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Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant *Rhizopus oryzae* lipase

Susan Hartwig Duarte [a,*], Gonzalo Lázaro del Peso Hernández [b], Albert Canet [b], Maria Dolors Benaiges [b], Francisco Maugeri [a], Francisco Valero [b]

[a] Laboratory of Bioprocess Engineering, Faculty of Food Engineering – UNICAMP, Campinas, Brazil
[b] Department of Chemical Engineering, School of Engineering – Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain

HIGHLIGHTS

- The yeast *Candida* sp. LEB-M3 may be a suitable feedstock for biodiesel production.
- The recombinant *Rhizopus oryzae* lipase immobilized was used as biocatalyst.
- Methanol stepwise addition showed to be a strategy to maintain lipase activity.

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ABSTRACT

The recombinant *Rhizopus oryzae* lipase (1–3 positional selective), immobilized on Relizyme OD403, has been applied to the production of biodiesel using single cell oil from *Candida* sp. LEB-M3 growing on glycerol from biodiesel process. The composition of microbial oil is quite similar in terms of saponifiable lipids than olive oil, although with a higher amount of saturated fatty acids. The reaction was carried out in a solvent system, and n-hexane showed the best performance in terms of yield and easy recovery. The strategy selected for acyl acceptor addition was a stepwise methanol addition using crude and neutralized single cell oil, olive oil and oleic acid as substrates. A FAMEs yield of 40.6% was obtained with microbial oils lower than olive oil 54.3%. Finally in terms of stability, only a lost about 30% after 6 reutilizations were achieved.

1. Introduction

The possibility of fossil energy depletion has become one of the most important issues discussed all over the world, due to energy consumption that is inevitable for human existence. This scenario takes to many alternatives to replace this exhaustible energy source (Xu et al., 2014). Biodiesel, usually produced by transesterification of triglycerides with methanol obtaining fatty acid methyl esters (FAMEs) in presence of a catalyst has shown to be an alternative to diesel fuel because its favorable features, environmental benefits and renewable biological resources origin. Among of the different raw materials, microbial oil, also called single cells oils, is a potential feedstock source (Duarte et al., 2014).

Between heterotrophic microorganisms, oleaginous yeasts, capable of accumulating high amount of lipids (>20% (w/w)) in its dried biomass, has important advantages due to its fast growth rate and high oil content. This new source has several advantages compared to other vegetable oils and animal fats: short life cycle, not depends of arable land, not competes with food sector, can be obtained independent of climatic factors and it is easier to scale-up (Li et al., 2008). Taking into account that the major economic factor to consider is the feedstock that increases the final cost to biodiesel production around 75–80%, if we compare microbial oils with vegetables oils, the use of cheap carbon sources as residual carbon sources, diminish significantly the cost of yeast oil production (Li et al., 2008; Demirbas, 2009).

Microbial oil has been produced by several research groups using different microorganisms including microalgal, fungi and bacterial oil, different substrates, like residues from industry and agriculture with low cost and nutritional value to the microorganism (Teo et al., 2014; Wang et al., 2014; Ruan et al., 2012; Karatay and Donmez, 2010). Recent studies in our group demonstrated that the yeast *Candida* sp. LEB-M3 contain high amounts of lipids (about 50% w/w) when it is grown on crude glycerol obtained as byproduct from biodiesel synthesis (Duarte et al., 2013). The biodiesel

* Corresponding author at: Department of Food Engineering, Faculty of Food Engineering, University of Campinas – UNICAMP, Rua Monteiro Lobato 80, Barão Geraldo, CEP 13083-862 Campinas, SP, Brazil. Tel.: +55 19 35214052.
E-mail address: susanduarte@hotmail.com (S.H. Duarte).

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production with this raw material challenges researchers to develop more efficient and environmental friendly processes.

It is well known that homogeneous alkali-catalysis method has been conventionally applied to biodiesel production. However, it presents some drawbacks and requires a rigorous feedstock specification, such as low contents of water and free fatty acids, to prevent the soap generation. An alternative to avoid these problems is the development of an enzymatic bioprocess catalyzed by lipases. This bioprocess approach works at mild operation conditions and allows the possibility to use raw materials with a high amount of free fatty acids, as in this present work, where the microbial oil used has significant amount of such compounds. The high cost of the biocatalyst has to be minimized using it immobilized on different supports allowing the reutilization and enhancing enzyme stability (Hwang et al., 2014; Robles-Medina et al., 2009).

Lipases can be categorized into three classes based on their specificity or selectivity such as region- or positional specific lipases; fatty acid type specific lipases and specific lipases for a certain class of acylglycerols (mono-, di-, or triglycerides) (Teo et al., 2014). Previous studies of lipases catalyzing the production of biodiesel from vegetable oil have proved that the yields of FAMEs are influenced by the solvent system used into the reaction (Fjerbaek et al., 2009). The addition of organic solvent improves the lipase stability and can improve the mass transfer of substrate because it reduces the viscosity in the enzymatic transesterification. It has been reported that surface properties of lipases such as hydrophobicity and charge distribution are the mainly factors to provide lipase stability in organic solvents (Chakravorty et al., 2012). Among all organic solvents, hydrophobic solvents are preferred as compared to hydrophilic solvents in order to maintain the minimum amount of water in lipase surrounding and to avoid its inactivation by loss of water in the enzyme structure essential to lipase activity (Nasaruddin et al., 2014). Besides, the enzyme inactivation that occurs when the alcohol captures essential water molecules required for lipase activity, can be overcome increasing the solubility of alcohol and oil by organic solvent addition (Hwang et al., 2014).

