Algal Research 28 (2017) 16–23

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Conversion of *Chlamydomonas* sp. JSC4 lipids to biodiesel using *Fusarium heterosporum* lipase-expressing *Aspergillus oryzae* whole-cell as biocatalyst

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

[a] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan
[b] Graduate School of Science, Technology and Innovation, 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
[d] State Key Laboratory of Urban Water Resource and Environment, School of Municipal and Environmental Engineering, Harbin Institute of Technology, China
[e] L'Institut national de la recherche agronomique, 135, Avenue de Rangueil, 31077 Toulouse Cedex 4, France

ARTICLE INFO

Keywords:
Microalgae
Lipid composition
Biodiesel
Lipase
Immobilized enzymes

ABSTRACT

Lipid from *Chlamydomonas* sp. JSC4 was used as a feedstock for biodiesel production. The lipid was found to contain high amounts of phospholipids and free fatty acid in addition to the triglycerides. Two enzymatic methods for the efficient conversion of the heterogenous lipid to fatty acid methyl esters (FAME) were carried out. The method using either a lipase cocktail containing *Candida cylindracea* lipase and *Thermomyces lanuginosus* lipase combination (m I) or immobilized *Fusarium heterosporum* lipase-expressing *Aspergillus oryzae* whole-cells (m II) were both successful. However, the method using lipase cocktail showed 30.8% relative stability after the fourth batch, whereas the whole-cell biocatalyst showed 98.1%. Although the whole-cell biocatalyst tolerated a wide range of water content, an exploration of the effect of water-methanol interaction on the biocatalytic process showed that 24% water and 7:1 methanol to oil ratio is more favorable for FAME production. A higher initial methanol consumption rate facilitated a more stable system with the whole-cell biocatalyst, producing over 97% FAME in 32 h. The efficient conversion of a highly heterogenous substrate in the presence of high amounts of water could be an effective technique for the enzymatic conversion of microalgal lipids.

1. Introduction

Most of the previously established biodiesel production processes work efficiently for the conversion of triglycerides, which happen to be the main components of plant and animal oils. Unfortunately, most lipids produced by oleaginous organisms contain high levels of polar lipids in the form of phospholipids and glycolipids, which are an integral part of the cell membrane [1]. It was reported that some strains of microalgae contain as high as 28% phospholipids of the total lipid weight [1]. In addition, there are high levels of free fatty acids (FFA) in these oils, which are usually produced through hydrolysis by endogenous enzymes or through oxidation by peroxides present in the cells of the organism [2,3]. In the presence of more than 2.5% (w/w) FFA in the oil feedstock, the conventional method for biodiesel production, which involves the use of chemicals such as NaOH, is marred by the competitive saponification reaction. This leads to the formation of undesirable soaps, low biodiesel output and complicated post-processing. This challenge can be eliminated by acid pretreatment, but the high temperatures required for this procedure compromises the biodiesel fuel energy balance. In addition, further processing steps are necessary for the removal of the water by-product before the base catalysis can be applied [4].

An alternative technology using enzymes for the conversion of lipids to biodiesel could overcome this challenge. This generally involves the use of triacylglycerol lipases (EC 3.1.1.3), which are hydrolases that act on the ester bonds in carboxylic esters. This technology further improves the environmental friendliness of biodiesel production and offers a better biodiesel fuel energy balance with its low energy input [5,6]. There are a number of these lipases, with each having properties that differ from the other. This unique property prevents side reactions, which lead to unwanted by-products. However, this also leads to limitations in biodiesel production, as the feedstock consists of varying components [4]. Also, due to the high sensitivity of lipases, polar compounds, such as phospholipids, contribute to the deactivation of the lipases. Hence the high levels of phospholipids and the heterogeneous nature of microalgal lipids could hinder the enzymatic conversion

Abbreviations: BSP, biomass support particle; FFA, free fatty acid; TAG, triglyceride; FAME, fatty acid methyl ester; FHL, *Fusarium heterosporum* lipase; m I, method 1; m II, method 2

* Corresponding author.
E-mail address: akondo@kobe-u.ac.jp (A. Kondo).

http://dx.doi.org/10.1016/j.algal.2017.10.003
Received 9 February 2017; Received in revised form 5 October 2017; Accepted 5 October 2017

method. Ways to overcome this limitation may include the use of a single lipase that is capable of converting the relevant components in the feedstock, or by the use of a mixture of two or more lipases with different properties. The combination of commercial formulations of *Thermomyces lanuginosus* lipase (TLL) and *Rhizomucor miehei* lipase (RML) improved the biodiesel production from palm oil from 44% to 80% [7]. Amoah et al. [8] reported the improvement of biodiesel production from 21.1% to 88.1% from an oil feedstock containing 5% phospholipid using a lipase AY-TLL cocktail.

