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Second- and third-generation biodiesel production with immobilised recombinant *Rhizopus oryzae* lipase: Influence of the support, substrate acidity and bioprocess scale-up

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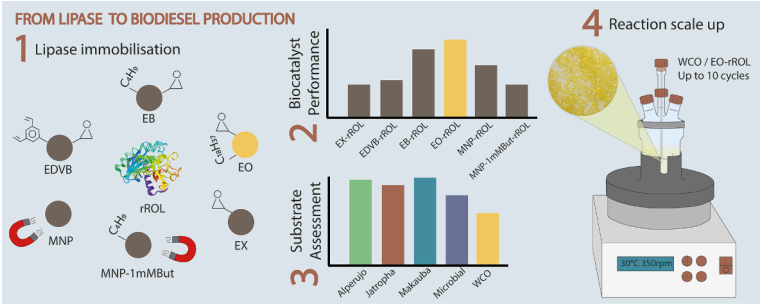
HIGHLIGHTS

- *Rhizopus oryzae* lipase proved an excellent biocatalyst for biodiesel production.
- Hydrocarbon chains on the support increased the biocatalyst operational stability.
- Low oil acidity negatively influenced biocatalyst operational stability.
- Acyl-acceptor concentration and oil acidity had a synergistic effect.
- Biodiesel production from waste cooking oil was successfully scaled up.

GRAPHICAL ABSTRACT



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ABSTRACT

Rhizopus oryzae lipase immobilised onto differently functionalised polymethacrylate (Purolite®) and magnetite superparamagnetic supports was assessed as a catalyst for biodiesel production with pomace oil. The presence of surface hydrocarbon chains increased the operational stability of the biocatalysts supported on Purolite® and superparamagnetic particles up to 9 and 2 times, respectively. By contrast, the presence of functional groups had no effect on the initial transesterification rate, which was twice higher with the lipase immobilised onto Purolite®. Also, functionalising Purolite® with epoxide and octadecyl groups led to the highest biodiesel and volumetric productivity. This biocatalyst with other substrates including makauba, jatropha, waste cooking oil, and microbial oil, led to similar initial reaction rates. However, simply raising substrate acidity from 0.5 to 2% increased the operational stability of the biocatalysts 15 times. A synergistic effect between acyl-acceptor concentration and substrate acidity was observed. The transesterification reaction was successfully scaled up to 50 mL.

1. Introduction

Global warming is an unavoidable process that requires a change of paradigm to enable a climate-neutral society and avoid environmental collapse. In this scenario, a wide variety of clean energy sources have the potential to jointly replace polluting fossil fuels (Connolly et al., 2016).

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One such source is biodiesel, which is likely to play a major role in the process by virtue of its substantial advantages; thus, this clean fuel is biodegradable, renewable, non-toxic, usable by existing engines, locally produced and carbon–neutral —carbon in biodiesel exhaust is recently fixed from the atmosphere (Ranganathan et al., 2008; Rodrigues et al., 2016). Biodiesel is a mixture of mono-alkyl esters of long-chain fatty acids obtained by transesterification of a wide range of oily substrates and classified as first-, second- or third-generation biodiesel —the latter two are designated "advanced biodiesel" in European Directive 2015/1513. First-generation biodiesel is produced from edible oils, consumption of which poses severe ethical problems. On the other hand, second- and third-generation biodiesel are obtained from non-edible oils (e.g., waste cooking oil, WCO), oils from plants growing in agriculturally unsuitable land (e.g., jatropha, makauba) (Hama and Kondo, 2013) or microbial oils respectively (Ma et al., 2018; Navarro López et al., 2016).

Global biodiesel production is primarily focused on first-generation biodiesel, the demand for which is expected to grow to 110.6 million barrels per day by 2035 (Ogunkunle and Ahmed, 2019) and hence to strengthen the ethical dilemma on this type of biofuel. In response, public institutions are taking steps to shift production from first- to second- and third-generation biodiesel. For instance, the European Union (EU/2015/1513) has amended its previous policies in this regard by aiming at decreased first-generation biofuel consumption and increased production of second- and third-generation biodiesel. Even so, most existing biodiesel production plants have been designed for first-generation biodiesel production, which is based on chemical transesterification in the presence of homogeneous basic catalysts. Under these conditions, the high content in free fatty acids (FFA) of second- and third-generation substrates facilitates soap formation unless they are previously neutralised. Alternative solutions involving transesterification with acids and heterogeneous solid catalysts have been proposed; most, however, are uneconomical, require more severe reaction conditions (e.g., higher alcohol-to-oil mole ratios) or result in lower reaction rates (Bonet-Ragel et al., 2018; Hama and Kondo, 2013; Ranganathan et al., 2008). Enzymatic transesterification with lipases (glycerol ester hydrolases, E.C.3.1.1.3) has emerged as an effective choice for biodiesel production without the previous shortcomings. In fact, lipases enable transesterification reactions with substrates containing high FFA concentrations under milder conditions, might avoid glycerol formation —an unwanted outcome for the biodiesel industry (Rodrigues et al., 2017) — and the need for solvents. Enzyme-based transesterification uses less energy and involves fewer downstream steps (He et al., 2018; Xu et al., 2017); however, lipases for use as biocatalysts by the biodiesel industry are expensive and unstable (especially in the presence of alcohols such as methanol, which diminishes enzyme reusability and compromises economic feasibility) (López-Fernández et al., 2019).

Immobilising enzymes provides an effective way of circumventing the shortcomings of biocatalysts by improving their stability against deactivation and enabling their reuse (Ariaeenejad et al., 2021; Madhavan et al., 2017). A great variety of lipase immobilisation methods have been reported including a number for biodiesel production. For instance, hydrophobic adsorption is broadly used on the grounds of the hydrophobicity of the active sites of lipases and their widely documented catalytic hyperactivation (Canet et al., 2016). Other strategies such as covalent immobilisation (Bonet-Ragel et al., 2015; Da Rós et al., 2010; Rodrigues et al., 2016), entrapment (Noureddini et al., 2005) and the use of cross-linked enzyme aggregates (CLEA) have also been used in search for highly efficient biocatalysts (Khanahmadi et al., 2016). Also, the previous approaches have been adapted for use with supports based on superparamagnetic nanoparticles (Cruz-Izquierdo et al., 2014) or with novel lipase-displaying whole-cell biocatalysts (Jin et al., 2013).

In this work, mature sequence of *Rhizopus oryzae* lipase (rROL) was expressed in the methylotrophic yeast *Komagataella phaffii*, which has been claimed as the most suitable cell factory for rROL production due to its secretion capacity, its lack of endogenous extracellular lipases or esterases and its ability to grow at high cell densities (ca. 100 g L^{-1} dry cell weight) in defined media (López-Fernández et al., 2020). rROL is a 1,3-regioespecific lipase which was used for enzymatic second- and third-generation biodiesel production. Its transesterification gives the corresponding mono-alkyl esters and, provided acyl-migration is controlled (Canet et al., 2017), 2-monoacylglyceride as well, thereby avoiding glycerol formation and giving a product with a high added value for the cosmetics and food industries (Luo et al., 2016). The lipase used here was covalently immobilised onto three glutaraldehyde-treated polymethacrylate-based supports containing both epoxide and hydrophobic functional groups. The effects of functional groups on lipase immobilisation and diffusional restrictions, and also on the initial reaction rate and operational stability of the catalyst, were assessed. Glutaraldehyde-treated superparamagnetic nanoparticles containing hydrophobic functional groups at variable densities were also used for covalent immobilisation of lipase and evaluation of biodiesel production. Tests were conducted by using *alperujo* oil (non-edible olive pomace oil with a high FFA content obtained in the olive extraction process; Bonet-Ragel et al., 2018), as a model substrate for transesterification. Subsequently, the best biocatalyst was used for production of second- and third-generation biodiesel from alternative substrates of industrial use, including jatropha, makauba, WCO, and microbial oils. Finally, transesterification with WCO was scaled-up to a 50 mL mini-laboratory reactor.

