Biodiesel production from crude Jatropha oil catalyzed by non-commercial immobilized heterologous *Rhizopus oryzae* and *Carica papaya* lipases

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Abstract

The aim of this study was to evaluate the feasibility of biodiesel production by transesterification of Jatropha oil with methanol, catalyzed by non-commercial *sn-1,3*-regioselective lipases. Using these lipases, fatty acid methyl esters (FAME) and monoacylglycerols are produced, avoiding the formation of glycerol

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as byproduct. Heterologous *Rhizopus oryzae* lipase (rROL) immobilized on different synthetic resins and *Carica papaya* lipase (rCPL) immobilized on Lewatit VP OC1600 were tested. Reactions were performed at 30ºC, with seven stepwise methanol additions.

For all biocatalysts, 51-65 % FAME (theoretical maximum= 66%) was obtained after 4 h transesterification. Stability tests were performed in 8 or 10 successive 4 h-batches, either with or without rehydration of the biocatalyst between each two consecutive batches. Activity loss was much faster when biocatalysts were rehydrated. For rROL, half-life times varied from 16 to 579 h. rROL on Lewatit VPOC 1600 was more stable than for rCPL on the same support.

Keywords: Biodiesel; *Carica papaya* lipase; Jatropha oil; *Rhizopus oryzae* lipase; *sn-1,3* regioselective lipase.

1. Introduction

Biofuels are a renewable alternative to fossil fuels that has lower greenhouse gas emissions. Several biofuel crops can be grown locally (including in marginal soils), helping countries to reduce their dependence on unstable foreign sources of fossil fuels. These potential environmental and social advantages of biofuels have led to some policy measures to support sustainable production. For instance, the Renewable Energy Directive (European Directive, 2009/28/E.C, 2009) forces EU Member States to achieve a minimum target of 10 % renewable energy in all the energy used in the transport sector by 2020

and a 7 % limit on food crop based biofuels. The fact that more than 95% of biodiesel production feedstocks come from edible oils, causes great concern because of the competition with the food supply chain. Consequently, there is now an increased interest in second generation biofuel crops, such as *Jatropha curcas* L., whose high oil content (27-45 % dry basis) is not suitable for consumption, because of the presence of toxic components (Makkar et al., 1998).

The most usual method in industry to transform oil into biodiesel is alkaline-catalyzed transesterification. However, this method has some disadvantages: uses large amounts of energy, the glycerol produced has low quality resulting in difficult and high-cost recovery and purification; alkaline catalyst is inactivated and removed by washing leading to the production of large amounts of alkaline effluents that must be treated. In addition, the free fatty acids present in the oil will form soaps by direct esterification with the catalyst (e.g. sodium hydroxide or sodium methoxide) leading to a lower biodiesel yield.

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are enzymes that, besides hydrolysis reaction, catalyze various synthetic reactions, including transesterification, when in low water activity media (Casas et al., 2012; Ferreira-Dias et al., 2013). The use of lipases as biocatalysts for biodiesel production has become more appealing, since lipases can act in mild temperature conditions, resulting in lower energy consumption, and with a wide diversity of raw materials, such as waste oils and fats with high levels of free fatty acids (FFA) and traces of water (Fan et al., 2012). Also, biodiesel recovery is easier since no emulsions are formed, less unit operations are needed and only small amounts of wastewater are produced. Furthermore, due to the high

selectivity of lipases, side-reactions with the formation of undesirable products, as well as soap formation occurring in alkaline-catalysis, are avoided, resulting in easier and environmentally friendly separation and purification processes (Juan et al., 2011).

The main reasons why lipases are not yet widely used in the industry are their cost and longer reaction time compared with alkaline catalysts. An essential strategy to lower the cost of the enzymatic process is the multiple reuse of the biocatalyst or its use in continuous bioreactors, which can be achieved by using immobilized enzymes. These biocatalysts must present both high transesterification activity and operational stability.

Lipase denaturation and inhibition by methanol (or ethanol) is currently observed during lipase-catalyzed transesterification. However, this problem can be overcome by stepwise addition of the alcohol along the reaction (Canet et al., 2014; Duarte et al., 2015; Kuo et al. 2015; Lotti et al., 2015; You et al 2013).Glycerol, the main byproduct of transesterification reaction, is one of the constraints for lipase-catalyzed transesterification efficacy. It adsorbs onto enzyme immobilization carriers, causing lipase deactivation and lowering the process efficiency (Hama et al., 2011). The use of *sn*-1,3-regioselective lipases to synthesize biodiesel and monoacylglycerols (MAG) simultaneously, avoiding the generation of glycerol, could be a solution for this problem (Calero et al., 2015; Canet et al, 2014; Verdugo et al., 2010). The MAG obtained can be used as emulsifiers in food, pharmaceutical and cosmetic industries.

