0.4–3.0 ml acetate buffer (4.0–30 wt.% water by substrate weight), the ME content reached approximately 80–90 wt.% after 72-h reaction. The highest ME content, 91.1 wt.%, was attained when the reaction mixture contained 1.5 ml buffer solution (15 wt.% water by substrate weight). In contrast, with 0.3 ml buffer solution (3.0 wt.% water by substrate weight) the ME content was only 54.4 wt.%, and with no addition of buffer solution the ME content failed to improve after reaching approximately 33 wt.%. An insufficient amount of water probably results...
in irreversible inactivation of lipase, which may be due to denaturation of the enzyme by methanol.

We previously reported that although the extracellular lipase from \textit{R. oryzae} cells is considered to exhibit 1(3)-regiospecificity, acyl migration can occur spontaneously in a water-containing system \cite{4}. The ME content using cells immobilized within BSPs reached the same level as that using the extracellular lipase. This clearly demonstrates that acyl migration occurred with the intracellular lipase of the immobilized cells as it did in the case of the extracellular lipase. Our findings suggest that \textit{R. oryzae} cells immobilized within BSPs can effectively catalyze the methanolysis reaction as a whole cell biocatalyst in water-containing systems.

In industrial applications using BSP-immobilized cells, they need to stable enough for repeated use. Thus, the stability of whole cell biocatalysts is currently under investigation.

4. Conclusion

In this study, to utilize immobilized \textit{R. oryzae} cells within BSPs as a whole cell biocatalyst for biodiesel fuel production, both the culture conditions for lipase production and methanolysis conditions were investigated. To enhanced the methanolysis activity of the immobilized cells olive oil or oleic acid added in the culture broth was significantly effective, but no glucose was necessary. The high methyl ester content over 90% obtained in water-containing systems was probably attributable to the spontaneous acyl migration.

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