2. Materials and methods

2.1. Materials

Olive pomace oil was a gift from Professor Eulogio Castro (University of Jaen, Spain); microbial oil from a modified strain of *Rhodosporidium toruloides* was supplied by Neol Biosolutions (Granada, Spain); jatropha and makauba oils were kindly donated by Professor Denise Freire of the Universidade Federal do Rio de Janeiro (Brazil); and WCO was obtained from a local public waste management company. All oils were centrifuged prior to use. Polymethacrylate matrix supports D6307, D6308 and D6309 were kindly supplied by Purolite® (King of Prussia, PA, USA). The colorimetric kit for enzymatic assay 11821729 was obtained from Roche (Mannheim, Deutschland) and bovine serum albumin standards (Ref. 11811345) were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Heptane, ethanol and methanol were purchased from Panreac (Barcelona, Spain). *n*-Butylamine solution, 3-aminopropyltriethoxysilane (APTS), ammonium sulphate, $NaBH_4$, $FeCl_2$, $FeCl_3$, standards of methyl/ethyl palmitate, methyl/ethyl stearate, methyl/ethyl oleate, methyl/ethyl linoleate, methyl linoleate and all unstated reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Lipase heterologous production

Recombinant *Rhizopus oryzae* lipase (rROL) formed by its mature sequence was produced by the Bioprocess Engineering and Applied Biocatalysis Group of the Universitat Autònoma de Barcelona (Barcelona, Spain) as described elsewhere (Arnau et al., 2010). Production runs were followed by centrifugation, microfiltration, ultrafiltration and lyophilisation of the culture broth to remove biomass and concentrate the enzyme (Guillén et al., 2012).

2.3. Lipase activity measurement and protein determination

Lipase activity was determined on a Cary 300 spectrophotometer from Varian (Mulgrave, VIC, Australia), using the Roche lipase colorimetric kit in 200 mM Tris-HCl buffer at pH 7.25 at 30 °C. Measurements were made in triplicate at 580 nm (Resina et al., 2004).

Protein concentrations were determined by using the Bradford method with bovine serum albumin as standard (Bradford, 1976).

2.4. Synthesis of magnetic nanoparticles (MNPs) and functionalisation with amino groups

Nanoparticles of magnetite (Fe_3O_4) were synthesised and functionalised with amino groups (MNPs-NH_2) as described elsewhere (Cruz-Izquierdo et al., 2014). All solutions used for MNPs synthesis and functionalisation were prepared in nitrogen-bubbled ultrapure water. The dry weight and concentration of MNPs were determined by using a VR-1/120/240 vacuum concentrator from Heto Lab Equipment (Zealand, Denmark).

2.5. Functionalisation of supports with aldehyde groups

Two types of immobilisation supports based on polymethacrylate and magnetite were used. The polymethacrylate-based supports included Purolite® D6307 with epoxide and butyl functional groups (EB); Purolite® D6308 with epoxide and octadecyl groups (EO) and Purolite® D6309 with epoxide and divinylbenzene groups (EDVB). All were conditioned as described elsewhere (Bonet-Ragel et al., 2015). Epoxide groups were converted into aldehyde groups with ethylenediamine first and glutaraldehyde then.

Previously functionalised MNPs-NH_2 were simply pretreated with glutaraldehyde following the protocol described elsewhere (Bonet-Ragel et al., 2015). The resulting aldehyde-functionalised MNPs (MNP-CHO) were further modified by incubation in a 0–1 M butylamine solution in 100 mM phosphate buffer pH 8 at room temperature for 2 h to obtain MNP-But-CHO. MNPs were washed three times with PBS after each step and stored at 4 °C until use.

2.6. Lipase immobilisation onto functionalised supports

Lipase was immobilised onto the above-described polymethacrylate- and magnetite-based supports by using a modified version of a previously reported method (Bonet-Ragel et al., 2015). Unless otherwise stated, 1 g of glutaraldehyde-treated support (dry weight for MNPs) was mixed with a 3500 AU lipase mL^{-1} solution at 4 °C for 42 h. The polymethacrylate biocatalysts thus obtained (EB-rROL, EO-rROL, EDVB-rROL) were vacuum-filtered and dried on silica gel, whereas the magnetite-based biocatalysts were recovered by application of a magnetic field, washed three times with PBS and concentrated to 2 mg mL^{-1}.

Schiff bases and unreacted aldehyde groups were reduced by incubating the supports with a 1 mg mL^{-1} $NaBH_4$ solution in 100 mM phosphate buffer at pH 8 at room temperature for 2 h (Cruz-Izquierdo et al., 2014).

The specific activity of the biocatalysts (immobilised AU mg $support^{-1}$) was calculated as the difference between those of the blank and supernatant solutions divided by the final weight (MNP dry weight) of biocatalyst. The immobilisation yield (IY) for the polymethacrylate supports was estimated by exposing them to lipase solutions at concentrations from 55 to 1000 AU mg $support^{-1}$:

$$IY\ (\%) = \frac{Biocatalyst\ \ specific\ \ activity}{offered\ \ activity\ \ units} x100 \quad (1)$$

2.7. Quantification of fatty acid methyl and ethyl esters

Fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) were quantified on a model 7890A gas chromatograph from Agilent (Santa Clara, CA, USA) equipped with a 19095 N-123 capillary column and an autosampler (Canet et al., 2014). The relative standard deviations (RSD) for the FAMEs and FAEEs never exceeded 3%.

2.8. Transesterification reactions

Reaction runs were conducted in 10 mL closed vials containing 8 g of oil at 30 °C that were placed in a KS 400 incubator from IKA (Staufen, Germany) and under orbital stirring at 350 rpm. Ethanol or methanol was added in 1, 5 or 10 pulses by splitting the stoichiometric volume of alcohol (2:1 alcohol/oil) into 1, 5 or 10 portions, respectively, that were added during the reaction to reach the theoretical maximum yield (Bonet-Ragel et al., 2018). The reaction was scaled up to a 50 mL Scharlau HME-R mini-reactor from Scharlab (Sentmenat, Barcelona, Spain) with mechanical stirring. The above-described procedures were performed with a final WCO mass of 40 g.

Olive pomace oil was used as model to investigate diffusional restrictions, the initial reaction rate and the operational stability of the biocatalysts. The best biocatalyst was used for further evaluation of the initial reaction rate and operational stability with alternative substrates including jatropha oil, WCO, makauba oil and microbial oil. All reactions components were pre-equilibrated for water activity, using saturated KOH (a_w = 0.093) overnight (a minimum of 16 h) (Bonet-Ragel et al., 2018).

The initial reaction rate of all evaluated biocatalysts was calculated by adding 0.16 mL of methanol to 8 g of pomace oil (viz., the stoichiometric amount needed to obtain a yield of ca. 14%) and a total amount of biocatalyst of 32 000 AU (Bonet-Ragel et al., 2015). An identical procedure was followed to assess diffusional restrictions but using an identical mass of biocatalyst (200 mg) with variable immobilised lipase activity.

Operational stability was assessed in duplicate tests by causing the biocatalyst to deposit in the vial bottom by decantation (Purolite®) or application of a magnetic field (magnetite) and removing the medium after each reaction run (Bonet-Ragel et al., 2018).

The optimum butylamine concentration for MNP-CHO functionalisation was established by assessing the operational stability of the modified biocatalysts (MNP-But-CHO). Test were conducted as described above but in triplicate, using a scaled-down reaction volume (1.5 mL vials) and only 1 pulse of ethanol.

2.9. Diffusional restrictions

The Weisz–Prater criterion, which is a dimensionless number used to assess internal diffusional restrictions (Harvey W. Blanch, 1997; Weisz and Prater, 1954) was calculated by substituting experimental data from the biodiesel reactions into Eq. (2):

$$\Phi = \frac{r_{obs}\ \rho_p}{D_{eff}\ \ C_{m,0}} \left(\frac{V_p}{A_p}\right)^2 \quad (2)$$

Where r_{obs} denotes transesterification rate (mol $g^{-1}_{particle}$ s^{-1}), ρ_p particle density (g cm^{-3}), V_P particle volume (cm^3), A_p particle area (cm^2), $C_{m,0}$ bulk concentration of methanol (mol cm^{-3}) and D_{eff} (cm^2 s^{-1}) the effective diffusivity coefficient as calculated from Eq. (3):

$$D_{eff} = \frac{D_{m,a}\ \varepsilon_p\ \sigma}{\tau} \quad (3)$$

$D_{m,a}$ (cm^2 s^{-1}) being the molecular diffusivity of methanol in the reaction medium (pomace oil) as estimated from the Nakanishi correlation (Poling et al., 2001), ε_p particle porosity, σ the constriction factor and τ particle tortuosity. Φ values under 0.3 ensure the absence of internal diffusional restrictions since the resulting effectiveness factor is close to unity (Harvey W. Blanch, 1997).

