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Enhancement of *n*-3 polyunsaturated fatty acid glycerides in Sardine oil by a bioimprinted cross-linked *Candida rugosa* lipase

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ABSTRACT

Considering the advantages of bioimprinting and carrier free immobilization, cross-linked enzyme aggregates (CLEA) were prepared by using bioimprinted *Candida rugosa* lipase (CRL) with Bovine serum albumin (BSA), Polyethyleneimine and glutaraldehyde. Effect of various factors such as CRL-Oleic acid ratio, CRL-BSA ratio, CRL- Polyethyleneimine ratio, glutaraldehyde loading, cross-linking time etc., on lipase activity recovery and aggregate yield were studied and optimized. This immobilized lipase (CRL-CLEA) was used for the selective hydrolysis of ester linkages of non-PUFA glycerides, with an aim to concentrate EPA and DHA glycerides in the Sardine oil. Imprinting with oleic acid in the presence of ethanol and Tween 60, and further immobilization with co-aggregates and cross-linking agent showed 10.4 times higher degree of hydrolysis compared to free enzyme. As result, 2.83-fold increase of *n*-3 PUFA content in deacidified oil was obtained by using CRL-CLEA. The resultant oil had negligible di- and triglycerides content, proving higher efficiency in hydrolysing ester bonds of fatty acids, other than *n*-3 PUFA. Reusability studies showed CRL-CLEA could be reused up to 5 runs without a substantial reduction in its performance. Improvement in degree of hydrolysis, thermostability, efficiency of hydrolysis and reusability were achieved due to bioimprinting and subsequent immobilization of CRL in the form of CLEA.

1. Introduction

Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) have been reported to play an important role in the human health [1]. The increasing world market for these *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) is because of its health benefits and its dietary requirement due to the inability of the human body to synthesize them. Several reports have reviewed and listed the benefits and methods to produce *n*-3 PUFA concentrates from the sources like marine algae, fish oil [2,3] and edible oils [4]. Review of literature suggests plenitude of methods for the production of *n*-3 PUFA from fish oil including urea complexation, low temperature crystallization, molecular distillation, iodolactonization, methods of salt solubility and liquid–liquid extraction and fractionation using sodium nitrate solution [4]. These methods, however suffer disadvantages of producing *n*-3 PUFA in the form of free fatty acids (FFA) which are nutritionally unfavourable, non-selective for the different fatty acids and employ extremes of reaction conditions. Compared to the chemical processes, enzyme mediated hydrolysis of oil provide diverse advantages like mild reaction conditions, increased specificity and reduced side reactions which leads to the energy and economic benefits [5]. Commercial lipases from *Candida rugosa*, *Geotrichum candidum*, *Humicola lanuginsa*, *Chromobacterium viscosum*, *Rhizomucor miehei*, *Aspergillus niger* and *Rhizopus delemar* have been expansively used for these purposes [6].

The usefulness of *Candida rugosa* lipase (CRL) for enhancement of *n*-3 PUFA from fish oil has already been reported [7]. CRL also has been used for the production of value added food products with medicinal properties because of their ability to catalyze reactions such as hydrolysis and *trans*-esterification [8] due to the tunnel like conformation of its active site [9]. These lipases remain active at the oil-water interface causing the release of FFA by the attachment of acyl group on the positive side chain and hydrogen ion on the negative side chain in the active site [8]. The presence of carbon–carbon cis-double bonds in EPA and DHA results in the bending of the chains enhancing the steric hindrance for the approaching lipase. This result in the selective hydrolysis of ester linkages of non-PUFA glycerides, in turn concentrating EPA and DHA due to the failure of CRL to cleave their ester linkages [7].

