Biodiesel production *via trans*-esterification using *Pseudomonas cepacia* immobilized on cellulosic polyurethane

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Abstract: In this work *Pseudomonas cepacia* lipase immobilized on cellulosic polyurethane was used as catalyst for biodiesel production *via trans*-esterification reactions in order to provide cost-effective methods of enzyme re-cycling. The efficacy of the immobilized enzyme catalyst at low loading (6.2 wt.%) and the effects of temperature, water content, and reaction time in model *trans*-esterification of glyceryl trioctanoate were investigated extensively. It was found that water was necessary for the reaction of glyceryl trioctanoate with ethanol to proceed. A high conversion of glyceryl trioctanoate (~70%) was obtained at 35 °C, with only 5.0 wt.% of water content over a reaction period of 12 h.

Keywords: enzyme, *trans*-esterification reaction, catalysis, cellulosic polyurethane, biodiesel, sustainable

Introduction

Availability and fluctuating costs of fossil fuels continue to drive research into new biofuel production and associated biomass conversion technologies. The use of bio-derived diesel, namely fatty acid alkyl esters, has been extensively studied and validated for use as a replacement and/or supplement to traditional fossil-derived diesel, in part, due to their comparatively straightforward preparation. Biodiesel production is achieved through catalytic *trans*-esterification of vegetable oils, animal fats or microalgal oils with alcohols including methanol [1, 2], ethanol [3, 4], 1-propanol [5] and 2-butanol [6], a procedure needed in order to provide the necessary physical properties of the fuel to ensure engine compatibility [7].

Currently, these required *trans*-esterification processes are undertaken mainly using soluble alkaline metal-based catalysts, *e.g.* sodium hydroxide, sodium methoxide, potassium hydroxide or potassium methoxide, which give fast diffusion and reaction kinetics [8]. However, in the presence of the dissolved metal cations, saponification of free fatty acids also occurs, which lowers yields of the target bio-derived diesel. In contrast, although inorganic acid-catalyzed *trans*-esterification reactions for biodiesel production (using, for example HCl, H_2SO_4, H_3PO_4) are possible and not strongly affected by the presence of free fatty acids [9], industrial use of acid-catalyzed processes is problematic due to slow reaction rates and the corrosive nature of the acid. Furthermore, both homogeneous acid- and base-mediated biodiesel manufacturing processes are hindered by the difficulty in separation of the catalyst from the reaction mixture, something that is not only an issue for downstream use of the bio-derived diesel itself, but also for the recovery of the co-produced glycerol, which is an increasingly important commodity chemical in its own right [10].

In order to circumvent some of the problems surrounding soluble acid- and base-mediated *trans*-esterification reactions, a range of alternative heterogeneously-catalyzed *trans*-esterification strategies for bio-derived diesel production have been explored, with enzymatic

catalysts having started to emerge as promising candidates, although their application remains in its infancy [11]. This nascent method of enzyme-mediated *trans*-esterification for biodiesel production has recently been reviewed by Norjannah [12]. In this area, the use of lipase triacylglycerol acylhydrolase (EC 3.1.1.3) has received considerable interest, as it has been shown to catalyze both the hydrolysis of triglycerides and *trans*-esterification reactions, thus potentially leading to a reduction in the number of necessary process steps required for bio-derived diesel manufacture [13-16]. Indeed, a number of studies have explored the production of bio-based diesel mediated by lipases, in particular with enzymes such as *Pseudomonas cepacia* and *Candida Antarctica* [17, 18]. Importantly, in contrast to traditional homogeneous chemical *trans*-esterification methodologies, enzyme-promoted *trans*-esterification is achieved at neutral pH, something that eliminates corrosive waste streams, prevents by-product soap formation, and facilitates both bio-derived diesel and glycerol recovery. Additionally, the overall life cycle for enzymatic routes to bio-derived diesel has relatively low energy consumption compared with traditional acid- or base-catalyzed *trans*-esterification reactions [8]. However, in general the catalytic performance of enzymes is significantly poorer than that achieved using soluble base catalysts, such as sodium hydroxide or potassium methoxide [19]. Furthermore, appropriate choice of reactant alcohol is crucial since methanol is known to act as a strong inhibitor of lipase catalytic activity and to decrease the enzyme's stability [20, 21]. For example, Shimada *et al.* have reported that during studies of immobilized *Candida Antarctica*-mediated *trans*-esterification of vegetable oil, methanol led to complete enzyme deactivation [22].

