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Converting oils high in phospholipids to biodiesel using immobilized *Aspergillus oryzae* whole-cell biocatalysts expressing *Fusarium heterosporum* lipase

Jerome Amoah[a], Shih-Hsin Ho[b], Shinji Hama[c], Ayumi Yoshida[c], Akihito Nakanishi[b], Tomohisa Hasunuma[b], Chiaki Ogino[a], Akihiko Kondo[a,*]

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

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ABSTRACT

The presence of phospholipids in oil has been a major hurdle in the production of biodiesel using immobilized *Aspergillus oryzae* whole-cell biocatalysts. A density of phospholipids within the range of 10–30% could reduce both the rate of production and the final yield of biodiesel. Phospholipids in the oil leads to the formation of water-in-oil phospholipid-based reverse micelles. The water that activates the enzymatic process is observed to be trapped inside these reverse micelles. This has resulted in the inactivation of the reaction systems and has subsequently led to the deactivation of the immobilized lipase by the extended residence time of the added methanol. A reaction system involving gentle agitation and higher amount of water was found to reduce the reverse micelles formation. This simple technique improved the conversion efficiency by approximately 3-folds, producing a final biodiesel of more than 90%, using immobilized *A. oryzae* whole cells expressing *Fusarium heterosporum* lipase. This demonstrates that, the above technique could be successfully applied to the enzymatic biodiesel conversion of oils containing high amounts of phospholipids such as that from microalgae.

1. Introduction

Depleting oil reserves, increasing dependency on scarce energy and environmental deterioration from the use of fossil fuels has led to the search of cleaner and more sustainable energy. Recent advances in biotechnology are paving the way for the production of various fuels from biomass, and biodiesel has become a plausible substitute for petroleum diesel. Biodiesel is a mixture of Fatty Acid Methyl Esters (FAME) and can be directly used in existing infrastructure without modification. Biodiesel now accounts for a 78% share of the total biofuel consumption in Europe's transportation industry [1].

The cost of biodiesel production requires a cheap form of feedstock and this has shifted the feedstock to various sources [2]. Several microorganisms, such as microalgae, have been targeted as a highly promising feedstock for the commercial production of biodiesel [3,4]. Chemical conversion of microalgae oils to biodiesel has been successfully carried out by various researchers [5,6]. However, the subsequent environmental issues and the high energy requirements associated with this method make it questionable [7,8]. A more environmentally friendly and low-energy enzymatic method that has been successfully employed with various plant oils is thought to be a better alternative [9–11].

However, phospholipids represent a major setback in the application of enzymatic conversion methods to microalgae oil. Phospholipids constitute as much as 30% of the total lipid weight of the oil from these microorganisms, and are known to have a negative effect on these enzymatic methods [12,13]. The phospholipids in microbial oils are known to possess highly significant portions of C:16 and C:18 polyunsaturated fatty acids, which are the best of fatty acid chains required for a high-quality biodiesel [14,15]. Watanabe et al. [16] reported that, even with of less than 1% phospholipid, the performance of immobilized *Candida antarctica* lipase was negatively affected, and that degumming of the plant oil was required for a successful conversion to biodiesel. Unfortunately, the process of degumming which has been suggested to remove phospholipids from plants oils may not be attractive for the high levels of phospholipids in microalgae, as it could lead to a great

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

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loss in feedstock—not to mention, the complete degumming of such oils may not possible [12]. Li et al. [17,18] have performed various studies on oils containing low amounts of phospholipids using free lipase, and found that the presence of phospholipids alone within the range investigated (2%) can accelerate the hydrolysis of oils, but that the co-existence of phospholipids and methanol leads to detrimental effects on free lipase.

Despite these findings, no report has documented the used of immobilized lipases for the production of biodiesel from high phospholipid-containing oils in a solvent-free system. In addition, the FAME production from oil containing amount of phospholipids that correspond to that of microbial oils using whole-cell immobilized lipases is yet to be investigated. Immobilization of lipases offers the added advantages of easy product separation, reduced contamination of product and enzymes, rapid termination of the enzyme-substrate reaction, and immobilization may also alter the activity of the lipase [19,20]. This points out a significant difference between free lipase and immobilized lipase, which suggests that applying the general conclusions from free lipase to immobilized lipases may not be warranted.

However, the industrial use of immobilized lipases has faced criticism because immobilization process often adds significant cost to the lipase, hence increasing the cost of this process [21]. To this end, Hama et al. [22] developed immobilized recombinant *Aspergillus oryzae* whole-cell biocatalysts for the production of biodiesel. These whole-cell immobilized biocatalysts differ from other immobilized catalysts in that the enzyme cultivation step and the immobilization step are combined in a single unit process, which requires no further purification. This drastically reduces the cost and duration of the enzyme preparation. Due to the simplicity and cost effectiveness of this form of immobilized lipase, it would be advantageous if this could be successfully used to convert oils with high phospholipid content.

