Biotechnology Reports 28 (2020) e00535

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Immobilisation of *Candida rugosa* lipase on a highly hydrophobic support: A stable immobilised lipase suitable for non-aqueous synthesis

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ARTICLE INFO

Article history:
Received 19 March 2020
Received in revised form 26 September 2020
Accepted 28 September 2020

Keywords:
Candida rugosa lipase
Enzyme stability
Glyceride synthesis
Interfacial adsorption
Restructured lipids

ABSTRACT

Lipase from *Candida rugosa* (CrL) was immobilised on highly hydrophobic, octadecyl methacrylate resin (Lifetech™ ECR8806M) via interfacial adsorption. The aim was to produce a stable biocatalyst suitable for use in a range of lipid-modifying reactions. Immobilisation was carried out in 10 mM phosphate buffer (pH 6.0) over 24 h at 21 °C. High protein binding of 58.7 ± 4.9 mg/g dry support accounted for ~53 % of the applied protein. The activity recovery against tributyrin was 74.0 ± 1.1 %. The specific activity of immobilised CrL against tributyrin was considerably higher than that of Novozym® 435, at 1.79 ± 0.05 and 1.08 ± 0.04 U/mg bound protein, respectively. Incubation with high concentrations (10 % w/v) of both Triton X-100 and SDS resulted in only a small reduction in immobilised lipase activity. Solvent-free synthesis of glycerides by the FFA-saturated immobilised CrL was successful over 6 reaction cycles, with no apparent loss of activity.
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1. Introduction

Interest in lipases has grown steadily over the past three decades owing to their current and potential uses in a range of applications, including cleaning products, modified foods and flavour development [1,2]. Of particular interest are molecular transformations of lipids. These transformations include reactions such as hydrolysis, transesterification and esterification, for the production of biodiesel and the synthesis of structured lipids [3,4]. Lipases are used in their 'free' form (i.e. unbound in solution) in some applications, such as flavour development in foods or inclusion in cleaning products [5]. But, for many large-scale industrial applications, immobilisation of the enzyme onto solid supports is required as it offers a number of advantages. Practical advantages of immobilisation include the ability to reuse the enzyme in multiple reaction cycles, reducing the cost of processing, and recovery of the enzyme post reaction to avoid contamination of the final product [3,6]. Immobilization is a key enabling technology for the commercial application of biocatalysts for green and sustainable manufacture of chemicals [7]. Regarding the properties of lipases, immobilisation often improves the stability of the bound enzyme with respect to temperature or solvent exposure [6,8]. Furthermore, carefully designed immobilisation can increase lipase activity by fixing the enzyme in the open "active" form [9], tailor enantioselectivity for the resolution of racemic compounds [10,11], reduce inhibition by restricting inhibitor access to binding sites [8,12], and even purify the lipase [9,13–15].

Amongst the many immobilised lipase preparations available commercially, Novozym® 435 is one of the most stable and active, and is the most commonly used lipase preparation in the literature [13,16]. Novozym® 435 consists of lipase B from *Candida antarctica* (CaLB) interfacially adsorbed on Lewatit VP OC 1600, a macroporous, divinylbenzene crosslinked methacrylate support [17,18]. It has been described as a 'perfect biocatalyst' due to its high stability and reaction versatility. The enzyme is particularly suitable for synthetic reactions, such as esterification in non-aqueous media (e.g. for the production of flavour esters) and production of optically pure compounds [16]. However, Novozym® 435 does have limitations in its substrate specificity, and can be prone to enzyme desorption under certain conditions, as well as

Abbreviations: 4-MUHP, methyl 4-methylumbelliferyl hexylphosphonate; 4-MU, 4-methylumbelliferone; C18M, octadecyl methacrylate resin (Lifetech™ ECR8806M); CaLB, lipase B from*Candida antarctica*; CrL, *Candida rugosa* lipase; FFA, free fatty acid.

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https://doi.org/10.1016/j.btre.2020.e00535
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mechanical fragility and dissolution of the support in several organic media [16]. Therefore, there is potential opportunity to augment the range of industrial synthesis reactions with other immobilised lipase preparations.

Candida rugosa (also known as *C. cylindracea*) produces at least five closely related lipases. All isoenzymes consist of 534 amino acids with >70 % homology and molecular weights of ∼60 000 g/mol [19–22]. The isoenzymes differ in their isoelectric points, carbohydrate content, substrate specificity and hydrophobicity [21,22]. Commercial *C. rugosa* lipase (CrL) preparations vary in purity and isoenzyme composition. Lipase accounts for ∼64 % (w/w) of protein in the preparation from Amano Enzyme Inc. used in this study (Section 2.4), with an isoenzyme composition of 74 % Lip1 and 26 % Lip3 [23]. While advantageous for improved biocatalytic performance and reproducibility, purification of individual isoforms by precipitation, ultrafiltration, ion-exchange and affinity chromatography is time-consuming and often expensive, with low yields of the target enzyme [21].

CrL is an important biocatalyst as it performs both hydrolysis and synthesis with high efficiency [24]. It is also an excellent model to study lipase immobilisation, as it has been characterised extensively in both free and immobilised forms [20,24–26]. The lipase possesses a unique configuration, with the active site positioned at the entrance of a deep tunnel that is buried inside the protein structure [20,27]. The lipase is non-regioselective, and has a preference towards medium chain length saturated fatty acids and 18-carbon mono and polyunsaturated fatty acids. Long chain polyunsaturated fatty acids, such as docosahexaenoic and eicosapentaenoic acids, are hydrolysed poorly, likely resulting from steric hindrance of the ester bond due to the extensive unsaturation [22,28–31]. Like most lipases, CrL undergoes interfacial activation in the presence of water-insoluble substrates [20,32]. Interfacial activation is described as the conformational change (movement of the 'lid' to expose the active site) lipases undergo as a result of adsorption to an interface, and the accompanying increase in activity against an insoluble substrate (e.g. long-chain triglyceride) [33–35]. Although using interfacial activation to distinguish between lipases and esterases has been questioned [36], it has been shown that under the right conditions it occurs with most lipases, including those with very small lids, such as CaLB [37,38].

CrL has been immobilised on many different supports, e.g. hydroxyapatite [24], polypropylene [26,29], chitosan [39], synthetic hydrogels [40], several ionic supports and hydrophobic octyl-Sepharose [28], as well as highly hydrophobic octadecyl-Sepabeads [41]. Support-free immobilisation approaches (e.g. cross-linked enzyme aggregates) have also been used [30]. The specific strategy to produce an active and stable immobilised lipase depends on the target reaction and nature of the reaction medium to be used. Hydrolytic reactions involving emulsions or biphasic systems generally require the lipase to be attached via covalent or ionic interactions rather than physical adsorption to hydrophobic surfaces. This is to avoid desorption of the lipase into the more hydrophobic medium. For example, Morais Júnior et al. immobilised CrL on a range of supports via different mechanisms and used them to hydrolyse omega-3 polyunsaturated fatty acids from fish oil [28]. They achieved higher immobilisation efficiency and catalytic performance when the lipase was immobilised on ionic supports, compared to adsorption on a hydrophobic octyl support. However, for synthetic reactions in low-water or non-polar organic solvent media, immobilisation via adsorption on hydrophobic supports is preferred. The lipase undergoes interfacial activation/conformational change upon immobilisation on hydrophobic supports [9,14,42,43].

