Systemic Concocting of Cross-Linked Enzyme Aggregates of Candida antarctica Lipase B (Novozyme 435) for the Biomanufacturing of Rhamnolipids

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Abstract In the present study, Candida antarctica lipase B was immobilized on amine-functionalized silica microspheres as cross-linked enzyme aggregates (CLEA) and utilized for the biomanufacturing of rhamnolipids (RL). Lipase CLEA synthesized under optimized conditions of 2.0:1.0 by volume of silica microsphere/enzyme concentration, a 1.0:2.5 (v/v) ratio of enzyme/2-propanol, 7 mM glutaraldehyde concentration, when incubated at pH 9.0 and 40 °C, for a cross-linking time of 30 min were observed to exhibit superior biocatalytic properties and a maximum enzyme load of 770 U g−1. Lipase CLEA exhibited enhanced pH stability in acidic and alkaline media and increased temperature resistance as compared to free lipase. Both free and CLEA lipases were used to synthesize RL in different solvent systems. After 12 h, from initiation of the esterification, the degree of esterification (molar conversion yield) reached 46% and 71% in the batch mode. 1H and 13C nuclear magnetic resonance (NMR) and high-performance liquid chromatographic (HPLC) analysis confirm RL production by CLEA lipase. The CLEA showed greater confrontation to enzyme-mediated bioprocess approach as compared to its soluble counterpart and exhibited excellent RL production and catalytic activity even after its tenth successive reuse.

Keywords Candida antarctica lipase B · Silica microspheres · Immobilization · Cross-linked enzyme aggregates · Rhamnolipids

Introduction

Lipases (E.C. 3.1.1.3) have gained wide popularity due to their distinct catalytic stability, selectivity, tolerance to non-natural reaction conditions, and broad substrate specificity (Ansorge-Schumacher and Thum, 2013). They typically catalyze reverse synthesis reactions such as esterification, transesterification, and interesterification reactions, and a number of elementary reaction types such as alcoholysis, ammoniolysis, and perhydrolysis, making them industrially dominant enzymes (Ansorge-Schumacher and Thum, 2013; de María et al., 2005; Idris and Bukhari, 2012). Among the Candida species known, Candida antarctica B (CaLB) lipase is widely used for biocatalytic purposes, due to its enantioselectivity, enormous possibility of applications in various fields, and high activity in both
hydrolysis and synthesis reactions (Idris and Bukhari, 2012). Because of their ubiquitous nature and considerable industrial potential, these sophisticated lipases remain a hot spot for various studies, with one of their potential applications being the synthesis of rhamnolipids (RL), the best characterized bacterial surfactant.

RL, primarily a crystalline acid, are composed of β-hydroxyl fatty acids comprising a rhamnose sugar molecule at the carboxy terminus (Irorere et al., 2017). RL, and many known amphiphilic glycolipids, are good foaming and wetting agents and are able to increase the aqueous solubility of hydrophobic compounds, making them excellent solubilizing and emulsifying agents for diverse applications (Sekhon Randhawa and Rahman, 2014). These third generation surface-active compounds have an edge over both the first generation alkylpolyglycosides (APG) and the second generation sophorolipids, due to their high potential to reduce the surface tension beyond 31 mN m$^{-1}$ (Pöhnlein et al., 2015).

The age-old method of production of RL by Pseudomonas aeruginosa produces two congeners (mono-RL and di-RL), which complicates the downstream processing and thereby affects their surface tension properties. As an alternative, production of the surface-active compounds via a biocatalytic route is advantageous as it efficiently produces a single congener by esterification of carbohydrates with fatty acids (Pöhnlein et al., 2015). Even though there are several reports concerning the production of biosurfactants using lipases and esterases, lipases are considered industrially significant as they esterify long-chain fatty acids (C6-C10) (Chahiniana and Sarda, 2009). Utilizing this benefit of lipase, Nott and coworkers engaged several lipases for the production of novel RL and established that CaLB is the most effective in the process (Akong and Bouquillon, 2015; Nott et al., 2013). However, on an industrial scale, the complete potential of the free CaLB cannot be utilized due to the lack of long-term operational stability, coupled with difficulty in recovery and reusability (Cao, 2005; Idris and Bukhari, 2012). This, however, can be grappled with the efficient immobilization of CaLB.

Immobilization enhances the catalytic activity and operational stability of an enzyme, in addition to its facile separation from the product and convenient handling (Idris and Bukhari, 2012; Mateo et al., 2007; Sheldon et al., 2005), which lowers the process cost, making the final product highly economical (Idris and Bukhari, 2012). The formation of cross-linked enzyme aggregates (CLEA), in specific, has significantly higher potential compared to other immobilization techniques, due to several advantages such as increased stability enabled by irreversible binding to the support, and improved catalytic activity, in addition to being an efficient solution to the leaching problem faced with the adsorption process (Cao, 2005; Lopez-Serrano et al., 2002; Mateo et al., 2007; Sheldon et al., 2005).

