Exploring substrate specificities of a recombinant *Rhizopus oryzae* lipase in biodiesel synthesis

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ABSTRACT

The alcoholysis of triolein was used to explore the specific features of a recombinant *Rhizopus oryzae* lipase (rROL) for biodiesel synthesis. For this purpose, different acylglycerols were compared as substrates in lipase-catalysed transesterification. rROL was shown to be more than 4-fold more specific towards 1-monoolein than triolein; in contrast, rROL did not accept 2-monoolein as substrate, concluding that it is highly 1,3-positional specific. Comparing ethanol and methanol as acyl-acceptors, it was observed that the latter caused more lipase inactivation. Regarding alcohols, it was also demonstrated that acyl migration occurred in moderate alcohol concentrations.

The characteristic lipase interfacial activation was also studied in the case of rROL. Thus, experiments comparing mono- and biphasic systems were carried out, achieving more than 20-fold initial alcoholysis rate when a mono-organic phase was used, which shows that rROL does not need interfacial activation to perform efficient biodiesel synthesis.

1. Introduction

Biodiesel is a mixture of fatty acids monoalkyl esters (FAMEs) and a biodegradable and renewable alternative fuel source with several advantages versus current diesel fuel [1,2]. It is produced industrially from short chain alcohols – i.e. acyl-acceptor – and triacylglycerols – i.e. acyl-donor –, typically vegetable oils using alkali catalysis – a process widely referred to as transesterification – [3].

Compared to the alkali process, lipase-catalysed transesterification has several advantages; mainly that lipases perform their conversions in milder conditions [4] and are able to catalyse both transesterification and esterification, which allows the use of cheaper raw materials with high amounts of free fatty acids for biodiesel production [5]. For these reasons, this process has drawn huge scientific attention during the last ten years [6]. However, the use of lipases in biodiesel synthesis has many limitations and drawbacks, therefore in recent years there has been an increasing number of scientific publications studying different lipase transesterification aspects. One of the main bottlenecks for the industrial application of the enzymatic process is the high cost of the catalyst – i.e. lipase – compared to the alkali catalyst, such as NaOH [6].

The different substrates, intermediates, by-products and products throughout the transesterification have an effect both on the reaction progress and on the synthesis activity of the lipase; thus, the monitoring and control of these species is crucial to optimising lipase biodiesel synthesis. In the case of the acyl-donors, several lipid-class species participate throughout the transesterification reaction, as shown in Figure 1. Initially, triacylglycerols are almost the sole compound, appearing later di- and monoacylglycerols as intermediates, which are also consumed. Bearing in mind that lipases exhibit different kinds of selectivities towards different lipid-class species [7,8], during transesterification different reaction rates will be obtained depending on the predominant species at each moment and the specificity of the lipase towards it.

Moreover, a wide range of lipases are 1,3-positional selective, which means that these enzymes are specific to hydrolyse ester bonds positions 1 or 3 in triacylglycerols [4]. Several examples of transesterifications described in the literature used this type of lipase from *R. miehei* [9–11], *R. oryzae* [10,12–14], *P. fluorescens* [10] or pig pancreatic lipase [15]. Although the utilization of these lipases in theory leads to a maximum theoretical yield of 66.7%, higher yields were often observed [10,13,14,16,17], due to acyl migration, which promotes the conversion of 1,2- to 1,3-diacylglycerols and 2- to 1(3)-monoacylglycerols, achieving an overall conversion to glycerol and biodiesel. Nevertheless, glycerol is related to several drawbacks, such as loss of lipase activity [18] and technical problems in diesel engines [15]. In contrast, monoacylglycerols are a by-product of interest, with several applications in the food and pharmaceutical industries –especially as emulsifiers – [19,20] and recently it has been reported that their presence in final biodiesel enhances its lubricity [15,21].

Regarding alcohols, although ethanol and methanol are widely recognised to have stronger negative effects on lipase comparatively to other alcohols, they are the most commonly used as acyl-acceptors in biodiesel synthesis, especially methanol because of its economic feasibility and accessibility [1,22,23]. Nevetheless, some lipases are more tolerant to methanol than others, such as those from *Pseudomonas* genus, while *C. rugosa* lipase, for instance, appears to be more methanol sensitive [23]. So, the awareness of the alcohol tolerance of a lipase is fundamental in keeping a high catalytic activity of the enzyme.

Moreover, free fatty acids can also participate in transesterification if they are present in the initial substrate or released after triacylglycerols hydrolysis as a consequence of water presence in the reaction medium. Water content in the reaction medium also plays an important role in lipase-catalysed reactions. First, a minimum amount of water is needed to keep lipase conformation, but an excess of water may lead to hydrolysis [5]. Furthermore, lipases are widely known to express generally higher catalytic activities in organic-aqueous interfaces than in monophasic systems – a phenomenon known as interfacial activation – [24–26]. Thus, water is not only crucial in keeping lipase conformation, but also allowing it to exhibit a high activity.

In this present study, the behaviour and the specificities of rROL towards different substrates were explored in transesterification reactions. Quantification analysis of free oleic acid, methyl and ethyl oleate, 1(3)- and 2-monoolein, 1,3- and 1,2-diolein and triolein have been made in order to understand the whole transesterification process and also to study acyl migration in the different reactions carried out. In addition, experiments comparing the lipase specificity towards different alcohols (i.e. ethanol and methanol) and acylglycerols (1-monoolein and triolein) were performed. Moreover, the effect of the organic-aqueous interface on the catalytic activity of the rROL was also explored, studying mono- and biphasic reaction systems and using the enzyme both in a freely soluble and immobilised form. This work has been done with a recombinant 1,3-positional selective *Rhizopus oryzae* lipase (rROL), which was cloned and expressed in *Pichia pastoris* [27], and previously used in several biodiesel synthesis studies [3,12,28,29] and also in other lipase biotransformations [30–32]. Experiments using a commercial *R. oryzae* lipase (ROL) were also carried out in order to compare the two lipases.

2 Materials and methods

2.1. Materials

High-grade n-hexane, n-tetradecane, methanol and methyl *t*-butyl ether were purchased from Merck (Darmstadt, Germany) and n-heptane from VWR Chemicals (Stockholm, Sweden). Analytical grade ethanol (99.5%) was obtained from Solveco (Rosersberg, Sweden). Ethyl oleate, methyl oleate and oleic acid were from Aldrich. Silica gel and *p*-nitrophenyl butyrate were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Relizyme OD403/S was from Resindion S.r.l. (Binasco, Italy). *N*-methyl-*N*-trimethylsilyheptafluorobutyramide (MSHFBA) was obtained from Macherey-Nagel (Düren, Germany) and triolein from Larodan Fine Chemicals (Malmö, Sweden), which contain traces of diolein and free oleic acid. Other chemicals were of analytical grade.

2.2. Lipase

Recombinant *R. oryzae* lipase (rROL) was produced in the Bioprocess Engineering and Applied Biocatalysis research group in the Universitat Autònoma de Barcelona (UAB) as stated in previous work [3]. The enzyme was produced by fed-batch cultivation of a recombinant *P. pastoris* strain using methanol as inductor. The culture broth was centrifuged and microfiltered to remove biomass, after which the supernatant was concentrated by ultrafiltration in a Centrasette® system from Pall Filtron (New York, USA) equipped with an Omega membrane of 10-kDa cut-off and subsequently dialysed against 10 mM Tris-HCl buffer at pH 7.5 and thereafter lyophilised.

Commercial *R. oryzae* lipase (ROL; product number 80612-25G) was purchased from Sigma-Aldrich.

2.3. Lipase activity

The hydrolytic activity of the lipase was analysed via spectrophotometric assay based on *p*-nitrophenyl butyrate hydrolysis, as described in a previous study [33]. The absorbance of the p-nitrophenol formed was measured continuously at a wavelength of 400 nm in a thermostat spectrophotometer at 25°C using pH 7 sodium phosphate buffer. *p*-nitrophenyl butyrate initial concentration was 0.4 mM in the cuvette and the enzyme concentration was chosen so that it allowed linear absorbance measurements for 2 minutes.