An additional disadvantage of enzyme-mediated *trans*-esterification is the high cost of lipases, something that is further compounded by the fact that their recovery and re-use is difficult. So, in order to provide cost-effective methods of enzyme re-cycling, efforts have been made to prepare and utilize solid-supported lipases. To this end, a range of inorganic particles

[23, 24], natural macromolecules [25-27], and polymers [28, 29] have been explored as supports for lipases, with many diverse immobilization methods having been developed, such as adsorption [30, 31], covalent binding [32], affinity immobilization [33, 34], and entrapment [35, 36]. Choosing the most suitable immobilization method has become an important factor for increasing enzymatic *trans*-esterification efficiency; potential support materials and methods for lipase immobilization have been recently reviewed by Sumitra *et al.* [37].

Cellulosic polyurethane has received particular attention as an appropriate support material due to its demonstrated biocompatibility, as well as its hydrogen bond-forming potential, which can enhance its mechanical and surface properties [38]. For example, hydrophilic cellulosic polyurethane variants (possessing poly(ethylene adipate)diol units) favor enzyme loading by adsorption compared with related materials with relatively hydrophobic surfaces bearing *poly*(propylene) glycol motifs [39]. Such hydrophobic supports have been shown to reduce enzymatic activity as a result of unfolding of the hydrophobic core of the enzyme towards the surface of the support [40].

Thus, with a view to developing a recyclable immobilized enzymatic catalyst for bio-derived diesel production *via trans*-esterification, in this paper we describe the immobilization of *Pseudomonas cepacia* lipase (PCL) on cellulosic polyurethane by adsorption and explore the efficacy of the resulting heterogeneous, supported enzyme as a catalyst in model *trans*-esterification reactions, exploring the effects of temperature, water content, and reaction time. For this study PCL was regarded as the enzyme of choice since it has previously been reported that *Pseudomonas cepacia* is reasonably tolerant towards methanol, a substrate of particular importance in commercial fatty acid upgrading to bio-derived diesel [41].

Results and discussion

Cellulosic polyurethane provided by Sea Marconi is made from hemicellulose cross-linked by diisocyanate linkages. The resulting material has both hydroxyl and urethane groups on its surface, which can be used as potential sites through which enzymes may be grafted, offering a means of accessing a heterogeneous catalyst of relevance to *trans*-esterification reactions. Consequently, with a view to generating an immobilized form of the enzyme *Pseudomonas cepacia*, a portion of this cellulosic polyurethane material was treated, under appropriate conditions, with the lipase and the resulting material subsequently tested in catalytic *trans*-esterification reactions.

Analysis of polymer-supported *Pseudomonas cepacia* lipase. A high immobilization yield of PCL of 88.0 % is obtained, which was calculated by measuring the difference in the PCL concentration of the supernatant before and after the immobilization process. Attempts were subsequently made to analyze the resulting material by FTIR spectroscopy since it has been shown to be a powerful tool for assessing the conformation of lipase *via* the three amide signature bands [44]: 1645 cm^{-1} (C-O stretch), 1583 cm^{-1} (N-H bend and C-N vibration), ~1434 cm^{-1} (N-H bend, C-C and C-N vibrations) [43]. The FTIR spectra of cellulosic polyurethane and cellulosic polyurethane immobilized *Pseudomonas cepacia* lipase samples are shown in Figure 1. However, the vibrational bands for the urethane bonds of cellulosic polyurethane overlap those of the lipase amide bands, which make it difficult to use FTIR to confirm the conformation of the immobilized PCL, although the spectra do show the presence of PCL on the cellulosic polyurethane.

