

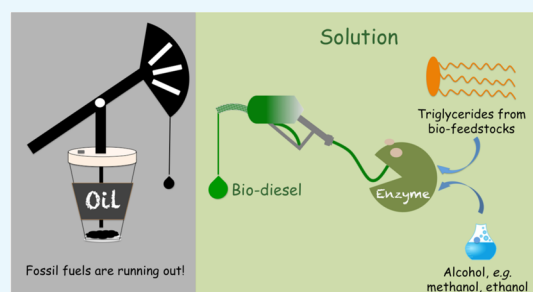
# Biodiesel Production via Trans-Esterification Using *Pseudomonas cepacia* Immobilized on Cellulosic Polyurethane

Li Li,<sup>†,‡,Ⓜ</sup> Philip W. Dyer,<sup>†,‡</sup> and H. Christopher Greenwell<sup>\*,†,§</sup>

<sup>†</sup>Centre for Sustainable Chemical Processes, Department of Chemistry, <sup>‡</sup>Department of Chemistry, and <sup>§</sup>Department of Earth Sciences, Durham University, South Road, Durham DH1 3LE, U.K.

## Supporting Information

**ABSTRACT:** In this work, *Pseudomonas cepacia* lipase immobilized on cellulosic polyurethane was used as a catalyst for biodiesel production via trans-esterification reactions in order to provide cost-effective methods of enzyme recycling. 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.



## INTRODUCTION

Availability and fluctuating costs of fossil fuels continue to drive research into new biofuel production and associated biomass conversion technologies. The use of bioderived 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, because of its comparatively straightforward preparation. Biodiesel production is achieved through catalytic trans-esterification of vegetable oils, animal fats, or microalgal oils with alcohols including methanol,<sup>1,2</sup> ethanol,<sup>3,4</sup> 1-propanol,<sup>5</sup> and 2-butanol,<sup>6</sup> a procedure needed in order to provide the necessary physical properties of the fuel to ensure engine compatibility.<sup>7</sup>

Currently, these required trans-esterification processes are undertaken mainly using soluble alkaline metal-based catalysts, for example, sodium hydroxide, sodium methoxide, potassium hydroxide, or potassium methoxide, which give fast diffusion and reaction kinetics.<sup>8</sup> However, in the presence of the dissolved metal cations, saponification of free fatty acids also occurs, which lowers yields of the target bioderived diesel. In contrast, although inorganic acid-catalyzed trans-esterification reactions for biodiesel production (using, e.g., HCl, H<sub>2</sub>SO<sub>4</sub>, and H<sub>3</sub>PO<sub>4</sub>) are possible and not strongly affected by the presence of free fatty acids,<sup>9</sup> industrial use of acid-catalyzed processes is problematic because of 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 bioderived diesel itself but also for the recovery of the coproduced glycerol, which is an increasingly important commodity chemical in its own right.<sup>10</sup>

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 bioderived diesel production have been explored, with enzymatic catalysts having started to emerge as promising candidates, although their application remains in its infancy.<sup>11</sup> This nascent method of enzyme-mediated trans-esterification for biodiesel production has recently been reviewed by Norjannah.<sup>12</sup> 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 bioderived diesel manufacture.<sup>13–16</sup> Indeed, a number of studies have explored the production of biobased diesel mediated by lipases, in particular with enzymes such as *Pseudomonas cepacia* and *Candida antarctica*.<sup>17,18</sup> 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 bioderived diesel and glycerol recovery. Additionally, the overall life cycle for enzymatic routes to bioderived diesel has relatively low energy consumption compared with traditional acid- or base-catalyzed trans-esterification reactions.<sup>8</sup> 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.<sup>19</sup> Furthermore, appropriate choice of reactant alcohol is crucial because methanol is known to act as a strong inhibitor of lipase catalytic activity and to decrease the enzyme's stability.<sup>20,21</sup> For

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example, Shimada et al. have reported that during studies of immobilized *C. antarctica*-mediated trans-esterification of vegetable oil, methanol led to complete enzyme deactivation.<sup>22</sup>

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 reuse are difficult. Therefore, in order to provide cost-effective methods of enzyme recycling, efforts have been made to prepare and utilize solid-supported lipases. To this end, a range of inorganic particles,<sup>23,24</sup> natural macromolecules,<sup>25–27</sup> and polymers<sup>28,29</sup> have been explored as supports for lipases, with many diverse immobilization methods having been developed, such as adsorption,<sup>30,31</sup> covalent binding,<sup>32</sup> affinity immobilization,<sup>33,34</sup> and entrapment.<sup>35,36</sup> 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 Datta et al.<sup>37</sup>