Abbreviations: BSA, bovine serum albumin; CLEA, cross-linked enzyme aggregates; CRL, *Candida rugosa* lipase; CRL-CLEA, bioimprinted cross-linked CRL aggregates; DHA, docosahexaenoic acid; DOH, degree of hydrolysis; EPA, eicosapentaenoic acid; FFA, free fatty acids; MA, myristic acid; *n*-3 PUFA, *n*-3 polyunsaturated fatty acids; OA, oleic acid; PA, palmitic acid; PEI, polyethyleneimine

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The use of commercial soluble enzymes leads to the wastage and limited reuse of the enzymes. To avoid this, designing of enzyme preparations with high activity and stability has attracted consideration over native enzymes. Enzyme immobilization, lipid coating and bioimprinting are the recently employed techniques to modify lipase [10]. Bioimprinting is known to increase the activity of enzymes by the increased electrostatic and hydrogen bonding interactions between the surface residues of the enzymes [11]. It is based on the fact that the lid that covers the active site of the enzyme can be opened at the interface in the presence of various imprint molecules [11]. Many literatures suggest the use of imprinted enzymes for hydrolysis in the anhydrous conditions where the enzymes are rigid and maintain an imprint molecule induced conformation [10]. Navarro and Braco [12] used nine different bioimprinted lipases to carry out alcoholysis and esterification and reported improved performance of lipase in general. Fishman and Cogan [11] in their studies proved the effectiveness of obtaining highly active enzymes in organic solvents by imprinting lipases with fatty acids. Yan et al. [13] described the use of a combined modification method including bioimprinting, pH tuning, lipid coating, salt activation, and immobilization improved the activity of the lipase in anhydrous organic solvents.

Among the various immobilization techniques, carrier bound and carrier free immobilizations are the most promising ones used for the improvement in the stability and reuse of the enzymes. Since carrier bound immobilization exhibit reduced volumetric activity of the biocatalyst, there is increasing interest in carrier free immobilization since it manifests a very high catalytic activity by maximizing the volumetric productivity due to the absence of extra carrier [14]. Three different strategies are employed for the production of carrier free cross-linked biocatalysts of which the preparation of cross-linked enzyme aggregates (CLEA) offers many advantages [15]. Numerous carrier free immobilization procedures have been retrospected by Cao et al. [16]. Lipases from *Candida rugosa*, *Penicillin acylase*, *Thermomyces lanuginosa*, *Candida Antarctica*, *Pseudomonas cepacia* have been used for the preparation of the CLEA [17].

Considering the advantages of carrier free immobilization of bioimprinted lipase enzyme, an attempt has been made to immobilize bioimprinted commercial CRL. As already described, several researchers reported good performance of bioimprinted lipases in non-aqueous environment. However, reports on the successful use of bioimprinted enzyme in aqueous environment are rather scarce. Thus, an effort was made in the present work to maintain the imprinted characteristics of the CRL in an aqueous milieu by cross-linking the CRL with glutaraldehyde in the presence of co-aggregates, to produce an enzyme for the use in aqueous systems by laying focus on the positive aspects of both bioimprinting and immobilization. The present work comprises two parts. The objective of the first part was to optimize the preparation of cross-linked enzyme aggregates (CLEA) along with its characterization. Second part focuses on the optimization of parameters to enhance degree of hydrolysis (DOH) so as to get *n*-3 PUFA enriched oil. The performance of CRL-CLEA was studied and compared with free-CRL. Finally, the reusability of CRL-CLEA was also investigated.

2. Materials and methods

2.1. Materials

Crude fish oil procured from Mukka Fish Oil Industries Ltd. (Mangaluru, India) was refined according to the method followed by Charanyaa et al. [1]. *Candida rugosa* lipase (CRL) (≥700 unit/mg solid) was purchased from Sigma Aldrich, India. isopropanol, *n*-hexane, ethanol, diethyl ether, sodium hydroxide, hydrochloric acid, boron trifluoride in methanol (10%), ammonium sulphate, acetone and phenolphthalein indicator (analytical grade) were purchased from Merck, India and used without further purification. Bovine serum albumin (BSA), polyethyleneimine (PEI), glutaraldehyde and oleic acid (OA), sodium borohydride were purchased from Himedia, India, Sigma Aldrich, India, Merck, India, and Spectrum, India, respectively. Solvents like isopropanol and acetonitrile of chromatographic grade were purchased from Merck, India and were used for chromatographic analysis without further purification.