CLEA are carrier-free immobilized enzymes that have high volumetric productivity and low-cost, owing to the exclusion of expensive carrier molecules (Sheldon et al., 2005). While CLEA of different forms of lipase are extensively investigated (Cruz-Izquierdo et al., 2014; Gupta et al., 2009; Kartal et al., 2011; Kartal and Kilinc, 2012; Khanahmadi et al., 2015; Kim et al., 2007; Lai et al., 2012; Lee et al., 2008; Lopez-Serrano et al., 2002; Rehman et al., 2016; Tudorache et al., 2013; Yu et al., 2006), there are only a few reports regarding CaLB CLEA (Cruz et al., 2012; Devi et al., 2009; Galvis et al., 2012; Hillal et al., 2004; Hobbs et al., 2006; Kapoor and Gupta, 2012; Lopez-Serrano et al., 2002; Schoevaart et al., 2004; Torres et al., 2013; Wilson et al., 2006). However, due to their lack in mechanical resistance, it is difficult to handle CLEA particles over repetitive uses (Kumar et al., 2014). Recovery of the CLEA is also complex, due to the high viscosity of some of the precipitants, which makes recovery of the immobilized enzyme difficult on a large scale (Garcia-Galan et al., 2011). These properties limit the continuous use of CLEA in industrial applications. Alternative solutions have been proposed to obtain a biocatalyst with favorable mechanical properties, namely, further immobilization of CaLB CLEA on various supports (e.g., membranes, silica, and magnetic particles) with better mechanical properties (Hillal et al., 2004; Kim et al., 2007; Lai et al., 2012; Lee et al., 2008; Tudorache et al., 2013). Among the solid supports reported to date, silica materials appear to be the ideal candidates for enzyme immobilization due to their controllable morphologies, porosities, and ease of functionalization (Kim et al., 2007).

In this study, the immobilization of CaLB as CLEA on silica microspheres was studied, with a special focus on the parameters—cross-linking time, pH, temperature, ratio of particle to enzyme, and glutaraldehyde concentration, for optimization, to identify their impact on lipase activity, when loaded onto the silica microspheres in the batch mode. Furthermore, a CaLB-assisted biocatalytic approach was developed for the synthesis of RL. In addition, characterization studies for identifying the stability of free and CLEA lipase in different microenvironments (pH, solvents) were carried out, along with their reusability in the production of RL.

Experimental

Materials and Methods

Lipase B from C. antarctica ($\geq 5000$ LU G$^{-1}$ of liquid), 4-nitrophenyl palmitate (pNPP), glutaraldehyde (25 wt.%
in H2O), monorhamnolipids (90%), 1-tetradecanoic acid (Myristic acid), 2-Methyl-2-Butanol (2M2B), and Molecular sieve (50 Å) were purchased from Sigma Aldrich (St. Louis, MO, USA). The bicinchoninic acid (BCA) assay kit was purchased from ThermoFisher Scientific Inc. (Waltham, MA, USA). Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), ethyl acetate, ethyl butyrate, acetonitrile, 2-propanol, ethanol, acetic acid, 1-butanol, and butyric acid of analytical grade were purchased from Fisher Scientific (Ottawa, ON, Canada). High-purity recombinant α-Rhamnosidase (prokaryote) and L-rhamnose assay kits were from Megazyme Int. Ireland Ltd. (Wicklow, Ireland).

Amorphous Silica Microspheres

The MAT 540 supports were supplied by Materium Innovations (Granby, QC, Canada), which comprised silica microspheres, with an average pore size of 16.74 nm, a pore volume of 1.06 cm3 g−1, and a BET surface area of 253.71 m2 g−1. The support material was also surface functionalized up to 350 μmoles of amine groups per g.

Microorganisms and Culture Conditions

For the antimicrobial activity, Bacillus subtilis MTCC 441, Staphylococcus aureus MTCC 3160, Escherichia coli MTCC 443, and Pseudomonas fluorescence MTCC 103T were procured from Microbial Type Culture Collection (MTCC), India and maintained in Tryptic soy agar plates at 4 °C.

Enzyme Activity

The biocatalytic activity of lipase was estimated by spectrophotometrically observing the hydrolysis of pNPP to 4-nitrophenol (pNP) and palmitate, at 410 nm (εmax = 15,000 M−1 cm−1). Hundred microliters of lipase or sample were added to eight hundred microliters of 0.1 M phosphate buffer, pH 8.0, followed by the addition of hundred microliters of pNPP (0.05 M in acetonitrile) and incubation for 5 min at 40 °C. Two milliliters of 0.1 N Na2CO3 were then added to the reaction mixture to stop the reaction. The pNPP hydrolysis was then estimated spectrophotometrically by measuring the absorbance at 410 nm (Spectra Max Plus 384, Molecular Devices Corp., Sunnyvale, CA, USA). One unit (U) of activity of lipase was defined as the amount of enzyme required to produce 1 μmol of pNP at pH 8.0 and 40 °C. All experiments were performed in triplicate and the average values were considered. The amount of protein in the sample was determined by the BCA assay at 562 nm using a UV–Vis Spectrophotometer (Walker, 1994).