2.4. Recombinant R. oryzae lipase immobilisation

rROL was immobilised by adsorption on Relizyme OD403/S. 0.7 g of lyophylised powder rROL were dissolved in a volume of 50 ml 5mM phosphate buffer at pH 7 under magnetic stirring at 4°C for 1 hour; then the solution was centrifuged and the supernatant kept for immobilisation. Meanwhile, 2.8 g of Relizyme OD403/S were mixed with 40 ml of water-acetone (50/50 v/v) solution for 30 min; after that the solution was removed by vacuum-filtration and washed several times with water to remove acetone. The pre-treated support – i.e. Relizyme OD403/S – was then mixed with the supernatant lipase solution for 7 hours at 4°C under mild agitation. Then the immobilisation was stopped by vacuum-filtration, rinsing the immobilised lipase with 200 ml of the initial buffer. Finally, the biocatalyst was dried to constant weight in a silica gel desiccator and stored at -20°C until use.

2.5. 1- and 2-monoolein preparation

2-monoolein was prepared according to previous published work [33]. 1-monoolein was synthesised by 2-monoolein acyl migration by adding 0.1 g silica gel and 3 ml heptane to 0.3 g 2-monoolein and setting the reaction at 70°C under 1000 rpm agitation in a thermoshaker for 24 hours. The silica was removed from the reaction medium by vacuum filtration and the solvent by rotary evaporator. 1-monoolein was stored at -20°C.

2.6. Transesterification experiments

All reactions were carried out in 4.5 ml glass vials in a thermoshaker at 37°C under 1000 rpm agitation. Duplicates of each experiment were conducted. Three types of transesterification experiments using triolein as substrate were performed, varying lipase preparation and reactions systems: free rROL in an organic-aqueous solution – i.e. biphasic system –, immobilised rROL in an organic-aqueous solution – i.e. biphasic system – and immobilised rROL in organic solution – i.e. monophasic system –. Reactions were prepared with 0.265 g triolein dissolved in 1.6 ml heptane (160 mM triolein) – i.e. the organic phase. Different triolein to acyl acceptors molar ratios were studied, adding different amounts of ethanol and methanol at the beginning of the reaction. 0.05 g lyophilised powder rROL were dissolved in 1 ml water, centrifuged and 0.4 ml of the supernatant were added to the reactions for free rROL catalysed reaction experiments. For the experiments with immobilised rROL, the same total lipase activity as in free rROL catalysis experiments was used, adding approximately 0.08 g of

biocatalyst in each reaction; 0.4 ml water were added to some of them – corresponding to the immobilised rROL in a biphasic system experiment.

2.7. Comparison recombinant/commercial lipase

All reactions were carried out in 4.5 ml glass vials in a thermoshaker at 37ºC under 1000 rpm agitation. Duplicates of the experiments were done. Transesterification comparison reactions using triolein, 1-monoolein or 2-monolein as substrates were performed dissolving it in 1.6 ml heptane to an initial concentration of 100 mM. These experiments were catalysed with 0.1 ml of free rROL and free ROL solution preparations, prepared as previously described. Compared to the previous transesterification experiments described above, the initial substrate concentration and the amount of lipase liquid preparation used were reduced (100 instead of 160 mM and 0.1 instead of 0.4 ml, respectively) in order to avoid the formation of emulsions. 0.026 ml ethanol were added as acyl acceptor. In the case using 2-monoolein, a control reaction was set with the same conditions but without lipase, in order to quantify and discount the acyl migration from the results.

2.8. Sample preparation and GC analysis

Sample preparation and GC analysis were almost the same as previously described [10]. Oleic acid, methyl and ethyl oleate, 1- and 2-monoolein, 1,2- and 1,3-diolein and triolein concentrations in the samples were analysed in a 430-GC gas chromatograph with an auto-sampler CP-8400, both from Varian (Agilent Technologies Inc., Santa Clara, USA), equipped with a flame ionisation detector and a capillary column (CP8907 VF-1 ms 15 m x 0.25 mm x 0.25 µm) from Agilent Technologies. Helium was used as a carrier gas and the temperature of both detector and injector was 350ºC. The starting oven temperature was 180ºC; it was maintained for 2.5 minutes; then the temperature was increased by 10ºC/min to a temperature of 340ºC and held for 26 minutes.

Samples were withdrawn at selected times from the organic phase of the reaction media and derivatised by silylation by adding an equal volume of MSHFBA and incubating them at room temperature for 30 min. An equal volume of ethanol was added afterwards to stop the silylation reaction. Samples were then diluted with a n-tetradecane solution (10 mM in hexane), used as internal standard.

3. Results and discussion

Fig. 1 shows the complete transesterification process from triolein to glycerol as a succession of single acylglycerol alcoholysis reactions (Fig. 1, reactions 1,2,3,6 and 7), catalysed by a non-regioselective lipase. However, the alcoholysis from 2-monoolein to glycerol (Fig. 1, reaction 3) is negligible when using 1,3-selective *R. oryzae* lipase, and thus acyl migration reactions (Fig. 1, reactions 4

and 5) are needed to reach all the way to glycerol. A corresponding reaction scheme could be drawn for hydrolysis with water and oleic acid replacing alcohol and alkyl oleate, respectively.

3.1. Effect of organic-water interface on rROL catalytic activity

Lipases in nature usually catalyse reactions at organic-aqueous interfaces and actually these enzymes display higher catalytic activity under such conditions than in a homogeneous solution; this phenomenon is known as interfacial activation, as previously mentioned [26]. In biodiesel production, quite promising results have been obtained with the lipase dissolved in the aqueous phase of a biphasic system with the oil as organic phase [34,35]. Nevertheless, immobilisation is an attractive methodology to enhance lipase activity and facilitate lipase reuse [26]. Here, three different systems with different rROL preparations were studied: free rROL (biphasic system), immobilised rROL without added water (monophasic-organic phase) and for comparison also immobilised rROL with water added (biphasic system).

Comparing both biphasic systems (Table 1), the initial rates were higher for free lipase regardless of alcohol type, most probably because the immobilised system added more mass transfer limitations – the particles – to the liquid-liquid ones – i.e. the interface between organic and aqueous phases. However, the highest rates were achieved for reaction systems catalysed by immobilised rROL without added water, being around 20 times higher than those of biphasic systems for both alcohols. This suggests that for the immobilised lipase no organic-aqueous interface is necessary to achieve high lipase alcoholysis activity. It is important to mention that even in biphasic systems, initially there was also formation of free oleic acid due to the presence of water, but it reached a much smaller concentration than the one for oleate esters (data not shown in Table 1).

3.2. Acyl-acceptor preference

Initial reaction rates for biphasic systems increased slightly with increasing alcohol to triolein molar ratio, except for the case of free lipase with ethanol, in which it remained constant (Table 1).

Another important result from the monophasic-organic system reactions is that reaction rates decreased dramatically when the alcohol to triolein molar ratio was increased from 3 to 6, especially in the case of methanol, where the initial reaction rate decreased from 9.5 to 0.3 mM/min (Table 1). This might be explained by the fact that adding more alcohol to the system generated a separate non-soluble alcohol phase which caused enzyme inactivation. In the biphasic systems, the alcohol was diluted in the aqueous phase, and thereby its inactivating effect was decreased. Lipases are easily inactivated in the presence of short-chain alcohols, which may interact with water molecules necessary to keep the native structure of the lipase [5].

3.3. Reaction time course and acyl migration

Product compositions in terms of oleic acid equivalents for the three systems at the end of the reaction are shown in Figs. 2, 3 and 4, respectively. Even though in both biphasic systems there was water, little oleic acid was formed compared to alkyl esters, and some hydrolysis occurred even in the reactions in organic systems (Fig. 4), probably because there was some remaining water inside the immobilised lipase.

Bearing in mind the 1,3-positional selectivity of the *R. oryzae* lipase, yields over 66.7% should not be expected. However, higher yield values were achieved, especially for reactions with immobilised enzyme without added water, because acyl migration allows the conversion of 2-monoolein to 1-monoolein, which can be easily used by rROL to produce glycerol. Analysis of glycerol in the reaction systems is difficult, so to further monitor the occurrence of these reactions, an acylglycerol balance was set up for each experiment, consisting of the sum of triolein, 1,2- and 1,3-diolein and 2- and 1-monoolein. This total amount of acylglycerols should be constant if further conversion to glycerol does not occur (reactions 3 and 7 in Fig. 1). On the other hand, if acyl migration occurs, rROL can alcoholyse 1-monoolein, thereby decreasing the total acylglycerol concentration. It can be concluded that acyl migration occurred in nearly all reactions, reaching 10.0 and 28.5% remaining acylglycerols for ethanol and methanol, respectively, in organic systems at a molar ratio of 3 (Table 2). One important aspect is that, despite achieving 81.2, 75.6 and 68.6% yields (Table 3) in the reactions with immobilised enzyme in an organic system, for alcohol to triolein ratios of 4.5 and 6 for ethanol and 4.5 for methanol, respectively, the remaining acylglycerols were still high – 54.0, 69.9 and 81.2%, respectively (Table 2). This observation is also in agreement with the product compositions of these experiments (Fig. 4), where the monoolein concentrations were higher than in other cases, meaning that only limited acyl migration had occurred, in contrast to the fast acyl migration when a molar ratio of 3 was used. These results agree with the ones found by Li et al. [36], who stated that increasing the polarity in the solvent phase decreased the acyl migration rate. So, the greater the amount of alcohol used, the higher the polarity in the reaction system and the slower the acyl migration. This phenomenon is observed in Figs. 5 and 6, which correspond to the first 3 hours' time course of the reactions catalysed by immobilised rROL in organic system when 3 and 6 alcohol to triolein molar ratios were used, respectively. In the case of the lowest alcohol to triolein molar ratio used (Fig. 5), 2-monoolein reached a maximum concentration at 30 minutes and started to decrease as a consequence of acyl migration, while in the highest alcohol molar ratio case (Fig. 6), 2-monoolein reached a maximum concentration at 60 minutes and remained almost constant thereafter.