In recent years, low-cost alternatives to commercial lipases have been developed in order to reduce process costs. The non-commercial heterologous *Rhizopus oryzae* lipase (rROL) has been produced and successfully used by

97 our group as catalyst for lipid restructuring (Nunes et al., 2011, 2012a; 2012b;
98 Simões et al., 2014; Tecelão et al., 2012b), for the production of bile acids or
99 corticoesteroid derivatives for pharmaceuticals applications (Quintana et al.,
100 2012, 2015) and also for biodiesel production (Bonet-Ragel et al., 2015; Canet
101 et al., 2014, 2016; Duarte et al., 2015). This recombinant lipase is a promising
102 new biocatalyst for biodiesel production that showed a 44-fold higher specific
103 activity compared to a commercially available lipase obtained directly from *R.*
104 *oryzae*, and a higher specificity towards the *p*-nitrophenol ester of long chain
105 length (Guillén et al, 2011).
106 *Carica papaya* lipase (CPL) is a naturally self-immobilized biocatalyst, since it is
107 attached to *Carica papaya* L. latex polymeric matrix. This low-cost biocatalyst
108 was also successfully used by us for enantioresolution (Rivera et al., 2013) and
109 the production of biopolymers (Sandoval et al., 2010), waxes (Quintana et al.,
110 2011) and human milk fat substitutes (Tecelão et al., 2012a). Efforts have been
111 made to isolate CPL unsuccessfully. Heterologous expression of this protein
112 shows as an alternative to overcome this problem (Rivera et al., 2013).
113 This study aims to: (i) produce Jatropha biodiesel (FAME) and
114 monoacylglycerols (MAG), by transesterification of Jatropha crude oil with
115 methanol, in stirred batch reactor and solvent-free media, catalyzed by non-
116 commercial *sn*-1,3 regioselective lipases, from microbial and plant origins,
117 immobilized in different supports and (ii) select the best immobilized biocatalyst
118 for Jatropha biodiesel production in terms of activity and operational stability.
119 The non-commercial heterologous *Rhizopus oryzae* lipase (rROL), immobilized
120 on Lewatit VPOC 1600, IRA-96, Lifetech™ ECR1030M, Lifetech™ ECR8285M,

Lifetech™ AP1090M; and the recombinant *Carica papaya* lipase (rCPL) immobilized on Lewatit VPOC 1600 were tested as biocatalysts.

2. Materials and Methods

2.1 Lipases

Two different non-commercial sn-1,3 regioselective lipases were tested: (i) the heterologous *Rhizopus oryzae* lipase (rROL), produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autonoma de Barcelona (UAB), Barcelona, Spain, and (ii) the heterologous *Carica papaya* lipase (rCPL), produced by the group of Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Guadalajara, Mexico.

rROL was produced by over-expression of the corresponding gene in a mutant strain of *Pichia pastoris*, according to Arnau et al. (2010) and Guillén et al. (2011). The rROL used in this study presented a hydrolytic activity of 178.8 U/mg of total protein according to the methodology developed by Resina et al. (2004).

rCPL, expressed extracellularly in *Pichia pastoris*, was produced by submerged fermentation in a rich medium at 30°C. Then, the culture broth was centrifuged and the supernatant was tested for lipase activity. Immobilized rCPL presents a hydrolytic activity of 30 U/mg of total protein.

2.2 Carriers

143 rCPL was immobilized by adsorption on Lewatit VPOC 1600. rROL was
144 immobilized by adsorption on different carriers: (i) Lewatit VP OC 1600, donated
145 by Lanxess, Germany; (ii) Lifetech^{TM} ECR1030M and (iii) Lifetech^{TM}
146 ECR1090M, gifts from Purolite. Also, rROL was immobilized by covalent binding
147 on: (iv) Lifetech^{TM} ECR8285M, kindly donated by Purolite, Wales, U.K; and (v)
148 Amberlite IRA 96, from Rhom and Haas, Philadelphia, USA. The main physical
149 and chemical properties of these five lipase immobilization carriers are
150 presented in Table 1.

151

152 **2.3 Jatropha oil extraction and characterization**

153 *Jatropha curcas* L. seeds were collected from healthy and ripened fruits
154 harvested in central Mozambique, in Sofala province (19º56'S; 34º24'E). The
155 whole seeds (not dehulled) were crushed with a hammer and the fraction
156 smaller than 2 mm diameter was mechanically extracted in a screw press, Täby
157 Press type 20 (Skeppsta Maskin AB, Sweden), as previously described
158 (Rodrigues et al., 2016).

159 The acidity (% of free fatty acids, FFA) of Jatropha oil was 3.7 %, and was
160 determined by titration, according to ISO standard 660:2009. This oil has 41.1
161 % of oleic acid, 38.8 % of linoleic acid and 11.6 % of palmitic acid, as major
162 fatty acids (Rodrigues et al., 2015).

163

164 **2.4 Methods**

165 **2.4.1 rROL and rCPL immobilization on Lewatit VP OC 1600 by adsorption**

rROL immobilization on Lewatit VP OC 1600 was carried out, as previously described by Tecelão et al. (2012b), by mixing the lipase powder with the carrier in 50 mL of 0.1 M phosphate buffer solution, at room temperature for 18 hours. The ratio lipase powder:support had been previously optimized for rROL (Tecelão et al., 2012b) and corresponds to 0.25 g of rROL (85.1 ± 10.5 mg of protein) per gram of Lewatit VP OC 1600. The beads were recovered by vacuum filtration and incubated, under gentle stirring with 25 mL of 2.5 % (v/v) glutaraldehyde solution, for 2 h at room temperature. The liquid phase was filtered and collected in order to determine protein content and evaluate immobilization yield. The immobilized lipase was rinsed twice with 50 mL of immobilization buffer solution, in order to remove the free enzyme and the liquid phase was collected for subsequent analysis. Beads were dried under reduced pressure for 10 minutes, transferred into a suitable container and kept refrigerated at 5 ºC until use.

rCPL was immobilized by direct adsorption on Lewatit VP OC 1600 (Sigma-Aldrich, Mexico) at 4°C, using 22 mg of total protein per g of support, without any subsequent treatment with glutaraldehyde. One of the reasons to select this hydrophobic support is because the natural support of papaya latex is also hydrophobic and can be used for lipase selective adsorption.