Preparation of CLEA Lipase

CLEA lipase immobilized on silica supports was prepared using a modified method of Kim and coworkers, with the use of 2-propanol as a precipitating agent, and glutaraldehyde as a cross-linking agent (Hilal et al., 2006; Kim et al., 2007). The stock concentrations of the enzyme and support, each, were considered as 0.43 mg mL−1, based on the protein concentration of liquid lipase. A mixture of the silica supports/CaLB lipase in the ratio of 2.0:1.0 (in pH 9.0 phosphate buffer) (based on protein concentration 0.43 mg mL−1) was prepared, to which a volume of 2-propanol, 2.5 times the total volume, was added. This was followed by the addition of glutaraldehyde solution to obtain a final concentration of 7 mM. The whole reaction mixture was then incubated for various time intervals followed by three phosphate buffer (pH 9.0) washes, to obtain the desired CLEA lipase. Enzyme load is given as apparent immobilized lipase activity in unit per gram of silica support used for immobilization in the following equation:

\[
\text{Enzyme load} \left( \frac{U}{g} \right) = \frac{\text{Apparent activity of CLEAs lipase} \left( \frac{U}{L} \right)}{\text{Support concentration} \left( \frac{g}{L} \right)}
\]

Characterization of Lipase Immobilized on Support

The optimal pH of free and CLEA lipase (10 U L−1) was determined by performing the pNPP hydrolysis reaction, in 0.1 M buffers in the pH range of 5.0–9.0 under standard conditions at 40 °C. The relative activity was obtained by considering the activity at each pH, relative to the highest activity obtained.

The stability of free and CLEA lipase in different pHs was determined by incubating the biocatalyst (10 U L−1) in 0.1 M buffers, in the pH range 6.0–9.0 for 240 min. pNPP hydrolysis was carried out under standard conditions to determine the lipase activity at every 30 min interval. Residual activity (%) was calculated by considering the percentile of lipase activity at each pH with respect to the initial lipase activity.

Optimum Temperature and Temperature Stability

pNPP hydrolysis reaction was carried out at 30–70°C under standard conditions (0.1 M, pH 8.0 phosphate buffer) to attain the activity of free and CLEA lipase (10 U L−1), to determine the optimal temperature for lipase activity. The relative activity was obtained by considering the activity at each temperature relative to the highest activity obtained. The effect of varying temperatures on the stability of free and CLEA lipase (10 U L−1) was studied by incubating the
enzyme in the temperature range of 40–60°C for 240 min, and the pNPP hydrolysis reaction was carried out under standard conditions at 30 min intervals. Residual activity (%) was calculated by considering the percentile of lipase activity at each temperature with respect to the initial lipase activity.

**Solvent Stability**

Stability of free lipase (100 U L⁻¹) and immobilized lipase (100 U L⁻¹), when incubated in different solvents, was evaluated. The solvent stability test was performed by incubating 1 mL of biocatalyst solution in equal volume of solvents (DMSO, THF, ethyl acetate, ethyl butyrate, 2M2B, and hexane). Enzymatic activity was measured for each sample after incubation for 12 h. Residual activity (%) was calculated by considering the percentile of lipase activity for each sample with respect to the initial lipase activity.

**Determination of Kinetic Parameters**

The kinetic parameters, Vₘₐₓ and Kₘ, of free and CLEA lipase (10 U L⁻¹) were determined by performing the pNPP hydrolysis reaction under standard conditions (0.1 M, pH 8.0 phosphate buffer, 40 °C) to determine the lipase activity, by using pNPP in the concentration range 0.01–6.0 mM as a substrate. The experimental data were fitted to the Lineweaver–Burk plot to obtain the kinetic parameters.

**Synthesis of RL from Rhamnosides Using Free and CLEA Lipases**

The process for the biocatalytic production of RL consisted of two steps (Nott et al., 2013). The first step involved the conversion of rhamnose to rhamnosides using the enzyme β-D-glucosidase, followed by lyophilization. Experiments were carried out in batch mode by reacting 10 mL of a mixture of 0.18 M of rhamnose with 100 U L⁻¹ of β-D-glucosidase. The enzymatic reactions were performed at pH 4.0 (0.1 M acetate buffer), and 40 °C for 12 h. The produced rhamnosides were dried with a speed vacuum apparatus (10 mbar, 40 °C) and the dried products were lyophilized. In accordance with Nott and coworkers, the second step of the process involved the CaLB-catalyzed esterification of rhamnosides with myristic acid, the products of which were purified to produce the RL (Nott et al., 2013). A typical reaction mixture consisted of rhamnosides (0.05 M) and fatty acids (0.30 M). A 50 g L⁻¹ 3Å molecular sieve was placed in a screw-capped glass vial with 10 mL of 2M2B and preincubated at 60 °C for 2 h using an orbital shaking water bath (120 rpm). The esterification reaction process was then initiated by adding 100 U L⁻¹ of the free or immobilized lipase to the homogeneous mixture. The reaction mixture was stirred at 200 rpm, at a temperature of 60 °C, kept constant for 24 h. The synthesized RL were extracted using an equal volume (v/v) of chloroform, followed by concentration using a rotary vacuum evaporator. The concentrated sample was resuspended in the mobile phase consisting of acetonitrile and water (55:45 v/v) with 0.03% trifluoroacetic acid and filtered through a membrane filter (0.45 μm); then, 20 μL of each aliquot was injected into the Agilent Technologies 1200 series high-performance liquid chromatographic (HPLC), coupled to an evaporative light scattering detector (Leitgeb and Knez, 1990; Nott et al., 2013; Sebatini et al., 2016). The percentage utilization of rhamnosides was calculated using the orcinol method and the L-rhamnose assay kit (Chandrasekar and Bemiller, 1980). Furthermore, the yield of the produced RL was quantified by calculating the residual fatty acid in the reaction solution, according to Sebatini and coworkers (Leitgeb and Knez, 1990; Sebatini et al., 2016). Subsequently, the effects of solvents, esterification time (2–48h), and temperature (35–60°C) on RL formation were investigated.