The recombinant 1,3 positional selective *Rhizopus oryzae* lipase (rROL) used in this work is able to synthesize biodiesel and monoacylglycerols simultaneously, not producing glycerol as final product, avoiding operational problems associated to the adsorption of the glycerol onto the support (Robles-Medina et al., 2009). The production of monoacylglycerols jointly with FAMEs increase the lubricity of the final biodiesel obtained, but also is a valuable product in food, pharmaceutical and cosmetics industries (Zhong et al., 2014). Moreover rROL produced in the cell factory *Pichia pastoris* showed a 44-fold higher specific activity compared to a commercially available lipase obtained directly from *R. oryzae* (Guillén et al., 2011). In a recent work, rROL immobilized on Sepabeads (similar to Relizyme OD403) showed a good performance in the production of biodiesel from olive oil (Canet et al., 2014). In addition, Sepabeads was concluded to be the best among other supports for esterification reaction by rROL (Guillén et al., 2012).

In the present work, biodiesel synthesis using extracted lipids from *Candida* sp. (LEB-M3) and rROL immobilized on Relizyme OD403 in a solvent system has been studied. In order to obtain an increase in FAMEs yield and to avoid a possible enzyme denaturation, a strategy of methanol stepwise addition was employed.

2. Methods

2.1. Microorganism cultivation and oil recuperation

The yeast *Candida* sp. LEB-M3, isolated from Pantanal, Brazil (Maugeri and Hernalsteens, 2007), was selected as an oleaginous yeast in previous study (Duarte et al., 2013). It was maintained in stock cultures at the Laboratory of Bioprocess Engineering (UNICAMP), on GYMP agar slants at 5 °C containing (g/L): 20 glucose, 5 yeast extract, 10 malt extract, 2 KH_2PO_4 and 20 agar, pH 5.5. Subsequently, two reactivated microbial culture slants were scraped with 10 mL of 0.1% peptone water each for removing the microorganism cells. Cells were transferred to Erlenmeyer flasks containing 180 mL of the growth medium composed of (g/L): 30 crude glycerol (obtained from the basic transesterification of a mixture: 80% soybean oil, 15% beef fat and 5% cottonseed oil with methanol, without previous treatment, kindly provided by Granol Anapolis company, Brazil), 7 KH_2PO_4, 2.5 Na_2HPO_4, 1.5 $MgSO_4{\cdot}7H_2O$, 1.4 yeast hydrolyzate, pH 6.0, which were maintained at 28 °C in flasks agitated at 180 rpm for 36 h. After that, cultivations were performed in Erlenmeyer flasks inoculated with 10% (v/v) and with the same inoculum cultivation conditions during 240 h. At the end, culture medium was centrifuged and the biomass was dried and treated with a 2 M HCl solution at 80 °C to cell wall rupture. The microbial oil was extracted using chloroform, methanol and water and measured by dry weight (Bligh and Dyer, 1959).

2.2. Microbial oil characterization

2.2.1. Thin layer chromatography

Free fatty acids, triglycerides, diglycerides, monoglycerides, FAMEs, carotenoids, sterol esters, sterols, tocopherols, and polar lipids (phospholipids, glycolipids and sphingolipids) in the extracted oil were identified and quantified by Thin Layer Chromatography (TLC). Chromatographic separation was developed in 10 cm × 10 cm silica-coated aluminum plates (Alugram Sil G/UV. Macherey–Nagel GmbH, Düren, Germany) using a solvent mixture of 88% (v) n-hexane, 11% (v) diethyl ether and 1% (v) glacial acetic acid. Visualization was carried out by staining with iodine. Digital image analyses of staining plates were performed with Un-Scan-It Gel 6.1 software (Silk Scientific Inc. Orem, UT, USA) and the lipid compositions were quantified by the corresponding calibration curves.

2.2.2. Fatty acids profile

The extracted lipid fraction was esterified to obtain the fatty acid methyl esters, according to the methodology proposed by Hartman and Lago (1973) and Metcalfe et al. (1966). Analyses were performed in duplicate using a Perkin Elmer gas chromatograph, Clarus 600, FID detector, Perkin Elmer Elite-225 capillary column (30 m × 0.25 mm × 0.25 μm). It was operated according to the following conditions: injector and detector temperature: 250 °C, initial oven temperature: 100 °C for 5 min, 230 °C for 20 min; carrier gas: helium, 1 mL/min, split 1:40; and injection volume of 0.4 μL. The methyl ester profile was quantified based on normalization of the relative peak areas (Basso et al., 2012).

2.2.3. Acidity index and iodine values

These parameters were determined according to official European methods EN 14101, and EN 14214, respectively.

2.3. Recombinant R. oryzae lipase production and immobilization

Recombinant *R. oryzae* lipase (rROL) was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB) (Arnau et al., 2010). The culture medium was centrifuged and micro-filtered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system (New York, USA) equipped with an Omega membrane with a 10 kDa cut-off, and subsequently dialyzed against 10 mM Tris–HCl buffer pH 7.5 and thereafter lyophilized (Guillén et al., 2011).