2.10. Substrate acidity

Total acidity was determined in accordance with the protocols in Commission Regulation (EEC) No 2568/91 Annex II, amended by Commission Regulation (EC) No 702/2007.

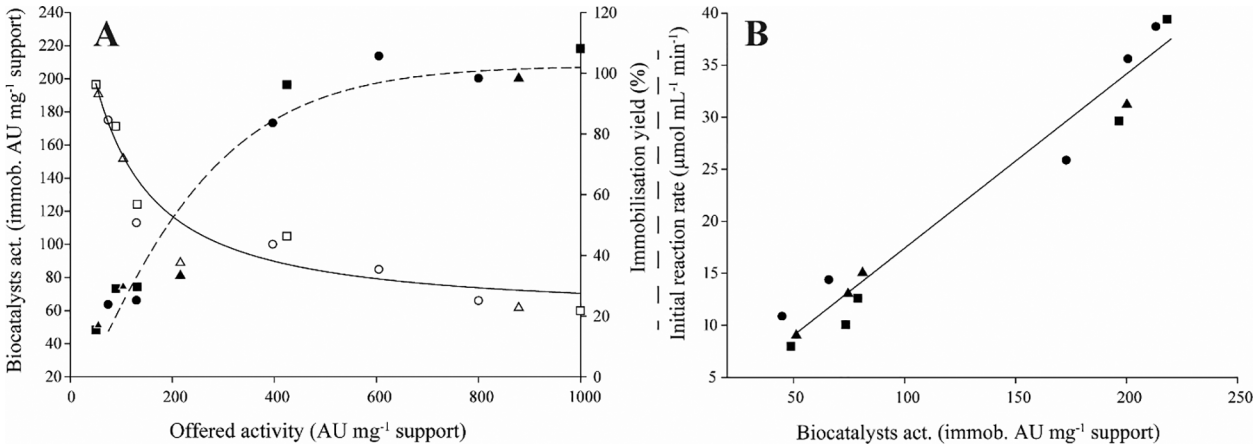

Fig. 1. (A) Biocatalyst activity (black symbols, ● ■ ▲) and immobilisation yield (white symbols, ○ □ △) at a variable offered activity. (B) Initial transesterification rate for each biocatalyst: EB-rROL (circles, ● ○), EO-rROL (squares, ■ □) and EDVB-rROL (triangles, ▲ △).

Table 1
Weisz–Prater criterion for pomace oil transesterification with methanol in the presence of various biocatalysts immobilised onto a polymethacrylate matrix. Φ and D_{eff} were calculated from Eq. (2) and (3), respectively, using σ = 1 and τ = 1.41 in the latter. Porosity (ε_p) and specific volume of the supports were 0.6 and 1.4 $cm^3\ g^{-1}$ support.

Parameters	Biocatalyst		
	EB-rROL	EO-rROL	EDVB-rROL
r_{obs} (mol s^{-1} cm^{-3}) × 10^7	6.44 ± 0.05	6.57 ± 0.02	5.20 ± 0.1
Biocatalyst weight (g)	0.2 ± 0.05	0.2 ± 0.03	0.2 ± 0.01
ρ_p (g cm^{-3})*	0.72	0.72	0.72
Specific area ($m^2\ g^{-1}$) *	152	139	59
r_{obs} (mol $g^{-1}_{particle}$ s^{-1}) × 10^5	2.89 ± 0.02	2.95 ± 0.01	2.33 ± 0.07
$C_{m,0}$ (mol cm^{-3}) × 10^4	4.41	4.41	4.41
$D_{m,a}$ ($cm^2\ s^{-1}$) × 10^6	1.18	1.18	1.18
D_{eff} ($cm^2\ s^{-1}$) × 10^7	5.04	5.04	5.04
Φ × 10^7	0.78 ± 0.05	0.95 ± 0.03	4.19 ± 0.08

*Data kindly provided by Purolite®.

3. Results and discussion

3.1. Lipase immobilisation onto polymethacrylate-based supports and diffusional restrictions

Recombinant *Rhizopus oryzae* lipase was covalently immobilised onto three glutaraldehyde-treated polymethacrylate-based Purolite® supports, namely: EB-rROL, EO-rROL and EDVB-rROL. Lipase solutions with an activity ranging from 55 to 1000 AU per mg support were used to evaluate the immobilisation yield and maximum lipase activity loading. All supports studied were identical in these parameters irrespective of their characteristics (Fig. 1A). Considering lipase immobilisation trend, offered activity values lower than 55 AU mg^{-1} support resulted in immobilisation yields of 100% and less than 90 AU mg^{-1} support in greater than 80%. As expected, increasing offered lipase activity decreased the immobilisation yield. Also, biocatalysts activity increased in proportion to a maximum lipase activity loading of 200 immobilised AU mg^{-1} support with all supports. Immobilised activity was maximal with lipase solutions of 400 AU mg^{-1} support, above which no further improvement was observed.

Mass transfer limitations in immobilised enzymes usually reflect in diffusional restrictions, whether external or internal. The latter are usually more severe with enzymes embedded in a solid porous matrix (Illanes, 2008) such as Purolite® supports. Diffusional restrictions were experimentally evaluated by examining the initial transesterification rate with methanol of biocatalysts containing variable amounts of immobilised enzyme (Fig. 1B). The proportional relationship observed suggested the absence of internal diffusional restrictions under the conditions studied. However, because mass transfer limitations may be an artefact on enzyme stability assessment (Klibanov, 1983; Ollis, 1972), the absence of restrictions was confirmed by calculating the Weisz–Prater Criterion (Φ). Table 1 shows the results for the three Purolite® biocatalysts with the highest immobilised activity. Φ was less than 0.3 in all cases, which supports the previous conclusion.

3.2. Polymethacrylate supports. Initial transesterification rate and operational stability

The catalytic performance of immobilised enzymes and the physico–chemical conditions of their microenvironment are strongly influenced by functional groups in the supports (Bolivar and Nidetzky, 2019). This led us to compare the initial reaction rate with methanol and operational stability, with both ethanol and methanol as acyl-acceptors, of the biocatalysts obtained by immobilising rROL onto Purolite® supports containing both epoxide and hydrophobic functional groups (viz., EB-rROL, EO-rROL and EDVB-rROL) with those of rROL immobilised onto a polymethacrylate-based support containing epoxide groups only (EX-rROL) (Bonet-Ragel et al., 2018).

The initial transesterification rate was similar with all polymethacrylate-based biocatalysts irrespective of the particular functional group —EB-rROL 24.9 ± 0.9, EO-rROL 25.1 ± 0.51, EDVB-rROL 24.4 ± 0.33 and EX-rROL 24.6 ± 0.78 μmol FAME min^{-1} cm^{-3}. Contrary to the expectations, these results showed that chemical differences in surface composition among supports had no effect on the initial reaction rate under the conditions studied.

On the other hand, the operational stability of the biocatalysts was dramatically influenced by the functional groups of the supports. Fig. 2A–2C shows the relative yield obtained by exposing each biocatalyst to a variable number of pulses of the different alcohols in consecutive reaction cycles. Operational stability with 1 pulse of methanol was not assessed owing to the low yield of the first reaction cycle by effect of deactivation of the enzyme (Fig. 2D) (Bonet-Ragel et al., 2018). Because rROL is a 1,3-regiospecific enzyme, the maximum expected yield was 66%. Using a stoichiometric amount of alcohol (viz., a 2:1 alcohol-to-oil mole ratio) avoided the presence of too high alcohol concentration in the reaction medium —and hence potentially adverse effects on the operational stability of the biocatalysts.