Unlike most proteins, lipases can be immobilised on hydrophobic supports under low ionic strength conditions, allowing immobilization, enrichment and stabilisation of the open form of lipase in one step [9,13,14]. It is also possible to alter lipase properties using supports with different hydrophobicity and internal morphology [43,44]. Both hydrophilic and hydrophobic resins can be functionalised with hydrophobic groups (e.g. octadecyl) to produce an optimal microenvironment for immobilisation. Although increasing the density and chain length (e.g. octadecyl vs. butyl) of functional groups increases the steric hindrance in the resin structure (via reduction of pore diameter, pore volume and surface area), the higher hydrophobicity generally results in increased lipase activity [45]. Interfacial adsorption is preferable to ionic and covalent binding because the lipase is in an active "open" form [8,41], as opposed to the "closed" structure seen with ionic and covalent binding. Using the interfacially adsorbed lipase for hydrolysis in a hydrophobic, aqueous emulsion can result in enzyme release from the support. Several solutions have been proposed to remedy this, including physical or chemical intermolecular crosslinking, and the use of hetero-functional supports where the lipase is immobilised via a combination of interfacial adsorption and covalent bonds [6,13,46]. In a non-polar solvent however, the use of hydrophobic interaction alone to immobilise and stabilise lipases is sufficient because the hydrophobic interfaces capable of desorbing lipase from the support are not favoured [47].

Palomo et al. immobilised CrL on a highly hydrophobic support (octadecyl-Sepabeads), with high activity and stability [41]. The enzyme was immobilised in an open form and the authors concluded that it was even more stable than multipoint covalent immobilisation of the closed form. However, the immobilised enzyme preparation was not tested for any synthetic reactions, and no reuse studies were reported. The present study describes the preparation and properties of CrL immobilised on octadecyl methacrylate (C18 M) from Purolite® Life Sciences (Lifetech™ ECR8806 M), another highly hydrophobic and robust support. This support has an optimal amount of octadecyl groups on its surface [45]. Although the activity and regioselectivity of *Thermomyces lanuginosus* lipase, *Rhizomucor miehei* lipase and CaLB immobilised on this support have been described [44,45,48–51], the combination of CrL with C18 M has not been reported to date. We compare the performance of immobilised CrL to that of Novozym® 435 and show that our immobilised lipase is very stable, that it can be used to synthesise mixed glycerides in non-aqueous media, and that it can be reused over multiple cycles for both hydrolysis and synthesis reactions.

2. Materials and methods

2.1. Chemicals and materials

Solid CrL preparation (Lipase AY "Amano" 30SD, ≥30,000 U/g, Amano LMAP method at pH 7.0) was sourced from Amano Enzyme Inc. (Japan) and used without further purification. This preparation contained 18.4 ± 0.4 mg protein/g (Lowry assay, Section 2.4). Novozym® 435 (11 100 Propyl Laurate Units/g) was purchased from Novozymes® (Denmark). This preparation contained 81.8 ± 3.1 mg bound protein/g dry support (elemental analysis, Section 2.5). The support resins Lifetech™ ECR8806 M (octadecyl methacrylate, C18 M) and Lifetech™ ECR1090 M (macroporous styrene) were purchased from Purolite® Life Sciences (Purolite Corporation, UK). Tributyrin (glyceryl tributyrate, ≥99 %) and triolein (glyceryl trioleate, ∼65 %) were purchased from Sigma-Aldrich® (USA). All chemicals and reagents were of analytical grade or higher.

2.2. Immobilisation of *Candida rugosa* lipase

CrL was immobilised on C18 M under low ionic strength conditions. Prior to immobilisation, the resin was washed with

Milli-Q® water, followed by 10 mM phosphate buffer pH 6.0, before being collected on a sintered glass funnel under vacuum. In a typical experiment, the lipase load solution (1.8 mg protein/mL phosphate buffer) was mixed with the damp resin in a beaker such that the applied protein was 110 mg/g dry support (~70 mg CrL/g dry support, as ~64 % of protein is lipase, Section 2.4), and considered the 'excess lipase loading'. This loading corresponded to ~0.0012 mol CrL/mol octadecyl groups on C18 M, assuming 1.03 mmol octadecyl/g dry support [45]. Immobilisations with protein applied at 20, 40, 60, 80 and 100 % of the maximum protein binding capacity (58.7 mg/g dry support, the concentration bound using the excess lipase loading) were also carried out. In all cases, binding was done at 21 °C for 24 h [45], mixed using a caged magnetic stirrer. A buffer pH of 6.0 allowed full lipase solubility as the pI of CrL used in this study is ~4.5 [22]. Previous studies on CrL immobilisation on hydrophobic supports were carried out under similar conditions [50,52].

Samples of the load solution and resin were taken during the course of immobilisation (after 2, 6 and 16 h) and analysed for protein concentration and activity. After 24 h, the final unbound material was recovered through a sintered glass funnel, the resin was washed with Milli-Q® water twice (each wash used five resin volumes of water), and the combined unbound fraction and washes were retained to measure protein concentration and activity. This activity was subtracted from the activity of the load solution to determine the theoretical specific activity of immobilised CrL, which was then used to calculate the activity recovery (Section 3.1). The resin was then washed with Milli-Q® water five more times (each wash used 10 resin volumes of water), with the liquid discarded. The damp resin was recovered as the immobilised lipase preparation and dried at 21 °C for 24 h in a desiccator under vacuum, before storage at 4 °C. Three separate immobilisations were carried out and the results reported as mean ± SE (Section 3.1). An identical experiment was carried out in the absence of the resin in order to establish the stability of free CrL under immobilisation conditions.

2.3. Lipase activity assays

2.3.1. Titrimetric assay (tributyrin)

Activities of the lipase load solution, unbound material and the immobilised preparations were measured against tributyrin using the Food Chemicals Codex method [53] with modifications. The aqueous substrate emulsion (17 mM or 0.5 % (v/v) tributyrin, 0.6 % (w/v) sodium caseinate and 0.05 % (w/v) lecithin) was prepared by homogenisation at 19 000 rpm for 2 min using an Ultra-Turrax® homogeniser. Reactions were carried out at 30 °C with 70 mL of the substrate emulsion in a stirred pH stat vessel (718 STAT Titrino, Metrohm, Switzerland). Enzyme sample was added and each reaction was monitored for 10–30 min, depending on the sample (e.g. dry resins containing immobilised lipase required 20–30 min assays for reproducibility). Sample volumes for the load solution and unbound material ranged from 10 to 50 μL (15–40 μg protein) per assay. Immobilised lipase was used at 40–70 mg (2−4 mg protein) per assay. The released FFAs were titrated with 0.01 M NaOH with the pH stat set at 8.0. The slope (mL 0.01 M NaOH/min) in the linear region of the titration curve was used to calculate the activity. One unit of activity was defined as 1 μmol butyric acid released/min under the assay conditions. Blank-rate activity was determined in the absence of enzyme.