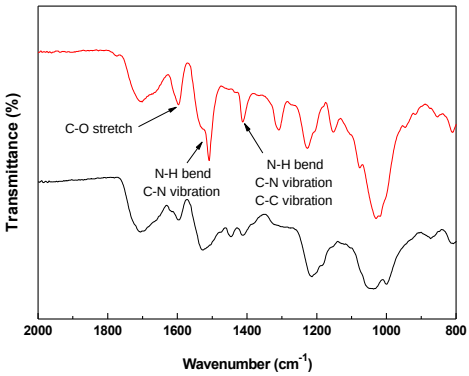
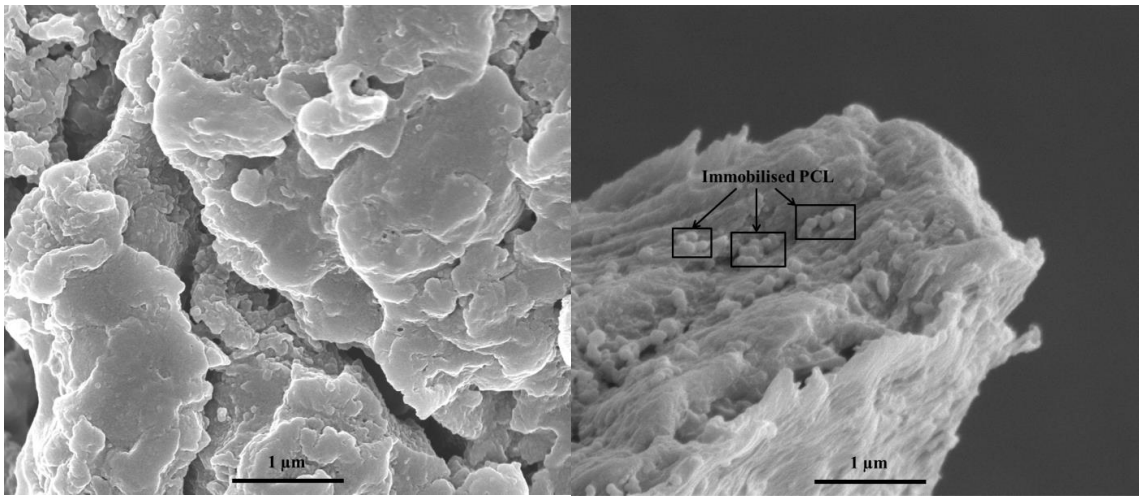


Figure 1. FTIR spectra of cellulosic polyurethane (− black) and cellulosic polyurethane immobilized *Pseudomonas cepacia* lipase sample (− red).

In order to further confirm the presence of the lipase on the polymeric support material, the morphologies of the cellulosic polyurethane before and after the PCL immobilization were investigated using SEM. Figure 2(2) shows the immobilized PCL attached to the surface of the polymer as spherical particles with a broad size distribution, something attributed to the formation of lipase aggregates [45, 46]. These spherical features were not observed on the pure cellulosic polyurethane.



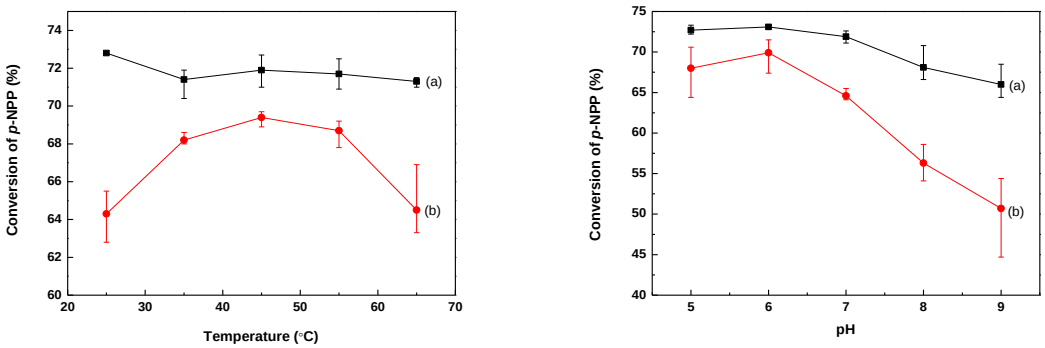
(1) (2)

Figure 2. Scanning electron microscopy (SEM) images of cellulosic polyurethane (1) and cellulosic polyurethane-immobilized PCL (2).

Effect of temperature and pH on the hydrolytic activity of immobilized *Pseudomonas cepacia* lipase. Before the immobilized PCL was used as a catalyst for *trans*-esterification, the effects of temperature and pH on its hydrolytic activity were investigated and compared with those from identical studies using the free lipase. The effect of temperature on the hydrolytic activity of free and immobilized lipase for *p*-NPP hydrolysis at pH 7.0 over the temperature range of 25-65 °C is shown in Figure 3(1). It was found that the hydrolytic activity of PCL decreased after immobilization, something that is attributed to the restricted conformational mobility of the enzyme structures on the support surface compared with free lipase [47, 48]. The hydrolytic activity of free PCL starts to decrease when reaction temperatures above 35 °C are employed, although notably, immobilized PCL retained its hydrolytic activity up to 55 °C, but for reactions conducted above this temperature a decrease in performance occurs. It is proposed that this loss in efficacy is not a result of leaching, rather it is attributed to the restricted conformational mobility of the enzyme structures following immobilization on the cellulosic support surface, which makes the enzyme sensitive to the increased temperature. Consequently, if the immobilized PCL was subject to leaching at this temperature, then this would lead to a relatively high hydrolytic activity. Whether this effect took place was explored through an analysis of the stability of the polymer support at 55 ºC for 16 h as studied by TGA (See ESI for details). Application of this heating cycle resulted only in a 3.1% of weight loss, which indicates that the polymer material used as the PCL support is very stable at 55 ºC.