The joint presence of epoxide/butyl and epoxide/octadecyl groups in supports EB and EO, respectively, substantially increased the operational stability of the biocatalysts relative to EX-rROL upon exposure to 1 pulse of ethanol and 5 of methanol addition strategies. By way of example, Fig. 2C shows the most extreme case, in which EB-rROL and

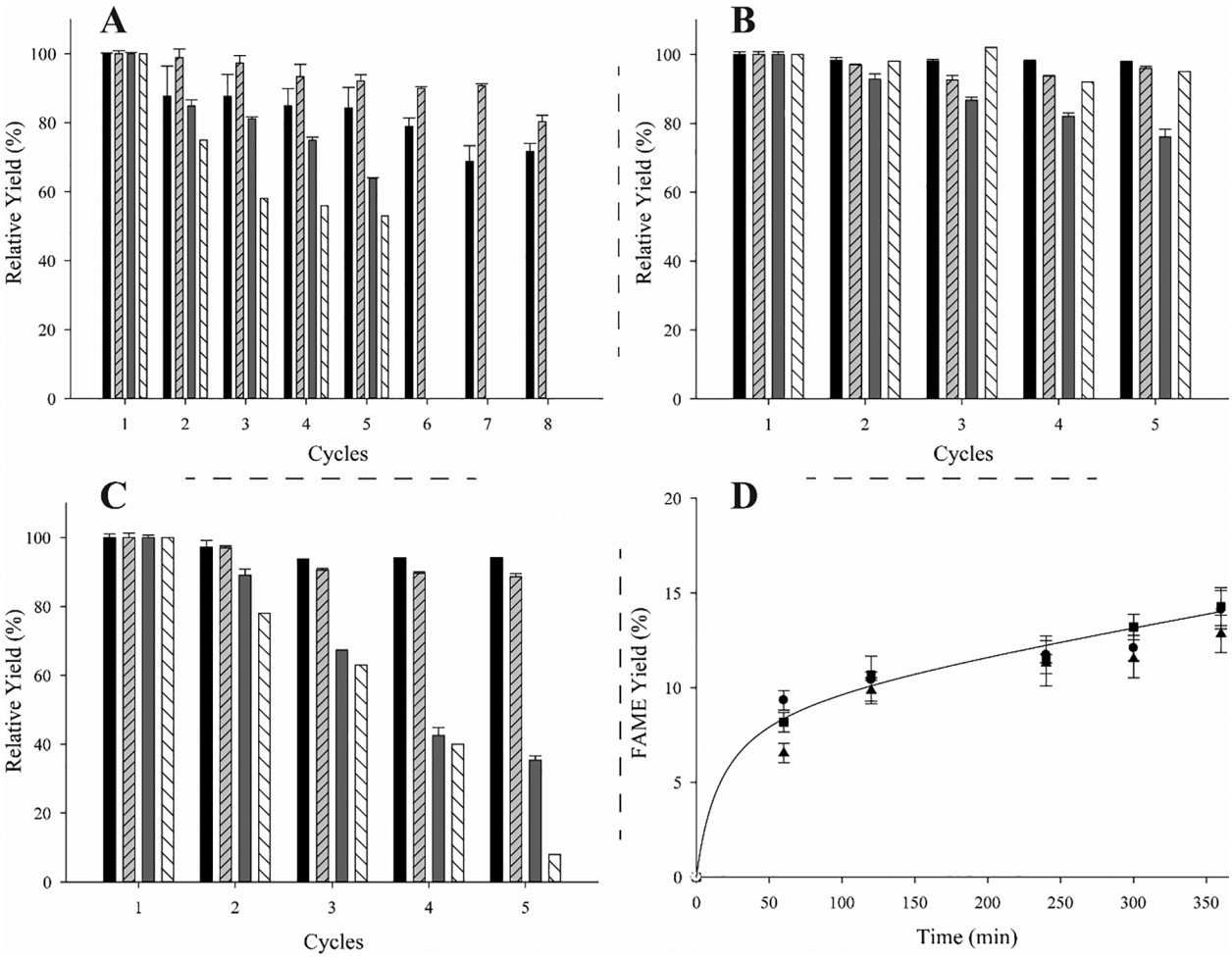

Fig. 2. Relative yield (%) in consecutive transesterification runs with biocatalysts immobilised on polymethacrylate-based supports upon exposure to (A) 1 pulse of ethanol, (B) 5 pulses of ethanol and (C) 5 pulses of methanol. The respective first reactions yields were taken to represent 100% yield. EB-rROL (black,), EO-rROL (up-striped grey,), EDVB-rROL (dark grey,) and EX-rROL (down-striped white,) (Bonet-Ragel et al., 2018). (D) First transesterification run with 1 pulse of methanol. The solid line represents the average FAME yield for the biocatalysts as a whole. EB-rROL (circles, ●), EO-rROL (squares, ■) and EDVB-rROL (triangles, ▲).

EO-rROL were roughly 9 times more stable than EX-rROL after 5 reaction cycles with 5 pulses of methanol. In contrast to EB-rROL and EO-rROL, EDVB-rROL exhibited no increase in stability —which was similar to that of EX-rROL. These results suggested that the increased stability of EB-rROL and EO-rROL was not a consequence of microenvironmental changes due the presence of hydrophobic functional groups —in fact, EDVB-rROL did not follow this trend—, but rather to that of surface hydrocarbon chains in the support possibly enhancing catalytic performance of the enzyme (Urrutia et al., 2018). In addition, EO-rROL was 20% more stable than EB-rROL against 1 pulse of ethanol (Fig. 2A), which testified to the positive effect of the hydrocarbon chain length on the operational stability of the biocatalysts.

The increased operational stability of EO-rROL and EB-rROL additionally made them resistant to 5 pulses of methanol. In fact, unlike EX-rROL and EDVB-rROL, both biocatalysts exhibited an identical relative yield close to 100% after 5 reaction cycles irrespective of the alcohol used (Fig. 2B and 2C) —being methanol a more powerful lipase inactivator than ethanol (Bonet-Ragel et al., 2018). However, neither EO-rROL nor EB-rROL resisted deactivation by 1 pulse of methanol (Fig. 2D), the final FAME yield being around 15% rather than the expected theoretical maximum: 66% (Fig. 2D).

Covalent lipase immobilisation causes the formation of Schiff bases through condensation of amino groups in lysines with aldehyde groups in the supports (Sheldon and van Pelt, 2013). Unless they have been reduced, formation of these bases is reversible in presence of water. However, EB-rROL performed identically in terms of initial reaction rate and operational stability with ethanol with reduced and unreduced Schiff bases. Consequently, there was no enzyme leakage (i.e., formation of Schiff bases was not reversed owing to the low water concentration in the transesterification medium and the well-known high complexity of the interaction between aldehyde groups in glutaraldehyde-treated supports and immobilised enzymes) (Sheldon and van Pelt, 2013).

