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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

Department of Food Engineering, Faculty of Food Engineering, University of Campinas-UNICAMP, Rua Monteiro Lobato 80, Barão Geraldo, CEP: 13083-862 Campinas, SP, Brazil

*susanduarte@hotmail.com, tel: +55 19 35214052

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.

Keywords: biodiesel, *Rhizopus oryzae* lipase, single cell oil.

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 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 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 *Rhizopus oryzae* (Guillén et al., 2011). In a recent work, rROL immobilized on Sepabeads (similar to Relyzyme 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 Relyzyme 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. MATERIAL AND 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 $MgSO_4.7H_2O$, 1.4 yeast hydrolyzate, pH 6.0, which were maintained at 28°C in flasks agitated at 180 rpm for 36 hours. After that, cultivations were performed in Erlenmeyer flasks inoculated with 10% (v/v) and with the same inoculum cultivation conditions during 240 hours. 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, tocoferols, 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 x 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 x 0.25 mm x 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 *Rhyzopus oryzae* lipase production and immobilization

Recombinant *Rhyzopus 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).

The immobilization of rROL was carried out by adsorption technique, using Relizyme OD403 as support (polymethacrylate) purchased from Mitsubishi Chemical. It was pre-treated by incubation in 100 mL of an acetone-water solution 50% (v/v) for 30 min, followed by solution removal by vacuum filtration. After that, 30 mL of a solution of 5 mM phosphate buffer at pH 7 containing an approximate rROL activity of 4,000 UA/mL was prepared, dissolving lyophilized rROL under magnetic stirring for 30 min at 4°C. The solution was then centrifuged for 10 min at 5,000 rpm and then the supernatant was mixed with pretreated support in a roller MoviROL (P-selecta, Abrera, Spain) for 12 h at 4°C. After that, the solution was removed by vacuum filtration. Immobilized biocatalyst was then dried with silica gel, at room temperature, in a desorber until its weight reached a constant value. Finally, the support with the immobilized lipase was stored at 4°C. The final immobilized rROL activity was 59

UA/mg biocatalyst. This value was calculated as the difference between the activity in the initial and final supernatant, divided by the weight of support (Canet et al., 2014).

2.4. Lipase activity

Lipolytic activity determination was carried out using the lipase colorimetric assay (kit from Roche diagnostics). The enzyme dissolved in buffer was mixed with 200 mM Tris-HCl buffer pH 7.25 and lipase kit for reaction monitoring along the time by spectrophotometer. The increase in absorbance at 580 nm was followed for 7 min with a Varian Cary 300 spectrophotometer. The absorbance increase per second was calculated from the slope of the curve and correlated to the lipolytic assay. One unit of lipase activity (UA) was defined as the amount of lipase necessary to hydrolyze 1 µmol of ester bond per minute under assay conditions (Resina et al., 2004; Canet et al., 2014).

2.5. Enzymatic reactions

The following methodologies were described for organic solvent selection and for methanol stepwise additions study. All reactions were done in 1.5 mL HPLC vials inside an incubator (IKA KS 400 ic, Satufen, Germany) at 30°C and under continuous stirring at 200 rpm. The reaction mixture contained 1:5 of oil:solvent solution (1 mL) and the amount of immobilized biocatalyst was calculated to achieve 4,000 UA (according to UA/mg of immobilized enzyme). The amount of methanol in each step (4 µL / 0.15:1 methanol:saponifiable lipids) was added to the reaction media to reach a maximum 14% of FAMEs yield to avoid the possible enzyme inactivation caused by the methanol. To determine the final amounts of methyl esters by gas chromatography, samples of the enzymatic reactions were withdrawn at predetermined time intervals according to Canet et al., (2014). For solvent selection, n-hexane, iso-octane and terc-butanol were studied. The reactions were performed in one step methanol addition by predetermined time intervals between 5 and 45 minutes. In order to avoid the inactivation of rROL by methanol and increase the final conversion of FAMEs, a strategy with stepwise methanol addition were performed during 240 minutes and the addition of methanol every 40 minutes (4 µL). The experiments were carried out with crude microbial oil (CMO), without any previous treatment, neutralized microbial oil (NMO) in this case all free fatty acids were neutralized with NaOH and then were removed, olive oil (OLO) and oleic fatty acid (OLA). For solvent selection only olive oil and crude microbial oil were studied.