Monoolein and diolein represented in Figs. 2, 3 and 4 are the sum of the isomers 1- and 2-monoolein, and 1,2- and 1,3- diolein, respectively. In all cases (except when using an alcohol to triolein molar ratio of 1 and 2 described in the next section), 2-monoolein was over 90% of the total monoolein and 1,2-diolein almost 100% of the total diolein (data not shown). This shows that the lipase-catalysed alcoholysis of the acylglycerols formed by acyl migration is fast enough to prevent building up high concentrations of them.

3.4. Transesterification at small alcohol to triolein molar ratios

The highest initial reaction rates were observed in an organic system at alcohol to triolein molar ratio of 3 and were 22.1 and 9.5 mM/min for ethanol and methanol, respectively (Table 1). Therefore, even lower molar ratios were tried to test whether even faster rates could be achieved. It was discovered that in the case of ethanol the decrease in the molar ratio did not enhance the initial reaction rate. In contrast, when the methanol to triolein molar ratio was reduced from 3 to 2, the initial rate was more than double, increasing from 9.5 to 21.0 mM/min, but it remained nearly constant when the molar ratio was further reduced to 1. Thus, the maximal initial reaction rates are nearly the same for both alcohols.

Figs. 7 and 8 show the time course of the reactions with a molar ratio of alcohol to triolein of 1 for both alcohols. In the reaction with ethanol, triolein was initially consumed to a concentration of 38 mM after 15 minutes, when its concentration started to increase to reach around 60 mM (Fig. 7). During the first 15 minutes rapid conversion of triolein and alcohol to alkyl ester, 1,2-diolein and 2-monoolein occurred. In this period a major part of the ethanol was consumed. Between 15 and 180 minutes acyl migration was prominent, directly seen as the formation of 1,3-diolein and to some extent 1-monoolein (Fig. 1, reactions 4 and 5). Most of the 1-monoolein formed was, however, converted rapidly to glycerol and ethyl oleate (Fig. 1, reaction 7) as indicated by the reduction in total acylglycerols. No accumulation of 1-monoolein was observed, because of the high lipase specificity towards this substrate as previously concluded, so lipase alcoholises it rapidly. Since the ethanol concentration was low during this period, ethanol necessary for the 1-monoolein alcoholysis must be obtained somehow from another reaction: 1,2-diolein reacted with ethyl oleate with the formation of triolein and ethanol (Fig. 1, reaction 1 backwards). This phenomenon is shown by the increase in triolein and decrease in 1,2-diolein occurring in parallel with the decrease in total acylglycerols (due to the formation of glycerol). After 180 minutes, acyl migration continued while otherwise only minor net changes were observed. Qualitatively the same behaviour was observed when using a methanol to triolein molar ratio of 1 (Fig. 8). Furthermore, these phenomena also occurred when an alcohol to triolein molar ratio of 2 was used (data not shown). In contrast, when molar ratios of alcohol to triolein of 3, 4.5 and 6 were used this phenomenon did not take place (Figs. 5 and 6 for 3 and 6 ethanol to triolein molar ratios; other data not shown), because there was enough acyl acceptor (alcohol) for all the fatty acids in the triolein substrate.

3.5. Comparison between recombinant and commercial R. oryzae lipase

The expression of recombinant enzymes in suitable host microorganisms is a widely used method to reduce difficulties and costs in their production. However, the properties of a recombinant enzyme are not always identical to those of the wild type enzyme. In the present study, substrate specificities of the recombinant and commercial lipases were examined and compared in order to elucidate possible differences. Both lipases, working in free form, had higher specificity towards ethanol than methanol but the difference was considerably larger for the recombinant enzyme.

Lipases are known to display significant differences in terms of acylglycerols specificities, regioselectivities and stereoselectivities [8,37]. In the present study, the ratios of the consumption rates of 1-monoolein versus triolein were 4.43 and 2.82 for rROL and ROL, respectively, which means that both enzymes are more specific for 1-monoolein than triolein, in agreement with the specificity of lipases from *C. antarctica B* and *R. miehei* but different from lipases from *R. arrhizus* and *T. lanuginosus* immobilised on polypropylene and used in methyl t-butyl ether without water addition [37]. Comparing recombinant and commercial *R. oryzae* lipase, it is clear that rROL is even more specific for 1-monoolein, which might be attributed to the presence of an esterase in the commercial ROL powder and also that rROL lacks the pre-pro-sequence of the mature commercial one, as described in a previous report [38]. Importantly, both lipases expressed negligible activity in alcoholysis of 2-monoolein, since 2-monoolein disappearance rates for both lipases were the same as in the control reaction without lipase and attributed to acyl migration. This shows that both lipases are highly 1,3-specific under these conditions.

4. Conclusions

The present work showed that recombinant *R. oryzae* lipase displays higher specificity towards 1-monoolein than triolein, being 4.43 bigger, and has negligible activity on 2-monoolein, which means that this lipase is strongly 1,3-positional selective. A similar behaviour was observed in the case of a commercial ROL.

Experiments studying the influence of the organic-aqueous interface on the interfacial activation of rROL were carried out. Immobilised rROL in a monophasic-organic system achieved more than 20-fold higher initial alcoholysis reaction rates compared to rROL experiments in organic-aqueous biphasic systems, which indicates that no interfacial activation is necessary to achieve high lipase alcoholysis activities.

Acyl migration was shown to occur especially at lower alcohol to triolein molar ratios, making it possible to reach yields higher than the expected 66.7% for a 1,3-specific lipase. Higher alcohol concentrations reduced the acyl migration rates and thereby the biodiesel yield.

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References

[1] Robles-Medina A, González-Moreno PA, Esteban-Cerdán L, Molina-Grima E. Biocatalysis: towards ever greener biodiesel production. Biotechnol Adv 2009;27:398–408.

[2] Tan T, Lu J, Nie K, Deng L, Wang F. Biodiesel production with immobilized lipase: A review. Biotechnol Adv 2010;28:628–34.

[3] Canet A, Bonet-ragel K, Benaiges MD, Valero F. Lipase-catalysed transesterification: Viewpoint of the mechanism and influence of free fatty acids. Biomass Bioenerg 2016;85:94–9.

[4] Gog A, Roman M, Toşa M, Paizs C, Irimie FD. Biodiesel production using enzymatic transesterification – Current state and perspectives. Renew Energy 2012;39:10–6.

[5] Aguieiras ECG, Cavalcanti-Oliveira ED, Freire DMG. Current status and new developments of biodiesel production using fungal lipases. Fuel 2015;159:52–67.

[6] Zhao X, Qi F, Yuan C, Du W, Liu D. Lipase-catalyzed process for biodiesel production: Enzyme immobilization, process simulation and optimization. Renew Sustain Energy Rev 2015;44:182–97.

[7] Borrelli GM, Trono D. Recombinant Lipases and Phospholipases and Their Use as Biocatalysts for Industrial Applications. Int J Mol Sci 2015;16:20774–840.

[8] Liu L, Gao C, Lan D, Yang B, Wang Y. Molecular basis for substrate selectivity of a mono- and diacylglycerol lipase from *Malassezia globosa*. Biochem Biophys Res Commun 2012;424:285–9.

[9] Calero J, Verdugo C, Luna D, Sancho ED, Luna C, Posadillo A, *et al*. Selective ethanolysis of sunflower oil with Lipozyme RM IM, an immobilized *Rhizomucor miehei* lipase, to obtain a biodiesel-like biofuel, which avoids glycerol production through the monoglyceride formation. N Biotechnol 2014;31:596–601.