2.2. Methods

2.2.1. Preparation of bioimprinted cross-linked CRL aggregates

Bioimprinting of CRL with OA was carried out as per Kahveci and Xu [18] with slight modifications. 75 mg of CRL was mixed with 0.5 mL of 0.1 M of phosphate buffer (pH 7). The imprint molecules like OA (0.5 mmol) were dissolved in 1 mL ethanol and 100 mg of tween 60 and was then added to the enzyme solution for imprinting and stirred for half an hour. The cross-linked aggregate of this bioimprinted enzyme was prepared by the method described by Vaidya et al. [19] with minor modifications. Commercial CRL (75 mg) was bioimprinted by incubating 820 U of CRL (200 µL) with varying quantities of OA and incubated at 18 °C for 30 min with continuous stirring for half an hour [11]. To this enzyme mixture, BSA was added and stirred for an hour by incubating the mixture at 18 °C, 300 rpm. Polyethyleneimine (PEI), a well-known co-aggregator of enzymes was added (5% of 25 mg/mL) and stirred for an hour. Then glutaraldehyde (40–400 µL; 25%, v/v), which is the cross-linking agent was added and stirred for an hour. 1 mL (100 mM) of sodium borohydride was added to this enzyme mixture and allowed to react for 15 min to remove the schiffs bases that might have formed during the course of cross-linking. Thereafter, the mixture was centrifuged at 3600 x g for 15 min to separate cross-linked enzyme aggregate pellets. The pellet obtained was washed thrice with distilled water and the product obtained was lyophilized for 16 h at −30 °C to get CRL-CLEA. A flow chart of the steps involved in the preparation of CRL-CLEA is shown in Fig. 1.

2.2.2. Optimization studies

Indian Sardine oil was refined as reported by Charanyaa et al. [1] and the obtained high purity oil (Table 1) was taken for optimization of parameters for enzyme catalyzed hydrolysis. Different reaction parameters such as CRL-OA volume ratio (1:1, 1:2, 1:3, 1:4, 1:5), CRL-BSA volume ratio (1:1, 1:2, 1:3, 1:4, 1:5), CRL-PEI volume ratio (1:1, 1:2, 1:3, 1:4, 1:5), glutaraldehyde quantity (40, 100, 160, 220, 280, 340, 400 µL) and cross-linking time (15, 30, 45, 60, 120, 180 min) were optimized by one factor at a time approach. Two assessment parameters, activity recovery (Eq. (1)) and aggregate yield (Eq. (2)) were considered in arriving at an optimum condition for the production of CRL-CLEA, as described by Vaidya et al. [19].

$$Activity\ \ recovery = \frac{A_{CLEA}}{A_{Free} \times V_{Free}} \times 100 \tag{1}$$

$$Aggregate\ \ yield = \left(100 - \left[\frac{A_{Residual} \times V_{Residual}}{A_{Free} \times V_{Free}}\right]\right) \times 100 \tag{2}$$

where A_{CLEA} is the activity as depicted by CRL-CLEA; A_{Free} is the activity of free-CRL; V_{Free} is the volume (mL) of the free-CRL involved in the preparation of CRL-CLEA; $A_{Residual}$ is the activity of the residual CRL supernatant; $V_{Residual}$ is the volume (mL) of the residual CRL remained after the formation of the CRL-CLEA. The experiments were performed in duplicates and the mean values of the results were presented.