2.4.2 rROL immobilization on Lifetech™ AP1090M and Lifetech™ ECR1030M by adsorption

188 The immobilization of rROL on Purolite ECR resins was performed based on
189 the Purolite Application Guide - Purolite ECR Enzyme Immobilization
190 Procedures, with some modifications.

191 For lipase immobilization on Lifetech™ AP1090M and Lifetech™ ECR1030M
192 macroporous styrenes, the resin was previously equilibrated by washing it with
193 phosphate buffer solution (pH = 7, 0.05 M) and then filtered. A resin/buffer ratio
194 of 1/1 v/v was used.

195 The lipase (0.25 g of rROL per gram of wet resin) was dissolved in buffer
196 solution in a ratio of 1/4 (w/v) resin/buffer. Then, Purolite ECR resins were
197 added to the lipase solution and the mixture was gently stirred with the resin for
198 24 hours at room temperature. After, the liquid phase was filtered and collected,
199 in order to determine the protein content in the liquid and evaluate the
200 immobilization yield. The immobilized enzyme was washed with the
201 immobilization buffer (ratio resin/buffer of 1/1, w/v), the immobilized lipase was
202 filtered under reduced pressure and kept refrigerated at 5 ºC.

203

204 **2.4.3. rROL immobilization on Lifetech™ ECR8285M epoxy acrylate by**
205 **covalent binding**

206 The resin equilibration was carried out following the same procedure described
207 for Lifetech™ AP1090M and Lifetech™ ECR1030M resins. rROL was also
208 dissolved in immobilization buffer in a ratio of 0.25 g of rROL per gram of wet
209 resin and the ratio resin/buffer of 1/4 (w/v) was also used.

The mixture of lipase solution with the Purolite Lifetech™ ECR8285M resin was placed under gentle stirring at room temperature for 18 hours. After, the stirring was stopped and the solution was left static for another 20 h. Then, the liquid phase was filtered and collected for subsequent analysis, and the immobilized lipase was washed once with buffer. The immobilized enzyme was kept refrigerated at 5 ºC.

2.4.4 rROL immobilization on Amberlite™ IRA 96 by covalent binding

The methodology used for immobilizing rROL on anion exchange resin Amberlite™ IRA96 is based on the method described by Wang et al. (2010) with some modifications, as follows: 5 g of Amberite™ IRA 96 were added to 50 mL of deionized water and put under gentle stirring for 30 minutes at 50 ºC. The support was washed three times with 25 mL of NaOH aqueous solution 1 M, alternating with 25 mL of HCl aqueous solution 1 M. The anion exchange resin was then equilibrated by immersion in 100 mL of sodium phosphate buffer solution 0.2 M (pH = 7.5). After, Amberlite™ IRA 96 was mixed together with rROL dissolved in 10 mL of sodium phosphate buffer solution 0.2 M (pH = 7.5), at room temperature and under magnetic stirring for 4 h. The ratio lipase powder: support used was also 0.25 g of rROL per gram of Amberlite™ IRA 96. After, particles were filtered under reduced pressure and then brought into contact with 0.5 % (v/v) glutaraldehyde aqueous solution using 25 mL of glutaraldehyde solution per gram of support. The immobilized lipase was rinsed three times with 15 mL of immobilization phosphate buffer solution. Beads were dried in a desiccator, transferred into a suitable container and stored at 5 ºC.

2.4.5 Protein assay

The method described by Bradford (1976) was used to determine the total amount of protein immobilized on the resins, using bovine serum albumin from Sigma-Aldrich, Saint Louis, USA, as a standard. The immobilization yield was defined as the difference between protein amount in the initial lipase solution (before the immobilization support was added), and the residual protein present in the supernatant after immobilization (as well as in the subsequent washing solutions), divided by the protein content in the initial lipase solution.

2.4.5. Time-course transesterification reactions catalyzed by rROL and CPL

Transesterification reactions were carried out in 25 mL cylindrical glass reactors for 48 h, at 30°C, and under magnetic stirring. Reaction conditions, were the same as previously optimized by Canet et al. (2014), for biodiesel production from olive oil by rROL immobilized in octadecyl-Sepabeads: 4% (w/w) water content in the reaction medium, substrate molar ratio (methanol:Jatropha oil) of 3:1 and seven methanol additions. A load of 5% (w/w) of biocatalyst in relation to the amount of Jatropha oil (10 g) was used. Samples were taken before every methanol addition (after 0, 30, 60, 90, 120, 150 and 180 min) and at the end of the reaction, and stored at -18°C for subsequent analyses.

2.4.6 Operational Stability Tests

The operational stability of the immobilized rROL on different resins or rCPL on Lewatit VPOC 1600 was evaluated during consecutive 4 h batches, carried out under the same reaction conditions of time-course transesterification experiments (c.f. 2.4.5.). At the term of each batch, the biocatalyst was removed from the reaction medium by vacuum filtration. After, it was (i) immediately added into fresh reaction medium and reutilized in the next batch (total of 10 batches) or (ii) rehydrated with 10 mL of 0.1 M sodium phosphate buffer solution (pH 7.0), filtered under reduced pressure, added into fresh medium and used in the subsequent batch (total of 8 batches). Samples were collected at the end of each batch and stored at -18 ° C until further analysis.

