Bioresource Technology 180 (2015) 47–53

Contents lists available at ScienceDirect

Bioresource Technology

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

Biodiesel production from microalgae oil catalyzed by a recombinant lipase

Jinjin Huang, Ji Xia, Wei Jiang, Ying Li *, Jilun Li

State Key Laboratories for Agro-biotechnology and College of Biological Sciences, China Agricultural University, Beijing 100193, PR China

HIGHLIGHTS

- *Chlorella vulgaris* oil is an excellent substrate for biodiesel production.
- Lipase GH2 can be efficiently catalytic microalgae oil into FAME and FAEE.
- A simple process flow – "one step method".

ARTICLE INFO

Article history:
Received 26 September 2014
Received in revised form 20 December 2014
Accepted 22 December 2014
Available online 30 December 2014

Keywords:
Recombinant lipase
Microalgae oil
Esterification reaction
Biodiesel
Conversion efficiency

ABSTRACT

A recombinant *Rhizomucor miehei* lipase was constructed and expressed in *Pichia pastoris*. The target enzyme was termed Lipase GH2 and it can be used as a free enzyme for catalytic conversion of microalgae oil mixed with methanol or ethanol for biodiesel production in an n-hexane solvent system. Conversion rates of two major types of biodiesel, fatty acid methyl ester (FAME) and fatty acid ethyl ester (FAEE), reached maximal values (>90%) after 24 h. The process of FAME production is generally more simple and economical than that of FAEE production, even though the two processes show similar conversion rates. In spite of the damaging effect of ethanol on enzyme activity, we successfully obtained ethyl ester by the enzymatic method. Our findings indicate that Lipase GH2 is a useful catalyst for conversion of microalgae oil to FAME or FAEE, and this system provides efficiency and reduced costs in biodiesel production.

1. Introduction

There is increasing interest in alternative or new energy sources because of the environmental impacts and declining supplies of fossil fuels. Biodiesel, a sustainable, nontoxic, biodegradable diesel fuel substitute, can be used in current diesel car infrastructure without major engine modifications (Luque et al., 2010). Biodiesel is usually obtained by transesterification of fat and vegetable oils with alcohol (usually methanol or ethanol) in the presence of a catalyst, with resulting production of a fatty acid methyl ester (FAME) or fatty acid ethyl ester (FAEE) that can be used as a biofuel (Almeida et al., 2012).

The major factor limiting development and use of biodiesel is its high production cost, of which feedstock expense accounts for >75% (Lim and Teong, 2010). Feedstock prices are a crucial factor in strategies to make biodiesel competitive with fossil fuels. Three "generations" are considered in the development of biodiesel feedstocks. First generation feedstocks were based on edible vegetable oils, *e.g.*, soybean and sunflower oils (Ahmad et al., 2011). Second generation feedstocks, intended to reduce dependence on edible vegetable oils, were based on nonfood sources such as jatropha, jojoba oil, waste oil, recycled oil, and animal fats (Ahmad et al., 2011; Pinzi et al., 2014). Second generation feedstocks can be produced from non-agricultural land, thereby eliminating competition with food production. Nevertheless, development of first and second generation feedstocks is limited by high cost, inefficiency, and unsustainability (Ahmad et al., 2011; Balat and Balat, 2010; Leung et al., 2010). Research attention is now focused on the third generation biodiesel feedstock: microalgae. In comparison with first and second generation feedstocks, microalgae is considered a more promising alternative source for biodiesel production because of its high oil productivity, rapid reproduction, no requirement of arable

Abbreviations: FAME, fatty acid methyl ester; FAEE, fatty acid ethyl ester; MAG, monoacylglycerol; 1,3-DAG and 1,2-DAG, diacylglycerols; TAG, triacylglycerol; FFAs, free fatty acids; C/M, chloroform:methanol (v/v).

* Corresponding author. Tel.: +86 10 62733751.
E-mail address: yingli528@vip.sina.com (Y. Li).

http://dx.doi.org/10.1016/j.biortech.2014.12.072
0960-8524/

land or fresh water, and enrichment in useful or valuable co-products (Pinzi et al., 2014; Minowa et al., 1995).

Production of biodiesel from microalgae involves several steps, including cell cultivation, cell harvesting, oil extraction, and biodiesel synthesis (Goncalves et al., 2013). Many research studies have focused on cultivation of microalgae for increased lipid productivity, but relatively few have addressed the chemical or enzymatic conversion of microalgae oil to biodiesel (Lai et al., 2012a).

Enzymatic conversation methods typically have lower operating costs and higher product purity than do chemical methods. Some lipases have been for enzymatic conversion of microalgae oil to biodiesel, *e.g.*, Novozym® 435 (Da Ros et al., 2012), extracellular lipase from *Bacillus* sp. (Sivaramakrishnan and Muthukumar, 2012), *Penicillium expansum* lipase (Lai et al., 2012b), immobilized *Burkholderia* lipase (Tran et al., 2013), and immobilized *Candida* sp. 99–125 lipase (Li et al., 2007).

Methanol has traditionally been the alcohol most commonly used in biodiesel production, but there is now increasing emphasis on use of ethanol for production of FAEE. Both FAME and FAEE preparation methods have unique advantages and disadvantages (Mohamad Firdaus et al., 2014). Methanol is less expensive than ethanol, but oil is less soluble in methanol, and methanolysis involves limited mass transfer during the transesterification process. Ethanol is safer to use, renewable, has higher oil solubility, and the mass transfer limitation is not as great; however, ethanol is more expensive than methanol (Zhang et al., 2014).