2.3.2. Titrimetric assay (triolein)

In addition to tributyrin, activities of the lipase load solution and immobilised CrL preparations were measured against a predominantly long chain triglyceride substrate — a crude triolein preparation (Sigma-Aldrich®). The lipid class composition of this substrate was 63 % triglyceride, 33 % diglyceride and 3 % monoglyceride, as determined by TLC-FID (Iatroscan) analysis using the method of Bettjeman et al. [54]. The fatty acid composition was C18:1 (60 %), C16:0 (16 %), C18:2 (10 %), C14:0 (8 %), C18:0 (5 %) and C12:0 (1 %), as determined by GC–MS analysis of fatty acid methyl esters using the method of Killeen et al. [55]. The substrate emulsion consisted of 20 mM or 1.75 % (v/v) triolein, 2 % (w/v) gum arabic, and 0.45 % (w/v) sodium cholate. Assays were carried out as for the tributyrin assay.

2.4. Protein concentration assay

Protein concentrations were determined using the method of Lowry et al. [56] with some modifications to the concentration of reagents and incubation times. Bovine serum albumin (BSA) was used as the standard.

A 2100 Bioanalyzer™ system (Agilent) run using an Agilent Protein 230 Kit was used to measure the approximate percentage of protein that was lipase. The peak area that correlated to the molecular weight of CrL from the chromatogram generated by electrophoretic separation was calculated as a percentage against all other peaks. It was found that ~64 % (w/w) of protein in the soluble CrL preparation was lipase.

2.5. Elemental analysis of bound protein

Elemental analysis (Carlo Erba Elemental Analyser EA 1108, University of Otago, New Zealand) was used as a direct method to determine the concentration of protein bound to the immobilisation supports in both immobilised CrL and Novozym® 435. The protein concentration was calculated from the nitrogen content and molecular weight of CrL (662 mol N/mol enzyme and 57 149 g/mol, respectively) and CaLB (389 mol N/mol enzyme and 33 022 g/mol, respectively).

2.6. Active site titration of immobilised *Candida rugosa* lipase

The functional active site concentration of immobilised CrL was measured using the lipase inhibitor methyl 4-methylumbelliferyl hexylphosphonate (4-MUHP), as described in Nalder et al. [57]. The method applies correction curves to account for the binding of released 4-methylumbelliferone (4-MU) by C18 M, with the total 4-MU concentration used to determine the concentration of lipase active sites. In addition, the starting concentration of the inhibitor was in large excess in order to account for any losses due to its possible adsorption to the support. The inhibition assay was also used to measure: 1. the concentration of active lipase as a function of total protein concentration bound on the support (Section 3.2); and 2. The concentration of active lipase present after each reaction cycle during enzyme reuse experiments (Section 3.4). Similarly to immobilised CrL, when Novozym® 435 was used for the synthesis of mixed glycerides (Section 3.4), the binding of the released 4-MU by the support in Novozym® 435 (divinylbenzene crosslinked methacrylate) was taken into account using the correction curve for this support [57].

2.7. Confocal laser scanning microscopy

A Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) was used to visualise the distribution of different CrL concentrations adsorbed onto the C18 M and styrene supports. Fluorescamine (50 mg/mL) was dissolved in acetone and the solution reacted with immobilised lipase for 3 min. The reacted material was then washed twice with acetone to remove unbound probe. The resins were then imbedded in paraffin wax before being sliced (4 μm) using a CUT 4055 microtome (microTec Laborgeräte

GmbH, Germany). The slices were mounted on glass slides in polyvinyl alcohol. Samples were excited at 405 nm and images collected using an emission bandwidth of 420–507 nm.

2.8. Desorption of immobilised Candida rugosa lipase

The stability of immobilised CrL was assessed by attempting to desorb the lipase using selected detergents (Triton X-100 and SDS) and solvents (acetone, ethanol and dimethyl sulfoxide (DMSO)). Detergent concentrations ranged from 0.1 to 10% w/v (Section 3.3), while the solvents were applied undiluted. Fully dry immobilised lipase (0.2 g) was incubated with 10 mL of each reagent for 90 min at 21 °C with continuous mixing on a lab roller. Milli-Q® water and 10 mM phosphate buffer, pH 7.0, were used as control treatments. At the end of incubation, the supernatant was recovered by filtration through a 0.45 μm PTFE filter (Gelman Acrodisc®) to remove any fine resin particles. The immobilised lipase was rinsed with Milli-Q® water five times and dried in a desiccator under vacuum before residual activity was determined using the tributyrin assay. The protein content and activity against tributyrin were measured in the supernatant. The protein content in Triton X-100 and SDS supernatants was calculated using the standard curves prepared in the presence of corresponding SDS or Triton X-100 concentrations. All experiments were carried out in triplicate and the results reported as mean ± SE.

2.9. Batch cycling reactions

2.9.1. Hydrolysis of tributyrin

Tributyrin, a short-chain triglyceride substrate that is readily hydrolysed by most lipases, was used in preliminary cycling trials with both immobilised CrL and CaLB (Novozym® 435). A tributyrin emulsion consisting of 17 mM (0.5 % v/v) tributyrin, 0.6 % (w/v) sodium caseinate and 0.05 % (w/v) lecithin in 0.25 M Tris−HCl buffer (pH 8.0) was prepared by homogenisation at 19 000 rpm for 2 min using an Ultra-Turrax® homogeniser. The emulsion was left to stir continuously at 40 °C. Fully dry immobilised CrL or Novozym® 435 (6.0 g of each, providing 588 and 530 U of activity against tributyrin, respectively) were combined with 90 mL of the emulsion in a beaker and the hydrolysis carried out at 40 °C with stirring at 300 rpm. One hydrolysis cycle lasted 60 min. At the end of each cycle, the reaction mixture was separated from the resin (immobilised lipase) by vacuum-filtration through a sintered glass funnel. The resin was then washed with 2.0 L of warm (45 °C) water and recovered for the next cycle. A total of 10 cycles were carried out in triplicate for each enzyme. After each cycle, a sample of the washed resin (0.20 g damp resin) was taken and dried in a desiccator under vacuum (0.11 g dry weight). The activity of the dry sample was measured against tributyrin (as described in Section 2.3.1) and reported as mean ± SE for each cycle.

2.9.2. Saturation of immobilised Candida rugosa lipase and Novozym® 435 with free fatty acids prior to cycling

FFAs were prepared by saponification of crude triolein and used to coat/saturate the immobilised lipases. Typically, 50.0 g triolein was combined with 250 mL of 1 M KOH in 95 % ethanol, and refluxed for 1 h. The slightly cooled mixture was transferred to a separating funnel and combined with 300 mL of Milli-Q® water at 50 °C and 50 mL of 6 M HCl to acidify the mixture to pH < 1.5. The mixture was mixed well twice and the phases left to separate. The bottom aqueous layer was discarded. The top FFA-containing layer was then washed with 300 mL of Milli-Q® water at 50 °C and the bottom layer discarded. The water wash was repeated three more times, after which the pH of the FFA mixture was >5, and 44.0–44.5 g of FFAs were obtained.