Cellulosic polyurethane has received particular attention as an appropriate support material because of its demonstrated biocompatibility, as well as its hydrogen bond-forming potential, which can enhance its mechanical and surface properties.<sup>38</sup> 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.<sup>39</sup> Such hydrophobic supports have been shown to reduce enzymatic activity as a result of unfolding of the hydrophobic core of the enzyme toward the surface of the support.<sup>40</sup>

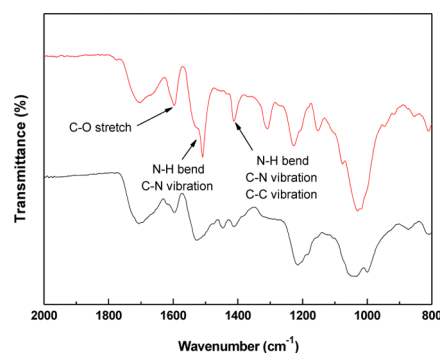
Thus, with a view to developing a recyclable immobilized enzymatic catalyst for bioderived diesel production via trans-esterification, in this paper, we describe the immobilization of *P. cepacia* lipase (PCL) on cellulosic polyurethane by adsorption and explore the efficacy of the resulting heterogeneous,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

## 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 *P. 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 PCL.** 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 Fourier transform infrared (FTIR) spectroscopy because it has been shown to be a powerful tool for assessing the conformation of lipase via the three amide signature

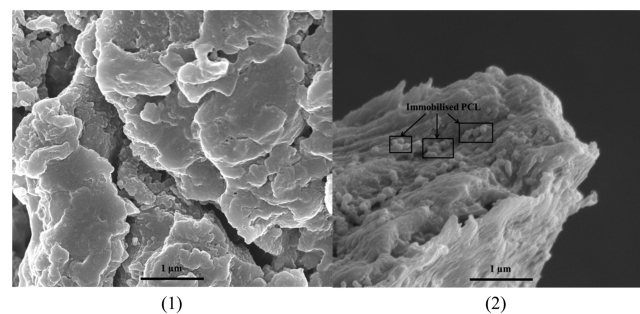
bands:<sup>44</sup> 1645  $\text{cm}^{-1}$  (C–O stretch), 1583  $\text{cm}^{-1}$  (N–H bend and C–N vibration), and  $\sim 1434$   $\text{cm}^{-1}$  (N–H bend, C–C and C–N vibrations).<sup>43</sup> The FTIR spectra of cellulosic polyurethane and cellulosic polyurethane-immobilized PCL samples are shown in Figure 1. However, the vibrational bands for the



**Figure 1.** FTIR spectra of cellulosic polyurethane (– black) and cellulosic polyurethane-immobilized PCL sample (– red).

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.

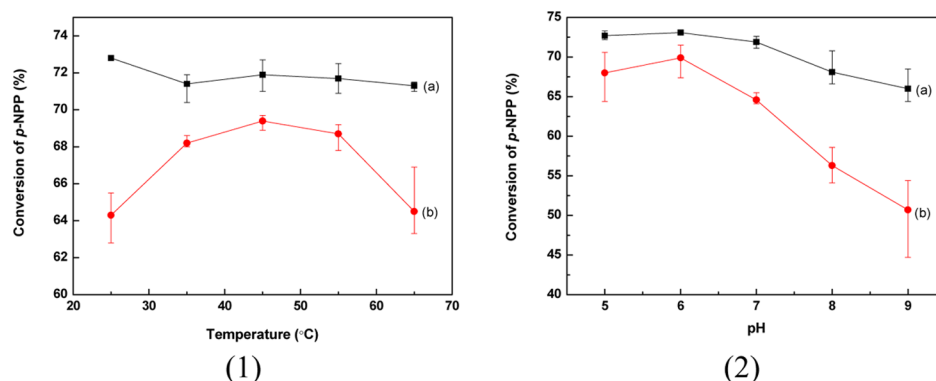
In order to further confirm the presence of the lipase on the polymeric support material, the morphologies of the cellulosic polyurethane before and after PCL immobilization were investigated using scanning electron microscopy (SEM). Figure 2(2) shows the immobilized PCL attached to the surface of the



**Figure 2.** SEM images of cellulosic polyurethane (1) and cellulosic polyurethane-immobilized PCL (2).

polymer as spherical particles with a broad size distribution, something attributed to the formation of lipase aggregates.<sup>45,46</sup> These spherical features were not observed on the pure cellulosic polyurethane.

**Effect of Temperature and pH on the Hydrolytic Activity of Immobilized PCL.** 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 lipases 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.<sup>47,48</sup> The



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

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 their 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 °C for 16 h as studied by thermogravimetric analysis (TGA) (see the [Supporting Information](#) 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 °C.