[10] Mangas-Sánchez J, Adlercreutz P. Highly efficient enzymatic biodiesel production promoted by particle-induced emulsification. Biotechnol Biofuels 2015;8:58.

[11] De Vasconcellos A, Paula AS, Luizon Filho RA, Farias LA, Gomes E, Aranda DAG, *et al*. Synergistic effect in the catalytic activity of lipase *Rhizomucor miehei* immobilized on zeolites for the production of biodiesel. Microporous Mesoporous Mater 2012;163:343–55.

[12] Canet A, Dolors Benaiges M, Valero F. Biodiesel Synthesis in a Solvent-Free System by Recombinant *Rhizopus oryzae* Lipase. Study of the Catalytic Reaction Progress. J Am Oil Chem Soc 2014;91:1499–506.

[13] Kaieda M, Samukawa T, Matsumoto T, Ban K, Izumot E, Fukuda H. Biodiesel Fuel Production from Plant Oil Catalyzed by *Rhizopus oryzae* Lipase in a Water-Containing System without an Organic Solvent. J Biosci Bioeng 1999;88:627–31.

[14] Wang Y, Shen X, Li Z, Li X, Wang F, Nie X, *et al*. Immobilized recombinant *Rhizopus oryzae* lipase for the production of biodiesel in solvent free system. J Mol Catal B Enzym 2010;67:45–51.

[15] Caballero V, Bautista FM, Campelo JM, Luna D, Marinas JM, Romero AA, *et al*. Sustainable preparation of a novel glycerol-free biofuel by using pig pancreatic lipase: Partial 1,3-regiospecific alcoholysis of sunflower oil. Process Biochem 2009;44:334–42.

[16] Li W, Li R, Li Q, Du W, Liu D. Acyl migration and kinetics study of 1(3)-positional specific lipase of *Rhizopus oryzae*-catalyzed methanolysis of triglyceride for biodiesel production. Process Biochem 2010;45:1888–93.

[17] Hama S, Tamalampudi S, Suzuki Y, Yoshida A, Fukuda H, Kondo A. Preparation and comparative characterization of immobilized *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase for enzymatic biodiesel production. Appl Microbiol Biotechnol 2008;81:637–45.

[18] Hama S, Yoshida A, Tamadani N, Noda H, Kondo A. Enzymatic production of biodiesel from waste cooking oil in a packed-bed reactor: An engineering approach to separation of hydrophilic impurities. Bioresour Technol 2012;135:417–21.

[19] Monteiro JB, Nascimento MG, Ninow JL. Lipase-catalyzed synthesis of monoacylglycerol in a homogeneous system. Biotechnol Lett 2003;25:641–4.

[20] Zhong N, Cheong L-Z, Xu X. Strategies to obtain high content of monoacylglycerols. Eur J Lipid Sci Technol 2014;116:97–107.

[21] Verdugo C, Luna D, Posadillo A, Sancho ED, Rodríguez S, Bautista F, *et al*. Production of a new second generation biodiesel with a low cost lipase derived from *Thermomyces lanuginosus*: Optimization by response surface methodology 2011;167:107–12.

[22] Zhao T, No DS, Kim Y, Kim YS, Kim IH. Novel strategy for lipase-catalyzed synthesis of biodiesel using blended alcohol as an acyl acceptor. J Mol Catal B Enzym 2014;107:17–22.

[23] Lotti M, Pleiss J, Valero F, Ferrer P. Effects of methanol on lipases: Molecular, kinetic and process issues in the production of biodiesel. Biotechnol J 2014;10:1–9.

[24] Verger R. “ Interfacial activation ” of lipases: facts and artifacts. Trends Biotechnol 1997;15:32–8.

[25] Buchholz K, Kasche V, Bornscheuer UT. Biocatalysts and Enzyme Technology. Wiley-VCH; 2005.

[26] Adlercreutz P. Immobilisation and application of lipases in organic media. Chem Soc Rev 2013;42:6406–36.

[27] Minning S, Serrano A, Ferrer P, Solá C, Schmid RD, Valero F. Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. J Biotechnol 2001;86:59–70.

[28] Duarte SH, del Peso Hernández GL, Canet A, Benaiges MD, Maugeri F, Valero F. Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant *Rhizopus oryzae* lipase. Bioresour Technol 2015;183:175–80.

[29] Bonet-Ragel K, Canet A, Benaiges MD, Valero F. Synthesis of biodiesel from high FFA alperujo oil catalysed by immobilised lipase. Fuel 2015;161:12–7.

[30] Martínez-Martínez M, Alcaide M, Tchigvintsev A, Reva O, Polaina J, Bargiela R, *et al*. Biochemical Diversity of Carboxyl Esterases and Lipases from Lake Arreo (Spain): A Metagenomic Approach. Appl Environ Microbiol 2013;79:3553–62.

[31] Quintana PG, Canet A, Marciello M, Valero F, Palomo JM, Baldessari A. Enzyme-catalyzed preparation of chenodeoxycholic esters by an immobilized heterologous *Rhizopus oryzae* lipase. J Mol Catal B Enzym 2015;118:36–42.

[32] Guillén M, Benaiges MD, Valero F. Biosynthesis of ethyl butyrate by immobilized recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris*. Biochem Eng J 2012;65:1–9.

[33] Mangas-Sánchez J, Serrano-Arnaldos M, Adlercreutz P. Effective and highly selective lipase-mediated synthesis of 2-monoolein and 1,2-diolein in a two-phase system. J Mol Catal B Enzym 2015;112:9–14.

[34] Cesarini S, Diaz P, Nielsen PM. Exploring a new, soluble lipase for FAMEs production in water-containing systems using crude soybean oil as a feedstock. Process Biochem 2013;48:484–7.

[35] Nordblad M, Silva VTL, Nielsen PM, Woodley JM. Identification of critical parameters in liquid enzyme-catalyzed biodiesel production. Biotechnol Bioeng 2014;111:2446–53.

[36] Li W, Du W, Li Q, Li R, Liu D. Dependence on the properties of organic solvent: study on acyl migration kinetics of partial glycerides. Bioresour Technol 2010;101:5737–42.

[37] Šinkuniene D, Adlercreutz P. Effects of regioselectivity and lipid class specificity of lipases on transesterification, exemplified by biodiesel production. J Am Oil Chem Soc 2014;91:1283–90.

[38] Guillén M, Benaiges MD, Valero F. Comparison of the biochemical properties of a recombinant lipase extract from *Rhizopus oryzae* expressed in *Pichia pastoris* with a native extract. Biochem Eng J 2011;54:117–23.

Figure captions

Figure 1

Complete alcoholysis of triolein to glycerol and free fatty acids (reactions 1, 2, 3, 6 and 7). Acyl migration phenomenon converts 1,2-diolein and 2-monoolein to 1,3-diolein and 1(3)-monoolein (reactions 4 and 5), respectively. If a 1,3-positional selective lipase is used, reaction 3 is negligible. Hydrolysis has the same reactions scheme, with water instead of alcohol and free fatty acids instead of alkyl esters.

Figure 2

Product composition after 24 hours. Alcoholysis of triolein catalysed by 0.4 ml free rROL formulation, using 0.265 g triolein in 1.6 ml heptane and different ethanol and methanol to triolein molar ratios at 37°C and 1000 rpm agitation. Legend: A=3 ethanol to triolein molar ratio, B=4.5 ethanol to triolein molar ratio, C=6 ethanol to triolein molar ratio, D=3 methanol to triolein molar ratio, E=4.5 methanol to triolein molar ratio and F=6 methanol to triolein molar ratio. Legend bars: Grey bar=oleic acid, Black bar=ethyl oleate, White bar=methyl oleate, Diagonal-striped bar=monooleins, Striped bar=dioleins and Dotted bar=triolein.

Figure 3

Product composition after 24 hours. Alcoholysis of triolein catalysed by immobilised rROL, using 0.265 g triolein in 1.6 ml heptane, 0.4 ml water and different ethanol and methanol to triolein molar ratios at 37°C and 1000 rpm agitation. Legend: A=3 ethanol to triolein molar ratio, B=4.5 ethanol to triolein molar ratio, C=6 ethanol to triolein molar ratio, D=3 methanol to triolein molar ratio, E=4.5 methanol to triolein molar ratio and F=6 methanol to triolein molar ratio. Legend bars: Grey bar=oleic acid, Black bar=ethyl oleate, White bar=methyl oleate, Diagonal-striped bar=monooleins, Striped bar=dioleins and Dotted bar=triolein.