Activity recovery of CRL-CLEA was calculated as the ratio of activity of CLEAs to the activity of free-CRL. The conditions of the activity recovery and aggregate yield measurements like time and the enzyme loads were prudently selected to assure linear activity responses [20,21]. The precipitable protein content (PP) which was determined by the co-aggregation using polyethyleneimine (PEI) was used for calculating the activity of the free-CRL. The amount of PP in CRL-CLEA was determined by mass balance by considering the amount of PP lost

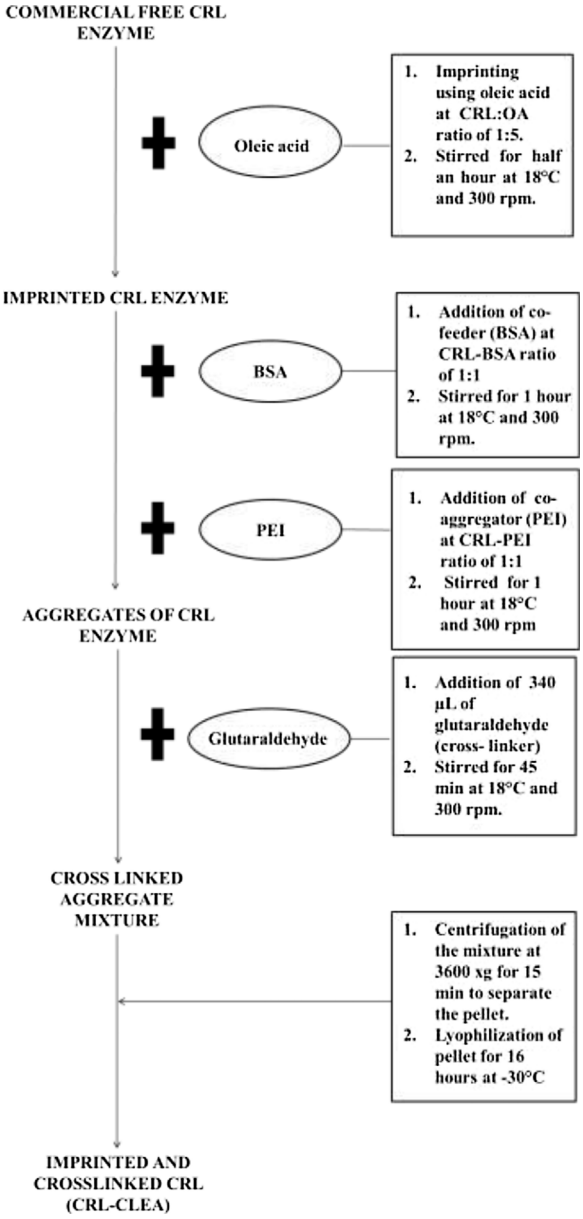


Fig. 1. A schematic representation of the preparation of bioimprinted and cross-linked CRL aggregates.

Table 1
Composition of refined Indian Sardine oil.

Parameters	Values
Phospholipid (ppm)	5.66
Iron (ppm)	BDL[a]
Copper (ppm)	0.1
Mercury (ppm)	BDL[a]
Fatty acids (% w/w)	
$C_{14:0}$	26.18
$C_{16:0}$	46.87
$C_{18:1}$	5.06
$C_{18:2}$	5.76
$C_{20:5}$ (EPA)	11.81
$C_{22:6}$ (DHA)	6.1

[a] BDL-Below detection limit.

in the supernatant and washings. The values of the activity recovery and aggregate yields were calculated using the formulae as represented in Eqs. (1) and (2).

2.2.3. Determination of lipase activity

CRL activity was determined by performing controlled hydrolysis of tributryin substrate by mixing 1:1 (w/w) ratio of tributyrin to water. The buffered enzyme solution was then added to the emulsion for 15 min and the hydrolyzed substrate was analyzed for the amount of FFA of tributyrin released by titrating hydrolyzed tributyrin against 0.1N potassium hydroxide (KOH). The end point (pink color) was observed by adding phenolphthalein solution as the indicator. One unit of enzyme activity (U) was expressed as the amount of CRL required to liberate one µmole of FFA of tributyrin per min at 30 °C.

