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Improved performance of a packed-bed reactor for biodiesel production through whole-cell biocatalysis employing a high-lipase-expression system

Ayumi Yoshida[a], Shinji Hama[b], Naoki Tamadani[b], Hideki Fukuda[c], Akihiko Kondo[a,*]

[a] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
[b] Bio-energy Corporation, Research and Development Laboratory, 2-9-7 Minaminanamatsu, Amagasaki 660-0053, Japan
[c] Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

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ABSTRACT

To improve enzymatic biodiesel production, we developed a packed-bed reactor (PBR) system using recombinant cells, into which a strong enolase promoter and 5′ untranslated region were introduced. *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase was immobilized within biomass support particles (BSPs) during cultivation and used directly as a whole-cell biocatalyst. BSP-immobilized *A. oryzae* carrying three copies of the expression cassette showed a higher performance than previously developed cell systems, resulting in 87.5% conversion after 10 passes in PBR. The performance was also affected by operational factors including residence time and methanol feeding. After optimization, the PBR system attained a final methyl ester content of 96.1% with a residence time of 140 min per pass and stepwise addition of 4.25 molar equivalents of methanol to oil for 6 passes. Moreover, lipase activity was maintained for 5 batch cycles. Therefore, the developed PBR employing a high-lipase-expression system is considered useful for improving enzymatic biodiesel production.

1. Introduction

Biodiesel fuel (fatty acid methyl esters), which is produced by methanolysis of triglycerides, has received considerable attention as an alternative fuel for diesel engines. Of the several processes so far proposed, chemical catalysis using alkalis and acids has been employed widely, but has some drawbacks: the catalyst used has to be removed from the product; wastewater treatment is required; and free fatty acids and water interfere with the reaction [1,2]. To overcome these drawbacks, many attempts have recently been made to develop an enzymatic process using (extra- or intracellular) lipase as a catalyst. A considerable advantage compared with chemical methods is the elimination of complex downstream processes including the removal of the catalyst and salt. However, most of the enzymatic methods using extracellular lipase require procedures for the purification of enzymes, leading to an obstacle for practical application [1].

The direct use of intracellular lipase as a whole-cell biocatalyst is a promising approach. In particular, a technique utilizing lipase-producing microorganisms immobilized within porous biomass support particles (BSPs; [3]) requires no complex procedures for enzyme purification and immobilization because cells can be immobilized within BSPs as a natural consequence of their growth [4]. Thus far, BSP-immobilized *Rhizopus oryzae* has become a promising candidate, as shown by extensive research studies [5–7], which provided important knowledge including the requirement of a certain amount of water and stepwise methanol addition to prevent irreversible lipase inactivation. Moreover, to improve enzymatic biodiesel production, *Aspergillus oryzae* was transformed using several recombinant lipases. A previous study showed that the immobilized recombinant *A. oryzae* carrying *Fusarium heterosporum* lipase (FHL) exhibits a high efficiency of converting triglycerides to methyl esters because FHL expressed efficiently by *A. oryzae* exhibits enzymatic characteristics for methanolysis different from those of *R. oryzae* carrying the wild-type lipase (the w-ROL strain) [8]. However, the specific methanolysis activity of recombinant *A. oryzae* generated using a P-No8142 promoter [13,14] is insufficient for practical application.

Previously, a novel *A. oryzae* whole-cell biocatalyst generated using a high-lipase-expression system was developed to improve biodiesel production [9]. In this system, a strong enolase promoter harboring a functional *cis* element (P-*enoA*142; [10]) and a 5′ untranslated region of a heat-shock protein (Hsp-UTR; [11]) were introduced to increase lipase expression level. In this study, we firstly attempted to use recombinant *A. oryzae* employing the aforementioned system, into which increased copy numbers of the expression cassette were introduced. We herein show that the developed whole-cell biocatalyst attains a higher performance than previously developed biocatalysts.

* Corresponding author. Tel.: +81 78 803 6196; fax: +81 78 803 6196.
E-mail address: akondo@kobe-u.ac.jp (A. Kondo).

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To further show the applicability to enzymatic biodiesel production, we developed a packed-bed reactor (PBR) system, in which a reactant solution is pumped through a column containing BSP-immobilized cells and the effluent from the column is recycled into the same column with a stepwise addition of methanol. PBR, one of the most commonly used reactor types in biotechnology, has an advantage of a low shear stress on immobilized enzymes with a high bed volume [12]. Thus, the PBR system herein developed would provide an important basis for the establishment of continuous biodiesel production with long-term enzyme stability. However, regarding a recombinant *A. oryzae* whole-cell biocatalyst, there has been no report on the performance of PBR for enzymatic biodiesel production. In this study, we evaluated the catalytic performance of the developed whole-cell biocatalysts in PBR. The best performance was obtained by using recombinant *A. oryzae* carrying three copies of the FHL-expression cassette. We then investigated the basic key factors related to reaction efficiency in enzymatic biodiesel production.