Prior to triolein hydrolysis cycling, immobilised CrL (17.0 g dry preparation) was saturated with the FFAs in two steps: 1. FFA (10.4 g) and 90 mL Milli-Q® water were added to the resin in a beaker and stirred for 2 h at 35 °C. The resin was recovered by vacuum-filtration through a fine plastic mesh (pore size <50 μm) and washed with water (40 °C). The resin was then dried in a desiccator under vacuum and weighed; 2. The binding was repeated with another 10.4 g FFA in 90 mL water, followed by water wash, recovery of the FFA/water mixture and drying. After each step, the activity against tributyrin was measured for the resin and the recovered FFA/water mixture, while the protein content (Lowry assay) was measured in the mixture only. The desorption of FFAs by emulsifiers (used to prepare the triolein emulsion, Section 2.9.3) was assessed by mixing the dry saturated resin with 250 mL of the emulsifier solution consisting of 2 % (w/v) gum arabic, 0.45 % (w/v) sodium cholate in 0.25 M Tris−HCl buffer pH 8.0, for 60 min at 40 °C. The emulsifier solution turned cloudy, suggesting desorption of FFAs. The cloudy solution was recovered, the resin rinsed with a further 150 mL of the fresh solution, followed by 1.5 L of water at 40 °C, dried fully, weighed, and the activity against tributyrin measured. The resin was mixed once more with 250 mL of the emulsifier solution for 60 min, recovered as above and dried. This preparation was used in the triolein hydrolysis cycling.

Prior to mixed glyceride synthesis cycling, immobilised CrL and Novozym® 435 (10.0 g of each) were saturated with the FFAs following the 2-step protocol above. The desorption of FFAs by glycerol (used for glyceride synthesis, Section 2.9.4) was assessed by mixing the dry saturated immobilised lipases with 200 mL of 20 % (w/w) glycerol solution for 60 min at 40 °C. The resins were recovered and rinsed with a further 150 mL of the fresh glycerol solution, followed by 1.5 L of water at 40 °C, dried fully and weighed. The activity of the resins and recovered FFA/glycerol mixtures was measured against tributyrin, and the protein content (Lowry assay) was measured in the mixtures only. The resins were then mixed once more with 200 mL of the glycerol solution for 60 min, recovered and dried. The preparations were used in the glyceride synthesis cycling.

2.9.3. Hydrolysis of triolein

A triolein emulsion consisting of 70 mM (6 % v/v) triolein, 2 % (w/v) gum arabic, and 0.45 % (w/v) sodium cholate in 0.25 M Tris−HCl buffer (pH 8.0) was prepared by homogenisation at 19 000 rpm for 2 min using an Ultra-Turrax® homogeniser.The emulsion was left to stir continuously at 40 °C. Fully dry, FFA-saturated immobilised CrL (6.0 g, providing 450 tributyrin activity units (U)) was combined with 90 mL of the emulsion in a beaker and the hydrolysis carried out at 40 °C with stirring at 300 rpm. One hydrolysis cycle lasted 60 min. At the end of each cycle, the reaction mixture was recovered by vacuum-filtration through a plastic mesh (pore size <50 μm). The lipid from the reaction mixture was recovered by first acidifying the mixture to pH < 1.5 followed by hexane extraction, and analysed by TLC-FID (Iatroscan) using the method of Bettjeman et al. [54]. The resin (immobilised CrL) was washed with2.0 L of warm (45 °C) water and recovered for the next cycle. A total of six cycles were carried out in triplicate. After each cycle, a sample of the resin (0.28 g damp resin) was taken and dried in a desiccator under vacuum (0.15 g dry weight). The activity against tributyrin was measured in the dry sample. The leftover sample was then rinsed with hexane three times to desorb all of the bound lipid, before measuring the concentration of active lipase in the sample (as described in Section 2.6). All the values were reported as mean ± SE for each cycle.

2.9.4. Synthesis of mixed glycerides

The synthesis of mixed glycerides (esterification reaction) was carried out under partial vacuum using a rotary evaporator. In a round-bottom flask, dried FFA-saturated immobilised CrL (3.5 g, providing 284 tributyrin U), or FFA-saturated Novozym® 435 (3.5 g, providing 201 tributyrin U) were combined with 6.0 g crude triolein FFAs and 0.55 g glycerol (~3.7 mol FFA : 1 mol glycerol). No solvent was used. The reactions were carried out under vacuum (100 mbar) at 40 and 60 °C for immobilised CrL and Novozym® 435, respectively. Each reaction cycle lasted 3 h. At the end of each cycle, the resin and reaction mixture were separated by filtration through a Porex® filter disc fitted inside a syringe. The resin was placed back in the round-bottom flask, fresh substrate added and the cycle repeated. A total of six cycles were carried out in triplicate. The reaction mixtures were analysed by TLC-FID (Iatroscan) for changes in lipid class composition. After each cycle, a sample of the resin (0.25 g) was taken and assayed for activity against tributyrin. The leftover sample was then rinsed with hexane three times to desorb all of the bound lipid, before measuring the concentration of active lipase in the sample. All the values were reported as mean ± SE for each cycle.

3. Results and discussion

3.1. Immobilisation of *Candida rugosa* lipase

When CrL was immobilised on C18 M under low ionic strength conditions, the soluble lipase activity decreased rapidly during the first 2 h of immobilisation. Correspondingly, the activity of the immobilised preparation increased in an exponential fashion, reaching 80 and 87 % after 2 and 6 h respectively (the activity after 24 h was assigned as 100 %). As the free lipase was stable under immobilisation conditions with no activity loss, the immobilisation was left to proceed for full 24 h. With ‘excess loading’ of CrL applied on the support, protein binding of 58.7 ± 4.9 mg/g dry support was achieved after 24 h (Table 1), relatively high when compared to lipase immobilisation studies in the literature. This accounted for ~53 % of the applied protein (110 mg/g dry support). For comparison, Verdasco-Martin et al. reported up to 34 mg bound protein/g support for three microbial lipases immobilised on C18 M [44]. Similarly, Cabrera et al. reported CaLB binding at 20–30 mg protein/g support for a range of hydrophobic supports, including octadecyl-Sepabeads [17]. Tacias-Pascacio evaluated six hydrophobic supports for the immobilisation of five microbial lipases with the highest binding of 47 mg protein/g support for *Rhizomucor miehei* lipase on C18 M [50].

By measuring the difference in specific activity against tributyrin (U/mg protein) between the lipase load solution applied on C18 M and the unbound fraction, it was calculated that 84 % of the bound protein was lipase. This is a substantial increase from ~64 % of protein in the load solution being lipase, indicating that the hydrophobic support selectively adsorbed lipase from a low ionic strength (10 mM) solution. Increasing the buffer concentration to 100 or 250 mM did not improve the affinity of C18 M for lipase. Although the higher ionic strength resulted in higher protein binding (61.6 and 69.6 mg/g dry support respectively), the specific activity of immobilised CrL was not affected, suggesting greater binding of both lipase and non-lipase proteins.