Figure 4

Product composition after 24 hours. Alcoholysis of triolein catalysed by immobilised rROL, using 0.265 g triolein in 1.6 ml heptane and different ethanol and methanol to triolein molar ratios at 37°C and 1000 rpm agitation. Legend: A=1 ethanol to triolein molar ratio, B=2 ethanol to triolein molar ratio, C=3 ethanol to triolein molar ratio, D=4.5 ethanol to triolein molar ratio, E=6 ethanol to triolein molar ratio, F=1 methanol to triolein molar ratio, G=2 methanol to triolein molar ratio, H=3 methanol to triolein molar ratio, I=4.5 methanol to triolein molar ratio and J=6 methanol to triolein molar ratio. Legend bars:

Grey bar=oleic acid, Black bar=ethyl oleate, White bar=methyl oleate, Diagonal-striped bar=monooleins, Striped bar=dioleins and Dotted bar=triolein.

Figure 5

Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 3 ethanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ●=ethyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dotted line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dotted line = 1,3-diolein), x = triolein, dotted line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)

Figure 6

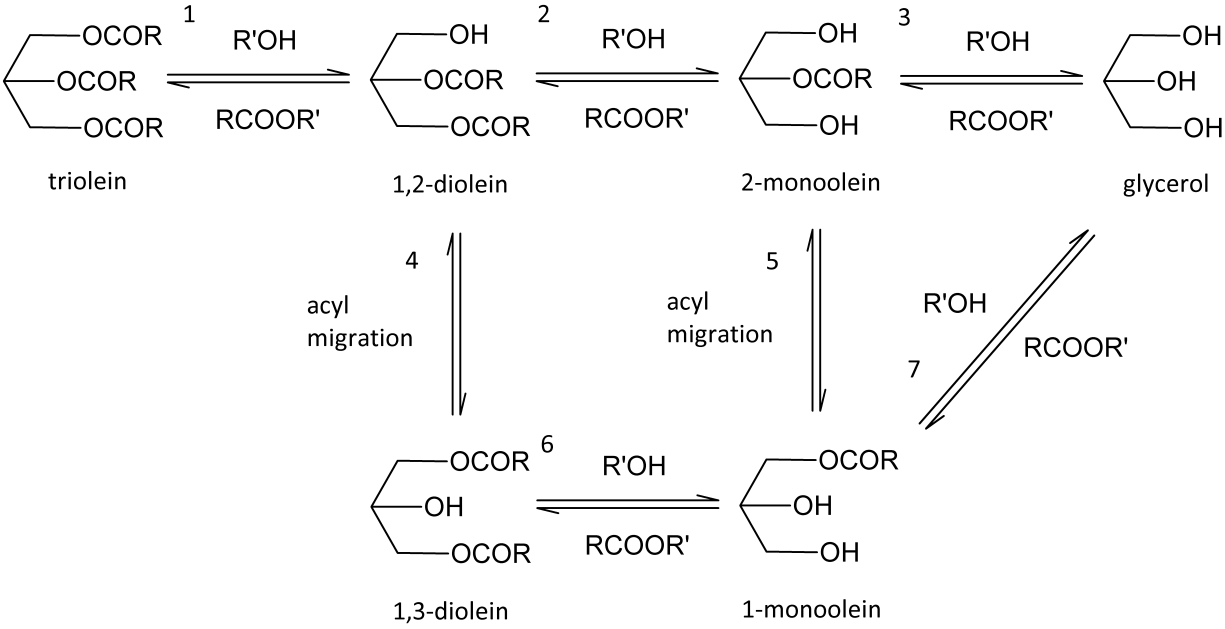
Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 6 ethanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ●=ethyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dotted line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dotted line = 1,3-diolein), x = triolein, dotted line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)

Figure 7

Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 1 ethanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ●=ethyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dotted line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dotted line = 1,3-diolein), x = triolein, dotted line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)