**Reusability Studies of CLEA of CaLB for the Synthesis of RL**

The reusability of CLEA lipase was studied by quantification of RL production, followed by assaying the pNPP hydrolysis under standard conditions (0.1 M, pH 8.0 phosphate buffer, 40 °C), for 10 consecutive cycles. A typical reaction mixture of rhamnoside solution consisting of 0.3 M fatty acid, 0.05 M acyl donor, and 50 g L⁻¹ molecular sieve was pretreated at 60 °C for 6 h under continuous agitation at 120 rpm. 200 mg CLEA-CaLB (1000 U L⁻¹) was added to the homogenous solution, which initiated the esterification process. After 12 h incubation at 60 °C, the reaction mixture was centrifuged at 4000 rpm for 30 min to retain the CLEA lipase as a pellet, followed by resuspension of the pellet in buffer. The recovered CLEA lipase was then reused for further batches of RL synthesis. Both the pellet and supernatant were assayed for pNPP hydrolysis reaction. The synthesized RL were extracted using equal volumes (v/v) of chloroform and then concentrated using a rotary vacuum evaporator.

**Characterization of the Synthesized RL**

A stalagometer was utilized to measure the surface tension of the RL, as described by Caykara and Birlik (2005). The tensile property of the synthesized biosurfactant was established using the contaminant-free, clean stalagometer, in which distilled water was used as a negative control, and 1000 ppm of the microbial RL was used as a
positive control. The tensile property was then calculated using the formula (Walter et al., 2010):

\[
\gamma = \gamma^\circ \left( \frac{n}{n_0} \right)
\]

where \(\gamma\) and \(\gamma^\circ\) are the surface tensions of the sample and reference solvent, respectively; \(n\) and \(n_0\) are the total drop weight of the sample and the reference solvent, respectively.

**Antimicrobial Activity**

The antimicrobial activity of the produced RL was analyzed against two gram positive (B. subtilis and S. aureus) and two gram negative bacteria (E. coli and P. fluorescens) by impregnating 1 mg mL\(^{-1}\) of the syringe-filtered surfactant onto a filter paper disc of diameter 5 mm. The study was performed with a negative control (water impregnated disc) and a positive control (standard RL from Sigma) by aseptically transforming the disc on the surface of Muller Hilton agar plates, inoculated with 24-h old culture. All the plates were incubated at 28 °C and the diameter of clear zones around the disc was measured in mm after 48 h (Ndlovu et al., 2017).

**Chemical Analysis of Synthesized RL**

The chemical structure of the produced RL was confirmed by nuclear magnetic resonance (NMR) analysis of the purified sample. For the analysis, the concentrated sample was suspended in deuterated DMSO and subjected to \(^1\)H NMR and \(^13\)C NMR for the structural analysis. Both \(^1\)H NMR and \(^13\)C NMR were performed using NMR 500 mHz (Bruker Biospin AG, Switzerland). The chemical shifts of \(^1\)H NMR and \(^13\)C NMR were represented in ppm.

**Results and Discussion**

**Optimization of Immobilization Parameters**

The immobilization of lipase by preparation of CLEA involves two consecutive steps—precipitation of enzyme using 2-propanol, followed by the cross-linking step using glutaraldehyde. Due to the presence of the amine-functionalized silica supports, the carrier behaves as a scaffold for the enzyme and the cross-linking is expected to occur not only among the amino groups in the lipase structure, but also with the surface amine groups on the particles. This step also enables the strong modification to enhance the enzyme characteristics, which can be achieved by selection of appropriate carriers, binding chemistry, and immobilization techniques (Cruz-Izquierdo et al., 2014; Gao et al., 2013; Hilal et al., 2006; Kim et al., 2007).

Experiments were performed by varying pH, temperature, contact time, glutaraldehyde concentration, and the ratio of particle to enzyme, resulting in high enzyme load on mesoporous silica. Figures 1 and 2 show the effect of the various parameters on optimizing the biocatalytic load of lipase on silica supports by the CLEA immobilization process. CLEA formation under alkaline conditions (pH 9.0, 0.1 M Glycine-NaOH buffer) led to increased activity and enzyme load (767.7 U g\(^{-1}\)) in comparison to that under acidic conditions (pH 4.0, acetate buffer) (Fig. 1a). It was also observed that a brief contact time of 30 min after the addition of precipitant and cross-linker enables high immobilization with less washing loss, which can be attributed to the availability of lipase for cross-linking. However, the significance of cross-linking time as an important parameter in the immobilization process was highlighted by the decrease in enzyme load to a very low value of 97.9 U g\(^{-1}\), with an increase in the cross-linking time to 24 h (Fig. 1b). Leaching of enzyme with high washing loss resulted under these conditions due to instability on prolonged incubation under shaking conditions (Hilal et al., 2006; Kim et al., 2007). The increase in cross-linking time to 24 h led to a loss of enzyme flexibility, activity and, stability (Fig. 1b). The loss of enzyme activity may be due to denaturation caused by the decrease in solubility of the enzyme in the surrounding medium during aggregation, because of the severe forces exerted on the structure of the enzyme (Schoevaart et al., 2004).