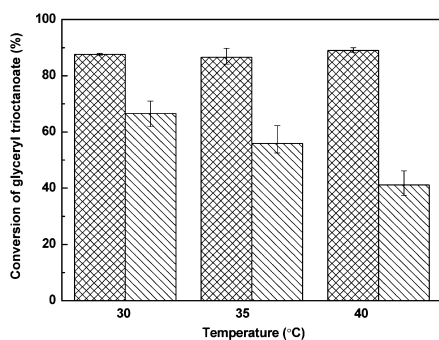
The impact of pH on the catalytic activity of the immobilized PCL was studied at 30 °C over the range of pH 5.0–9.0, with the results being shown in [Figure 3\(2\)](#). Here, the best hydrolytic activity is achieved between pH 5.0–7.0, albeit with levels lower than that achieved using the free lipase. 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.<sup>43,49</sup>

In summary, from the data presented in [Figure 3](#), it is evident that first, the free PCL is slightly more hydrolytically active than the immobilized PCL across the temperature and pH ranges tested. Second, the reactivity of the immobilized PCL is more sensitive to both temperature and pH than that of the free PCL. Because 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 PCL.** 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, under 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 *P. cepacia* is reasonably tolerant of methanol poisoning, in contrast to other readily available enzymes such as *Candida rugosa* and *Pseudomonas fluorescens*.<sup>41</sup> However, this was not found 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 because our work was focused purely on improving the reusability of 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, because we clearly demonstrate that any such inhibition process is 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.

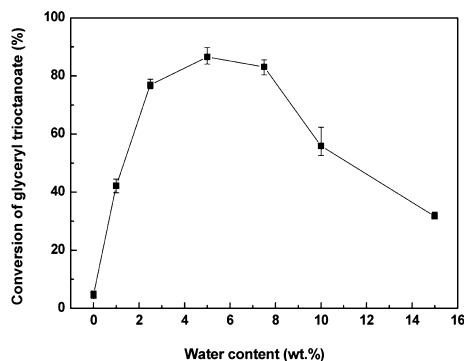
It has been reported that water has a strong influence on the catalytic activity and stability of lipase for lipase-catalyzed trans-esterification, especially when reactions are undertaken in predominantly nonaqueous media. The water is believed to play multiple roles including mass transfer of substrates and products, enhanced nucleophilicity and proton transfer at the active site, and solvent shell-mediated dynamics for accessing catalytically competent conformations.<sup>50,51</sup> 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.<sup>52</sup> Because glyceryl trioctanoate and water are indeed immiscible, catalytic tests were undertaken using the immobilized PCL in the presence of 5.0 and 10 wt % of added water; the results are presented in [Figure 4](#). Significantly higher conversions of glyceryl trioctanoate were obtained for reactions performed with 5.0 wt % of water, than those achieved with 10.0 wt % of water. It is believed that this is a competitive



**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.

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 % of water were essentially unaffected by changes in temperature. In contrast, at 10 wt % of 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 *Thermomyces lanuginosus*.<sup>53</sup>

Subsequently, a detailed investigation was carried out probing glyceryl trioctanoate/ethanol trans-esterification with different water contents, which were varied between 0 and 15.0 wt % at 35 °C for 24 h; results are shown in Figure 5. Notably,



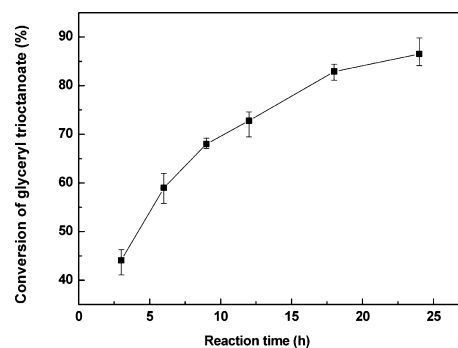
**Figure 5.** Effect of water content on trans-esterification of glyceryl trioctanoate with ethanol at 35 °C for 24 h. The mass ratio of glyceryl trioctanoate to catalyst was 20:1, and the molar ratio of glyceryl trioctanoate to ethanol was 1:4.

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 and 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 leads to only 2.6% conversion of glyceryl trioctanoate, confirming the role of immobilized PCL in catalyzing trans-esterification.

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.<sup>17,54</sup> It is believed that initial triester 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.