Figure 8

Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 1 methanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ○=methyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dotted line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dotted line = 1,3-diolein), x = triolein, dotted line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)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OH
OCOR
OH
OH
OH
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1,2-diolein
2-monoolein
glycerol
4
acyl
migration
5
acyl
migration
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OCOR
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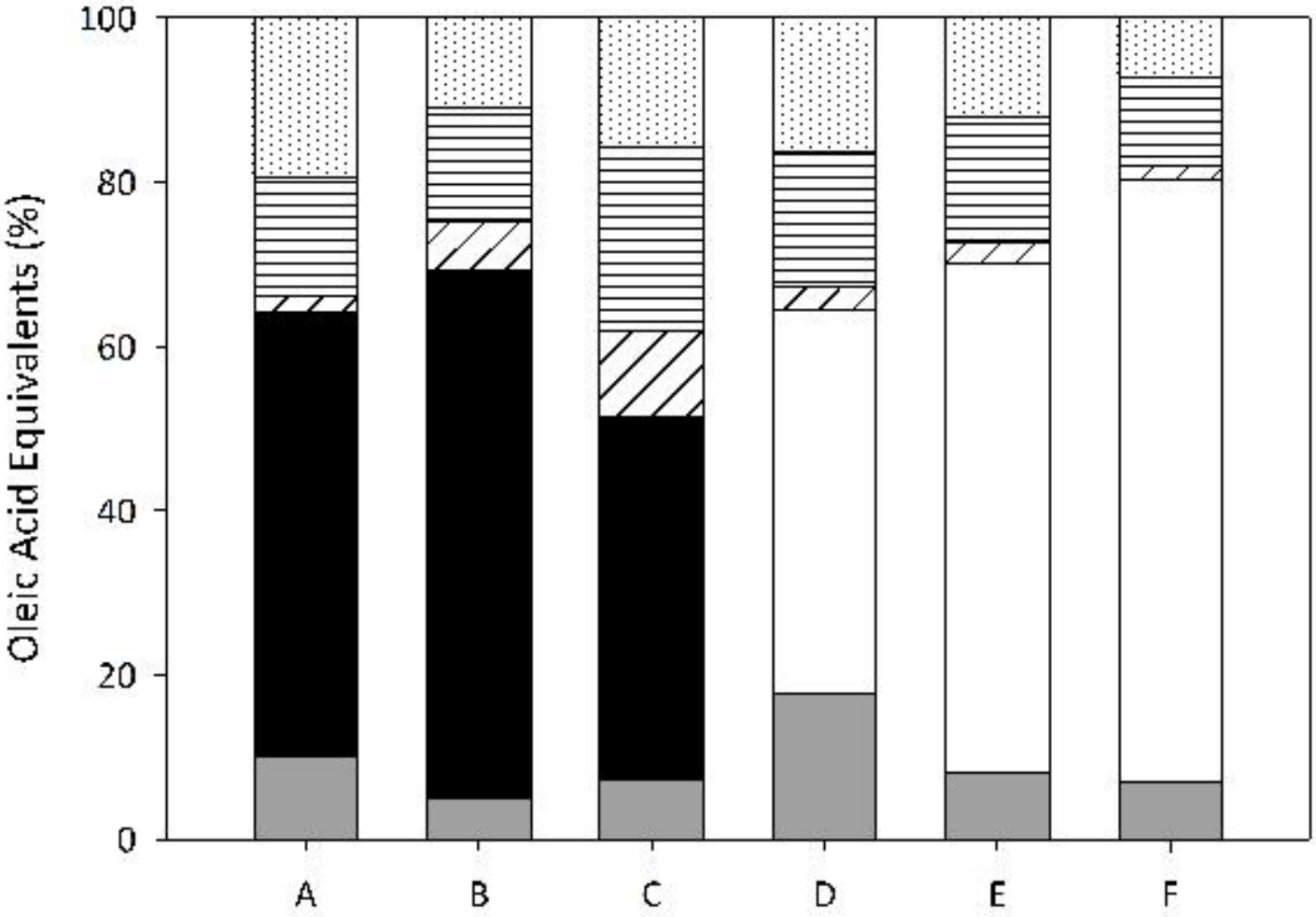
OH
1,3-diolein