2.6. Fatty acid methyl esters and oleic acid analysis

Methyl esters and oleic acid sample concentration were analyzed with a 7890A gas chromatography (Agilent Technologies, Santa Clara, USA) equipped with a capillary column 1909BD-113 (30 m x 0.32 mm x 0.25 µm) and an auto-sampler. The column temperature was held at 190°C for 9 min, raised to 250°C at 20°C/min and kept at this temperature for 13 min. The temperature of the injector and the flame ionization detector were 250 and 300°C, respectively. Helium was used as the carrier gas at a constant flow of 0.9 mL/min. Standards of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate and oleic acid were purchased from Sigma-Aldrich (St Louis, USA).

3. RESULTS AND DISCUSSION

3.1. Microbial oil characterization

In Table 1 the composition of extracted lipids from microbial oil and the olive oil are presented and compared. Not all lipids obtained from microbial biomass are suitable for biodiesel production. Only saponifiable lipids and free fatty acids can be converted into FAMEs, which can be used as biodiesel if they comply with the current standards (ASTM D6751 in the United States or EN 14213 and 14214 in the European Union). The total lipids obtained from biomass cultivation were 27% (w/w) and 2.82

g/L of lipid concentration. The saponifiable lipids and free fatty acids were around 97.9 (wt%) of the total lipids extracted from the yeast biomass, with free fatty acids, diglycerides and triglycerides as the main components.

If these results are compared with those obtained from olive oil, and neutralized microbial oil, which not contained free fatty acids, the amount of triglycerides is higher in both oils 91.6 ± 0.4 and 83.6 ± 0.3 respectively against 75.7 ± 2.3 (wt%) of crude microbial oil. The non-saponifiable lipid fraction consisted of small amounts of carotenoids, retinoids, sterols and tocopherols for all samples are around 2 (wt%). The acid index of crude microbial oil was 10 mg KOH/g, which determines an acid-catalyzed process for chemical biodiesel production, more suitable for producing biodiesel than an alkali one in order to avoid yield losses from free fatty acid neutralization (Yan et al., 2010). The alternative is an enzymatic catalytic bioprocess catalyzed by lipases.

Table 2 shows the fatty acid profile for the saponifiable lipids and free fatty acids extracted from *Candida* sp. and olive oil. As can be seen, the microbial oil and the olive oil contain similar types of fatty acid. However, the olive oil does not have heptadecanoic and heptadecenoic acids in its composition. These fatty acids are very difficult to find in the nature, and only it is possible to be found in some bacteria and yeast. The crude microbial oil has a higher amount of saturated fatty acids than the olive oil, especially palmitic acid (19.2% versus 11.8%). However the olive oil has a higher content of monounsaturated fatty acids, oleic acid, than crude microbial oil (74.2% versus 56.45%). The high amount of palmitic inherent to the FAMEs derived from these fatty acids produces a high oxidative stability, but also the high amount of oleic acid of the FAMEs produces good fuel properties at low temperatures, which is an advantage in winter operation (Ramos et al., 2008).

The iodine value is a measure of the unsaturation level and, therefore, only depends on the oil used as the raw material (Davies and Hossain, 2009). Thus, the result of iodine value for the saponifiable lipids and the free fatty acids extracted from yeast was 75.8 mg I_2/100g, and for the olive oil 81.8 mg I_2/100g, both results were far below the specified a maximum limit of 120 mg I_2/100g in the European Union Standards for biodiesel. Finally, these results show that the microbial oil is quite similar to olive oil being a positive conclusion.

3.2. Organic solvent selection

The partition coefficient, log *P*, is used as an indicator of solvent polarity (hydrophilic character). Solvents with a log *P* value below 2.0 have not been considered appropriate because presents high polarity (Laane et al., 1987). In this work three different organic solvents were studied in reactions with immobilized rROL. FAMEs yield and stability of the biocatalyst were evaluated. The organic solvents studied presented different log *P* value, iso-octane (4.5), n-hexane (3.5) and terc-butanol (0.8) (Jin et al., 2013). Figure 1 shows the monitoring of FAMEs yield during the reaction time for olive and crude microbial oils using different organic solvents.