As opposed to the behavior seen with immobilization by covalent binding, no saturation is observed in the formation of CLEA lipase, with an increase in the ratio of particle/enzyme to 2:1 (Cruz-Izquierdo et al., 2014; Gao et al., 2013; Hilal et al., 2006; Kim et al., 2007). Formation of CLEA, under acidic conditions (pH 4.0), resulted in a 1.2-fold decrease in the biocatalytic load due to inactivity of lipase under these conditions (Cruz-Izquierdo et al., 2014). The comparison of experiments with a variation in the ratio of particle/enzyme (from 2.0:1.0 to 0.5:1.0) showed that even with a lower volume of particles added, the biocatalytic load remained as high as 770.3 U g\(^{-1}\), and 716.7 U g\(^{-1}\), at 40 °C, respectively (Fig. 1d). Without n-propanol, a 23.1 ± 4.8% reduction in the biocatalytic yield was observed along with a maximum washing loss of 57%. This result emphasizes the role of propanol in the selective extraction and immobilization of the enzyme. A maximum biocatalytic load of 767.7 U g\(^{-1}\) was observed under the following reaction conditions: cross-linking time of 30 min, pH 9.0, 40 °C, 2.0:1.0 ratio of particle/enzyme, and glutaraldehyde concentration of 7 mM (Fig. 2). A glutaraldehyde concentration of 7 mM is required for immobilization of the enzyme and a lower concentration is observed to lead to improper cross-linking, thus,
resulting in lower activity yields. On comparison between the different experiments, clear observations were made that the CLEA formation when performed under the same conditions but with a lower glutaraldehyde concentration of 1 mM resulted in a lower biocatalytic load of 686.2 U g\(^{-1}\), as a result of lesser cross-linking occurring between the enzyme and support (Cruz-Izquierdo et al., 2014; Kim et al., 2007).

**Free and Immobilized Enzyme Characterization**

**Optimum pH and pH Stability**

The effect of pH on the activity of free and CLEA lipase was studied (Fig. 3a). There was no shift observed at the optimum pH for the CLEA lipase formed (pH 8.0), in comparison with that of free lipase despite any conformational changes possibly occurring during immobilization (Mateo et al., 2007). It has been reported that the free Cal B lipase exhibits maximum activity under basic conditions (pH 7.0–8.0) due to the stability maintained by the structure and maintenance of the open, active structural configuration under these conditions (Fatiha et al., 2013). No
A difference in the activity between free and CLEA lipase was observed at pH 8.0.

To study the effect of a pH-based microenvironment on the enzyme, the stability of free and CLEA lipase at different pH was also explored. CLEA lipase exhibited a lower stability compared to free enzyme at pH 6.0, with 80% of the initial activity maintained after prolonged exposure of up to 240 min. This may be attributed to the conformational changes that could be occurring during the CLEA formation process, making the CLEA unstable under slightly acidic conditions of pH 6.0, despite maintaining high activity under basic conditions (Fatiha et al., 2013; Gupta et al., 2009) (Fig. 3b). As seen in Fig. 3c, under neutral pH conditions, the activity of free lipase increases to 1.9-fold, followed by a decrease with prolonged exposure. However, in the case of CLEA, the stability of the lipase was maintained in a pattern, similar to that seen at pH 6.0 (Lopez-Serrano et al., 2002). A total of 80% of the initial activity was observed to be maintained even after prolonged exposure. Under basic conditions of pH 8.0, when incubated for 240 min, the stabilities of both free and CLEA lipase were maintained at 80% of the initial activity (Fig. 3d). The free lipase, however, was observed to lose stability under alkaline conditions of pH 9.0, with only 20% of the initial activity maintained (Fig. 3e). In contrast, CLEA lipase exhibited an improved stability of 90% of the initial activity, under alkaline conditions, due to the rigidity of the structure achieved with cross-linking (Gao et al., 2013). The high stability of CLEA lipase under alkaline conditions for prolonged periods of time, therefore, widens the possibility to explore their use in a number of applications that involve the bleach, detergent, paper, and pulp industries, which typically operate under extreme, basic conditions (Ansorge-Schumacher and Thum, 2013; de María et al., 2005; Idris and Bukhari, 2012).