The impact of pH on the catalytic activity of the immobilized PCL was studied at 30 °C over the range of pH 5.0-9.0, with the results being shown in Figure 3(2). Here, the best hydrolytic activity is achieved between pH 5.0-7.0, albeit with levels lower than that achieved using the free lipase. Further increase in the reaction pH leads to a significant attenuation in the

hydrolytic activity of the immobilized PCL. This is attributed to alteration of intermolecular interactions of the lipase, such as dispersion and H-bonding between the enzyme and the polymer support, which result in deleterious conformational changes of the enzyme, as reported previously [43, 49].



(1) (2)

Figure 3. (1) Effect of temperature on the hydrolytic activity of free *Pseudomonas cepacia* lipase at pH 7 (a) and immobilized *Pseudomonas cepacia* lipase (b); (2) effect of pH on the hydrolytic activity of free *Pseudomonas cepacia* lipase (a) and immobilized *Pseudomonas cepacia* lipase (b). *p*-NPP = *p*-nitrophenylpalmitate

In summary, from the data presented in Figure 3, it is evident that, firstly, the free PCL is slightly more hydrolytically active than the immobilized PCL across the temperature and pH ranges tested. Secondly, the reactivity of the immobilized PCL is more sensitive to both temperature and pH than the free PCL. Since the hydrolytic activity of the immobilized PCL may potentially be attributed to leaching of PCL from the support and thus it may be expected that if leaching has occurred, then the highest leaching may align with the highest conversion activity at ~pH 5-7. Consequently, to explore this issue a PCL leaching experiment was performed at pH 7.

Leaching of *Pseudomonas cepacia* lipase. Tests to probe the influence of PCL leaching from the support upon catalytic activity were carried out in a phosphate buffer solution at pH 7.0, with stirring at 200 and 500 rpm at 35 °C for 24 h. After both tests, no PCL was detected in the supernatant liquid, which indicates that PCL was firmly immobilized on the cellulosic polyurethane.

Catalytic *trans*-esterification reactions. In prior studies it has been reported that *Pseudomonas cepacia* is reasonably tolerant of methanol poisoning, in contrast to other readily available enzymes such as *Candida rugosa* and *Pseudomonas fluorescens* [41]. However, this was found not to be the case for our new immobilized PCL although it should be noted that the previously reported experiments with free enzyme were not repeated since our work was focused purely on improving the reusability of *Pseudomonas cepacia* lipase (PCL) through immobilization. Incubation of the immobilized PCL was undertaken with either methanol or ethanol at 30 °C for 24 h, prior to hydrolytic activity testing. It was found that although the catalytic activity of the immobilized PCL was inhibited significantly by methanol, no inhibition was observed with ethanol. Additionally, a number of test reactions (reaction of glyceryl trioctanoate with methanol catalyzed by the immobilized PCL) were carried out to verify the reproducibility of the catalytic tests. It was found that in each case the conversion of the starting glyceryl trioctanoate in these test reactions was very low, less than 5.0%. As such, we propose that the immobilized PCL is somehow being inhibited by methanol. Thus, since we clearly demonstrate that any such inhibition process are significantly reduced when using ethanol in the place of methanol, our subsequent work reported in this paper focused exclusively on reactions of glyceryl trioctanoate with ethanol. Here, an excess of ethanol was used in order to maximize conversion (glyceryl trioctanoate/ethanol = 1:4). The mass ratio of glyceryl trioctanoate/immobilized PCL was kept at 20:1 for all reactions.

It has been reported that water has a strong influence on the catalytic activity and stability of lipase for lipase-catalyzed *trans*-esterification, especially when reactions are undertaken in predominantly non-aqueous media. The water is believed to play multiple roles including mass transfer of substrates and products, enhanced nucleophilicity and proton transfer at the active site, and solvent shell-mediated dynamics for accessing catalytically competent conformations [50, 51]. Furthermore, it has been demonstrated that lipase catalytic activity generally depends on the available interfacial area of the immiscible organic-water interface, which can be increased by the addition of water [52]. Since glyceryl trioctanoate and water are indeed immiscible, catalytic tests were undertaken using the immobilized PCL in the presence of 5.0 and 10 wt.% added water; the results are presented in Figure 4. Significantly higher conversions of glyceryl trioctanoate were obtained for reactions performed with 5.0 wt.% water, than those achieved with 10.0 wt.% of water. It is believed that this is a competitive reaction and that this effect can be attributed to fast hydrolysis of the acylenzyme intermediate, which is initially formed in the reaction before it can be trapped by ethanol. Furthermore, catalytic tests employing 5.0 wt.% water were essentially unaffected by changes in temperature. In contrast, at 10 wt.% water content conversion decreases as temperature is also increased. Consequently, an optimum water content is required to maximize the lipase activity as has been demonstrated previously for the *trans*-esterification reaction of canola oil catalyzed by immobilized *T. lanuginosus* [53].