3.3. *Magnetite-based supports*

Superparamagnetic nanoparticles (MNPs) have been widely used as supports for immobilised enzymes by virtue of their easy recovery by application of a magnetic field. The MNP matrix is non-porous, spherical-like solid magnetite (Fe_3O_4) that can be functionalised with a broad variety of chemicals for immobilisation purposes (Del Arco et al., 2021). In this work, MNPs containing aldehyde groups (MNP-CHO) were further functionalised with butylamine to obtain particles with both aldehyde and butyl groups. The particles were treated with butylamine solutions of eight different concentrations to obtain functionalised derivatives from MNP-CHO (the blank control, with no butyl groups) to MNP-1MBut-CHO (functionalised with the most concentrated butylamine solution). Functionalised nanoparticles were then used to immobilise rROL and the biocatalysts thus obtained were assessed for

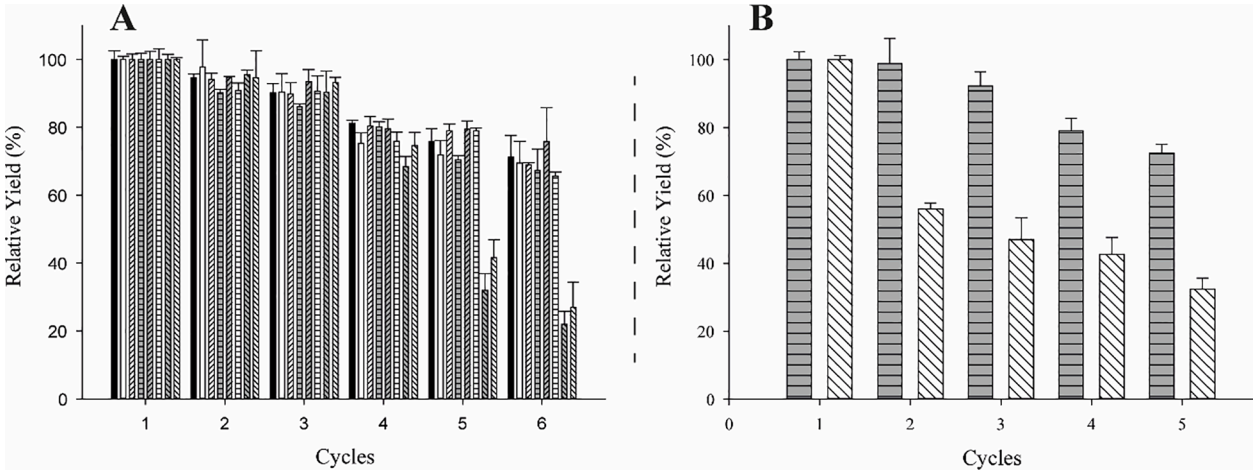

Fig. 3. (A) Relative yield (%) of consecutive transesterification runs (1 pulse of ethanol) in the presence of rROL immobilised on MNP-based supports functionalised with solutions ranging from 0 (blank control) to 1 M butylamine in 1.5 mL vials. The bars correspond to the functionalised MNPs obtained by using a butylamine concentration of 1 M (MNP-1MBut-rROL, black,), 100 mM (MNP-100mMBut-rROL, white,), 10 mM (MNP-10mMBut-rROL, up-striped white,), 1 mM (MNP-1mMBut-rROL, horizontally striped grey,), 100 μM (MNP-100μMBut-rROL, up-striped grey,), 10 μM (MNP-10μMBut-rROL, horizontally striped white,), 1 μM (MNP-1μMBut-rROL, down-striped grey,) and 0 M (MNP-rROL, blank, down-striped white,). (B) Relative yield (%) of consecutive transesterification runs (1 pulse of ethanol) with MNP-1mMBut-rROL (horizontally striped grey,) and MNP-rROL (blank, down-striped white,) in 10 mL vials. The respective first reactions yields were taken to represent 100% yield.

the influence of surface butyl groups in the support on biocatalyst performance.

Fig. 3A shows the results of the screening with ethanol as acyl-acceptor for the optimum butylamine concentration for use in 1.5 mL vials. As can be seen, MNPs functionalised with solutions containing a butylamine concentration above 10 μM provided biocatalysts more than twice as stable as those immobilised on MNPs treated with lower butylamine concentrations or no amine (blank). Also, no differences in operational stability were observed with butylamine concentrations above 10 μM. Therefore, a concentration of 1 mM was chosen to assess the influence on the initial transesterification rate and operational stability in 10 mL vials.

MNP-rROL and MNP-1mMBut-rROL exhibited a similar initial transesterification rate in 10 mL vials. As with the Purolite® biocatalysts, the presence of surface hydrocarbon chains on the support had no effect on the initial rate. However, the initial reaction rate with the MNP-based biocatalysts was roughly one-half that obtained with the Purolite®-based counterparts —MNP-rROL 10.3 ± 0.25 and MNP-1mMBut-rROL 12.2 ± 0.85 μmol FAME min^{-1} cm^{-3}. Operational stability (Fig. 3B) followed the same trend as in the screening tests (Fig. 3A), MNP-1mMBut-rROL being twice more stable than MNP-rROL after 5 consecutive reaction cycles. Whereas MNP-rROL and EX-rROL (i.e., two catalysts with homologous supports as regards functional groups) were similar in operational stability, MNP-1mMBut-rROL was 20% less stable than EO-rROL and 15% less so than EB-rROL against 1 pulse of ethanol addition strategy. Therefore, no further research was performed with superparamagnetic biocatalysts.

3.4. Biocatalyst performance: Productivity and volumetric productivity

In addition to initial reaction rate and operational stability, the biocatalysts were assessed in terms of productivity (μmol FAEE/FAME min^{-1}) and volumetric productivity (μmol FAEE/FAME min^{-1} cm^{-3}) in 5 consecutive reaction cycles for use at the industrial scale.

As can clearly be seen from Table 2, the biocatalysts immobilised onto supports functionalised with hydrocarbon chains performed better than those onto supports containing none irrespective of matrix type (polymethacrylate or magnetite nanoparticles). Therefore, the presence of hydrocarbon chains had a favourable effect on biocatalyst performance. Purolite®-based biocatalysts with surface hydrocarbon chains exhibited better productivity and volumetric productivity than MNP-based biocatalysts also containing surface chains (e.g., 20% higher with EO-rROL than with MNP-1mMBut-rROL). Also, productivity and volumetric productivity among Purolite®-based biocatalysts were 1.5–2 times greater in EO and EB-rROL than they were in EX-rROL with 1 pulse of EtOH and 5 of MetOH, respectively —differences were smaller with 5 pulses of EtOH as a result of the weaker adverse effect of this acyl-acceptor and of its application being split over several pulses (Bonet-Ragel et al., 2018). In addition, adding ethanol in 5 pulses resulted in roughly twice better productivity and volumetric productivity than using a single pulse with any biocatalyst. Further research splitting the amount of alcohol in more pulses was not performed as it would reduce biodiesel productivity (Bonet-Ragel et al., 2018).

The productivity, volumetric productivity and operational stability results led us to choose EO-rROL and 5 pulses of either alcohol for further testing.

Table 2
Productivity and volumetric productivity of polymethacrylate- and magnetite-based biocatalysts exposed to a variable number of pulses of methanol or ethanol.

Biocatalyst	Productivity (μmol min^{-1})			Volumetric productivity (μmol min^{-1} cm^{-3})		
	EtOH 1 pulse	EtOH 5 pulses	MetOH 5 pulses	EtOH 1 pulse	EtOH 5 pulses	MetOH 5 pulses
EB-rROL	38.27 ± 1.96	74.89 ± 0.36	48.76 ± 0.30	0.86 ± 0.04	1.68 ± 0.02	1.10 ± 0.01
EO-rROL	41.56 ± 0.91	73.43 ± 0.42	48.04 ± 0.35	0.94 ± 0.02	1.65 ± 0.01	1.08 ± 0.02
EDVB-rROL	34.83 ± 0.28	67.07 ± 0.39	34.25 ± 0.38	0.78 ± 0.01	1.51 ± 0.01	0.77 ± 0.01
EX-rROL	23.00 ± 0.33	60.36 ± 0.40	19.62 ± 0.33	0.52	1.36 ± 0.02	0.44 ± 0.02
MNP-rROL	22.16 ± 0.66			0.50 ± 0.02		
MNP-1mMBut-rROL	35.36 ± 1.50			0.79 ± 0.03		

Table 3
Percent acidity of alternative oily substrates and initial transesterification rate with EO-rROL.

Substrate	Acidity (%)	Initial reaction rate (μmol FAME min^{-1} cm^{-3})
Makauba oil	12.16 ± 0.85	21.4 ± 0.15
Jatropha oil	7.85 ± 0.22	24.5 ± 0.22
Microbial oil	2.47 ± 0.19	22.06 ± 0.34
WCO	0.77 ± 0.13	22.8 ± 0.68
Olive pomace oil	18.93 ± 0.8	24.4 ± 0.33

3.5. Alternative substrates: second- and third-generation biodiesel

Although oil pomace was used as a model substrate to assess biocatalysts performance, the potential usefulness of this substrate for the food industry could still stir the ethical debate between food and fuels. We therefore chose to investigate alternative oily substrates such as non-edible vegetable oils from makauba and jatropha, microbial oil from *Rhodosporidium toruloides* and WCO.