**Optimum Temperature and Temperature Stability**

The free lipase, when studied at varying temperatures, exhibited higher activity at 40 °C. While CLEA lipase showed improved activity at an optimum temperature of 60 °C.
Similar results of shift in the optimum temperature of Cal B lipase have been reported highlighting the stabilization of immobilized enzyme at higher temperatures, due to rigidity of the structure achieved with immobilization (Arroyo et al., 1999). Thermal stability of free and CLEA lipase, when studied, exhibited excellent stability of CLEA lipase at higher temperatures, when compared to that of free lipase (Fig. 4b–d). At 40 °C, the free lipase was observed to have increased stability in comparison to the CLEA lipase with 80% of the initial activity being maintained (Fig. 4b). As shown in Fig. 4c, at 50 °C, a minimal reduction in activity (10%) was observed on prolonged exposure, indicating its conformational stability. At a higher temperature of 60 °C, the stability of free lipase decreased to 30% of the initial activity, while CLEA exhibited a high thermal stability with about 90% of the initial activity retained. Thermal inactivation, typically related to unfolding of the enzymes, is evidently prevented by the immobilization of enzyme, with restriction of conformational changes (Arroyo et al., 1999). The critical role played by the support particles in stabilizing the active conformation of the lipase, assisted by the strong multipoint attachments between the lipase molecules, increases the rigidity of the enzyme, in turn reducing the heat extensional deformations at the lipase-active site (Zhang et al., 2003). Thermostable lipases find promising applications in various industries involved in synthesis of biosurfactants, oleochemical industry, and paper manufacturing (Poojari and Clarson, 2013).

**Kinetic Parameters**

The apparent kinetic parameters for free and CLEA lipase, as denoted in Table 1, show a sixfold increase in the \( K_m \) value for CLEA lipase when compared to that of free lipase. This increase in \( K_m \) may be attributed to the structural changes in the enzyme, due to immobilization, or the lower accessibility of pNPP to the active sites of the enzyme. A high \( K_m \) value indicates the presence of more substrate molecules saturating the enzyme, depicting the lower affinity of CLEA lipase to the pNPP substrate. This trend might be a result of the conformational changes and steric hindrances induced by the immobilization of the enzyme. Consequently, due to the lower affinity, a 41.7% decrease in the hydrolytic rate (\( V_{\text{max}} \)) for CLEA lipase was also observed (Table 1). The turnover number (\( K_{\text{cat}} \)) predicts that the maximal catalytic rate for free lipase is 37.5% higher than that for CLEA lipase, resulting in lower...
catalytic efficiencies ($K_{cat}/K_m$) for the CLEA when compared with that of the free lipase. These results could be attributed to several factors such as conformational changes on immobilization, steric hindrance or diffusional limitations caused by modification of the amine functional groups on the lysine amino acids at the structural active site (Devi et al., 2009; Poojari and Clarson, 2013).

Synthesis of RL from Rhamnosides Using Cal B Lipase

Most of the esterification reactions using CaLB involved the use of organic solvents due to their enhanced properties, specifically with respect to handling nonpolar substrates (Park et al., 2012). The selection of organic solvents for suspending the rhamnose is considered to play a crucial role in enhancing the rate of reaction (Li et al., 2010). Most of the esterification processes were studied in the presence of organic solvents or low-water organic solvents, because buffers are expected to limit the reaction rate by reducing the activity of enzymes. This can be attributed to the tendency of water molecules to bind to the catalytic hotspots of CaLB, and thus limiting the reaction by restricting the interaction between the substrate and the active sites. The free and immobilized biocatalysts were then studied to explore their solvent stability and RL yield. As depicted in Fig. 5, the free lipase, when incubated for 12 h in the different organic solvents (DMSO, THF, ethyl acetate, ethyl butyrate, 2M2B, and hexane), exhibited a decrease in stability. The products from the free CaB-catalyzed esterification reactions were then analyzed at predetermined time intervals using the orcinol method and HPLC, to confirm the esterification between the fourth hydroxyl group of rhamnoside and myristic acid over time. HPLC analysis of the resultant RL showed a major peak at the retention time of 2.547 min (Fig. 6). However, the standard RL (90% purified) showed two predominant results, with one major peak at 2.547 min and a small peak at 7.047 min. The present results correlate with those of the previous work of Mohammed et al. (2018).

Exposure of the CLEA lipase to 100% solvent facilitates the exhibition of 80% of the activity in 2M2B, with 50% of the residual activity maintained in the other solvents. The activity of the free lipase, however, was observed to be 20–39% of the residual activity when incubated in the solvents. It was reported previously that the activity/stability of an enzyme can be correlated with its log P value (the logarithm of solvent–water partition coefficient), one of the most important properties of a solvent. The log P values of the different solvents studied with respect to the stability of solvent highlighted the effects of log P of the solvents. As described by Park et al., 2015, in the case of a solvent with