According to Figure 1, n-hexane and iso-octane solvents presented similar final FAME yields for both substrates. When the initial rates were compared, olive oil presented always higher initial rate (0.31 mmol/h, 0.39 mmol/h and 0.31 mmol/h for iso-octane, terc-butanol and n-hexane, respectively) than microbial oil (0.19 mmol/h for all solvents). Although this solvent is more polar that the n-hexane and iso-octane and should dissolve better the polar lipids the final FAMEs yield reached was the worst affecting negatively to the biocatalyst along the time. Thus, this solvent was discarded in next step due to the lower FAMEs yield obtained with this solvent.

Figure 2 presents the stability of immobilized rROL with iso-octane and n-hexane into the reaction medium using olive oil, after three reutilizations of 30 minutes. The reutilization of immobilized rROL in olive oil reaction showed that this enzyme is stable in iso-octane and n-hexane even after three utilizations with a low loss of activity, because these solvents protect the water surrounding the lipase that retain their optimal conformation of the enzyme (Fjerbaek et al., 2009).

The necessity of final solvent recovery can be a drawback to the process. However, the following aspects should be considered: solvent recovery is a common practice in the chemical catalyzed production of biodiesel and it is necessary in all cases to remove the excess of methanol; the boiling point of n-hexane is low (69°C) and near to methanol boiling point (65°C), resulting an easy separation of the solvent together with the methanol (Royon et al., 2007). The iso-octane boiling point is higher than the n-hexane boiling point (99ºC versus 69ºC). Thus, higher energy consumption and cost (about 6 fold higher) for its recovery is necessary. For these reasons n-hexane was selected as solvent for the next experiments.

3.3. Methanol stepwise additions

Enzymatic biodiesel production presents a difficult problem regarding the low tolerance of lipase toward acyl acceptors (i.e., methanol). The use of the organic solvent helps to reduce the methanol lipase inactivation since the methanol is solubilized in solvent preventing the enzyme water capture by methanol, but methanol can affect anyway the enzyme and it should not be added in excess (Fu and Vasudevan, 2010).

The monitoring of methyl esters yield along the time is shown in Figure 3. The vegetable oil was studied to compare the results obtained with a traditional feedstock to biodiesel synthesis and acid oleic to verify the behavior of the reaction in presence only of free fatty acids. As it was expected, the highest FAMEs yield was obtained using free fatty acids as substrate when a direct synthesis of FAMEs occurring. This is due to the fact that the esterification reaction occurs directly on FFA and is faster than transesterification where first the enzyme needs to break ester bonds (Al-Zuhair, 2005).

OLO reached higher final yield than NMO and CMO. It can occur because the microbial oil has a higher amount of sterol esters and polar lipids besides the unsaponifiable matter (Table 1) that could be the responsible of the poor access of the substrate into the enzyme, and subsequently, can decrease the final yield. Although, the final yield of CMO and NMO are quite similar, the conversion at times lower than 80 minutes for CMO is higher than NMO, associate to the fact that initially the CMO has a higher amount of free fatty acids, which causes that the reaction was faster.

Also, it is important to mention that because of the regiospecificity of rROL that acts in the locations 1 and 3 of TAG, the maximum yield achieved cannot be over of 66% of FAMEs. The final FAMEs yield obtained of OLO (54,3%) are nearby to the theoretical maximum yield (66%), little lower values were obtained for CMO and NMO, around 40%, however the reaction is not finished.

Moreover, the fractioned methanol additions strategies is an efficient process because tend to generate more continuous evolutions in methyl ester synthesis and therefore avoid periods of time where there is a slow synthesis of methyl ester caused for methanol exhaustion. Figure 4 shows the formation of the different methyl esters over time for each oil (CMO, OLO, NMO). Hence, the results show that the lipase used, has no preference for any particular free fatty acid, reacting simultaneously with them over time (Canet et al., 2014).

3.4. Biocatalyst stability

In order to know a possible enzyme inactivation due to methanol and solvent presence, the biocatalyst was removed from the reaction media after 240 minutes of reaction by vacuum filtration and used again. The results are shown in Figure 5. The reducing of the yield even after 24 hours of contact with large amount of non-reacted methanol and n-hexane was about 30% for both oils. Thus, methanol and n-hexane had a moderate inactivation effect on the immobilized lipase under the studied conditions. Moreover, the solubility of methanol in the alkyl esters is greater than in the oil, and then the inhibitory effect of methanol is large at the beginning of the reaction, but with increasing oil conversion it decreases and, consequently, limits enzyme deactivation (Shimada et al., 2002). Then, crude microbial oil presented a similar behavior, in terms of stability than OLO, showing that this substrate could be an alternative to vegetable oils in biodiesel production.