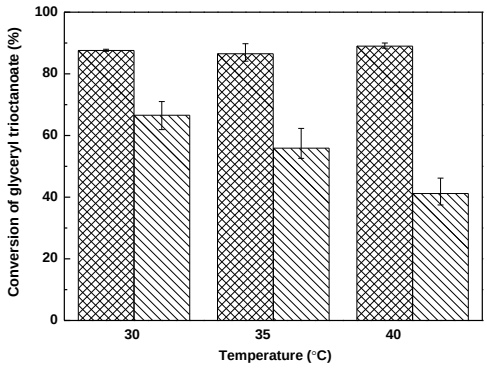


Figure 4. Results of *trans*-esterification of glyceryl trioctanoate with ethanol performed over 24 h at various temperatures with (▨) 5.0 wt.% and (▧) 10.0 wt.% of water. The mass ratio of glyceryl trioctanoate to catalyst was 20:1 and the molar ratio of glyceryl trioctanoate to ethanol was 1:4.

Subsequently, a detailed investigation was carried out probing glyceryl trioctanoate/ethanol *trans*-esterification with different water contents, which were varied between 0-15.0 wt.% at 35 °C for 24 h; results are shown in Figure 5. Notably, only 4.7% conversion of glyceryl trioctanoate was achieved in the absence of water. Substrate conversion increased significantly to 42% when the water content was only increased slightly to 1.0 wt.%, and increased to 77% in the presence of 2.5 wt.% of water. Indeed, good conversions of glyceryl trioctanoate were obtained when the water content lies in the range between 2.5-7.5 wt.%. By way of comparison, a blank test was carried out in the absence of immobilized PCL at 35 °C for 24 h with 5.0 wt.% of added water, which lead to only 2.6% conversion of glyceryl trioctanoate, confirming the role of immobilized PCL in catalyzing *trans*-esterification.

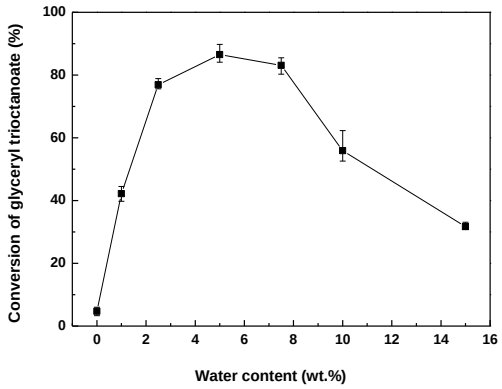
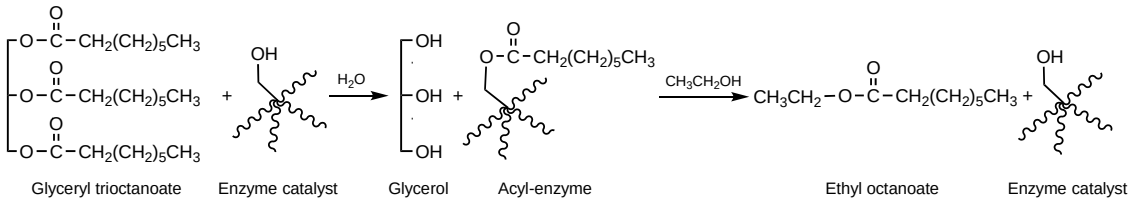


Figure 5. Effect of water content on *trans*-esterification of glyceryl trioctanoate with ethanol at 35 °C for 24 h. The mass ratio of glyceryl trioctanoate to catalyst was 20:1 and the molar ratio of glyceryl trioctanoate to ethanol was 1:4.