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Free lipase</th>
<th>CLEA lipase</th>
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<tbody>
<tr>
<td>$V_{max}$ (U L$^{-1}$)</td>
<td>88.49 ± 0.10</td>
<td>51.52 ± 0.47</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>0.002 ± 0.01</td>
<td>0.012 ± 0.02</td>
</tr>
<tr>
<td>$K_{cat}$ (mM of pNPP per mg of enzyme min$^{-1}$)</td>
<td>2.05 ± 0.01</td>
<td>1.28 ± 0.08</td>
</tr>
<tr>
<td>$K_{cat}/K_m$ (mg of enzyme$^{-1}$ min$^{-1}$)</td>
<td>773.58 ± 0.48</td>
<td>106.60 ± 0.52</td>
</tr>
</tbody>
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Fig. 5 Rhamnolipid production and solvent stability profiles for free and CLEA lipase of different organic solvent systems. Residual activity of free and CLEA lipase after incubation for 12 h in organic solvents at pH 8.0 and 60 °C. The experiments were done in triplicate and the error bar represents the percentage error in each set of determination.
a negative log P value, the solvent cannot access the enzyme due to phase separation (Park et al., 2012). On the other hand, with water miscible solvents having a positive log P value, the water molecules at the enzyme’s surface would be stripped out due to hydrophilic interactions that lead to inactivation, or denaturation of the enzyme (Park et al., 2012). Li and coworkers explained that the stability of the enzyme depends on the hydrogen bond between the Asparagine (Asp) and Histidine (His) amino acid residues present at the active site of the CaLB, and breakdown of the bond due to the solvent interactions was considered to reduce the activity of the CaLB (Li et al., 2010). It was observed that, RL yields in DMSO and 2M2B were as high as 45–70% with both the free and CLEA lipase (Fig. 5). However, the polar solvent 2M2B was preferred over DMSO because of its universal utility in food industries and because of its higher polarity, as previously reported by Akong and Bouquillon (2015) and Gandhi et al. (2000). On the other hand, THF being an ideal solvent for esterification was limited due to its interaction while assaying synthesized esters.

The esterification process was optimized for maximizing the yields of the RL, by studying varying incubation times and temperatures (Fig. 7). The degree of esterification reached a maximum for both free CaLB and immobilized CaLB (46% and 71%, respectively), 12 h after initiation of the reaction, after which no significant changes were observed due to the reaction reaching its equilibrium (Fig. 7a). The increase in the yield using CLEA lipase can be explained by the increase in the thermal stability and maintenance of equilibrium of water activity (aW), which are factors known to influence the enzyme activity (Gandhi et al., 2000; Sheldon et al., 2005). Moreover, with progression of the esterification reaction, the water molecules produced increasingly inhibit the enzyme activity by masking the active sites (Nott et al., 2013). This phenomenon is thought to be involved with both the free and immobilized enzymes, when the reactions were performed without the

![HPLC chromatogram of biocatalytically synthesized rhamnolipids. The standard product was commercially available monorhamnolipids (Sigma Aldrich, R-90)](image)

![Optimization of the various parameters involved in the biocatalytic synthesis of rhamnolipids using free and immobilized biocatalyst. (a) Esterification time of CALB for the production of rhamnolipids keeping the temperature—60 °C as constant. (b) Incubation temperature of CaLB for the production of rhamnolipids keeping the time 12 h as constant the experiments were done in triplicate and the error bar represents the percentage error in each set of determinations)](image)
molecular sieves. However, complete removal of the water molecules leads to a reduction in the enzyme activity (Leitgeb and Knez, 1990) and aW ranging between 0.09 and 0.96 is observed to have an appropriate effect (Gandhi et al., 2000). The reduction in the yields can be explained by the involvement of unfavorable conditions and the production of surfactants, which interfere with the enzyme activity (Gandhi et al., 2000). However, an increase in the incubation temperature to 55 °C (Akong and Bouquillon, 2015) leads to a twofold increase in the yield of RL (Fig. 7b); although a further increase in the temperature leads to a rapid destabilization of the enzyme in both cases (Akong and Bouquillon, 2015; Yu et al., 2008). The continuous shaking regime and maintenance of optimum conditions were considered as the factors determining the reduction in esterification time.

Reusability Studies of CLEA of CaLB in the Synthesis of RL

Reusability of a biocatalyst is considered as one of the determining factors in evaluating the efficiency of the immobilized biocatalyst. The residual activity of CLEA lipase, studied by applying consecutive pNPP hydrolysis reaction cycles, decreased from 97% to 70% in the second cycle, likely due to superficial immobilization of CaLB and continuous washing, which led to leaching out of the enzyme (Fig. 8). Furthermore, the residual activity decreased to 35% after 10 consecutive cycles, which can be attributed to the inactivation of the enzyme over multiple washes (Devi et al., 2009). This decrease in residual activity of CLEA lipase, in turn, decreased the esterification efficiency from 70% to 16% over 10 cycles of RL synthesis (Fig. 8). Even though the CLEA lipase exhibited stability with a minimal loss in the biocatalytic activity (70%) up to the third cycle, prolonged incubation at a high temperature of 60 °C resulted in a decrease in the thermal stability of the CLEA lipase, which in turn lowered the biocatalytic activity. Moreover, the decrease in biocatalytic activity can also be dictated by the swelling of the matrix during the prolonged incubation in organic solvents, which further decreased the yield of RL (Gandhi et al., 2000).

Measurement of Surface Tension of Synthesized RL

One of the elemental characteristics of a functional surfactant is the reduction of surface tension and a standard surfactant reduces the surface tension of water from 72 to 22 mN m⁻¹ (Aparna et al., 2011). In the present study, the tensile properties of the produced RL were studied and it was observed that the surface tension of water reduced from 72 to 23 mN m⁻¹ against the microbial RL, with a surface tension of 28 mN m⁻¹. The surface tension further decreased with the increase in the RL yield, which was followed by the saturation in surface tension at the critical micelle concentration (Kłosowska-Chomiczewska et al., 2017).