4. Conclusion

The results indicate that *Candida* sp. LEB-M3 oil may be a suitable feedstock for biodiesel production using rROL lipase immobilized on Relyzyme OD403 in a solvent system, obtaining similar results than vegetable oil, although the composition of microbial oil has the presence of heptadecanoic and heptadecenoic acids, sterol esters and polar lipids. Methanol stepwise addition showed to be an excellent strategy to maintain lipase activity with a lost not higher than 30% after 6 reutilizations. Finally, the use of glycerol from biodiesel production to growth *Candida* sp. LEB-M3 is important to reduce the economic impact of enzymatic biodiesel production approach.

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Figure captions

Figure 1: Monitoring of FAMEs yield (%) for (a) olive oil and (b) crude microbial oil from *Candida* sp. LEB-M3 oil during catalyzed biodiesel synthesis by immobilized rROL in different organic solvent media.

Figure 2: FAMEs yield (%) for olive oil reaction with immobilized rROL in iso-octane and n-hexane media during three cycles utilizations of 30 minutes reaction.

Figure 3: FAMEs yield (%) obtained in different substrates used for biodiesel synthesis in n-hexane media catalyzed by rROL with methanol additions.

Figure 4: Monitoring of methyl ester concentration in the reaction medium, showing the composition of each methyl ester. The data was obtained from methanol stepwise additions experiments for (a) CMO (b) OLO (c) NMO.

Figure 5: Relative FAMEs yield (%) of olive oil and crude microbial oil transesterification reaction catalyzed by rROL after 5 reutilizations (1440 minutes).

Tables and Figures

Table 1: Composition of lipids extracted from *Candida* sp. LEB-M3 and olive oil.

Lipid classification	**Type of lipid**	**Crude microbial oil (wt%)**	**Neutralized microbial oil (wt%)**	**Olive oil (wt%)**
Free fatty acids and saponifiable lipids	Free fatty acids	6.7 ± 1.0	n.d. *	n.d.*
	Monoglycerides	3.4± 0.4	3.0± 0.1	1.9± 0.3
	Diglycerides	6.6 ± 0.2	5.7 ± 0.2	3.7 ± 0.2
	Triglycerides	75.7 ± 2.3	83.6 ± 0.3	91.6 ± 0.4
	Sterol esters	3.4 ± 0.7	4.7 ± 0.4	0.3 ± 0.1
	Polar Lipids	2.1± 0.1	1.1± 0.0	0.4± 0.1
	Total saponifiable lipids	**97.9**	**98.1**	**97.9**
Non-saponifiable lipids	Carotenoids	n.d. *	n.d. *	0.9± 0.2
	Sterols and tocopherols	0.6± 0.1	0.6± 0.1	0.3± 0.1
	Retinoids	1.5± 0.0	1.3± 0.0	0.9± 0.2
	Total Non-saponifiable lipids	**2.1**	**1.9**	**2.1**

* n.d.: no detected.

Table 2: Fatty acid composition in the saponifiable lipids and free fatty acids from microbial oil, *Candida* sp. LEB-M3 and olive oil.

Fatty acid		**Microbial oil (wt%)**	**Olive oil (wt%)**[1]
Palmitic acid	16:0	19.2	11.8
Palmitoleic acid	16:1	7.45	1.5
Heptadecanoic acid	17:0	0.6	n.d. *
Heptadecenoico acid	17:1	2.37	n.d. *
Stearic acid	18:0	3.73	2.7
Oleic acid	18:1	56.43	74.2
Linoleic acid	18:2	9.15	8.5
Linolenic acid	18:3	0.85	0.7
Others		0.23	0.6

[1] Akoh et al., 2007

* n.d.: no detected.