A number of prior studies have concluded that *trans*-esterification of triglycerides catalyzed by lipases in the presence of optimal quantities of water occurs in two-stages as illustrated in Scheme 1 [17, 54]. It is believed that initial tri-ester hydrolysis occurs releasing glycerol, forming an acyl-enzyme intermediate in which the acyl moiety of the substrate is transiently attached to a hydroxy group of the enzyme. Subsequently, the acyl-enzyme intermediate is trapped by ethanol and forms the ethyl octanoate product. This is consistent with our observed dependence of catalysis on maintaining an optimal water content.



Scheme 1. Suggested pathway for *trans*-esterification reactions of glyceryl trioctanoate with ethanol catalyzed by the immobilized PCL catalyst to produce ethyl octanoate.

Since a relatively high conversion of glyceryl was obtained at 35 °C for 24 h with 5.0 wt.% of H_2O, the conversion of glyceryl trioctanoate with ethanol was investigated with different reaction times under the same process conditions. Subsequently, the conversion of glyceryl trioctanoate was found to be relatively low over the first 3 h of reaction (44%), but increased to 73% after 12 h, Figure 6. Increasing the reaction time further to 24 h led only to an additional 14% substrate conversion. Consequently, all further catalytic tests for the *trans*-esterification of glyceryl trioctanoate mediated by immobilized PCL were performed for a period of 12 h. The relatively low degree of substrate conversion and long reaction time of these initial studies indicates that the immobilized PCL is considerably less active for catalyzing *trans*-

esterification than traditional homogeneous base catalysts. It is noteworthy, however, that in the present study the immobilized PCL was used with a relatively low lipase loading of 6.2 % and, furthermore, the mass ratio of glyceryl trioctanoate to immobilized PCL was kept relatively low, at 20:1. In contrast, it has been reported that a relatively high conversion of soybean oil can be achieved, but this occurs only with a higher PCL loading of ~13.6% and a lower mass ratio of soybean oil to immobilized PCL of 10:3 [52].

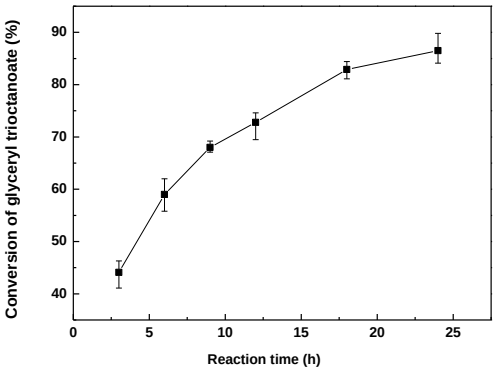


Figure 6. Results of *trans*-esterification of glyceryl trioctanoate at 35 °C with 5 wt.% of water as a function of reaction time. The mass ratio of glyceryl trioctanoate to catalyst was 20:1 and the molar ratio of glyceryl trioctanoate to ethanol was 1:4.

Since the primary rationale for choosing an immobilized form of PCL for this type of *trans*-esterification reaction was to enhance the catalyst stability and to facilitate its separation from the reaction mixture and subsequent reuse, recycling tests using the immobilized PCL system were carried out. This screening was undertaken at 35 °C for 12 h with 5 wt.% of water, with a constant mass ratio of glyceryl trioctanoate to immobilized PCL of 20:1. After each reaction, the catalyst was separated by filtration and washed thoroughly with water and then freeze-dried prior to reuse; immobilized PCL was recycled four times and the catalytic performance was recorded (Figure 7). After the first recycling test, the conversion of glyceryl trioctanoate

decreased by 11.9%, with then a further 8.3% and 6.5% decrease from the second and third recycling tests, respectively. The conversion of glyceryl trioctanoate dropped by 42% after four re-use cycles. Although the origins of the loss in activity are not readily apparent, we propose that this may be attributed to the washing after each recycling test, or catalyst inhibition resulting from the presence of the intermediate product, octanoic acid. Further studies are needed to understand the factors affecting the longevity of the immobilized PCL.

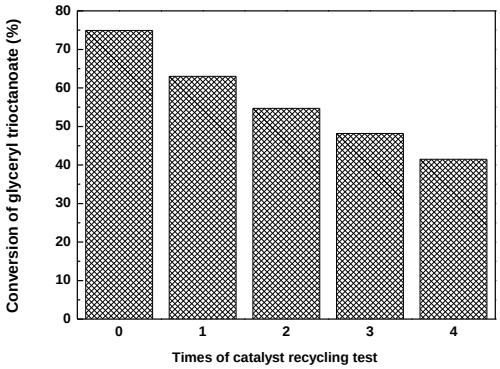


Figure 7. Recycling tests using the immobilized *Pseudomonas cepacia* lipase for *trans*-esterification of glyceryl trioctanoate at 35 °C for 12 h with 5 wt.% of water. The mass ratio of glyceryl trioctanoate to catalyst was 20:1 and the molar ratio of glyceryl trioctanoate to ethanol was 1:4.