It was considered that each biocatalyst has 100 % of its original activity, at the end of the first batch. In order to describe the deactivation kinetics of biocatalysts, each experimental point (FAME yield), at the end of each batch n, was converted into the fraction of the original activity, i.e. its residual activity. The residual activity (A_{res}, %) after each reuse was calculated as the ratio between FAME yield of batch n, divided by FAME yield observed in the first batch, and multiplied by 100. The fit of lipase deactivation models to the experimental data was performed using “solver”, a tool included in Microsoft Excel for Windows, by minimizing the sum of squares of errors between the experimental data and those estimated by the respective model. The following deactivation models, first order exponential decay (eq. 1) and two-component first order exponential decay (eq 2), were tested:

$$A_{res} = ae^{-kn} \qquad \text{(Eq. 1)}$$

$$A_{res} = a\, e^{-k_1 n} + b\, e^{-k_2 n} \qquad \text{(Eq. 2)}$$

where, k, k_1 and k_2 are deactivation coefficients (n^{-1}).

The kinetic constants were obtained by non-linear regression analysis for the tested models. Also, the operational half-life of the biocatalyst ($t_{1/2}$), *i.e.* the time after which the activity of the biocatalyst is reduced to 50 %, was estimated by the deactivation model fitted to the experimental results.

2.4.7 Analysis of reaction products

With the purpose of monitoring the transesterification reaction kinetics, the determination of MAG, diacylglycerols (DAG), triacylglycerols (TAG) and FAME contents was carried out for each sample, based on the European standard EN 14105: 2011, with some modifications. This European standard refers only to the detection of trace amounts of glycerol, MAG, DAG and TAG in purified biodiesel (FAME). Therefore, it was necessary to adapt the methodology to be able to follow the transesterification kinetics.

The preparation of the samples was carried out according to Faustino et al. (2015). Samples were derivatized with N-methyl-N-trimethylsilyl-tri-fluoroacetamide (MSTFA) to convert the -OH groups to -OSi $(Me)_3$ groups

The sample analysis was performed on a GC Agilent Technologies 7820A, equipped with an on-column injector and a flame ionization detector. The capillary column used for sample analysis was a J & W DB - 5HT (15 m x 0.32

303 mm x 0.10 mm). The main operating conditions of the equipment were the
304 same used by Faustino et al. (2015) in a study about the production of human
305 milk fat substitutes, by acidolysis of tripalmitin with camelina oil FFA, catalyzed
306 by rROL. All compounds with retention times equal or higher than 25 min were
307 considered as TAG; DAG and MAG were assumed as the compounds with
308 retention times between 22 and 25 min or 17.8 and 21 min, respectively. FAME
309 presented retention times between 11 and 17 min.

310 Calibration curves for methyl oleate (retention time of 12.7 min) and triolein
311 (retention time of 35.2 min) were established, in order to quantify each group of
312 compounds (%, w/w), using mononodecanoin as internal standard (Mono C19;
313 retention time of 19.4 min).The masses of partial acylglycerols (MAG and DAG)
314 were calculated using the equations from the European standard EN
315 14105:2011. FAME yield (%) was defined as the ratio between the amount of
316 methyl esters formed and the total amount of fatty acids (free and esterified in
317 MAG, DAG and TAG) in the oil at the beginning of the reaction.

318

319 **3. Results and Discussion**

320 **3.1 Immobilization yield**

321 rROL and rCPL were immobilized on synthetic resins by adsorption, since it is
322 an economic and easy immobilization technique that maintains lipase activity
323 and specificity. rROL was also immobilized on ECR8285 M and Amberite^{TM} IRA
324 96 by covalent binding, which is considered one of the most efficient technique
325 for enzyme immobilization, due to the formation of chemically stable covalent

linkages between the different functional groups of the lipases and the active functionalities of the carrier.

Table 2 shows the immobilization results in terms of yield and amount of immobilized protein. The highest immobilization yield was achieved with rCPL in Lewatit VPOC 1600 (98 %), which was higher than the value observed for rROL in the same support (77.2 %). It is worthy to notice that the amount of immobilized rCPL protein is much lower than that of rROL immobilized in Lewatit VP OC 1600.

With respect to rROL, the immobilization yields were similar for Lewatit VPOC 1600, ECR1030M and ECR8285M (77.2-79.5 %) and slightly lower for AP1090M (70.1 %). The lowest immobilization yield was observed for IRA-96. The immobilization method used (adsorption or covalent binding) seems not to affect the immobilization yield, evaluated in terms of immobilized protein.

3.2. Time-course of the transesterification reactions catalyzed by rROL and rCPL

The results obtained after 48 h batch transterification reactions with methanol and catalyzed by rROL immobilized on Lewatit VP OC 1600, IRA-96, ECR1030M, ECR8285M, AP1090M or rCPL on Lewatit VP OC 1600 are presented in Fig. 1.