The measured specific activity against both triglyceride substrates was very low in immobilised CrL compared to the free enzyme (Table 1). This is because only the lipase on the support surface was able to access the substrate, with large triglyceride emulsion droplets unable to enter the pores of the support. This is discussed further in Section 3.2. However, from the difference in activity against tributyrin between the load and unbound fractions (an approach reported consistently in the literature), we calculated the theoretical specific activity of immobilised CrL to be 618 ± 42 U/mg bound protein (Table 1). This represents an increase of ~31 % compared to the free enzyme. The theoretical activity recovery of immobilised CrL against tributyrin was 74.0 ± 1.1 % of the applied activity (Table 1). For comparison, Verdasco-Martin et al. reported theoretical activity recoveries of 56 and 94 % respectively for *Rhizomucor miehei* lipase and CaLB immobilised on C18 M [44]. Like the free enzyme, immobilised CrL hydrolysed tributyrin more efficiently than triolein (Table 1), retaining its preference for substrates containing shorter chain saturated fatty acids. The measured specific activity against tributyrin was 1.79 ± 0.05 U/mg bound protein, which is considerably higher than that of Novozym® 435 against the same substrate (1.08 ± 0.04 U/mg bound protein).

Immobilisation of lipases on hydrophobic surfaces inevitably results in the loss of some activity due to enzyme deformation and steric hindrance caused by various interactions (e.g. strong hydrophobic forces contributing to adsorption). Titration of the active site concentration of CrL immobilised on C18 M, with the irreversible inhibitor 4-MUHP, showed that ~56 % of the active sites were functional [57]. The fraction of active CrL molecules is high compared to previously published results on immobilised microbial lipases. For example, when CaLB was immobilised on epoxy-activated macroporous poly(methyl methacrylate) Amberzyme beads at various loadings, only 13–25 % of the bound enzyme was found to be active [18]. A related study immobilised CaLB by adsorption onto macroporous poly(methyl methacrylate), improving the fraction of active molecules to 40–45 % [58].

3.2. Distribution and activity of immobilised *Candida rugosa* lipase on support beads as a function of bound protein concentration

Confocal microscopy was used to visualise the distribution of CrL bound on C18 M, with three different lipase concentrations

Table 1
Properties of *Candida rugosa* lipase immobilised on Lifetech™ ECR8806 M. The enzyme was applied to the support under ‘excess loading’ conditions (110 mg protein/g dry support) over 24 h. Values are mean ± SE of three separate immobilisations.

Sample	Bound protein (mg/g dry support)		Activity against tributyrin (U/g dry support)	Specific activity against tributyrin (U/mg protein)		Specific activity against triolein (U/mg protein)	Tributyrin activity recovery (%)
	By difference [a]	From elemental analysis [b]		Measured	Theoretical [c]	Measured	
Free CrL	n/a	n/a	n/a	472 ± 15	n/a	301 ± 12	n/a
Immobilised CrL	58.7 ± 4.9	51.2 ± 7.6	105.0 ± 8.4	1.79 ± 0.05	618 ± 42	0.94 ± 0.03	74.0 ± 1.1

[a] Calculated using the Lowry method, from the difference between the applied protein concentration in the lipase load and that in the combined unbound and wash fractions.
[b] Calculated from the nitrogen content and molecular weight of CrL (662 mol N/mol enzyme and 57 149 g/mol, respectively) using the elemental analysis data.
[c] Calculated from the difference in activity between the load and unbound fractions.

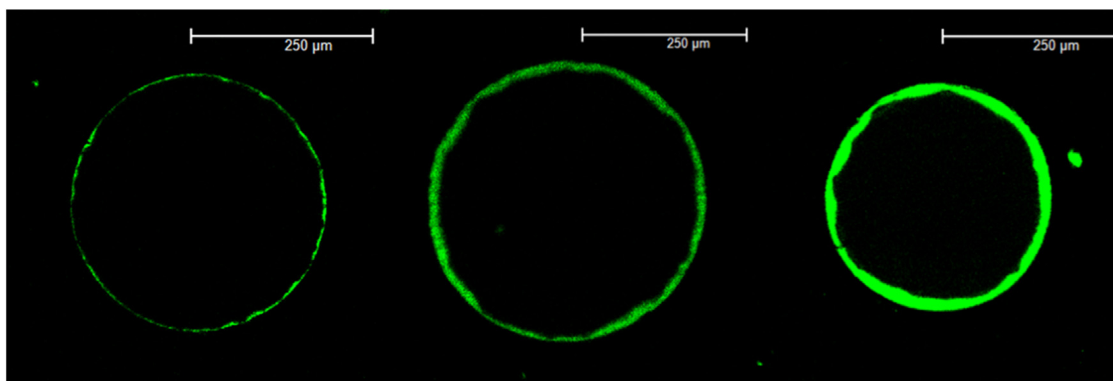

Fig. 1. Confocal microscopy images of CrL immobilised on C18 M beads when 20, 60 and 100 % (L to R) of maximum protein binding capacity was loaded on the support. Sample preparation and imaging was carried out as described in Section 2.7.

immobilised on the support. In each case, the majority of the enzyme appeared to be concentrated in the region near the surface of C18 M beads, with only a small amount present in the internal structure of the bead (Fig. 1). This suggests shallow penetration of the enzyme inside the pores of the support, despite the relatively large pore size of C18 M (500–700 Å). A similar distribution with the majority of enzyme bound near the surface was also observed for CaLB immobilised on Amberzyme (235 μm particle size, 220 Å pore size) [18]. In another study by Chen et al. [58], the penetration of CaLB inside the 250 Å pores of macroporous poly(methyl methacrylate) increased as the particle (bead) size decreased. Thus, smaller beads allowed greater lipase penetration compared to the larger beads with identical pore size. The reason for this is as yet unknown but suggests that in our study, the relatively large particle size (300–710 μm) of C18 M could have an effect of limiting the penetration of CrL inside the pores of the support. The depth of CrL penetration inside C18 M beads increased with an increase in bound protein concentration (Fig. 1).

To compare the distribution of CrL immobilised on C18 M with that on another support of different pore size, we immobilised the lipase (at excess loading) on a hydrophobic styrene support with the same particle size (300–710 μm), but with much larger pores (900–1100 Å). The lipase was expected to penetrate inside the styrene beads more freely than with C18 M. This does not appear to have been the case and overall C18 M beads appear to have more lipase bound (Fig. 2) despite the smaller pore size (500–700 Å). The images in Fig. 2 show some penetration of lipase into the interior of both types of bead but once again the indication is that the bulk of binding occurs in a region just below the bead surface. It is likely that C18 M resin bound more lipase than styrene because of its higher hydrophobicity. Low protein binding on styrene (26.9 and 19.7 mg/g dry support, by difference and elemental analysis, respectively) is in agreement with the very limited lipase penetration inside the beads.