Conclusions

This preliminary study exemplifies that enzyme-mediated *trans*-esterification of triglycerides can offer an environmentally benign, process-efficient option for bio-derived diesel production through improved ease of separation of catalyst from the reaction, coupled with an acid-/base-free waste stream. In this study, *Pseudomonas cepacia* lipase (PCL) was successfully immobilized on cellulosic polyurethane, with the resulting system showing good thermal stability and moderate water tolerance. The immobilized PCL was successfully used as a catalyst, at low loading (6.2 wt.%), for *trans*-esterification of glyceryl trioctanoate with

ethanol. It was found that water was necessary for the conversion of the triester, with acceptable conversions of ~75% being obtained at 35 °C, with 5.0 wt.% of water and a reaction period of 12 h. This preliminary catalysis performance data indicate the promise of immobilized enzymes in bio-derived fuel production. However, more research is required to understand why the immobilized PCL is more sensitive to methanol than ethanol. Similarly, the origins of the relatively quick deactivation of the immobilized PCL over the four repeat recycling test needs to be better understood, in particular looking at the potential for catalytic inhibition by reaction products. Building on these results, immobilized *Pseudomonas cepacia* lipase will be used as the catalyst for *trans*-esterification of extracted non-polar microalgal oil in future research.

Experimental section

Catalyst preparation. *Pseudomonas cepacia* lipase (PCL) with activity ≥30,000 U/g, Triton X-100, *p*-nitrophenyl palmitate (*p*-NPP), bovine serum albumin (BSA), glyceryl trioctanoate, ethyl octanoate, and dihexyl ether were purchased from Sigma-Aldrich. Potassium phosphate (tribasic) and sodium carbonate were obtained from Sigma-Aldrich, and 4-nitrophenol and phosphoric acid from Alfa Aesar. BCATM protein assay reagent A and Piercei® BCA protein assay reagent B were purchased from Thermo Scientific. Cellulosic polyurethane was kindly donated by Sea Marconi Technology SAS, Italy. Deionized water was used to prepare all aqueous solutions, unless stated otherwise.

In order to remove possible residual impurities from the cellulosic polyurethane, the material (2 g) was incubated with ethanol (20 ml) for a period of 20 h at 25 °C, followed by a thorough washing with potassium phosphate buffer solution (60 ml, 0.01 M, pH 7, 0.1% (v/v) Triton X-100). The resulting pre-treated polymer support was added to a PCL solution (30 ml, 5 mg/ml in a potassium phosphate buffer solution) and the mixture incubated at 30 °C for 24 h, during which time the vessel was shaken at 150 rpm. Subsequently, the mixture was separated by

centrifugation (4000 rpm) at 15 °C for 15 min. The resulting polymer-immobilized lipase material was washed thoroughly with potassium phosphate buffer to remove any unbound lipase, followed by vacuum drying of the solid material for 24 h, which was then stored at 5 °C until required. The supernatant removed by centrifugation was combined with the washings; the concentration of residual (C_x) free PCL in this solution was then determined using the BCA protein assay method [42]. The immobilization yield was calculated by measuring the difference in the PCL concentration of the supernatant before (C_0) and after (C_x) the immobilization process according to the following equation:

$$\text{Immobilization yield (\%)} = (C_0 - C_x) \times 100\%/C_0$$

The PCL concentrations in the supernatant before and after the immobilization were 5 mg/ml and 580.3 μg/ml, respectively. This corresponds to an immobilization yield of PCL of 88.0 % and hence to a lipase loading on the cellulosic polyurethane of 6.2 % by mass.

Instrumentation. Fourier transform infrared (FTIR) spectra were recorded from 800 to 2000 cm^{-1} on a Perkin Elmer 1600 Series instrument with 8 cm^{-1} resolution. Electron micrographs were obtained using a Hitachi SU70 analytical scanning electron microscope (SEM). Thermogravimetric analyses were conducted using a Perkin Elmer Pyris 1 TGA, purged with air. Gas chromatographic (GC) analyses were performed with a Shimadzu GC-2014 gas chromatograph with AOC-20i auto injector and AOC-20s auto sampler, equipped with a flame ionization detector (FID) and a capillary column (HP-5, length 30 m, ID 0.25 mm and film thickness 0.25 μm); H_2 was used as the carrier gas. The column was heated with a temperature profile of 40 to 300 °C at 20 °C/min, and subsequently held for 2 min. The temperature of the injection port and FID were set at 230 and 305 °C, respectively.