The highest methyl ester production rates were observed in the beginning of the transesterification reactions. After the second methanol addition, reaction progress was slower and quasi-equilibrium was attained in less than 4 h for all

the biocatalysts tested. No glycerol was detected along the reactions. Thus, acyl migration was not produced.

The maximum percentage of FAME (%, w/w) in the reaction medium obtained with rROL immobilized on Lewatit VPOC 1600, IRA-96, ECR1030M, ECR8285M or AP1090M and rCPL immobilized on Lewatit VPOC 1600, was 64.5, 63.8, 64.8, 60.6, 60.4 and 51.7 %, respectively. These results are very close to the theoretical maximum FAME production, which is 66 (mol-%) for *sn*-1,3 regioselective lipases. They do not directly reflect the amounts of immobilized protein in the supports. In fact, for rROL, similar FAME production was observed when this lipase was immobilized in Lewatit VP OC 1600, IRA-96 or ECR1030M, while IRA-96 showed the lowest protein load (Table 2). With rCPL, 51.7 % FAME was obtained, in spite of the low amount of immobilized protein (21.6 mg/g Lewatit VP OC 1600). Probably, a higher rCPL load in the support would increase FAME yield.

More important than the amount of immobilized protein is the catalytic activity of this protein. Also, deactivation and/or steric hindrance on the lipase conformation occurring during immobilization, as well as internal diffusion effects during the reaction may be responsible for the different results observed.

The maximum amount of MAG varied from 1.5 % with rCPL immobilized in Lewatit VP OC 1600 to 27.9 % in rROL immobilized on Lewatit VP OC 1600.

In a study carried out by Canet et al. (2014), rROL immobilized in octadecyl-Sepabeads was successfully used as catalyst for the transesterification of virgin olive oil with methanol. Reaction conditions were the same as described in the present study and a 50.3 % FAME yield was achieved in 3 hour reaction. This

373 value is similar to that obtained with rCPL in our study. Also, Duarte et al.
374 (2015) produced biodiesel from yeast oil and olive oil using the same rROL
375 immobilized in Relizyme OD403 (polymethacrylate) as catalyst, in a solvent
376 system, with stepwise methanol addition. However, a lower FAME yield (40.6%)
377 was obtained with yeast oil as substrate, when comparing with olive oil (Canet
378 et al., 2014; Duarte et al., 2015) and Jatropha oil, in our study.

379 The transesterification of jatropha oil with methanol has been also carried out by
380 non-regioselective lipases, namely *Burkholderia cepacia* lipase immobilized on
381 modified attapulgite (You et al., 2013) and free recombinant *Candida rugosa*
382 lipase isozymes (Kuo et al., 2015), also using stepwise methanol addition.
383 When *Burkholderia cepacia* lipase was used, 94 % of biodiesel yield was
384 attained after 24 h reaction at 35 ºC (You et al., 2013). With *C. rugosa* lipase
385 isozymes, a maximum of 95.3 % FAME yield was obtained after 48h reaction at
386 37 ºC (Kuo et al., 2015).

387 In fact, in the studies on the production of biodiesel from jatropha oil, using non-
388 regioselective lipases as catalysts for the transesterification reaction, either in
389 solvent or solvent-free systems, FAME yields between 75 and 98 % have been
390 attained after 24 to 90 h reaction times (Juan et al., 2011) .

391 In our study, reaction equilibrium was attained after 4 h transesterification with
392 all the biocatalysts tested. This result, together with the absence of free glycerol
393 in the reaction medium, is highly beneficial in terms of industrial scale-up of the
394 process and reaction implementation in continuous bioreactors.

395

396 **3.4 Operational stability of the tested lipases**

397 The short reaction time needed to attain equilibrium, together with the high
398 FAME yields obtained with all biocatalysts tested are very interesting results.
399 However, a high operational stability of the biocatalyst is also a key-factor for
400 industrial implementation of the process.

401 Thus, the operational stability of rROL and rCPL immobilized in different
402 supports was assayed in 10 or 8 consecutive batches, as previously described
403 (c.f. 2.4.6). The duration of each batch (4 h) was selected from the results
404 obtained in the 48 h time-course transesterification. Since biocatalyst
405 inactivation by dehydration is currently described (Nunes et al., 2012b; Tecelão
406 et al., 2012b), lipases were rehydrated between batches.

407 Residual activities along reuses, deactivation models fitted to the experimental
408 data and estimated half-life times for the biocatalysts tested, are presented in
409 Fig. 2 and Table 3. Lipase stability was found to be dependent on the
410 characteristics of immobilization matrices. The behaviour of rROL in ECR8285M
411 and rehydrated rROL in Lewatit VP OC 1600 can be described by a two
412 component first-order decay model. The inactivation profiles of rROL in
413 AP1090M, ECR1030M or IRA-96 and rCPL in Lewatit VP OC 1600, could be
414 well described by a first-order deactivation model.