Table 3 shows the acidity of each alternative substrate. The values for vegetable oils from makauba (Aguieiras et al., 2014) and jatropha (Jain and Sharma, 2010) are consistent within previous reports. However, the acidity of these oils depends markedly on factors such as the harvest and storage conditions (Souza et al., 2016). The acidity of microbial oil from *R. toruloides* is also similar to previously reported values (Singh et al., 2018). Although the acidity of WCO falls within the typical ranges (less than 0.5% for refined oils and 0.5–15% for used oils; Lam et al., 2010), its low value suggests that it was not extensively cooked —the presence of FFAs in waste oil is mainly a result of oxidative, hydrolytic and thermolytic reactions during frying (Hama et al., 2013).

The presence of FFAs has been reported to have a favourable effect on the initial reaction rate and the operational stability of enzymes during transesterification (Bonet-Ragel et al., 2015). Although EO-rROL exhibited a similar initial rate irrespective of substrate acidity (Table 3) —what might be explained because not only the initial FFA content is crucial, but also the immediately generated FFAs (Lin et al., 2011)— this property had a marked effect on its operational stability. With ethanol as acyl-acceptor in the transesterification reaction, oil acidity had no appreciable effect (Fig. 4A) because this alcohol is known to scarcely inactivate lipase. With methanol, a more powerful lipase inactivator (Bonet-Ragel et al., 2018), FFAs did influence the results (Fig. 4B). Thus, with the least acidic substrate (WCO), the operational stability of the biocatalyst was rather poor —more than 95% of the initial relative yield was lost after only 5 reaction cycles. Meanwhile, substrates with acidity >2% maintained at least 60% of the initial value —roughly 30 times more than the relative yield obtained with WCO. Therefore, substrate acidity strongly influenced the operational stability of the biocatalyst. Also, the results are suggestive of a synergistic effect of the alcohol

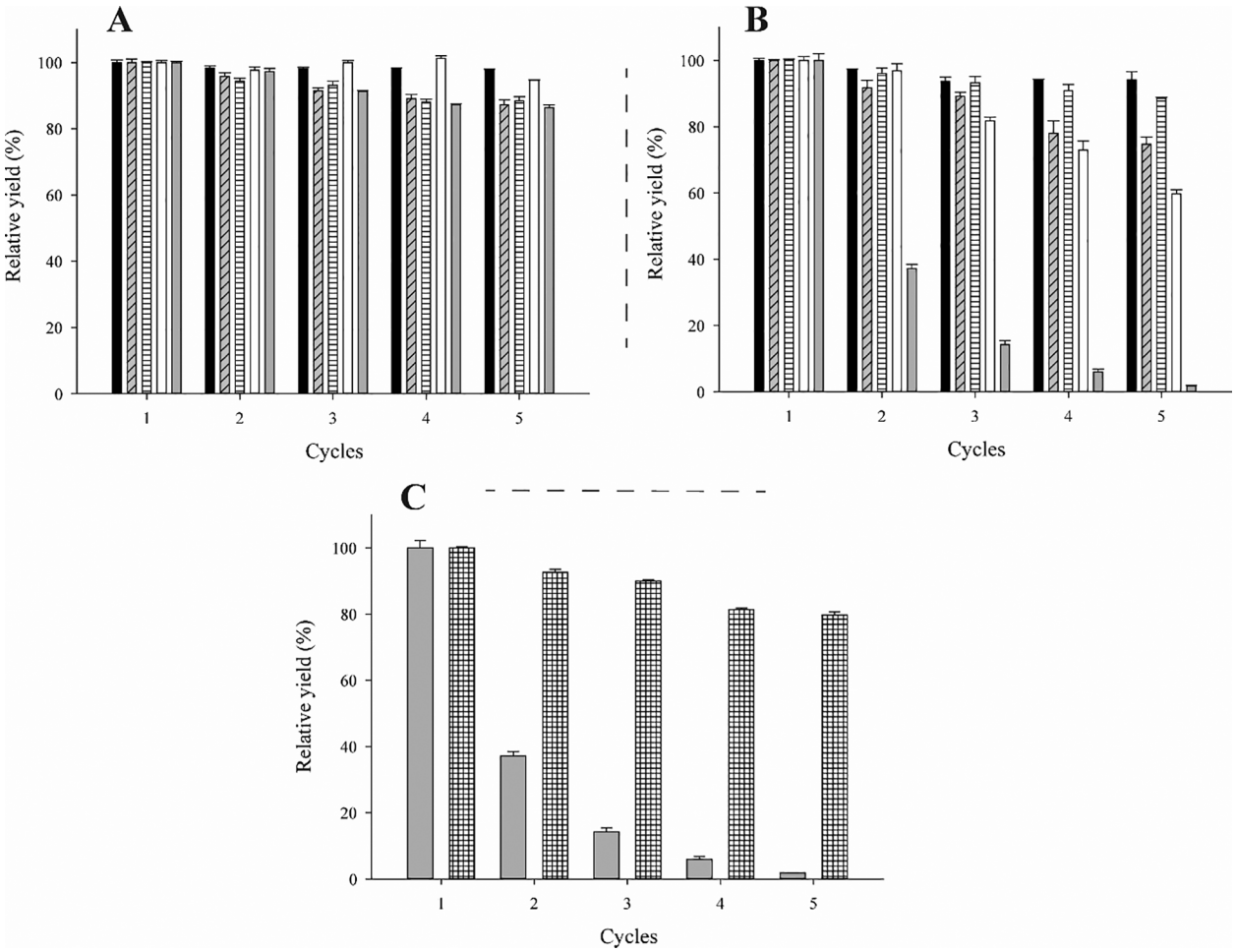

Fig. 4. Relative yield (%) of consecutive transesterification cycles of various types of oil with 5 pulses of ethanol (A) or 5 of methanol (B) in the presence of biocatalyst EO-rROL. The bars correspond to pomace (black,), makauba (up-striped grey,), jatropha (striped white,), microbial (white,) and WCO (grey,). (C) Comparison of the relative yield (%) obtained with 5 (grey,) and 10 pulses of methanol (squared white,) and WCO as substrate. The initial reactions yields were taken to be 100%.

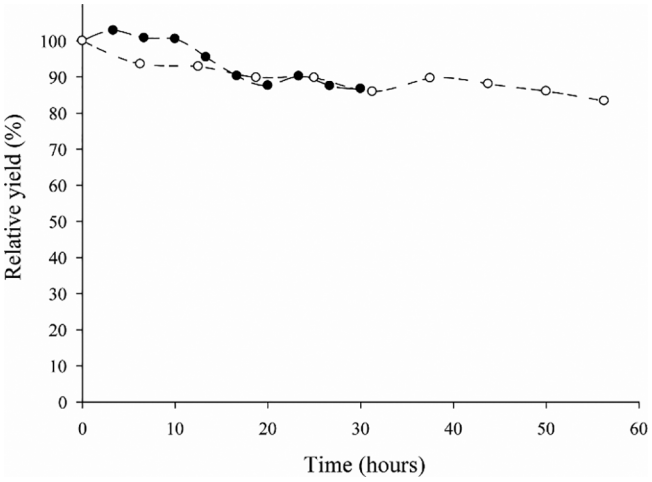

Fig. 5. Relative yield (%) of consecutive transesterification cycles of WCO with 5 pulses of ethanol (black circles, ●) and 10 of methanol (white circles, ○) in the presence of biocatalyst EO-rROL. The times on the x-axis are those at which each cycle was started. The initial reactions yields were taken to be 100%.

concentration and substrate acidity on deactivation of the biocatalysts. This hypothesis was checked by using a reduced concentration of methanol in the reaction medium (specifically, by splitting the amount used in 10 pulses) with WCO as substrate. As can be seen from Fig. 4C, the operational stability of the biocatalyst was 20 times greater under these conditions. Therefore, the origin of the synergistic effect was the increased polarity of the reaction medium in the presence of FFAs, which acted as a buffering agent for the high polarity of methanol —and hence for its also high deactivation capacity (Canet et al., 2016).