1-monoolein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B
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D
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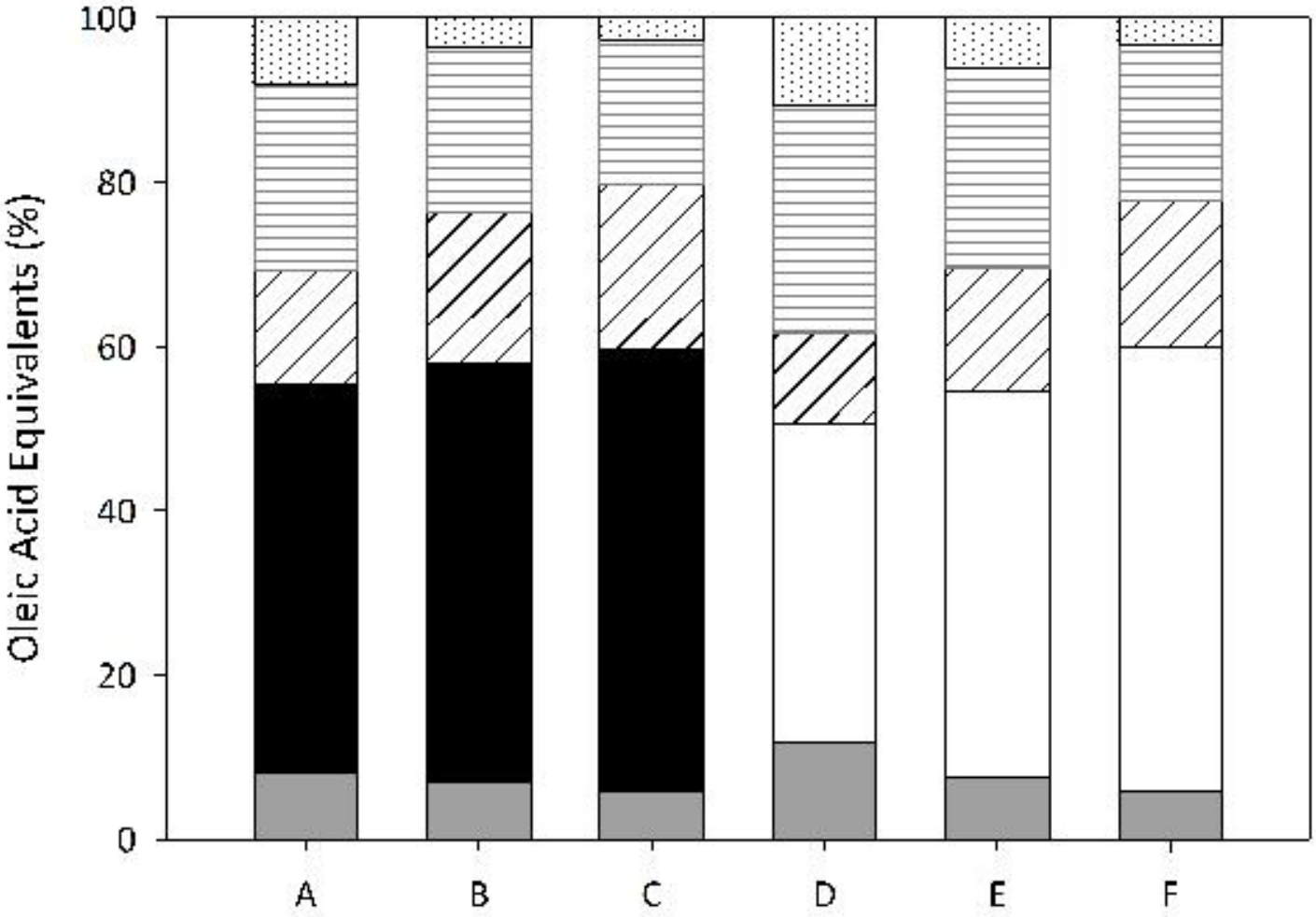
F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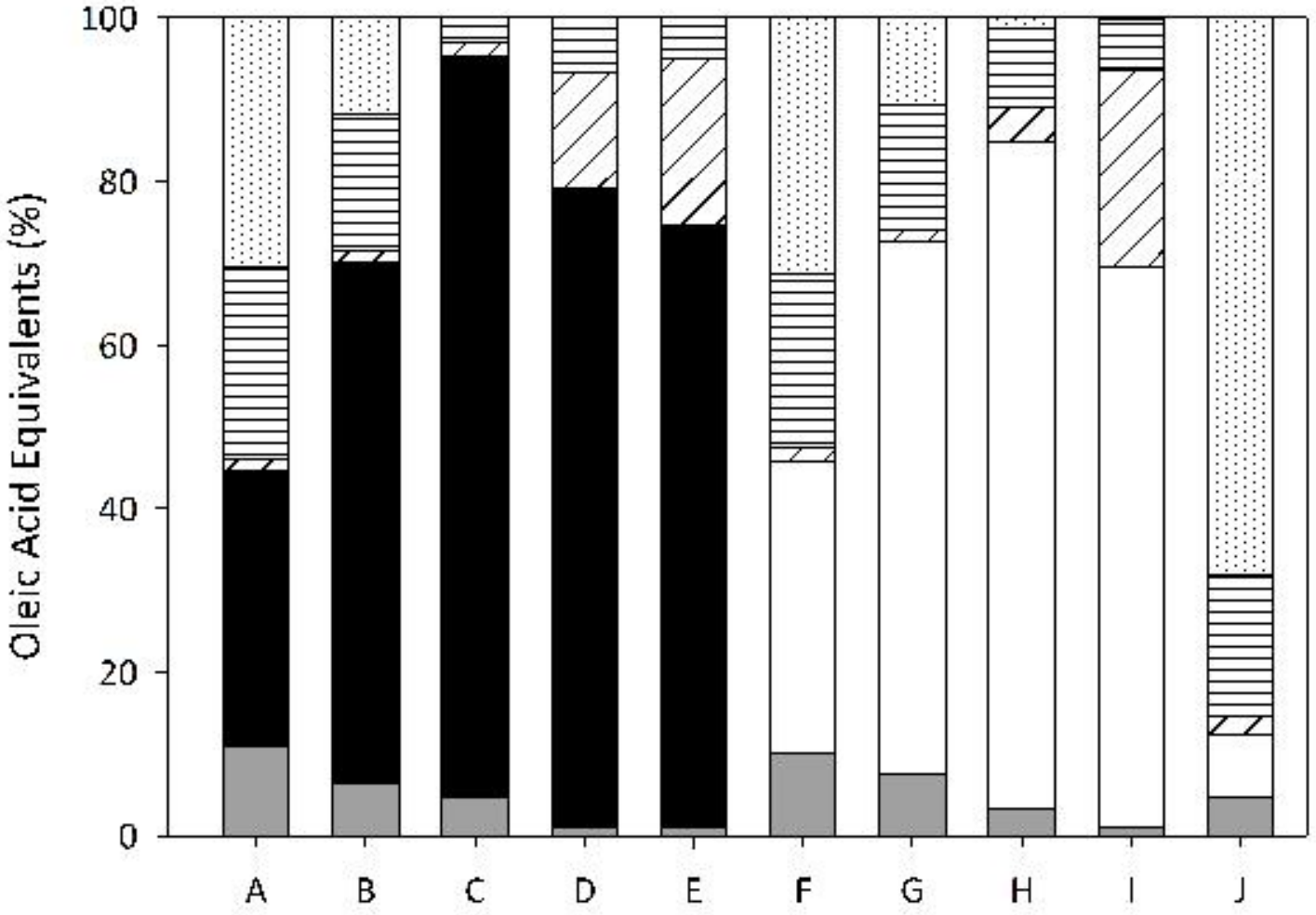
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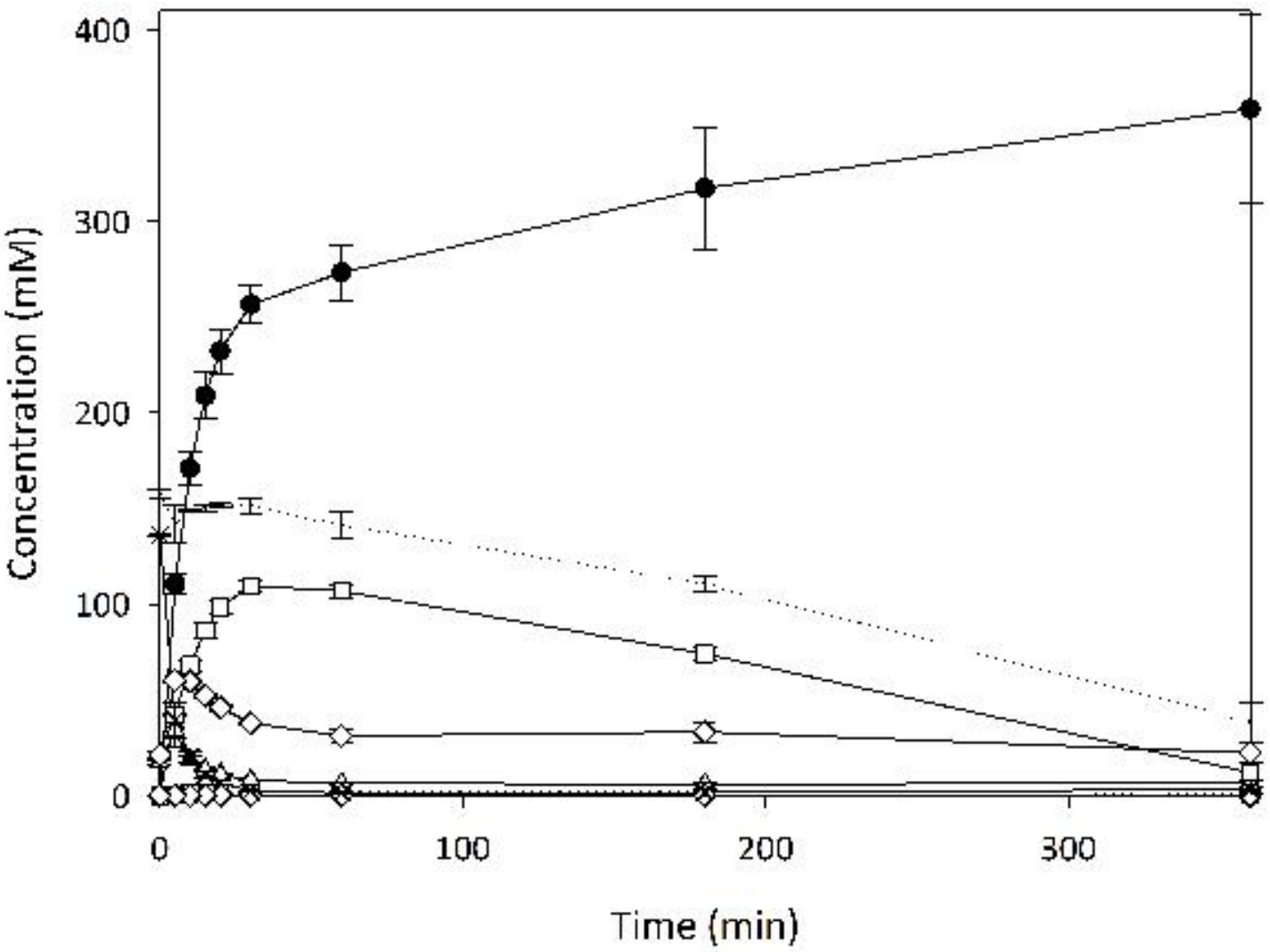
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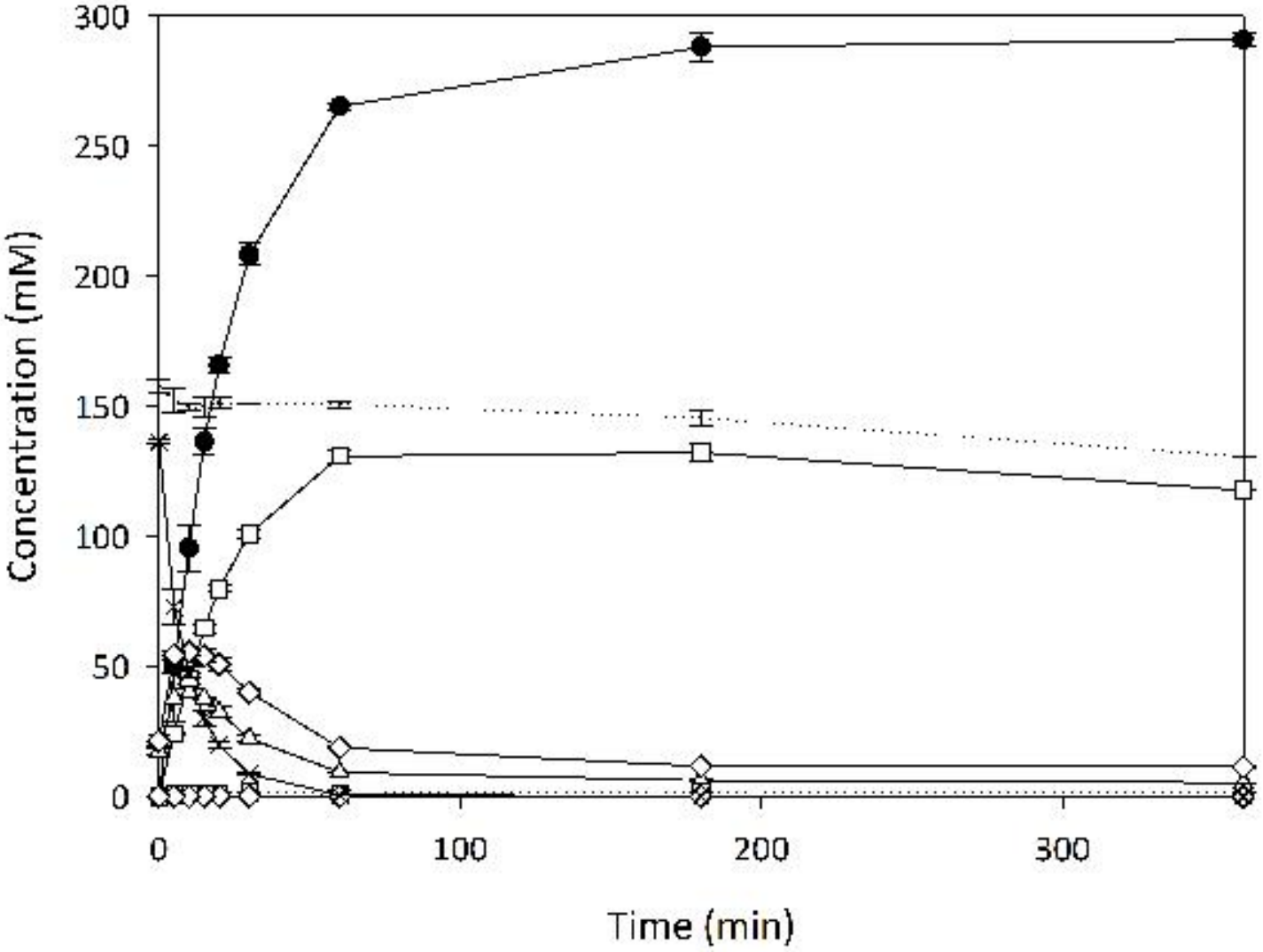
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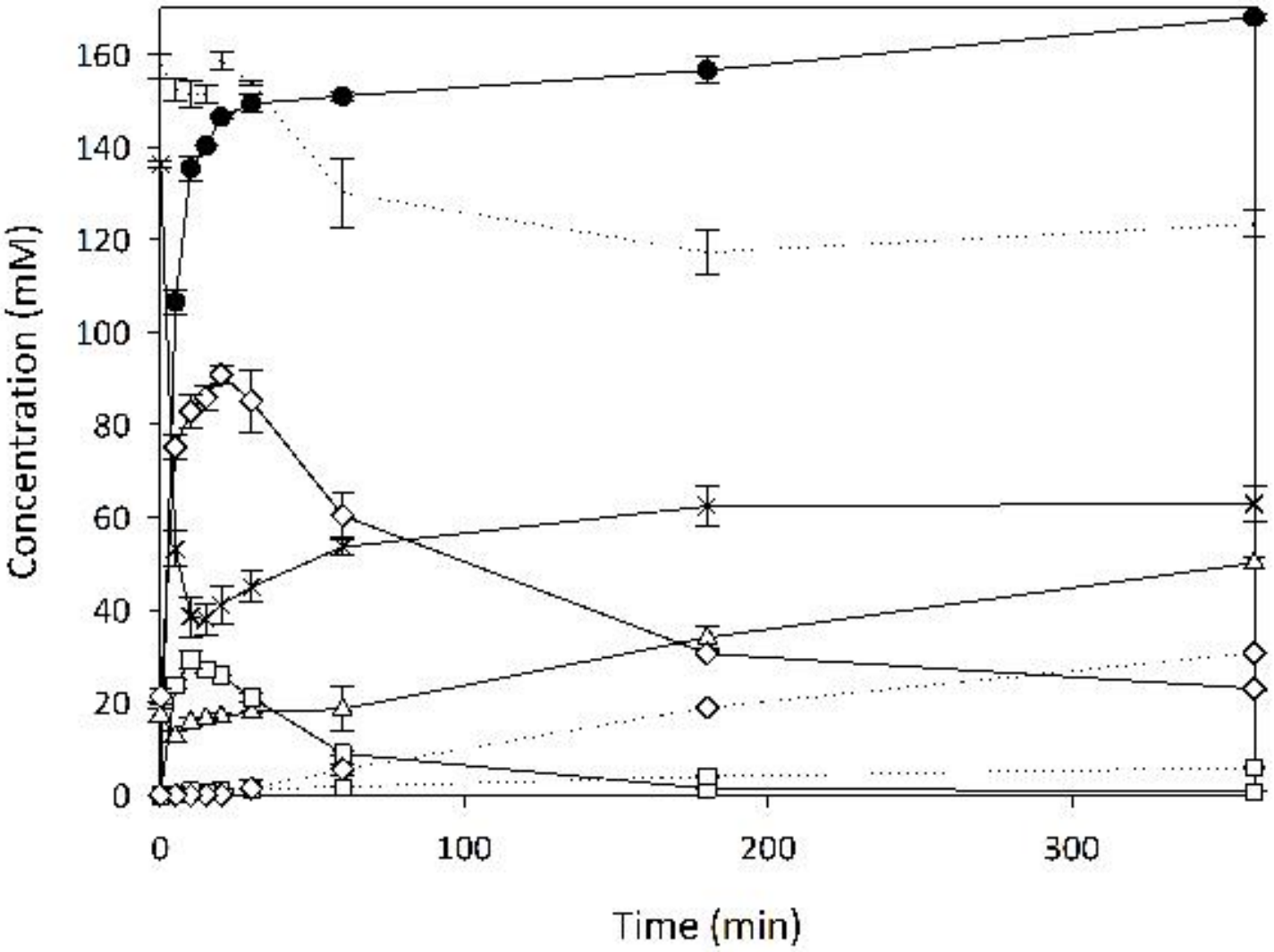
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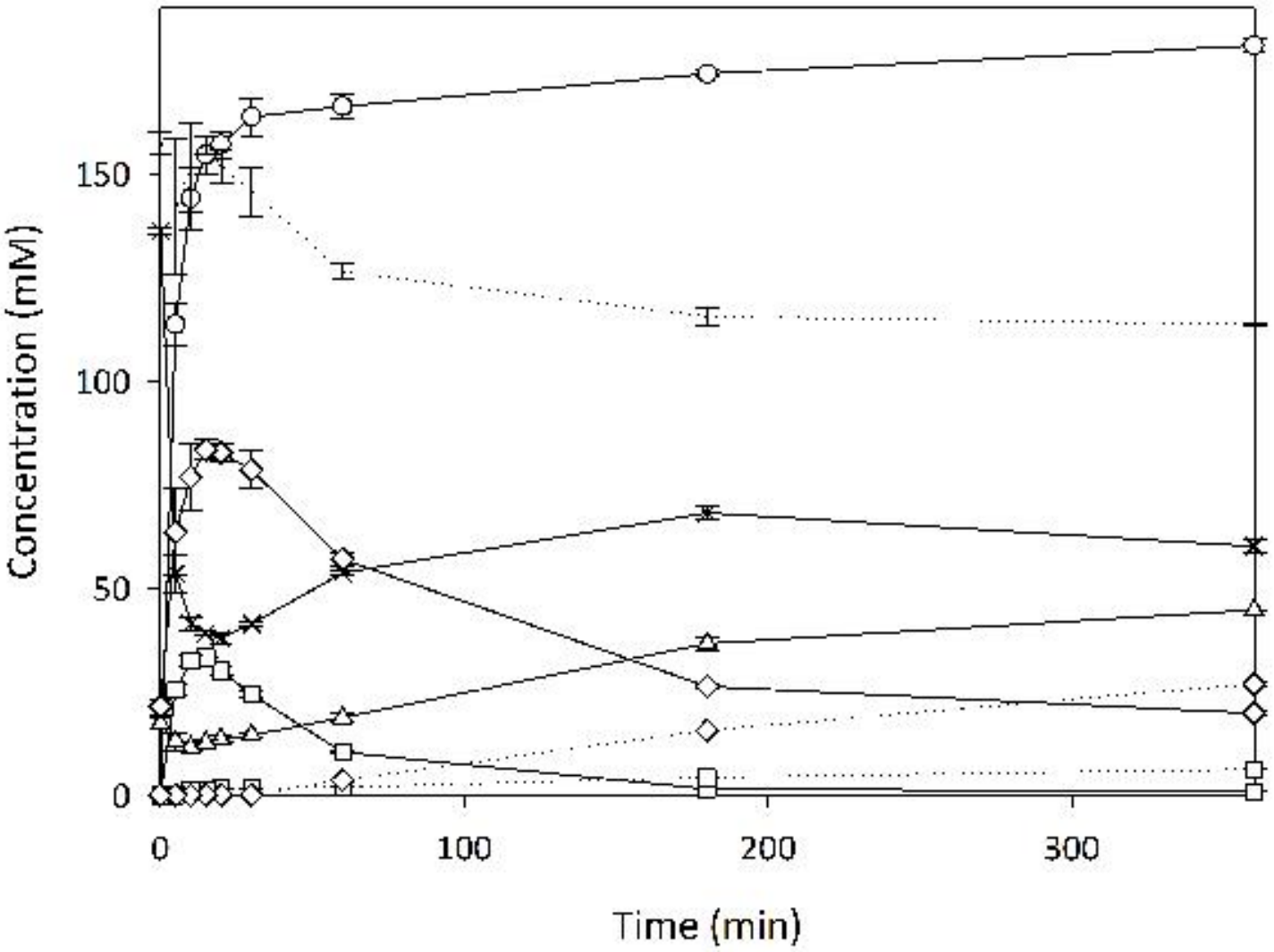
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100
200
300
Time (min)