The present study investigated the effect that the high amount of phospholipids (30%) in microbial oils exerts on the enzymatic biodiesel production by immobilized *A. oryzae* whole cell biocatalyst expressing *Fusarium heterosporum* lipase. Two major factors were surveyed: agitation and water concentration. These factors were expected to significantly affect the reaction system. This allowed for the demonstration of a simple technique to overcome the low biodiesel conversion efficiency caused by the presence of high phospholipid content.

2. Methods

2.1. Materials and strains

The host strain, *A. oryzae* NS4 (niaD-, sC-), was derived from the wild-type strain [23]. Soybean refined oil and soybean phospholipids were obtained from Wako Pure Chemical industries (Osaka, Japan).

2.2. Construction of lipase expression vector and transformation of A. oryzae

pSENSU-FHL harboring a P-enolA142 and a 5′UTR of *Hsp12* with an FHL gene and an *sC* gene as a selectable marker was constructed as described in a previous study [24]. Briefly, the gene encoding *F. heterosporum* lipase (FHL) was amplified from pNAN8142-FHL by PCR using FHL-F1 (5′-TCGCAAACATGATGCTCGTCCTATCTCTTC-3′) and FHL-R1 (5′-GCTCTAGACTAAATCATCTGCTTAACAAAT-3′) as primers. An amplified fragment was digested with *XbaI* and inserted into a pSENSU plasmid and was then digested again using *PmlI* and *XbaI*. The cells of *A. oryzae* transformant carrying pNAN8142-FHL were then transformed with pSENSU-FHL on a Czapek-Dox (CD)-NO_2-methionine selection plate [22]. The obtained lipase was then designated r-FHL.

A. oryzae biocatalyst expressing mono- and diglycerol lipase was prepared by isolating mdlB gene from *A. oryzae* niaD300 using two primers, fw-SalI (5′-TGGTCGACATGCGCTTCCTCTCCGGCTTCGTTTCTGTT-3′) and rv-SphI (5′-GTGCATGCTTAGCGCAATGGCAATCCAGGACCCTTGCA-3′). The fragments amplified by PCR were digested with *SalI* and *SphI* and inserted into the vector pSENSU. The constructed plasmid (pSENU-mdlB) was then integrated into *A. oryzae cells*. The obtained lipase was then designated r-mdlB.

2.3. Immobilized A. oryzae whole-cell biocatalyst preparation

The *A. oryzae* strains were initially cultured on a Czapek-Dox (CD) agar plate at 30 °C for 6 days. The spores were harvested with 5 ml of distilled water and aseptically inoculated into a 500 ml Sakaguchi flask containing approximately 850 mg of reticulated polyurethane foam BSPs (Bridgestone Corporation, Osaka, Japan) in 100 ml of DP medium. The BSPs used for the fungal cell immobilization had an average cuboid size of 6 mm × 3 mm × 3 mm and pore size of 50 pores per linear inch. The fungal strain was cultivated at 30 °C for 96 h on a reciprocal shaker at 150 oscillations per min. The cells were naturally immobilized in the pores of the BSPs during cultivation, and the immobilized whole cells were separated from the culture broth by simple filtration. The BSP-immobilized whole cells were washed with distilled water, lyophilized for 48 h and used for biodiesel conversion.

2.4. Lipase-mediated FAME production

Soybean oil was spiked with various amounts of phospholipids to simulate the high phospholipid content of crude oils and subjected to sonication to ensure homogeneity. The lipase-catalyzed methanolysis was carried out in screw-capped cylindrical glass tubes. The reaction mixture consisted of oil (with or without phospholipids), distilled water, immobilized *A. oryzae* whole-cell biocatalysts, and the reaction was initiated via the addition of an initial amount of methanol (1:1 molar ratio of the oil). The reaction proceeded in a block rotator (Nissin Thermo Block Rotator SN-06BN) at 30 °C with a rotation speed of 7.5 (an arbitrary scale on the instrument) with or without pre-agitation. To avoid the general deactivation of the immobilized lipase by methanol, the remaining methanol was added step-wise at 24, 48 and 72 h (1:1 molar ratios of the oil at each time leading to a total of 1:4). Samples were taken at specified times to determine the FAME production during the course of the reaction. For repeated use, the immobilized whole cells were either washed with tap water or reused directly without any treatment and applied on a fresh reaction mixture.

2.5. Gas chromatography analysis

Fatty Acid Methyl Esters produced during the course of the reaction were measured via gas chromatography. Samples taken at specified times were centrifuged at 12,000 × *g* for 5 min at 4 °C, and the upper layer was analyzed using GC-17A (Shimadzu, Kyoto, Japan) equipped with a DB-5 capillary column (0.25 mm × 15 m) (J&W Scientific, USA), an auto-sampler and a flame ionization detector. During the analysis, the temperatures of the injector and detector were set at 245 and 320 °C, respectively. The column was configured at a temperature program with an initial temperature of 150 °C for 0.5 min, raised to 300 °C at 10 °C/min and maintained at this temperature for 10 min. The FAME composition in each reaction mixture was reported as the percentage of the oil in the reaction mixture using tricaprylin as an internal standard.