Whole cell biocatalysts, a cheaper form of lipases, have been recently used for biodiesel production. Over 90% biodiesel yield has been produced from various plant oils using whole cell biocatalysts *Fusarium heterosporum* lipase, *Candida antarctica* lipase and *Rhizopus oryzae* lipase [9–11]. In our previous work, *F. heterosporum* lipase-expressing *Aspergillus oryzae* was found to achieve over 90% FAME production from a model oil containing 30% phospholipid [12]. These lipases are cheaper due to their simple production via a simultaneous cultivation and immobilization process. An in-depth understanding of these lipases could facilitate their industrial application in microalgal oil conversion. Whole cell yeast biocatalyst immobilized on waste sugarcane bagasse could achieved 85.29% biodiesel from *Chlorella salina* using methyl acetate as the acyl acceptor [13]. It was reported that biodiesel yields of 68.2% and 50.3% were produced from the marine microalgae DY54 and *Chlorella* sp., respectively, using mold-fungus JN7 whole cell biocatalyst [14]. An intensive work on the properties of biodiesel from *Scenedesmus obliquus* produced by whole cell *Aspergillus niger* was carried out [15]. Although most of the properties met the standards, the reuse of the lipase was poor. Despite these works, the operational parameters for the use of whole cell biocatalysts need to be further understood to prevent issues of deactivation, improve lipase reuse, and reduce reaction time, among others.

In this work, the improvement of lipid production in *Chlamydomonas* sp. JSC4 by acetate addition was performed. In the production of biodiesel from the produced algal lipid using whole cell *A. oryzae* expressing *F. heterosporum* lipase, the active sites of the catalyst were enhanced under mild agitation while using a step-wise methanol addition. Once the activated sites were boosted, a 1-time methanol addition was used to explore the interaction between water content and methanol concentration on the lipase. An understanding of this interaction was used to improve the reaction rate.

2. Methods

2.1. 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 [16]. 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. The obtained lipase was then designated rFHL.

2.2. 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 (biomass support particles, BSPs) (Bridgestone Corporation, Osaka, Japan) in 100 mL of DP medium (2 g glucose, 2 g polypeptone, 100 mg KH_2PO_4, 50 mg $MgSO_4.7H_2O$). 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.

2.3. Cultivation of Chlamydomonas sp. JSC4

2.3.1. Culture medium and strains

The *Chlamydomonas* sp. JSC4 strain used in this work was isolated from the coast of southern Taiwan. The cells were initially precultured in a 1 L modified Bold 6 N medium that consisted of 0.375 g $NaNO_3$, 0.0383 g K_2HPO_4, 0.075 g $MgSO_4.7H_2O$, 0.088 g KH_2PO_4, 0.025 g NaCl, 0.025 g $CaCl_2.2H_2O$ and 20 g sea salt [17]. 2% CO_2 was supplied at a rate of 0.06 vvm (where vvm is the litres of gas passing through 1 L of medium per minute) and the cells were cultivated at 30 °C ± 2 for 96 h with approximately 250 μmol photons $m^{-2} s^{-1}$ illumination.

2.3.2. Photobioreactor operation

The precultured cells were then transferred to a 30 L open system bubble column photobioreactor (PBR) (200 mm × 1228 mm) at an inoculum size of 100 mg/L. The cells were cultured in modified Bold 3 N medium (22.50 g $NaNO_3$, 1.15 g K_2HPO_4, 2.25 g $MgSO_4.7H_2O$, 2.64 g KH_2PO_4, 0.75 g NaCl, 0.75 g $CaCl_2.2H_2O$ and 600 g sea salt) with 250 μmol photons $m^{-2} s^{-1}$ single sided illumination (FHF32EX-DH fluorescent lamp, Mitsubishi Electric Corp., Tokyo, Japan). As the main carbon source, 2% CO_2 was continuously fed into the culture at a rate of 1.2 L/min and this also served as the sole agitation force for the culture. The rate of CO_2 feed was monitored by measuring the difference between the CO_2 concentrations in the influent and effluent streams of the PBR using a GM70 CO_2 detector (Vaisala, Tokyo, Japan). The culture was maintained at 30 °C ± 2 for 10 days and samples were taken at regular intervals to determine culture density, nitrate concentration, lipid accumulation rate, cell growth rate and contamination monitoring. In order to improve the lipid production, 2 and 4 g/L of sodium acetate were added after nitrogen depletion (day 2). To measure the nitrogen concentration, samples were taken from the culture broth and filtered with a 0.22 μm pore size filter. The absorbance of the filtrate was determined at 220 nm (UVmini-1240, Shimadzu Corp., Kyoto, Japan) and the nitrate concentration deduced using a preconstructed calibration curve.

After cultivation, the agitation was stopped and the cells were allowed to settle under their own weight. Approximately 75% of the supernatant medium was discarded and the remaining culture containing the cells was centrifuged at 7600*g* for 5 min. The cells were lyophilized for further processing.

2.4. Extraction and characterization of lipids from Chlamydomonas sp. JSC4

The lyophilized cells were subjected to cell breaking by sonication using an ultrasonic disruptor UD-201 (Tomy Seiko Co., Ltd., Tokyo, Japan). Approximately 10 mL of distilled water was added to about 0.5 g of the dried cells. The equipment was set to an output of 35 and 60 s pulses with each pulse followed by 60 s cooling in ice bath. The total sonication time was 5 min. 7.5 mL each of methanol and hexane were added to the disrupted cells, mixed vigorously and centrifuged at 2330*g* for 5 min. The upper layer was characterized by gas chromatography and thin layer chromatography and the crude extract was further used for transesterification.