Antimicrobial Activity of Synthesized RL

Antimicrobial activity of the enzymatically synthesized RL was compared with microbial RL (R-90 Sigma) from P. aeruginosa. The results obtained, in terms of clear zone formation, showed a similar pattern of inhibition by the standard and produced RL against B. subtilis and S. aureus, with an average of 2.3 ± 0.14 and 1.2 ± 0.41 mm diameter inhibition, respectively (Table 2). However, in contrast, an approximate 1.3 fold increase in the inhibition of gram negative bacteria by the enzymatically synthesized RL was

<table>
<thead>
<tr>
<th>Rhamnolipid (1 mg mL⁻¹)</th>
<th>Inhibition zone in mm</th>
<th>Gram positive bacteria</th>
<th>Gram negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram positive bacteria</td>
<td>Bacillus subtilis</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Bio-catalytically synthesized RL</td>
<td>2.2 ± 0.24</td>
<td>1.21 ± 0.10</td>
<td>2.72 ± 0.41</td>
</tr>
<tr>
<td>Commercial RL from Pseudomonas aeruginosa (Sigma Aldrich R90)</td>
<td>2.2 ± 0.14</td>
<td>1.18 ± 0.41</td>
<td>1.51 ± 0.34</td>
</tr>
</tbody>
</table>
recorded against microbial RL. This projected the presence of other congener form(s) of RL as impurities in the microbial RL and their role in reducing the antimicrobial activity. The current results support the previous reports that the presence of di-RL in the microbial RL, as a congener form, induces a reduction in the antimicrobial activity against gram negative bacteria, by inhibiting the cell membrane penetration of RL against gram negative bacteria, (Bharali and Konwar, 2011; Das et al., 2014).

Chemical Analysis of Synthesized RL

The structure of the enzymatically synthesized RL was studied by subjecting the resultant RL to NMR analysis. The NMR data were interpreted with reference to the previous works (Akong and Bouquillon, 2015; Monteiro et al., 2007; Nott et al., 2013; Wei et al., 2005).

1H NMR ((CD3)2S=O),500 MHz): δ1H 0.853 rhamnose (3H, s, CH3), 1.231–1.240 (20H, overlap, (CH2)n=), 2.00 (2H, CH2CH2COO), 2.3–2.4 (2H, Overlap, −COO−CH2), 2.5 (2H, overlap, COO−CH2CH3), 3.2 (1H(Rhamnose−3H)−OH), 3.4–3.6 (3H, overlap, 2’,3’,5’ −H), 4.0 (2H, broad, CH2COOCH2CH2), and 4.6 (1H, d, rhamnose (O) CH2CH2−).

13C NMR ((CD3)2S=O),500 MHz): δ 14.3 ppm (−CH3), 28.8–31.7 (CH2CH2−CH2), 32.13 (−CH2COO), 34.65 (−COOCH2CH2−), 68.8 (rhamnose (O)CH2CH2), 69.54 (rhamnose (CH(CH3)−)), 71.73 (rhamnose(CH(CH3)CH(OH)−), 72.4 (rhamnose(CH2CH2OH)), 102.40 (rhamnose (−CH(OH)CH (OCH2−))), and 171.5 (esters C=O), 173.9 (−COO);

In the present work, 1H NMR (Fig. S1a, Supporting information) and 13C NMR (Fig. S1b) spectra exhibited eight different spin systems (3.2 to 4.6) ppm and (68 to 103) ppm, indicating the presence of rhamnose in the produced RL. The presence of spin at 171 ppm strongly supported the formation of esters between rhamnose and the fatty acids. Similarly, the spin at (1–3) ppm and (14–35) ppm confirmed the structural presence of fatty acid. Based on the NMR patterns, the formation of esters in RL using CLEA lipase was confirmed.

Conclusions

With the advancement in enzyme technology, various engineering strategies are being developed aiming at the enhancement of biocatalytic stability and reusability. Out of the several techniques developed, the use of CLEA is such a technique that has significantly higher potential due to the irreversible binding of the enzyme to the support matrix. In the present study, various process parameters were optimized for production of CLEA lipase, and maximum biocatalytic activity was achieved under the following conditions during synthesis: pH 9.0, 40 °C, 0.5 h, 2:1 ratio of particle/enzyme, and 7 mM glutaraldehyde concentration. The promising characteristic properties of the CLEA lipase evidently exhibit its potential to be used in industrial applications where they can be utilized in a strategic approach for the production of RL from rhamnosides. The selective esterification of the fourth hydroxyl group of rhamnosides mediated by CLEA lipase led to a 69.23% yield of RL under the optimized conditions of 60 °C and an incubation time of 12 h. Being ecologically accepted and application oriented, these third generation biosurfactants have immense potential industrial applications and have made their way into the food industries, cosmetic industries, and pharmaceuticals.

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Conflict of Interest  The authors declare that they have no conflict of interest.

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