Figure 1

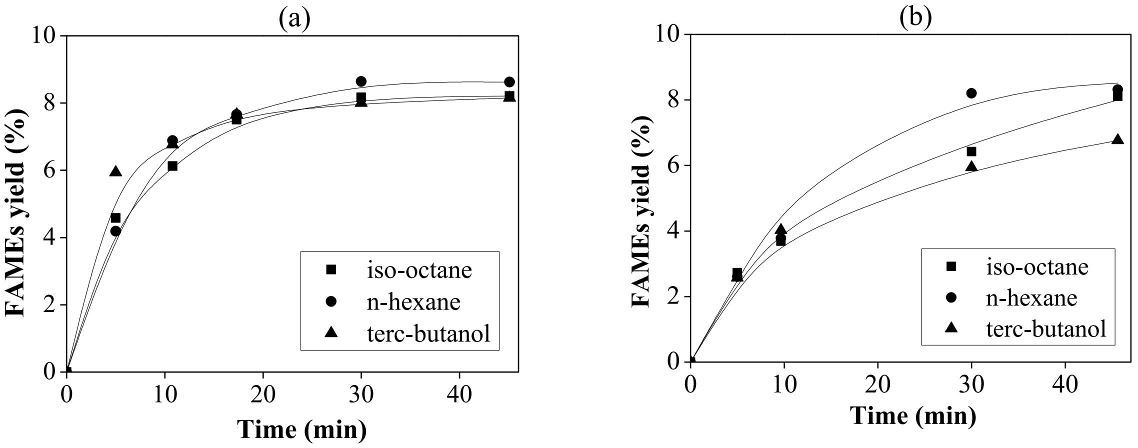
(a)
FAMEs yield (%)
0
2
4
6
8
10
Time (min)
0
10
20
30
40
iso-octane
n-hexane
terc-butanol
(b)
FAMEs yield (%)
0
2
4
6
8
10
Time (min)
0
10
20
30
40
iso-octane
n-hexane
terc-butanol

Figure 2

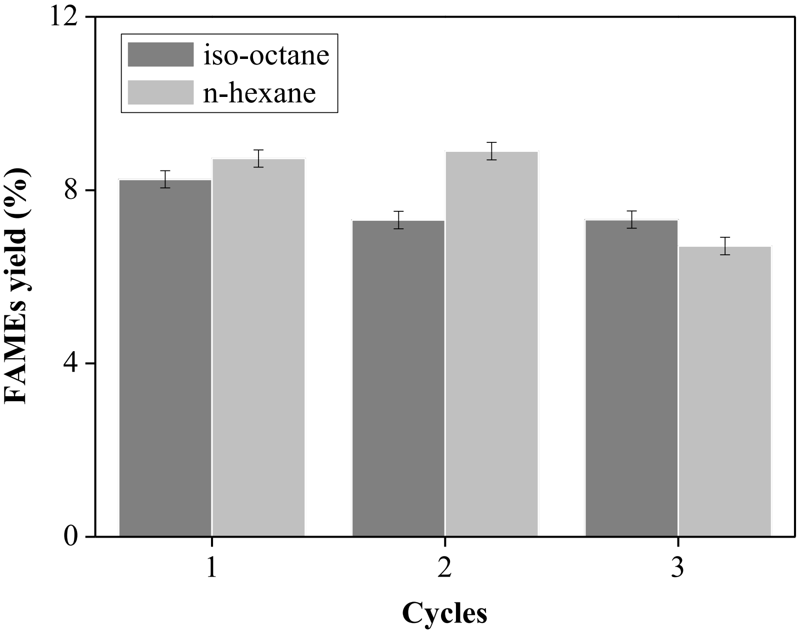

Figure 3

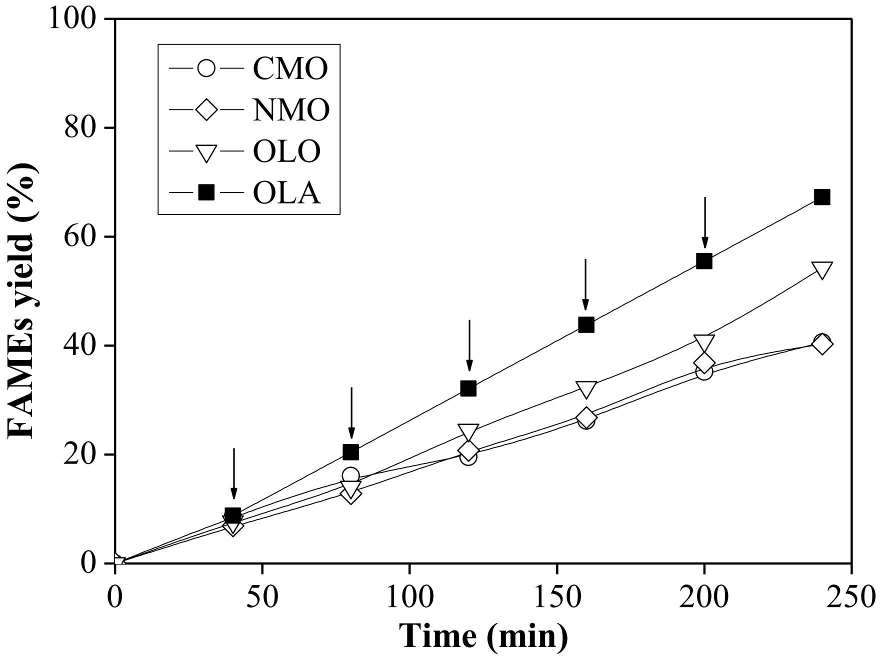
100
80
60
40
20
0
FAMEs yield (%)
0
50
100
150
200
250
Time (min)
CMO
NMO
OLO
OLA

