Bioresource Technology 211 (2016) 224–230

Contents lists available at ScienceDirect

Bioresource Technology

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

Lipase cocktail for efficient conversion of oils containing phospholipids to biodiesel

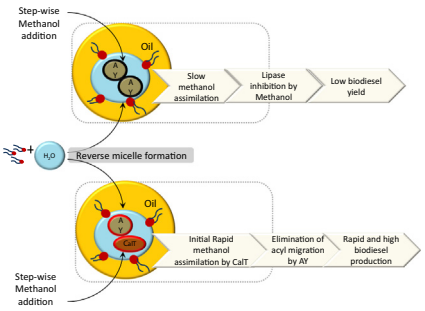
Jerome Amoah [a], Shih-Hsin Ho [b], Shinji Hama [c], Ayumi Yoshida [c], Akihito Nakanishi [b], Tomohisa Hasunuma [b], Chiaki Ogino [a], Akihiko Kondo [a,*]

[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

HIGHLIGHTS

- Biodiesel production from phospholipid-containing oil.
- Lipase AY was inhibited in the presence of 5% phospholipid.
- CalT and lipase AY gave the best combination.
- The lipase cocktail tolerated high methanol addition rate.
- 88.1% FAME was achieved in 2 h.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form 9 March 2016
Accepted 11 March 2016
Available online 14 March 2016

Keywords:
Biodiesel
Enzyme biocatalysis
Mixed lipase
Transesterification
Phospholipid

ABSTRACT

The presence of phospholipid has been a challenge in liquid enzymatic biodiesel production. Among six lipases that were screened, lipase AY had the highest hydrolysis activity and a competitive transesterification activity. However, it yielded only 21.1% FAME from oil containing phospholipids. By replacing portions of these lipases with a more robust bioFAME lipase, CalT, the combination of lipase AY–CalT gave the highest FAME yield with the least amounts of free fatty acids and partial glycerides. A higher methanol addition rate reduced FAME yields for lipase DF–CalT and A10D–CalT combinations while that of lipase AY–CalT combination improved. Optimizing the methanol addition rate for lipase AY–CalT resulted in a FAME yield of 88.1% at 2 h and more than 95% at 6 h. This effective use of lipases could be applied for the rapid and economic conversion of unrefined oils to biodiesel.

1. Introduction

Biodiesel, an alternative to fossil fuel keeps gaining attention in research due to its environmentally friendly nature among other advantages. It is a mixture of fatty acid methyl esters (FAME) that has conventionally been produced from the alkaline transesterification of animal and vegetable oils (Vyas et al., 2010). In advanced researches, a two-step method of esterifying high free fatty acid containing oil with acid catalysts before transesterification with the alkaline catalyst has been developed (Chen et al., 2012; Hancsok et al., 2004; Wang et al., 2006). This is to prevent the formation of soap. Other heterogeneous alkaline and acid catalysts such as CaO/Al_2O_3 and $Li\text{-}CaOFe_2(SO_4)_3$ have been developed over the past years for the production of biodiesel (Umdu et al., 2009; Endalew et al., 2011). Although these catalysts may offer some advantages over the conventional ones, the environmentally benign purpose of substituting fossil fuel with biodiesel is

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

http://dx.doi.org/10.1016/j.biortech.2016.03.062
0960-8524/

undermined. These chemicals cause corrosion to reactors and the post-processing needed to finalize the biodiesel production causes an equally environmental hazardous situation. To this end, current research in biodiesel production has been channeled to the use of EC 3.1.1.3 lipases.

EC 3.1.1.3 lipases are hydrolases that act on ester bonds in carboxylic esters. There are hundreds of these lipases and many of them from various microorganisms have been known to be applicable in the industrial production of biodiesel (Li et al., 2011; Ghaly et al., 2010). A well-known bacterium for lipase production is *Burkholderia glumae* (Jaeger and Reetz, 1998). *Thermomyces lanuginosus* and *Rhizopus oryzae* are popular lipases from fungi and *Candida rugosa* is a common yeast for lipase production (Nordblad et al., 2014; Oda et al., 2005; Chang et al., 1994). These lipases can either be used in the immobilized form or in the liquid form. The use of immobilized lipases offers an advantage of easy product separation and reuse of lipase. Unfortunately, on the industrial scale, the immobilization of purified lipase adds significant cost to the biodiesel production and the immobilization of lipases often alters the desired specificity of the lipase (Guldhe et al., 2015; Christopher et al., 2014). Furthermore, immobilizing lipase leads to an increase in mass transfer resistance due to the solid nature of the enzyme and the liquid nature of the other reactants and this often leads to slower reaction times. This gives liquid lipases an urge over immobilized lipases.