2.3. Comparison of CRL-CLEA and free-CRL

2.3.1. Structural characterization by scanning electron microscopy (SEM)

The morphology of CRL-CLEA during different stages of reusability studies was obtained by studying the micrograph on JEOL JCPDS (Japan) operated under an accelerating voltage of 20 kV at a magnification of 2500×. The enzyme samples were lyophilized and coated with gold before being scanned under vacuum. The SEM images were captured in low vacuum and processed using ImageJ software.

2.3.2. Optimization of sardine oil hydrolysis using free-CRL and CRL-CLEA

The reaction system containing 1 g of refined sardine oil and 1 g of water (1:1 w/w) was taken and homogenized using hand held continuous homogenizer (IKA T18 basic, Ultra- Turrax) for 15 min. It was then mixed with lyophilized CRL-CLEA and free-CRL incubated at 18 °C with a constant stirring on a magnetic stirrer at 300 rpm to start the hydrolysis reaction. The DOH of free-CRL and CRL-CLEA was determined after the incubation period and were compared. Various parameters like pH (6, 6.5, 7, 7.5, 8), temperature (30 °C, 40 °C, 50 °C, 60 °C), enzyme load (2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 10 mg/mL, 12 mg/mL) and oil-water ratio (1:1, 1:2, 1:3, 1:4, 1:5) were optimized by changing one parameter at a time, while keeping all the other parameters constant.

The DOH was determined by measuring the acid value of both hydrolyzed and unhydrolyzed oils as well as the saponification value of the unhydrolyzed oil according to the Eq. (3).

$$\text{Degree of hydrolysis } (DOH) = \left(\frac{AV_t - AV_0}{SV_0 - AV_0}\right) \times 100 \quad (3)$$

where SV_0 (mg of KOH/g of oil) and AV_0 (mg of KOH/g of oil) are the saponification value and the acid values of refined sardine oil before hydrolysis, respectively, and AV_t (mg of KOH/g of oil) is the acid value of the hydrolyzed oil at the optimized reaction time.

The hydrolysis was performed under the optimized reaction conditions of free-CRL and CRL-CLEA for 15 min, after which the reaction was stopped by adding 15 mL of methanol. The hydrolyzed oil was subjected to methanol extraction, to eliminate the FFA released during the process. The solvent extracted oil (hydrolyzed) was studied for its fatty acid composition using GC which was compared with the fatty acid composition of the refined oil (unhydrolyzed). The experiment was done under vacuum conditions in order to prevent oxidation of the oil. The acid value of oil was calculated according to the official method of American Oil Chemist's Society (AOCS) methodologies [22], (Cd3d-63).

2.3.3. Analysis of hydrolyzed oil

After enzymatic treatment, 15 mL of methanol was added and the aqueous methanol solution containing enzyme and FFA was discarded. The oil was subjected to methanol extraction to remove released FFA [1]. After the solvent traces were evaporated and dried as per the procedure mentioned by Ichihara and Fukubayashi [23], the residue was esterified with borontrifluoride methanol agents and analyzed by GC (Trace 3330 GC Ultra, Thermo Electron Corporation) equipped with a flame ionization detector (FID), split/splitless injector and DB-5 column (30 m x 0.25 mm x 0.2 µm). The GC conditions were maintained

as per the procedure followed by Charanyaa et al. [1]. Chrome Cad software was used for the analysis of the chromatograms.

Triglycerides, diglycerides and monoglycerides present in deacidified hydrolyzed oil was estimated by HPLC. The oil sample (8 μL) was dissolved in 4 mL of isopropanol (chromatographic grade) and analyzed by HPLC with a pump (LC-20AD, Shimadzu Co, Kyoto, Japan) in the presence of RPC18 column using ELSD (Gilson). The sample was analyzed by using a gradient program in accordance to the method followed by Aoki et al. [24] employing a mixture of isopropanol and acetonitrile mixture as the elution systems. The flow rate and the column temperature were 0.5 mL/min and 30 °C, respectively. The evaporation temperature, nebulizer temperature and gas flow rate of ELS detector were 110 °C, 80 °C and 1 mL/min, respectively.