3.6. Transesterification scale-up

Once WCO was found to be the most suitable substrate on the grounds of its low cost, high production and potential for use in accordance with the principles of circular economy (Hama et al., 2013; Olkiewicz et al., 2016), it was used to scale up the enzymatic production of biodiesel to a 50 mL mini-reactor for industrial proof of concept.

Transesterification runs in 10 mL vials under orbital stirring were successfully scaled up to a stirred tank reactor under mechanical stirring (Bonet-Ragel, 2018). As can be seen from Fig. 5, the relative yields obtained in consecutive cycles using 5 pulses of EtOH and 10 of MetOH with the laboratory-scale mini-reactor were similar to those obtained in 10 mL vials (Fig. 4). Therefore, the proposed enzymatic biodiesel production method can be easily implemented on an industrial proof of concept scale. Also, around 80% of the initial relative yield was maintained after 10 consecutive reaction cycles with either acyl-acceptor. Although few studies on the use of WCO in combination with *Rhizopus oryzae* lipase have been reported (López-Fernández et al., 2020), similar operational stabilities have been obtained with other substrates (Duarte et al., 2015; Su et al., 2014). Also, there was virtually no difference between using 5 pulses of EtOH and 10 pulses of MetOH, in order to minimise the negative synergistic effect of the alcohol concentration and substrate acidity on biocatalyst activity at the expense of longer reaction times, reduced productivity and increased operational costs for a potential biodiesel plant.

4. Conclusions

Rhizopus oryzae lipase proved a suitable biocatalyst for biodiesel production from various oily substrates. The hydrocarbon chain length was found to play a key role in increasing the operational stability of rROL immobilised onto polymethacrylate- and magnetite-based supports. Despite their easy recovery, magnetite-based biocatalysts were outperformed by Purolite®-based biocatalysts. Oil acidity and acyl-acceptor concentration proved crucial and synergistically influential on biocatalyst performance. The fact that the enzymatic transesterification of WCO with EO-rROL was successfully scaled up validated its potential for industrial implementation to enable biodiesel production from an inexpensive substrate and management of the waste under the circular economy principles.

CRediT authorship contribution statement

Josu López-Fernández: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. **Maria Dolors Benaiges:** Data curation, Methodology, Supervision, Validation, Writing - review & editing. **Francisco Valero:** Data curation, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.125233.

References

Aguieiras, E.C.G., Cavalcanti-Oliveira, E.D., De Castro, A.M., Langone, M.A.P., Freire, D. M.G., 2014. Biodiesel production from *Acrocomia aculeata* acid oil by (enzyme/enzyme) hydroesterification process: use of vegetable lipase and fermented solid as low-cost biocatalysts. Fuel 135, 315–321. https://doi.org/10.1016/j.fuel.2014.06.069.

Ariaeenejad, S., Motamedi, E., Hosseini Salekdeh, G., 2021. Application of the immobilized enzyme on magnetic graphene oxide nano-carrier as a versatile bifunctional tool for efficient removal of dye from water. Bioresour. Technol. 319, 124228 https://doi.org/10.1016/j.biortech.2020.124228.

Arnau, C., Ramon, R., Casas, C., Valero, F., 2010. Optimization of the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. Enzyme Microb. Technol. 46, 494–500. https://doi.org/10.1016/j.enzmictec.2010.01.005.

Bolivar, J.M., Nidetzky, B., 2019. The microenvironment in immobilized enzymes: methods of characterization and its role in determining enzyme performance. Molecules 24, 3460–3484. https://doi.org/10.3390/molecules24193460.

Bonet-Ragel, K., 2018. Enzymatic synthesis of biodiesel from high free fatty acid feedstock using a recombinant *Rhizopus oryzae* lipase. Universitat Autònoma de Barcelona, Cerdanyola del Vallés.

Bonet-Ragel, K., Canet, A., Benaiges, M.D., Valero, F., 2018. Effect of acyl-acceptor stepwise addition strategy using *alperujo* oil as a substrate in enzymatic biodiesel synthesis. J. Chem. Technol. Biotechnol. 93, 541–547. https://doi.org/10.1002/jctb.5399.

Bonet-Ragel, K., Canet, A., Benaiges, M.D., Valero, F., 2015. Synthesis of biodiesel from high FFA *alperujo* oil catalysed by immobilised lipase. Fuel 161, 12–17. https://doi.org/10.1016/j.fuel.2015.08.032.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Canet, A., Benaiges, M.D., Valero, F., Adlercreutz, P., 2017. Exploring substrate specificities of a recombinant *Rhizopus oryzae* lipase in biodiesel synthesis. N. Biotechnol. 39, 59–67. https://doi.org/10.1016/j.nbt.2017.07.003.

Canet, A., Bonet-Ragel, K., Benaiges, M.D., Valero, F., 2016. Lipase-catalysed transesterification: viewpoint of the mechanism and influence of free fatty acids. Biomass Bioenergy 85, 94–99. https://doi.org/10.1016/j.biombioe.2015.11.021.

Canet, A., Dolors Benaiges, M., Valero, F., 2014. Biodiesel synthesis in a solvent-free system by recombinant *Rhizopus oryzae* lipase. Study of the catalytic reaction