The activity of immobilised CrL against tributyrin (U/g dry support) remained constant regardless of the applied loading on the support (Table 2). This suggested that only surface bound lipase was actively involved in catalysis, with the lipase molecules inside the pores unable to access the substrate. This is not surprising considering that tributyrin is present as an emulsion, with the diameter of lipid droplets likely to be much larger than the pore size of the support. To verify this, the C18 M beads were ground into a fine powder using a mortar and pestle to better expose the lipase molecules inside the pores. The lipase activity of this powder increased with the concentration of bound enzyme (Table 2). This increase was not proportional, with the lowest % loading sample still retaining the highest specific activity, indicating that tributyrin substrate was still unable to access all of the enzymes.

The activity of lipase inside the pores of the support was confirmed by active site titration of immobilised CrL (as whole beads, not ground). Using 4-MUHP, the concentration of active lipase increased in proportion to the bound protein concentration

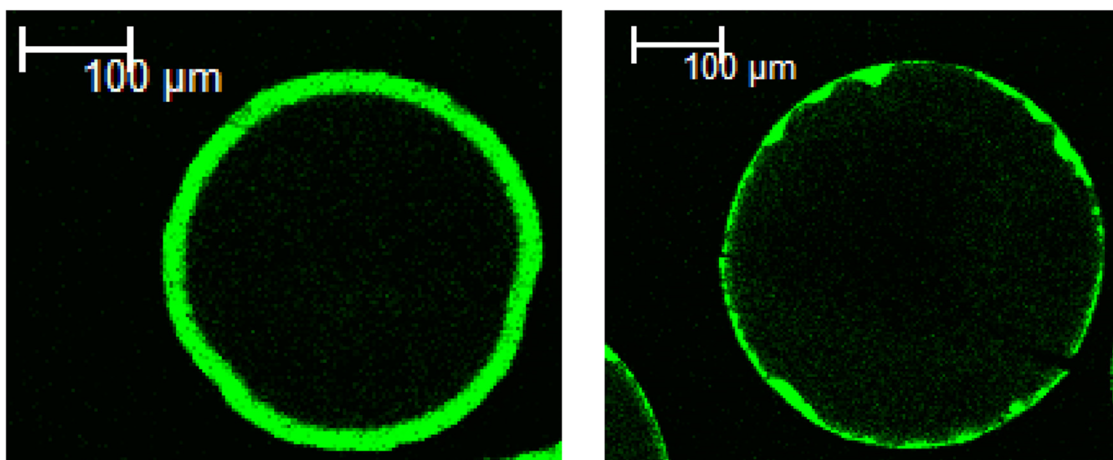

Fig. 2. Confocal microscopy images of CrL immobilised on C18 M at 'excess loading' (L) and styrene at 'excess loading' (R).

Table 2
Activity of immobilised *Candida rugosa* lipase (as whole beads and finely ground powders) at different concentrations of bound protein. Values are mean ± SE (n = 3).

Loading (% of max bound)	Bound protein (mg/g dry support) [a]	Activity against tributyrin (U/g dry support)	Specific activity against tributyrin (U/mg bound protein)	Activity against tributyrin after grinding the beads (U/g dry support)	Specific activity against tributyrin after grinding the beads (U/mg bound protein)
20	11.7 ± 1.3	103.4 ± 1.4	8.84 ± 0.12	387 ± 29	33.1 ± 1.1
60	28.7 ± 2.6	99.5 ± 4.7	3.46 ± 0.16	481 ± 33	16.8 ± 0.8
100	45.9 ± 4.0	99.3 ± 7.5	2.17 ± 0.16	636 ± 41	13.9 ± 0.7

[a] Calculated using the Lowry method, from the difference between the applied protein concentration in the lipase load and that in the combined unbound and wash fractions.

(Supplementary data, Fig. S1). Unlike the tributyrin emulsion, 4-MUHP is able to access all the lipase molecules due to its small size (facilitated by the presence of Triton X-100 in the assay [57]).

3.3. Effect of detergents and polar solvents on immobilised Candida rugosa lipase

Lipases are generally more stable in non-polar organic solvents than polar solvents due to a lack of solubility in the former, and the preservation of the water shell around the enzyme [59]. The strength of CrL adsorption on the highly hydrophobic C18 M support was therefore tested with polar organic solvents and detergents commonly used to denature/desorb immobilised lipases. Low desorption of protein was achieved with acetone, ethanol and DMSO (Fig. 3). Incomplete desorption (37 %) was achieved with 10 % Triton X-100, and with 1 and 10 % SDS (32 and 40 % respectively). Incomplete desorption after exposure to 10 % Triton X-100 is very different to the results reported by other researchers. For example, Fernandez-Lorente et al. were able to desorb different microbial lipases from butyl, hexyl and octyl supports using 0.2–0.6 % Triton X-100 [43]. CaLB was completely desorbed from Novozym® 435 using 2 % Triton X-100 [17]. Even when an octadecyl support was used, 3–4 % Triton X-100 was sufficient to fully desorb both CrL and CaLB [17,41].

In our studies, the activity of immobilised CrL was not affected greatly by incubation with detergents (Fig. 4), despite there being some apparent desorption from the support. The relatively low lipase activities in the recovered supernatants post detergent treatment indicate that it was mostly non-lipase protein that was desorbed. As an extreme example, the supernatant from incubation even with very high levels of Triton X-100 (10 %) contained ~50 % of the activity expected if all the desorbed protein was lipase. It appears that the strong interfacial adsorption and shallow penetration of CrL inside the pores of the support are responsible for its resistance to desorption and preservation of activity. Additional detergent treatments were not tested but would be expected to result in further enzyme desorption from C18 M.

More than 90 % of immobilised lipase activity was retained after incubation with Triton X-100 concentrations ≤1 % (data not shown). Acetone also had a small effect on the residual activity (Fig. 4). Only the highly hydrophilic solvents ethanol and DMSO (logP = -0.24 and -1.35 respectively) reduced the activity considerably, with DMSO inactivating the lipase completely. The loss of activity is due to enzyme denaturation caused by the removal of tightly-bound, essential water molecules from the enzyme's surface [60–62].

The remarkable resistance to desorption and high residual activity make CrL immobilised on C18 M an ideal candidate for use in non-aqueous (organic solvent or solvent-free) media for synthetic reactions.

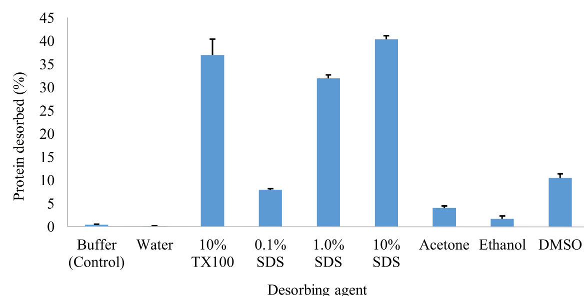

Fig. 3. Desorption of bound protein from immobilised CrL with organic solvents and detergents. The lipase was incubated with each desorbing agent for 90 min at 21 °C. Values are mean ± SE of three separate incubations.