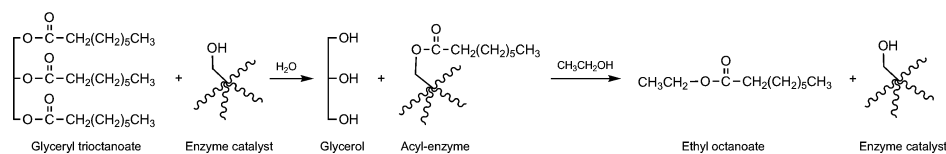
Because a relatively high conversion of glyceryl was obtained at 35 °C for 24 h with 5.0 wt % of H<sub>2</sub>O, the conversion of glyceryl trioctanoate with ethanol was investigated with different reaction times under the same process conditions. Subsequently, the conversion of glyceryl trioctanoate 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



**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.

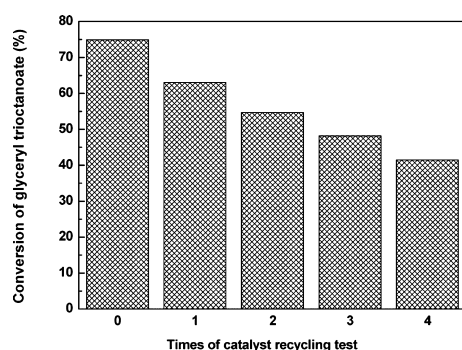
time further to 24 h led only to an additional 14% substrate conversion. Consequently, all further catalytic tests for the trans-esterification of glyceryl trioctanoate mediated by immobilized PCL were performed for a period of 12 h. The relatively low degree of substrate conversion and long reaction time of these initial studies indicate 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

### Scheme 1. Suggested Pathway for Trans-Esterification Reactions of Glyceryl Trioctanoate with Ethanol Catalyzed by the Immobilized PCL Catalyst To Produce Ethyl Octanoate



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.<sup>52</sup>

Because 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



**Figure 7.** Recycling tests using the immobilized PCL 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.

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 reuse cycles. Although the origins of the loss in activity are not readily apparent, we propose that this may be attributed to 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.

## CONCLUSIONS

This preliminary study exemplifies that enzyme-mediated trans-esterification of triglycerides can offer an environmentally benign, process-efficient option for bioderived diesel production through improved ease of separation of catalyst from the reaction, coupled with an acid-/base-free waste stream. In this study, 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 bioderived 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 PCL will be used as the catalyst for trans-esterification of extracted nonpolar microalgal oil in future research.

## EXPERIMENTAL SECTION

**Catalyst Preparation.** PCL with activity  $\geq 30\,000$  U/g, Triton X-100, *p*-nitrophenylpalmitate (*p*-NPP), bovine serum albumin, 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. BCA protein assay reagent A and Pierce 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 pretreated 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.<sup>42</sup> 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 immobilization were 5 mg/mL and 580.3  $\mu\text{g/mL}$ , 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.** FTIR spectra were recorded from 800 to 2000  $\text{cm}^{-1}$  on a PerkinElmer 1600 Series instrument with 8  $\text{cm}^{-1}$  resolution. Electron micrographs were obtained using a Hitachi SU70 analytical scanning electron microscope. TGAs were conducted using a PerkinElmer Pyris 1 TGA, purged with air. Gas chromatographic (GC) analyses were performed with a Shimadzu GC-2014 gas chromatograph with an AOC-20i auto injector and an 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  $\mu\text{m}$ );  $\text{H}_2$  was used as the carrier gas. The column was heated with a temperature profile of 40–300 °C at 20 °C/min and subsequently held for 2

min. The temperatures of the injection port and FID were set at 230 and 305 °C, respectively.

**Determination of Immobilized PCL Hydrolytic Activity.** Determination of the hydrolytic activity of the immobilized PCL was carried out according to the spectrophotometric protocol reported by Dhake et al.<sup>43</sup> 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 preheated to 30 °C, then a *p*-NPP 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<sub>2</sub>CO<sub>3</sub> 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

$$\begin{aligned} & \text{Amount of free PCL (mg)} \\ &= \text{immobilized PCL (10 mg)} \times \text{PCL loading (6.2 wt \%)} \end{aligned}$$

**Probing the Effect of Temperature and pH on Free and Immobilized PCL 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, 0.01 M K<sub>3</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub>, 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.

**PCL 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.

**PCL-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 % of 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

were 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 % of water. Blank experiments (without any immobilized PCL) were also carried out at 35 °C with 5 wt % of 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.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00110.

TGA results for cellulosic polyurethane (weight vs temperature; weight vs time) (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [chris.greenwell@durham.ac.uk](mailto:chris.greenwell@durham.ac.uk). Phone: +44(0)191 334 2324 (H.C.G.).

### ORCID

Li Li: 0000-0003-4878-6607

### Notes

The authors declare no competing financial interest.

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