progress. JAOCS. J. Am. Oil Chem. Soc. 91, 1499–1506. https://doi.org/10.1007/s11746-014-2498-y.
Connolly, D., Lund, H., Mathiesen, B.V., 2016. Smart energy Europe: the technical and economic impact of one potential 100% renewable energy scenario for the European Union. Renew. Sustain. Energy Rev. 60, 1634–1653. https://doi.org/10.1016/j.rser.2016.02.025.
Cruz-Izquierdo, Á., Picó, E.A., López, C., Serra, J.L., Llama, M.J., 2014. Magnetic cross-linked enzyme aggregates (mCLEAs) of *Candida antarctica* lipase: an efficient and stable biocatalyst for biodiesel synthesis. PLoS One 9, 1–22. https://doi.org/10.1371/journal.pone.0115202.
Da Rós, P.C.M., Silva, G.A.M., Mendes, A.A., Santos, J.C., de Castro, H.F., 2010. Evaluation of the catalytic properties of *Burkholderia cepacia* lipase immobilized on non-commercial matrices to be used in biodiesel synthesis from different feedstocks. Bioresour. Technol. 101, 5508–5516. https://doi.org/10.1016/j.biortech.2010.02.061.
Del Arco, J., Alcántara, A.R., Fernández-Lafuente, R., Fernández-Lucas, J., 2021. Magnetic micro-macro biocatalysts applied to industrial bioprocesses. Bioresour. Technol. 322, 124547 https://doi.org/10.1016/j.biortech.2020.124547.
Duarte, S.H., del Peso Hernández, G.L., Canet, A., Benaiges, M.D., Maugeri, F., Valero, F., 2015. Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant *Rhizopus oryzae* lipase. Bioresour. Technol. 183, 175–180. https://doi.org/10.1016/j.biortech.2015.01.133.
Guillén, M., Benaiges, M.D., Valero, F., 2012. Biosynthesis of ethyl butyrate by immobilized recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris*. Biochem. Eng. J. 65, 1–9. https://doi.org/10.1016/j.bej.2012.03.009.
Hama, S., Kondo, A., 2013. Enzymatic biodiesel production: an overview of potential feedstocks and process development. Bioresour. Technol. 135, 386–395. https://doi.org/10.1016/j.biortech.2012.08.014.
Hama, S., Yoshida, A., Tamadani, N., Noda, H., Kondo, A., 2013. Enzymatic production of biodiesel from waste cooking oil in a packed-bed reactor: an engineering approach to separation of hydrophilic impurities. Bioresour. Technol. 135, 417–421. https://doi.org/10.1016/j.biortech.2012.06.059.
Harvey W. Blanch, D.S.C., 1997. Biochemical engineering, second edition - CRC Press. Boca Raton, USA.
He, Y., Wu, T., Wang, X., Chen, B., Chen, F., 2018. Cost-effective biodiesel production from wet microalgal biomass by a novel two-step enzymatic process. Bioresour. Technol. 268, 583–591. https://doi.org/10.1016/j.biortech.2018.08.038.
Illanes, A., 2008. Enzyme biocatalysis: principles and applications, in: enzyme biocatalysis: principles and applications. 2008th edition. Springer. https://doi.org/10.1007/978-1-4020-8361-7.
Jain, S., Sharma, M.P., 2010. Kinetics of acid base catalyzed transesterification of *Jatropha curcas* oil. Bioresour. Technol. 101, 7701–7706. https://doi.org/10.1016/j.biortech.2010.05.034.
Jin, Z., Han, S.Y., Zhang, L., Zheng, S.P., Wang, Y., Lin, Y., 2013. Combined utilization of lipase-displaying *Pichia pastoris* whole-cell biocatalysts to improve biodiesel production in co-solvent media. Bioresour. Technol. 130, 102–109. https://doi.org/10.1016/j.biortech.2012.12.020.
Khanahmadi, S., Yusof, F., Chyuan Ong, H., Amid, A., Shah, H., 2016. Cocoa pod husk: a new source of CLEA-lipase for preparation of low-cost biodiesel: an optimized process. J. Biotechnol. 231, 95–105. https://doi.org/10.1016/j.jbiotec.2016.05.015.
Klibanov, A.M., 1983. Stabilization of enzymes against thermal inactivation. Adv. Appl. Microbiol. 29, 1–28. https://doi.org/10.1016/S0065-2164(08)70352-6.
Lam, M.K., Lee, K.T., Mohamed, A.R., 2010. Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: a review. Biotechnol. Adv. 28, 500–518. https://doi.org/10.1016/j.biotechadv.2010.03.002.
Lin, Y.H., Luo, J.J., John Hwang, S.C., Liau, P.R., Lu, W.J., Lee, H.T., 2011. The influence of free fatty acid intermediate on biodiesel production from soybean oil by whole cell biocatalyst. Biomass and Bioenergy 35, 2217–2223. https://doi.org/10.1016/j.biombioe.2011.02.039.
López-Fernández, J., Barrero, J.J., Benaiges, M.D., Valero, F., 2019. Truncated prosequence of *Rhizopus oryzae* lipase: key factor for production improvement and biocatalyst stability. Catalysts 9, 961. https://doi.org/10.3390/catal9110961.
López-Fernández, J., Benaiges, M.D., Valero, F., 2020. *Rhizopus oryzae* lipase, a promising industrial enzyme: biochemical characteristics, production and biocatalytic applications. Catalysts 10, 1277. https://doi.org/10.3390/catal10111277.
Luo, X., Ge, X., Cui, S., Li, Y., 2016. Value-added processing of crude glycerol into chemicals and polymers. Bioresour. Technol. 215, 144–154. https://doi.org/10.1016/j.biortech.2016.03.042.
Ma, Y., Gao, Z., Wang, Q., Liu, Y., 2018. Biodiesels from microbial oils: opportunity and challenges. Bioresour. Technol. 263, 631–641. https://doi.org/10.1016/j.biortech.2018.05.028.
Madhavan, A., Sindhu, R., Binod, P., Sukumaran, R.K., Pandey, A., 2017. Strategies for design of improved biocatalysts for industrial applications. Bioresour. Technol. 245 Part B, 1304–1313. https://doi.org/10.1016/j.biortech.2017.05.031.
Navarro López, E., Robles Medina, A., González Moreno, P.A., Esteban Cerdán, L., Martín Valverde, L., Molina Grima, E., 2016. Biodiesel production from *Nannochloropsis gaditana* lipids through transesterification catalyzed by *Rhizopus oryzae* lipase. Bioresour. Technol. 203, 233–244. https://doi.org/10.1016/j.biortech.2015.12.036.
Noureddini, H., Gao, X., Philkana, R.S., 2005. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. Bioresour. Technol. 96, 769–777. https://doi.org/10.1016/j.biortech.2004.05.029.
Ogunkunle, O., Ahmed, N.A., 2019. A review of global current scenario of biodiesel adoption and combustion in vehicular diesel engines. Energy Reports 5, 1560–1579. https://doi.org/10.1016/j.egyr.2019.10.028.
Olkiewicz, M., Torres, C.M., Jiménez, L., Font, J., Bengoa, C., 2016. Scale-up and economic analysis of biodiesel production from municipal primary sewage sludge. Bioresour. Technol. 214, 122–131. https://doi.org/10.1016/j.biortech.2016.04.098.
Ollis, D.F., 1972. Diffusion influences in denaturable insolubilized enzyme catalysis. Biotechnol. Bioeng. 14, 871–884. https://doi.org/10.1002/bit.260140603.
Poling, B.E., Prausnitz, J.M., O'Connell, J.P., 2001. The properties of gases & liquids - 5th edition. McGraw Hill Education. New York, USA. https://doi.org/10.1016/0894-1777(88)90021-0.
Ranganathan, S.V., Narasimhan, S.L., Muthukumar, K., 2008. An overview of enzymatic production of biodiesel. Bioresour. Technol. 99, 3975–3981. https://doi.org/10.1016/j.biortech.2007.04.060.
Resina, D., Serrano, A., Valero, F., Ferrer, P., 2004. Expression of a *Rhizopus oryzae* lipase in *Pichia pastoris* under control of the nitrogen source-regulated formaldehyde dehydrogenase promoter. J. Biotechnol. 109, 103–113. https://doi.org/10.1016/j.jbiotec.2003.10.029.
Rodrigues, A., Bordado, J.C., Dos Santos, R.G., 2017. Upgrading the glycerol from biodiesel production as a source of energy carriers and chemicals - a technological review for three chemical pathways. Energies 10, 1817–1853. https://doi.org/10.3390/en10111817.
Rodrigues, J., Perrier, V., Lecomte, J., Dubreucq, E., Ferreira-Dias, S., 2016. Biodiesel production from crude jatropha oil catalyzed by immobilized lipase/acyltransferase from *Candida parapsilosis* in aqueous medium. Bioresour. Technol. 218, 1224–1229. https://doi.org/10.1016/j.biortech.2016.07.090.
Sheldon, R.A., van Pelt, S., 2013. Enzyme immobilisation in biocatalysis: why, what and how. Chem. Soc. Rev. 42, 6223–6235. https://doi.org/10.1039/C3CS60075K.
Singh, G., Jeyaseelan, C., Bandyopadhyay, K.K., Paul, D., 2018. Comparative analysis of biodiesel produced by acidic transesterification of lipid extracted from oleaginous yeast *Rhodosporidium toruloides*. 3. Biotech 8, 434. https://doi.org/10.1007/s13205-018-1467-9.
Souza, G.K., Scheufele, F.B., Pasa, T.L.B., Arroyo, P.A., Pereira, N.C., 2016. Synthesis of ethyl esters from crude macauba oil (*Acrocomia aculeata*) for biodiesel production. Fuel 165, 360–366. https://doi.org/10.1016/j.fuel.2015.10.068.
Su, F., Li, G., Zhang, H., Yan, Y., 2014. Enhanced performance of *Rhizopus oryzae* lipase immobilized on hydrophobic carriers and its application in biorefinery of rapeseed oil deodorizer distillate. Bioenergy Res. 7, 935–945. https://doi.org/10.1007/s12155-014-9415-y.
Urrutia, P., Arrieta, R., Alvarez, L., Cardenas, C., Mesa, M., Wilson, L., 2018. Immobilization of lipases in hydrophobic chitosan for selective hydrolysis of fish oil: the impact of support functionalization on lipase activity, selectivity and stability. Int. J. Biol. Macromol. 108, 674–686. https://doi.org/10.1016/j.ijbiomac.2017.12.062.
Weisz, P.B., Prater, C.D., 1954. Interpretation of measurements in experimental catalysis. Adv. Catal. 6, 143–196. https://doi.org/10.1016/S0360-0564(08)60390-9.
Xu, J., Liu, C., Wang, M., Shao, L., Deng, L., Nie, K., Wang, F., 2017. Rotating packed bed reactor for enzymatic synthesis of biodiesel. Bioresour. Technol. 224, 292–297. https://doi.org/10.1016/j.biortech.2016.10.045.