415 The best results, in terms of operational stability, were observed when the
416 biocatalysts were reused without rehydration. The highest half-life value was
417 estimated for rROL immobilized in ECR1030M (579 h), followed by IRA-96 (381
418 h), AP1090M (270 h) and Lewatit VP OC 1600 (113 h). For rROL in ECR8285M
419 and rCPL in Lewatit VP OC 1600, the stability was much lower, with half-lives of
420 16 and 27.3 h, respectively. The lower stability exhibited by rCPL immobilized in

421 Lewatit VP OC 1600, compared to that of rROL in the same support, may be
422 explained by different protocols followed for immobilization. The treatment with
423 glutaraldehyde after rROL adsorption will promote the formation of stable
424 crosslink between the lipase and the matrix, as well as intermolecular bonds
425 between enzyme molecules, hindering the leakage of enzyme molecules along
426 operation (Tecelão et al., 2012b). Also, the inhibitory effect of methanol for rCPL
427 may be stronger than for rROL. When lipases were rehydrated between reuses,
428 a dramatic loss of activity was observed, probably due to the leaching of
429 enzyme molecules during hydration. Also, the presence of water molecules in
430 the support will increase its hydrophilicity, promoting methanol diffusion inside
431 the support, with the risk of reaching inhibitory concentrations at the
432 microenvironment of the enzyme.
433 When rROL immobilized in Lewatit VP OC 1600 was used as catalyst for the
434 production of (i) human milk fat substitutes (HMFS) by acidolysis of tripalmitin
435 with oleic acid, in solvent-free media, an increase in operational stability was
436 observed when the biocatalyst was rehydrated between reuses ($t_{1/2}$ increased
437 from 64 to 195 h) (Tecelão et al., 2012b). Similar behaviour was observed with
438 the same immobilized rROL used for the production of low calorie TAG: an
439 increase in $t_{1/2}$ from 49 to 234 h was observed after rehydration (Nunes et al.,
440 2012b). It worth to notice that in these studies carried out by Tecelão et al.
441 (2012b) and Nunes et al. (2012b), no water was added to the reaction medium.
442 In the present study, the addition of 4% water to the reaction medium shows to
443 be sufficient to maintain rROL activity. Conversely, the rehydration of rROL
444 immobilized in Eupergit C, also used for the production of low calorie TAG in

445 solvent-free media, resulted in a decrease in operational stability of the
446 biocatalyst (Nunes et al., 2011; 2012 b).

447 The operational stability of rROL in octadecyl sepabeads used by Canet *et al.*
448 (2014) in the transesterification of olive oil was evaluated after 2 and 21 h of
449 reaction time. No significant differences were observed in methyl esters yield
450 (%) when this biocatalyst was reused. As in the experiments without biocatalyst
451 rehydration between batches of the present study, methanol was not washed
452 out between batches. In view of that, methanol did not seem to inactivate rROL
453 in octadecyl-Sepabeads under the considered conditions. However, the same
454 lipase immobilized in Relizyme OD403 lost 30 % of its activity after 6
455 reutilizations of 4 h each in transesterification of yeast oil or olive oil (Duarte et
456 al., 2015).

457 Luna *et al* (2014) used a low-cost multipurpose additive for the food industry
458 (Biolipase-R, from Biocon-Spain), containing *Rhizopus oryzae* lipase, as
459 catalyst for the transesterification reaction of sunflower oil with ethanol. When
460 this enzyme preparation was covalently immobilized on amorphous
461 $AlPO_4$/sepiolite support with the *p*-hydroxybenzaldehyde linker, a conversion of
462 84.3 % of TAG into a blend of fatty acid ethyl esters (FAEE), MAG and DAG
463 was obtained, after 2h reaction at 30 ºC, using a molar ratio oil/ethanol of 1:6.
464 This biocatalyst did not show a significant loss of its initial catalytic activity for
465 more than five successive reuses of 2 h each.

466 The rROL used in the present study showed similar or even higher stability in
467 presence of methanol than Biolipase-R in presence of ethanol, which is an
468 alcohol with lower deactivation effect on lipases.

4. Conclusions

The non-commercial recombinant *sn*-1,3 regioselective lipases rROL and rCPL are promising catalysts for the production of biodiesel and MAG from crude Jatropha oil. Transesterification was rather fast and equilibrium was reached after 4 h-reaction with high FAME yields, varying from 51.7 to 64.8 %, which is very close to the theoretical maximum for *sn*-1,3 regioselective lipases (66%).

All biocatalysts were active during 10 consecutive batches without rehydration. However, when lipases were rehydrated between each two consecutive batches, the loss of activity was much faster. rROL in Lewatit VPOC 1600 showed to be more stable than rCPL in the same support.

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620 **Figure captions**

621

622 Figure 1. Evolution of fatty acid methyl ester (FAME), monoacylglycerol (MAG),
623 diacylglycerol (DAG) and triacylglycerol (TAG) concentrations in the reaction
624 medium, during the 48 hour transesterification reaction catalysed by rROL
625 immobilized in different supports or rCPL in Lewatit® VP OC 1600.

626

627 Figure 2. Operational stability of rROL immobilized in different supports or CPL
628 in Lewatit® VP OC 1600, with and without rehydration of the biocatalyst
629 between each consecutive 4-h batch, when transesterification of Jatropha oil
630 with methanol was performed.

631

632

633

Table 1. Main physical and chemical properties of synthetic resins tested for rROL or rCPL immobilization.