Table 1

Table 1. Initial rate of alkyl ester formation in triolein transesterification (mM /min)

System	Ethanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			1.1±0.1	1.0±0.1	1.0±0.0
Immobilised enzyme – biphasic			0.2±0.1	0.4±0.1	0.5±0.1
Immobilised enzyme – organic	21.3±0.5	24.1±0.7	22.1±1.0	15.7±0.0	10.0±0.4
System	Methanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			0.5±0.1	0.6±0.0	0.8±0.1
Immobilised enzyme – biphasic			0.3±0.1	0.3±0.0	0.4±0.1
Immobilised enzyme – organic	22.7±1.0	21.0±1.8	9.5±0.7	1.2±0.2	0.3±0.1

Table 2

Table 2. Ratio: Final Acylgycerols / Initial Acylglycerols (%)

System	Ethanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			50.3±1.7	49.7±8.9	94.2±5.9
Immobilised enzyme – biphasic			87.4±2.6	90.5±7.0	88.6±4.2
Immobilised enzyme – organic	79.6±2.7	43.5±1.8	10.0±2.3	54.0±1.9	69.9±2.2
System	Methanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			48.4±4.0	42.1±3.2	28.2±2.6
Immobilised enzyme – biphasic			82.4±9.2	86.3±4.4	83.3±9.2
Immobilised enzyme – organic	77.0±1.7	36.5±0.9	28.5±1.1	81.2±2.5	103.2±9.7

Table 3

Table 3. Alkyl oleate yield (%)

System	Ethanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			57.2±2.0	64.2±2.3	51.3±1.9
Immobilised enzyme – biphasic			50.0±0.8	52.3±5.0	54.0±2.3
Immobilised enzyme – organic	38.4±1.3	67.9±1.1	100.7±7.0	81.2±0.8	75.6±1.1
System	Methanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			45.9±1.7	61.0±2.9	71.2±2.5
Immobilised enzyme – biphasic			38.2±8.0	46.3±0.5	51.1±2.3
Immobilised enzyme – organic	40.5±1.3	63.3±1.7	82.6±1.7	68.6±1.5	7.8±4.8

CERTIFICATE OF ENGLISH EDITING

To whom it may concern,

This is to certify that the manuscript "**Exploring substrate specificities of a recombinant *Rhizopus oryzae* lipase in biodiesel synthesis**" has been duly proofread and edited for language.

Peter Howard Lindsey

07/12/2016

Rhizopus oryzae lipase is 4 times more specific towards 1-monoolein than triolein

Rhizopus oryzae lipase shows no need for interfacial activation for high activity

Acyl migration is enhanced as medium polarity is decreased