Figure 4

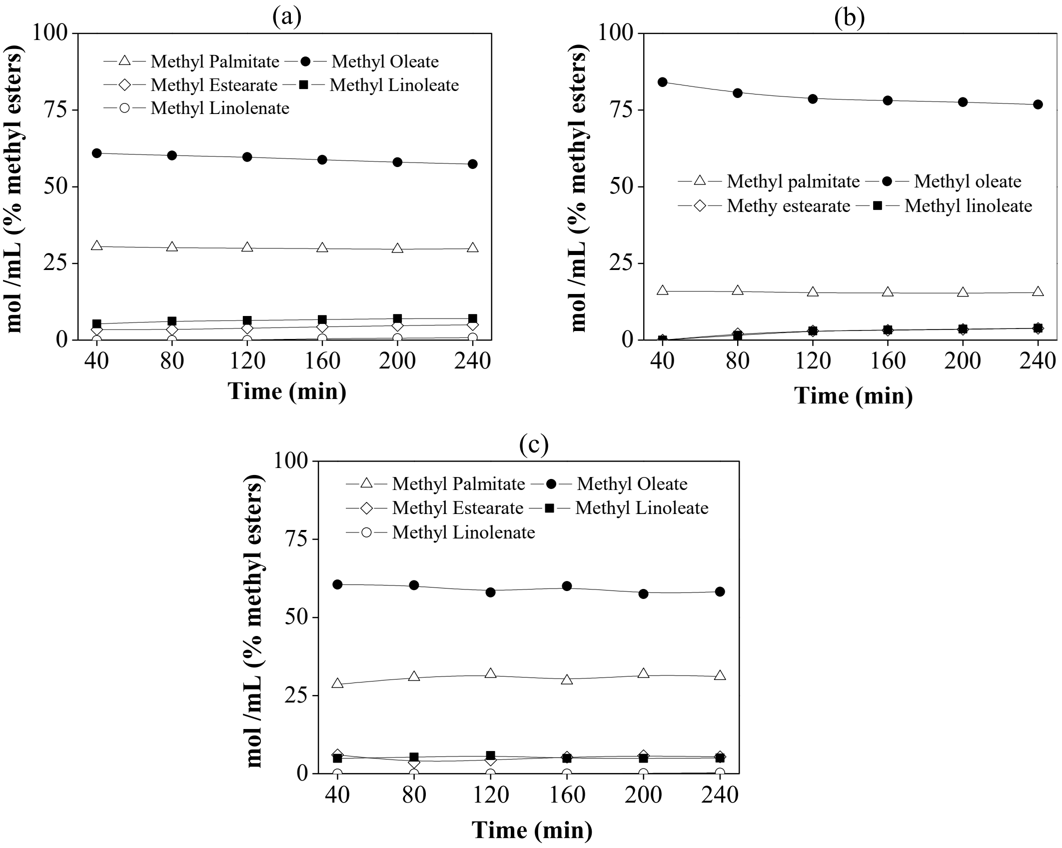
(a)
100
75
50
25
0
mol /mL (% methyl esters)
Methyl Palmitate
Methyl Oleate
Methyl Estearate
Methyl Linoleate
Methyl Linolenate
40
80
120
160
200
240
Time (min)
(b)
100
75
50
25
0
mol /mL (% methyl esters)
Methyl palmitate
Methyl oleate
Methy estearate
Methyl linoleate
40
80
120
160
200
240
Time (min)
(c)
100
75
50
25
0
mol /mL (% methyl esters)
Methyl Palmitate
Methyl Oleate
Methyl Estearate
Methyl Linoleate
Methyl Linolenate
40
80
120
160
200
240
Time (min)

Figure 5