Carrier	Polymer structure	Method of immobilization	Functional Group	Particle size range (mm)	Pore Diameter (A)	Structure/ Appearance
Lifetech™ ECR8285M	Epoxy/butyl methacrylate	Covalent binding	Epoxy	0.30 - 0.71	400 - 500	White, spherical, porous beads.
Lifetech™ AP1090M	Macroporous styrene	Adsorption	None	0.30 - 0.71	900- 1100	
Lifetech™ ECR1030M	DVB/ methacrylate	Adsorption	None	0.30 - 0.71	250	
Lewatit® VP OC 1600	DVB-crosslinked methacrylate	Adsorption	None	0.315 - 1.0	150	
Amberlite™ IRA-96	Styren/divinylbenzene copolymer	Covalent binding	Tertiary amine: at least 85 %	0.55 - 0.750	-	Tan, opaque, spherical beads

Table 2- Immobilization yields (± STD) and amounts of immobilized protein for rROL immobilized in different supports and rCPL immobilized in Lewatit VP OC 1600.

Biocatalyst	**Immobilization yield (%)**	**Immobilized protein (mg/g support)**
rROL in Lewatit VPOC 1600	77.4 ± 4.1	65.7
rROL in Amberlite IRA-96	58.8 ± 1.0	50.0
rROL in Lifetech ECR1030M	79.5± 1.5	67.6
rROL in Lifetech ECR8285M	78.4 ± 8.3	66.7
rROL in Lifetech AP1090M	70.1± 7.8	59.6
rCPL in Lewatit VP OC 1600	98.0 ±1.2.	21.6

Table 3. Deactivation model equations fitted to the experimental data and the estimated half-lives for rROL immobilized in different supports and rCPL in Lewatit® VP OC 1600 (n= batch number; 1 batch= 4 h).

Biocatalyst		Deactivation Model	Model Equation	Half life time (h)
rROL in Lifetech™ ECR8285M	Non rehydrated	Two component first-order	$A_{res} = 0.06e^{0.61n} + 115.20\ e^{-0.21n}$	16.0
	Rehydrated	Two component first-order	$A_{res} = 22178.04e^{-5.64n} + 23.55\ e^{-0.12n}$	4.7
rROL in Lifetech™ AP1090M	Non rehydrated	First-order	$A_{res} = 85.76\ e^{-0.008n}$	270.0
	Rehydrated	First-order	$A_{res} = 97.17\ e^{-0.17n}$	15.6
rROL in Lifetech™ ECR1030M	Non rehydrated	First-order	$A_{res} = 66.79\ e^{-0.002n}$	579.0
	Rehydrated	First-order	$A_{res} = 203.92\ e^{-0.73n}$	7.7
rROL in Amberlite™ IRA-96	Non rehydrated	First-order	$A_{res} = 88.51\ e^{-0.006n}$	380.7
	Rehydrated	First-order	$A_{res} = 294.02\ e^{-1.07n}$	6.6
rROL in Lewatit® VP OC 1600	Non rehydrated	First-order	$A_{res} = 80.91\ e^{-0.017n}$	113.0
	Rehydrated	Two component first-order	$A_{res} = 8.14\ e^{-0.10n} + 475.3\ e^{-1.65n}$	5.8
CPL in Lewatit® VP OC 1600	Non rehydrated	First-order	$A_{res} = 90.26\ e^{-0.087n}$	27.3
	Rehydrated	First-order	$A_{res} = 710.83\ e^{-1.96n}$	5.4