Determination of immobilized *Pseudomonas cepacia* lipase hydrolytic activity. A determination of the hydrolytic activity of the immobilized PCL was carried out according to the spectrophotometric protocol reported by Kishor *et al.* [43]. Immobilized lipase (10 mg

including the support) in potassium phosphate buffer solution (2.91 ml, 0.01 M, pH 7.0, 0.1% Triton X-100) was pre-heated to 30 °C, then a *p*-nitrophenylpalmitate solution (80 µl of 20 mM, dissolved in 2-propanol) was added to give a total volume for the mixture of 3 ml, which was then kept at 30 °C and stirred at 200 rpm for 5 min. Subsequently, Na_2CO_3 solution (1 ml, 0.1 M) was added to terminate the reaction. Finally, the solid component was removed by centrifugation and the absorbance at 410 nm due to the release of *p*-nitrophenol into the supernatant following the enzymatic hydrolysis of *p*-NPP was measured. Each lipase activity assay was performed in triplicate and mean values are reported. For comparison, the hydrolytic activity of free lipase was also tested. The same procedures were followed except that free lipase was used as the catalyst instead of the immobilized PCL. The amount of free PCL used for the hydrolytic activity test was calculated according to the following equation:

Amount of free PCL (mg) = immobilized PCL (10 mg) × PCL loading (6.2 wt.%)

Probing the effect of temperature and pH on free and immobilized *Pseudomonas cepacia* lipase stability. The temperature stability of the free and immobilized PCL was studied by incubation (for 60 min) with potassium phosphate buffer solutions (pH 7.0) at 25, 35, 45, 55 and 65 °C, followed by a hydrolytic assay (in triplicate) as detailed in the above section.

The effect of pH on free and immobilized PCL stability was investigated by incubating samples at 30 °C for 30 min in phosphate buffer solutions (2.91 ml, 0.01M K_3PO_4/H_3PO_4, with 0.1% Triton X-100) at pH values from 5.0 to 9.0, followed by hydrolytic assay, which were performed in triplicate and mean values reported. The masses of free lipase and immobilized PCL used were the same as detailed in the above section.

***Pseudomonas cepacia* lipase leaching test.** Leaching of immobilized PCL from the support was investigated in phosphate buffer solution (3 ml) at pH 7.0. Separate suspensions of

immobilized PCL (10 mg) were stirred at 200 and 500 rpm for 24 h, followed by centrifugation. The lipase concentration in the supernatant was tested using the BCA method.

***Pseudomonas cepacia* lipase-catalyzed *trans*-esterification reactions.** A screw-capped glass vial (14 ml) was charged with glyceryl trioctanoate (1.43 g), immobilized PCL catalyst (71.7 mg), with the mass ratio of glyceryl trioctanoate:catalyst = 20:1, and an amount of ethanol (molar ratio of glyceryl trioctanoate:ethanol = 1:4) and either 5 or 10 wt.% water. When the molar ratio of glyceryl trioctanoate/ethanol was set at 1:4, a 55.0% conversion of glyceryl trioctanoate was obtained. On increasing the molar ratio of glyceryl trioctanoate/ethanol from 1:4 to 1:6, the conversion of glyceryl trioctanoate only increased by 1.5%. Accordingly, the molar ratio of 1:4 was applied to all following reactions. In order to investigate the effect of reaction temperature on the thermal stability of the immobilized lipase a series of reactions were performed at 30, 35 and 40 °C for 24 h. To further investigate the effect of water on the catalytic activity of the immobilized lipase, a set of tests was carried out with different water contents in ethanol at 35 °C over a period of 24 h. It was found that immobilized PCL shows good activity for *trans*-esterification of glyceryl trioctanoate with ethanol at 35 °C when the water content was 5 wt.%. Accordingly, the conversion of glyceryl trioctanoate was investigated over different reaction times, each performed at 35 °C with 5 wt.% water. Blank experiments (without any immobilized PCL) were also carried out at 35 °C with 5 wt.% water; the conversion of glyceryl trioctanoate was only 2.6 % over a period of 24 h in this blank test. After each catalysis test reaction, the solid fraction was separated by centrifugation. The resulting liquid fraction was diluted with hexane to provide solutions for GC analysis, to which was added dihexyl ether (0.45 ml) as an internal standard, prior to analysis.

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Supporting Information

TGA results of cellulosic polyurethane (weight vs temperature; weight vs time).

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