2. Materials and methods

2.1. Strains and media

R. oryzae IFO4697 was routinely maintained on an agar slant made from 4% potato dextrose agar and 2% agar. Recombinant *A. oryzae* carrying pNAN8142-FHL (hereafter, the pNAN8142-FHL strain), which possesses the P-No8142 promoter [13,14], a gene-encoding FHL [8], and *niaD* as a selectable marker, was routinely maintained on Czapek-Dox (CD) medium [2% glucose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% KCl (w/v), 0.8 M NaCl, 0.001% (v/v) trace element solution (2% $CuSO_4{\cdot}5H_2O$, 1% $FeSO_4{\cdot}7H_2O$, 0.1% $ZnSO_4{\cdot}7H_2O$, 0.1% $MnSO_4{\cdot}7H_2O$, 0.1% $AlCl_3$, w/v)] containing 1.5% (w/v) agar. *A. oryzae* carrying pSENSU-FHL (hereafter, the pSENSU-FHL strain), which possesses the P-*enoA*142 promoter [10], Hsp-UTR [11], and *sC* as a selectable marker, was provided by Ozeki Co., Ltd. (Hyogo, Japan). A transformant carrying three copies of the expression cassette (determined by real-time PCR analysis) was selected and routinely maintained on 1.5% (w/v) agar-containing CD medium, in which $NaNO_2$ was used instead of $NaNO_3$. The recipients are derived from the wild-type strain RIB40 and their transformants were previously reported for both the pNAN8142-FHL [8] and pSENSU-FHL strains [9].

2.2. Preparation of BSP-immobilized cells

For cell immobilization within BSPs, 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) was cut into 6 mm cubes.

The spores of *R. oryzae* were aseptically inoculated to Sakaguchi flasks (500 ml) containing 100 ml of basal medium (3% olive oil, 7% polypeptone, 0.1% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, adjusted to pH 5.6). The cultures in flasks were incubated at 30 °C for 96 h on a reciprocal shaker (150 oscillations/min; amplitude, 50 mm). For *A. oryzae*, 100 ml of DP medium (2% glucose, 2% polypeptone, 0.5% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, adjusted to pH 6.8) was used. The cultures in flasks were incubated at 30 °C for 72 h or 96 h on a reciprocal shaker (120 oscillations/min; amplitude, 50 mm). The cells of the *R. oryzae* w-ROL strain and the *A. oryzae* pNAN8142-FHL and pSENSU-FHL strains became well immobilized within BSPs naturally during cultivation. The resulting BSP-immobilized cells were separated from the culture broth by filtration, washed with distilled water, and lyophilized for 24 h before use in subsequent experiments. Immobilized-cell weight within one BSP was measured as described previously [5].

2.3. Measurement of lipase methanolysis activity

For methanolysis in a screw-capped bottle, the reaction mixture contained 9.65 g of olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.35 g of methanol (one molar equivalent to oil), 0.5 ml of distilled water, and 25 BSPs retaining dry immobilized cells. The screw-capped bottles were incubated at 30 °C on a reciprocal shaker (150 oscillations/min; amplitude, 50 mm). The methanolysis activity liberating 1 μmol of methyl oleate per min was defined as 1 unit (U). The measurement was conducted in triplicate.

2.4. Methanolysis in PBR containing BSP-immobilized cells

2.4.1. Reactor setup

The PBR vessel consisted of a glass column (15 mm in internal diameter and 300 mm in height) and 1000 BSPs retaining dry cells. The vessel was equipped with a silicon tube and a cassette tube pump. The reaction mixture was supplied into the bottom of PBR. A schematic of the PBR system is shown in a previous paper [17]. However, as noted in the next section, recycling flow of the effluent is different.

2.4.2. Methanolysis reaction

For methanolysis in PBR, the reaction mixture contained 96.5 g of Shirashime oil (a mixture of rapeseed and soybean oils), an appropriate amount of methanol, and 5 ml of distilled water. The reaction mixture was emulsified by ultrasonication for 10 min prior to reaction. Methanolysis was performed at room temperature in PBR, where the effluent from a column was obtained at each pass at a fixed flow rate and mixed with an appropriate amount of methanol for the next pass. One pass was continued until the reservoir tank for the reaction mixture becomes empty. After one batch cycle (5–10 passes), BSP-immobilized cells were washed with water, dried under vacuum condition, and packed into the PBR vessel. Then, a fresh reaction mixture was supplied into PBR for the next batch cycle.

2.5. Gas chromatography

Samples, obtained from the reaction mixture or effluent from PBR, were centrifuged at 12,000 rpm for 5 min. The upper oil layer was analyzed using a GC-2010 gas chromatograph (Shimadzu Co., 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 comparison of their retention times with those of a standard solution. Tricaprylin served as the internal standard for measuring the contents of methyl ester (ME) and other components. 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 ME content was described in a previous paper [5].

3. Results and discussion

3.1. Methanolysis activity of BSP-immobilized cells

The performance of BSP-immobilized cells producing recombinant lipases was compared with that of cells producing native lipase in terms of their methanolysis activities (Table 1). The pNAN8142-FHL strain, which expresses FHL under the control of the P-No8142 promoter [13,14], showed a lower methanolysis activity than the