In a recent study, we used various strategies (optimization of signal peptide codons, addition of target gene propeptide, optimized gene dosage) to enhance expression level of a recombinant lipase (termed Lipase GH2) in the methylotrophic yeast *Pichia pastoris*. The unique properties of Lipase GH2 made it more suitable than other lipases for conversion of microalgae oil to FAME (Huang et al., 2014).

Depend on Lipase GH2 was obtained, the aims of the present study were to (i) evaluate the ability of Lipase GH2 in combination with ethanol to catalyze production of FAEE from microalgae oil; (ii) further optimize the reaction conditions and improve the conversion rate of FAME and FAEE by Lipase GH2 in a n-hexane system; (iii) comparison of the differences in FAME and FAEE preparation process by using Lipase GH2.

2. Methods

2.1. Enzyme preparation

Construction of a recombinant *P. pastoris* strain (mα-2pRML-X33) was described in our previous report (Huang et al., 2014). The strain highly expressed *Rhizomucor miehei* lipase (termed Lipase GH2), and this enzyme displayed high lipase activity. The strain was flask-cultured in BMGY/BMMY medium as described by Hu et al. (2013), and target protein expression was induced by daily addition of 1% methanol. Optical density (OD_{600}) and enzyme activity were measured throughout the culture period as described by Guan et al. (2010), except that our reaction buffer was 0.1 M sodium dihydrogen phosphate/citric acid, pH 6.0, 35 °C. After fermentation, the supernatant were collected by 5 min centrifugation at 6000 rpm and stored at 4 °C.

2.2. Microalgae oil extraction and detection

Chlorella vulgaris (CV) powder was kindly provided by Dr. Peng Pu (State Key Laboratory of Catalytic Material and Reaction Engineering, Research Institute of Petroleum Processing, SINOPEC, Beijing, China). Dried CV powder (0.5 g) was mixed with 20 mL deionized water. CV cell walls were disrupted by sonication (200 W, ultrasonic 5 s, intermittent 5 s, 60 times), and the disrupted cells were mixed with biphasic solvent (chloroform/methanol [C/M], 1:2) or hexane in a shaker (200 rpm, 30 min). The mixture was centrifuged (6000 rpm, 10 min) to form two layers. The upper hexane layer that contained microalgae oil was collected, and crude oil was obtained by evaporation in a hood at room temperature.

Free fatty acids (FFAs) in the extracted crude oil were analyzed by gas chromatography (GC) and thin-layer chromatography (TLC). In brief, 10 μL of the upper layer was mixed with 40 μL hexane, and 2 μL of the mixture was subjected to TLC (Guan et al., 2010). FFAs in the microalgae oil were analyzed using a GC system (model 6890A, Agilent Technologies; Santa Clara, CA, USA) equipped with a J&W DB-23 capillary column (60 m × 250 μm × 0.25 μm). Preparation of standards and samples, and testing conditions, were as described by Li et al. (2011). These experiments were performed at the Feed Detection Center of the Chinese Ministry of Agriculture.

2.3. Biodiesel preparation

Extracted crude oil as above was used as a substrate for biodiesel production using the organic solvent n-hexane. Short-chain alcohols (methanol or ethanol) were used separately as another substrate. Free Lipase GH2 was used as a biocatalyst. We performed a series of experiments to determine optimal reaction conditions (temperature, water content, alcohol/oil molar ratio, procedure for adding alcohol, Lipase GH2 amount). The following optimal reaction conditions were used, except when stated otherwise: 0.3 g microalgae oil, 90 μL methanol or 217 μL ethanol, 600 μL n-hexane, 150 μL Lipase GH2, incubation temperature 30 °C, shaking at 150 rpm for 24 h. Enzyme solution in reaction was recovered by centrifugal, then the enzyme solution was added in the next new reaction system to detected the reuse of Lipase GH2.

2.4. Detection of enzyme-catalyzed reaction products

FAME or FAEE in the reaction mixture were analyzed by a GC (model GC522, Wufeng, China) equipped with an HP-INNOWax capillary column (30 m × 0.25 mm × 0.25 μm; Agilent). Heptadecanoic acid methyl ester was dissolved in hexane (10 mg/mL) as internal standard. The reaction mixture was centrifuged (12,000 rpm, 5 min) and solvent was eliminated by drying. The dried sample (40 mg) was mixed thoroughly with 400 μL internal standard for GC analysis as described by Guan et al. (2010).

3. Results and discussion

3.1. Collection of efficient liquid enzyme

P. pastoris is frequently used as a host for expression of heterologous proteins because it can be tightly regulated by a eukaryotic promoter (alcohol oxidase I; AOX1) and can tolerate a broad pH range (3.0–7.0) (Soyaslan and Calik, 2011). To date, ~500 heterologous proteins have been expressed in *P. pastoris* systems (Yu et al., 2010).

R. miehei lipase is a strong 1,3-specific lipase customarily used for production of biodiesel. We recently generated a recombinant *R. miehei* lipase (Lipase GH2) in *P. pastoris* by addition of the target gene propeptide, optimization of signal peptide codons, and optimization of gene dosage (Huang et al., 2014). The recombinant *P. pastoris* strain contained two copies of the *R. miehei* lipase precursor gene, and displayed maximal lipase production in flask culture.

We cultured the recombinant strain in shake flasks as described in our previous report, and measured cell growth and enzyme