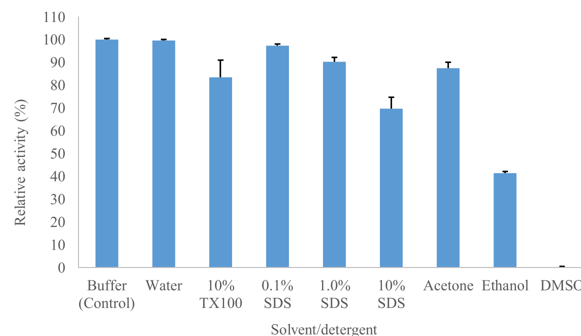

Fig. 4. Effect of organic solvents and detergents on the activity of immobilised CrL against tributyrin after incubation for 90 min at 21 °C. Values are mean ± SE of three separate incubations.

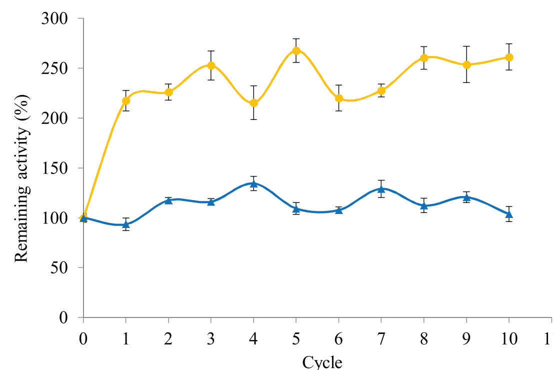

Fig. 5. Remaining activity of immobilised CrL (circles) and Novozym® 435 (triangles) after each cycle of tributyrin hydrolysis. Batch reactions were carried out at 40 °C with each cycle lasting 60 min. Values are mean ± SE (n = 3).

3.4. Batch cycling reactions

3.4.1. Hydrolysis of tributyrin

Tributyrin was used in preliminary cycling trials to assess the stability and reusability of immobilised CrL in an aqueous emulsion of relatively low hydrophobicity, that was unlikely to cause substantial enzyme desorption [63]. The activity of immobilised CrL did not decrease with progressive cycling. On the contrary, it more than doubled after the first cycle, then maintained a gradual upward trend with subsequent cycles (Fig. 5). For comparison, the activity of Novozym® 435 remained constant over the 10 cycles. The emulsion breakdown and strong odour of butyric acid after each cycle confirmed substrate hydrolysis. The degree of hydrolysis was not measured after each cycle, however a preliminary test before the start of cycling resulted in a very high degree of hydrolysis (> 90 %) after a 30 min reaction. This result indicated that a cycle duration of 60 min would be sufficient.

Previous studies using CrL interfacially adsorbed to various supports indicated an optimum temperature of ≤ 40 °C for prolonged or repeated reactions [28,29]. This temperature was used in our study for both the hydrolysis and synthesis cycling reactions, as it provided high reaction rates without compromising the stability of the enzyme.

The increase in activity of immobilised CrL following each cycle suggests a form of enzyme hyperactivation. Upon immobilisation on hydrophobic supports, hyperactivation has been shown to occur in lipases that possess a relatively large lid, due to the hydrophobic groups having an interface-like effect on the lipase [9,14]. Similarly, the tributyrin substrate offers a large interfacial area provided by the emulsion droplet [64], contributing to enzyme hyperactivation with each cycle.

Protein desorption after each cycle ranged from 1 to 2% of the total bound protein. Although we were unable to detect any lipase activity in the product mixture after each cycle, it likely contained both lipase and non-lipase protein. The small loss of lipase was not enough to affect the performance of the immobilised preparation.

3.4.2. Saturation of immobilised lipases with free fatty acids prior to cycling reactions

With immobilised CrL maintaining high levels of activity over the course of multiple tributyrin hydrolysis cycles, the hydrolysis of a long chain triglyceride substrate (crude triolein) was trialled. Unexpectedly, the first cycle of triolein hydrolysis resulted in a very low percentage of FFAs measured in the reaction mixture. This suggested low activity or that the released FFAs (mostly long chain and thus hydrophobic) were binding to the enzyme support. FFA binding was not an issue with the tributyrin trials as the butyric acid product is considerably more hydrophilic than the FFAs released from the hydrolysis of crude triolein. FFA binding was confirmed by hexane treatment to desorb lipids from the support, with FFAs accounting for 98 % of the desorbed lipid. This made it difficult to measure the extent of hydrolysis during each cycle using the immobilised CrL. As a means of making activity simpler to follow, saturation of the immobilised lipase with FFAs prior to cycling was investigated.

Following the initial binding of FFAs to the support, the preparation was rinsed with either the emulsifier solution (for triolein hydrolysis) or glycerol solution (for glyceride synthesis) until a constant weight was obtained. When immobilised CrL was saturated with FFAs prior to triolein hydrolysis cycling, the dry, rinsed resin weight was 23.2 g (36 % increase from the starting 17.0 g of immobilised CrL). FFA saturation of CrL and Novozym® 435 prior to synthesis cycling produced final dry resin weights of 12.7 g (27 % increase) and 17.1 g (71 % increase), respectively. The dissimilar FFA affinity of the two supports is likely due to differences in support hydrophobicity and the density of bound lipase on the support surface. The activity (tributyrin) of both immobilised CrL and Novozym® 435 was not affected by FFA saturation, and the total desorbed protein in the unbound FFA/water mixture accounted for < 1 % of bound protein in both enzyme preparations (data not shown).

To check that the amount of fatty acid bound on the support remained constant after each cycle, two tests were carried out before and after a reaction cycle. These were gravimetric analysis of the fully dried immobilised lipase, and hexane desorption and analysis of the bound lipid. A slight decrease (3.5 %) in the weight of immobilised lipase after a cycle indicated that no additional lipid binding occurred during the cycle, i.e. no FFAs were removed from the reaction mixture during the cycle. Similarly, 6 % less lipid was recovered by desorption after the cycle compared to the immobilised lipase before the cycle. Iatroscan analysis of the desorbed lipid confirmed the bound lipid composition was primarily FFAs (>95 %), with a small amount of glycerides (<5 %).

3.4.3. Hydrolysis of triolein

In contrast to the high remaining activity after each cycle of tributyrin hydrolysis, the activity of FFA-saturated immobilised CrL decreased gradually during cycling with triolein (Fig. 6). The amount of FFAs released and the concentration of active lipase after each cycle also showed a decreasing trend. The loss of activity is most likely due to extensive desorption of bound lipase by the more hydrophobic substrate emulsion during each reaction cycle [63]. This is a common problem with lipases immobilised via interfacial adsorption to hydrophobic supports. Lipase desorption can be reduced through intermolecular crosslinking and covalent anchors [6,13,46]. Virto et al. reported a similar decrease in activity over multiple cycles for the hydrolysis of animal fats and olive oil by CrL immobilised on microporous polypropylene [29]. The activity of *Humicola lanuginosa* lipase adsorbed onto Amberlite XAD-7 also decreased with repeated hydrolysis of olive oil [65].

3.4.4. Synthesis of mixed glycerides

Immobilised microbial lipases are commonly used to synthesise specialty lipids. As discussed in the introduction, Novozym® 435 is the most commonly used lipase preparation in the literature, and is particularly suitable for synthetic reactions in non-aqueous media [16]. The performance of FFA-saturated immobilised CrL was compared to that of FFA-saturated Novozym® 435 for the synthesis of mixed glycerides from crude triolein FFAs and glycerol.