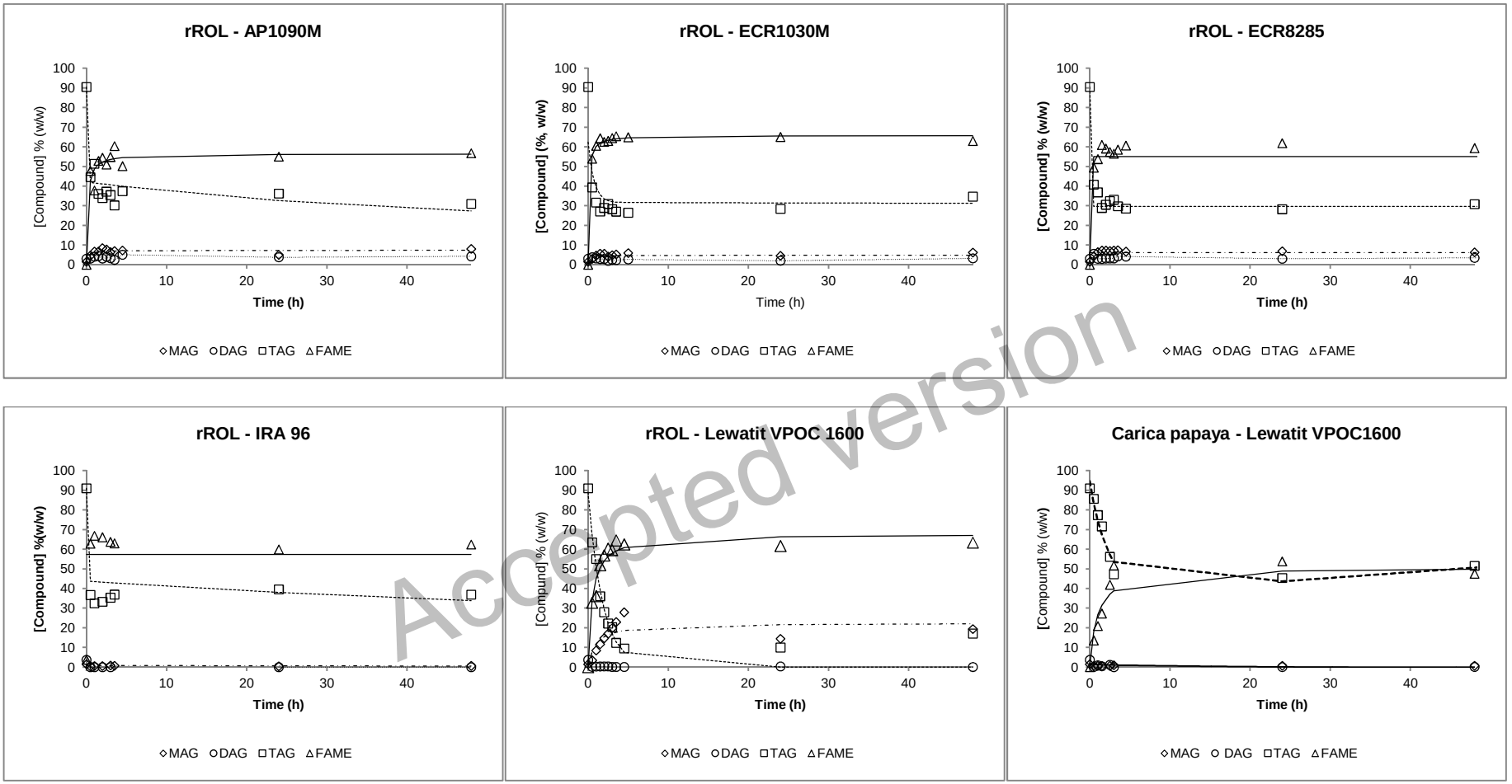

Fig. 1

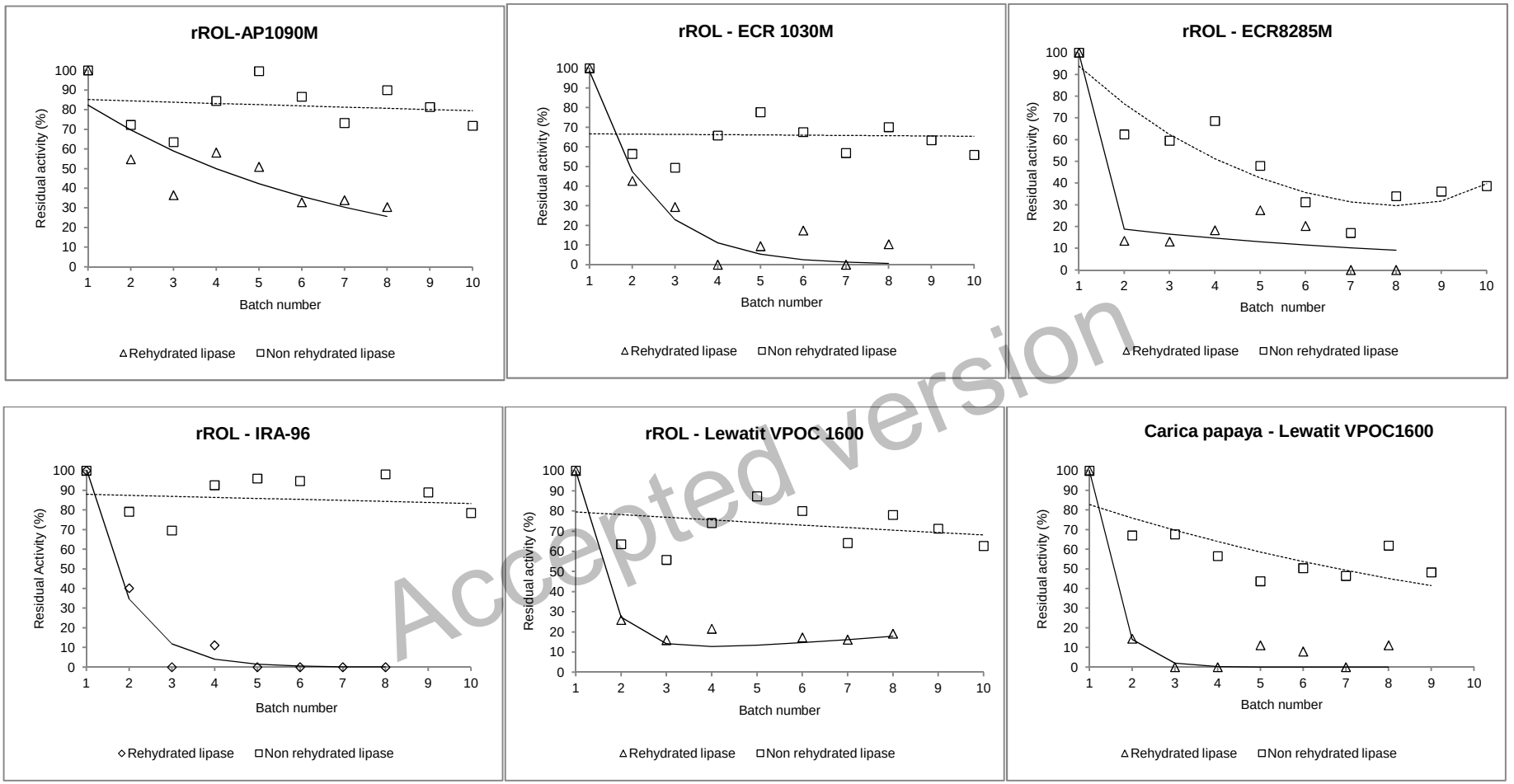

Fig. 2