The drift of attention towards the enzymatic biodiesel production is due to the fact that, enzymes can convert oils to biodiesel under very mild conditions of temperature and pressure, they are highly environmentally friendly and due to the unique specificities of lipases, the production of unwanted products are eliminated. Although the regiospecific nature of lipases eliminates the production of unwanted products, this specificity may also lead to low product yield as most of the oil substrate consist of a wide range of compounds. Generally, most oil substrates are made of triglycerides, diglycerides, monoglycerides and free fatty acids of various chain lengths. Some lipases are known to be sn-1,3 regiospecific which can cleave the ester at positions 1 and 3 of the triglyceride alone (Du et al., 2005; Hama et al., 2008; Guan et al., 2010). Others are known to act on di and monoglycerides only where some are only effective on free fatty acids (Hama et al., 2009; Adachi et al., 2011). Furthermore, with the aim of reducing the cost of feedstock, the use of unrefined oils are being considered for biodiesel production and this often contains high levels of free fatty acids as well as phospholipids, making the oil substrate even more complicated.

Phospholipids have been known to accelerate lipase inhibition with the formation of reverse micelles which alters the nature of the reaction mixture (Amoah et al., 2016; Li et al., 2014). Degumming of crude oil has been proposed for curbing this problem however, this adds additional cost, increases processing steps and causes loss of feedstock. Various common lipases have been used for the conversion of refined vegetable oil to biodiesel but their successful application in oils containing phospholipids is yet to be investigated. Adachi et al. (2013) reported the successful use of *C. rugosa* lipase for the hydrolysis of soybean oil for a two-step biodiesel conversion. Oda et al. (2005) also reported the use of an sn-1,3 regiospecific lipase where acyl migration was facilitated by an increased water content resulting in an efficient FAME production.

Due to the unique properties of individual lipases, the concept of lipase cocktail which involves the combination of two or more lipase has led to interesting results. Lee et al. (2011) found out that a combination of *C. rugosa* and *R. oryzae* lipases in supercritical carbon dioxide gave a better yield than the use of a single lipase. A combination of CalT, RML and CalB improved the conversion yield from olive oil to 95% in 18 h, up from the 50% for the best individual lipase (CalB) where as a combination of CalT and RML improved the conversion from palm oil to 80% (Poppe et al., 2015). In the ethanolysis of used palm oil, the combined use lipase AY and lipase AK gave a higher yield of biodiesel than using AK alone (Tongboriboon et al., 2010).

In this work, the concept of lipase cocktail is employed to reduce total lipase loading in order to reduce the cost of lipase. The first part deals with screening of 6 lipases commonly used in the food industry, and their applicability in biodiesel production from oil containing phospholipid is explored. Following this, a comparative analysis of several lipase cocktails is carried out and the best lipase combination based on reaction rate, final FAME yield and residual partial glyceride composition selected. Ultimately, the improvement of reaction rate for an efficient production of FAME from oil containing phospholipid is demonstrated.

2. Materials and methods

2.1. Materials and lipase strains

Callera Trans L, a liquid formulation of *T. lanuginosus* lipase (CalT) was obtained from Novozymes (Bagsvaerd, Denmark). Powdered lipases DF and MER from *R. oryzae*, AY from *Candida cylindracea* (formerly known as *C. rugosa*), R from *Penicillium roqueforti* and A from *Aspergillus niger* were obtained from Amano (Nagoya, Japan). Powdered lipase A-10D from *Rhizopus* sp., was obtained from Nagase Enzymes (Kyoto, Japan). Refined soybean oil and soybean phospholipids were obtained from Wako Pure Chemical industries (Osaka, Japan).

2.2. Lipase solution preparation

1 g each of 6 different powdered lipases was dissolved separately in 10 ml pH 7 phosphate buffer. They were set on Bioshaker at 30 °C and 200 rpm for 30 min. The activities of the obtained lipase solutions were tested and were further used for the transesterification reaction.

2.3. Lipase activity assay

The activity assays of the lipase for both transesterification and hydrolysis were carried out. The transesterification activities were tested using p-nitrophenyl butyrate (pNPB) as a chromogenic substrate. A stock solution was prepared by dissolving 5 μl of pNPB in 250 μl of ethanol and further diluting to 50 ml with distilled water. The lipase-hydrolytic activities at 35 °C of the various lipases were carried out by incubating for 10 min in a Bioshaker, Taitec, Japan. After incubation, 5% of trichloroacetate was added to terminate the reaction. The absorbance of the produced para nitrophenol (pNP) was measured at 400 nm (UV–Vis spectrophotometer, Shimadzu). 1 unit (U_2) of lipase activity was defined as the amount of lipase that liberates 1 μmol of pNP from pNPB per minute.

For hydrolysis, the reaction mixture contained 2 g of olive oil, 9 ml of 0.1 M acetate buffer (pH 5.6) and 1 ml of 0.05 M $CaCl_2$ in a 50 ml screw-cap bottle. It was stirred in a water bath at 250 rpm at 30 °C and the reaction was terminated by the addition of ethanol. The obtained emulsion was titrated against 0.1 NaOH till a pH of 10. This hydrolytic activity (U_1) is defined as the amount of lipase that liberates 1 μmol of FFA from olive oil at pH 10 and 37 °C.

2.4. Lipase-mediated methanolysis

Soybean oil was spiked with 5% (w/w) soybean phospholipid to simulate the presence of phospholipid in low quality crude oils and subjected to sonication to ensure homogeneity. The