Lipase-catalysed esterification is used widely for the synthesis of useful lipids, such as emulsifiers, flavours, pharmaceutical intermediates etc. As water is a side product of the reaction, it can accumulate in the immobilised lipase and reduce the synthetic activity. Molecular sieves are used traditionally to adsorb the released water and shift the equilibrium in the favour of synthesis.

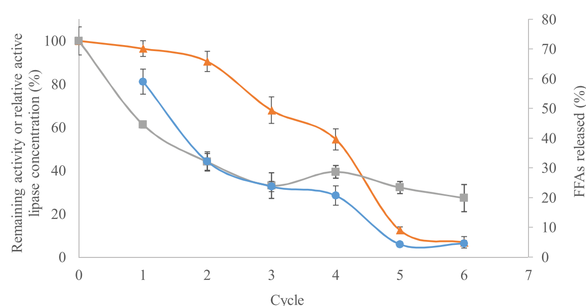

Fig. 6. Hydrolysis of triolein by FFA-saturated immobilised CrL. Batch reaction cycles of 60 min were carried out at 40 °C. FFAs released (circles) after each cycle, remaining activity (triangles) and concentration of active lipase (squares) relative to cycle 0. Values are mean ± SE (n = 3).

Another strategy is to use a highly hydrophobic support to repel the water [13,16,66]. In our study, the synthesis of mixed glycerides was carried out under vacuum (100 mbar) in order to remove the water product and drive the esterification forward. Continuous esterification was also facilitated by the highly hydrophobic C18 M support through its low water affinity.

Both enzymes maintained the starting activity over the six cycles, as indicated by the high percentage of FFAs consumed during each cycle (Figs. 7 and 8). Novozym® 435 also had a consistently high concentration of active lipase after each cycle (Fig. 8). However, for immobilised CrL, the concentration of active lipase decreased gradually with progressive cycles (Fig. 7). This suggests that access to the active site depends on the substrate. The reason why the inhibitor's access to the active site of CrL was compromised after each synthesis cycle is not clear at this stage. Nevertheless, the activity of immobilised CrL (against tributyrin) remained unchanged after each cycle (Fig. 7).

The performance of immobilised CrL is encouraging compared to the previous studies on the synthesis of various esters by immobilised lipases. *Thermomyces lanuginosus* lipase adsorbed on mesoporous poly-hydroxybutyrate lost 30 % of its initial activity after five successive cycles during the synthesis of short-chain alkyl esters of oleic acid [67]. The same lipase adsorbed on mesoporous poly-methacrylate retained its initial activity after 22 cycles of isoamyl oleate synthesis, followed by ~10 % decrease in activity over the next eight cycles [68]. In related studies on the kinetic resolution of synthetic intermediates by immobilised CaLB and *Burkholderia cepacia* lipase, reusability was effective over five to eight cycles [69,70].

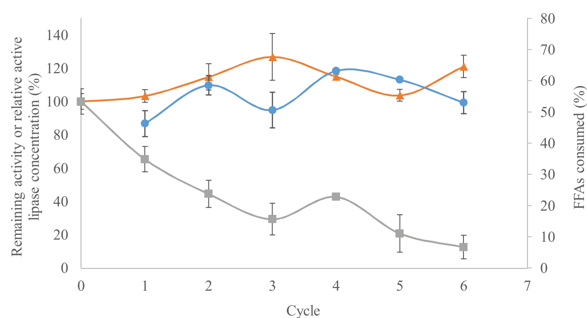

Fig. 7. Synthesis of mixed glycerides from crude triolein FFAs and glycerol by FFA-saturated immobilised CrL. Batch reaction cycles of 3 h were carried out at 40 °C under partial vacuum (100 mbar). FFAs consumed (circles) after each cycle, remaining activity (triangles) and concentration of active lipase (squares) relative to cycle 0. Values are mean ± SE (n = 3).

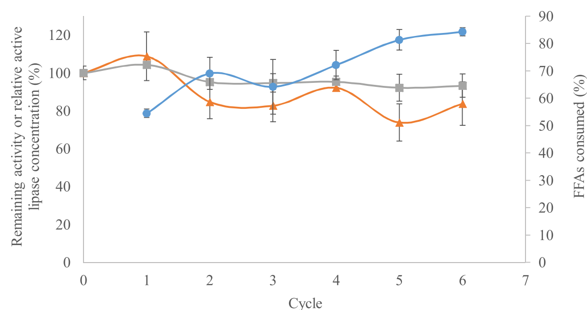

Fig. 8. Synthesis of mixed glycerides from crude triolein FFAs and glycerol by FFA-saturated Novozym® 435. Batch reaction cycles of 3 h were carried out at 60 °C under partial vacuum (100 mbar). FFAs consumed (circles) after each cycle, remaining activity (triangles) and concentration of active lipase (squares) relative to cycle 0. Values are mean ± SE (n = 3).

Commercial application of immobilised biocatalysts requires the enzyme to be bound strongly to a high mechanical strength, robust support of sufficiently large particle size [15]. CrL immobilised on C18 M meets these requirements and has demonstrated high synthetic activity and reusability. In light of these, our immobilised CrL preparation has potential industrial applications.

4. Conclusion

In this study, a very stable immobilised CrL preparation was formed using a highly hydrophobic octadecyl methacrylate resin (C18 M) as the support. Both non-ionic (Triton X-100) and ionic (SDS) detergents at high concentrations (up to 10 % w/v) did not desorb all of the lipase and caused only a small decrease in immobilised lipase activity, highlighting the enzyme's remarkable resistance to desorption from C18 M. Furthermore, immobilised CrL showed potential for use in the synthesis of restructured lipids, as it maintained high activity throughout six cycles of glyceride synthesis. It was also apparent that a FFA-saturation step prior to using the immobilised enzyme preparation in multiple hydrolysis and synthesis reaction cycles is necessary for accurate estimation of enzyme activity. This prevents the hydrophobic FFAs released from substrate hydrolysis, or present as starting material for synthesis, from binding to the support. The performance of immobilised CrL was comparable to that of Novozym® 435, however the immobilised CrL preparation offers a wider substrate selectivity, and greater resistance to desorption by detergents (i.e. Triton X-100) than Novozym® 435.

CRediT authorship contribution statement

Ivan Kurtovic: Conceptualization, Methodology, Project administration, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Tim D. Nalder:** Investigation, Visualization, Writing - original draft, Writing - review & editing. **Helen Cleaver:** Investigation, Data curation. **Susan N. Marshall:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors would like to acknowledge funding from The New Zealand Ministry for Business, Innovation and Employment (MBIE Programme 'Export Marine Products' C11X1307) and The Australian Research Council (ARC Linkage Programme LP140100722) that has made this research possible. We thank Daniel Killeen and Asli Card (Plant & Food Research, Nelson) for Iatroscan and GC–MS analyses, and Nigel Perry and Elaine Burgess (Plant & Food Research, University of Otago) for assistance with the elemental analyses.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00535.

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