The EPA and DHA content of deacidified hydrolyzed oil was estimated by LC–MS (Shimadzu Co, Japan). The samples for LC–MS were prepared similar to the method adopted for the preparation of samples for HPLC, and injected into the system with a pump (LC-20AD), UV/Vis detector (SPD–20 A), column oven (CTO-10 AS) and MS fitted with an electron spray ionization source (ESI) by full scan acquisition. The conditions of column, mobile phase, and flow rate and column temperature were the same as the conditions used for studying the contents of the refined and hydrolyzed fish oil in HPLC. The detection wavelength of PDA used was 250 nm. The ESI conditions in desolvation line temperature, heat block temperature, detector voltage, drying gas flow rate, interface current and voltage were 250 °C, 200 °C, 1 kV, 15 L/min, 0.2 μA, 4.5 kV, respectively. The analysis of the oil was made by studying both the positive and negative ion mode over the range of 100–1000 *m/z*.

2.4. Reusability of CRL-CLEA

To assess the reusability of CRL-CLEA, the enzyme was used repeatedly for multiple batches under optimal conditions of hydrolysis. After the specified optimal reaction time, the enzyme was separated by centrifugation, washed with distilled water twice and then added again to the fresh reaction mixture. The DOH was estimated after each cycle and DOH estimated in the first cycle was considered as 100%.

3. Results and discussions

3.1. Optimization of bioimprinted, carrier free cross-linked enzyme aggregate preparation

3.1.1. Effect of CRL: OA ratio

Among the different ratios of CRL:OA considered (1:1, 1:2, 1:3, 1:4, 1:5 v/v), 1:5 ratio showed the highest activity recovery and aggregate yield of 33.8% and 71.4%, respectively (Fig. 2A). Hence this was chosen as the optimal value and was used for all the further experiments. Interestingly, in the absence of OA poor values of activity recovery and aggregate yields (10.8% and 22.3%) were obtained. These results highlighted that CRL might have a high selectivity towards OA [13]. The advantage of using OA as the imprint molecule is that it is a liquid at the room temperature, which on mixing with ethanol and tween 60 could have got imprinted on the enzyme and increased the DOH. This could be due to the conformational changes in the site of CRL which matches the substrate molecule. To assess the effect of individual component of the reaction mixture and their combinations, DOH of CRL imprinted with ethanol, Tween 60, OA-Tween 60, OA-ethanol and Tween 60-ethanol was studied. DOH exhibited by OA-ethanol-Tween 60 was much higher than all other combinations (Supplementary Table S1). These results concur with the findings of Yan et al. [13], where they had reported imprinting with *Geotrichum* sp. lipase with OA in the presence of methanol and *tert*-butyl alcohol. As tunnel like conformation present in CRL is sterically suitable for OA [25], it can be concluded that OA could successfully imprint the CRL in the presence of ethanol and Tween 60. Foresti et al. [26] had confirmed the bioimprinting of CRL with OA for esterification of OA and ethanol.

3.1.2. Effect of CRL: BSA ratio

CRL: BSA ratio was found to be an important factor influencing activity recovery and the aggregate yields (Fig. 2B). With the increase in the CRL: BSA ratios, the activity recovery and the aggregate yields reduced drastically. It was demonstrated that the maximal activity recovery and aggregate yields (36.2% and 72.6%) were achieved at the ratio of 1:1. Any further decrease in the ratio (increase in the BSA concentration) led to the decrease in the activity recovery, possibly due to the formation of clusters with mass transfer limitations. On the contrary, only 7.1% of the activity was recovered in the absence of added BSA. Addition of BSA is known to facilitate CLEA preparation in cases in which the protein concentration of the enzyme preparation is low. The cross-linking agent, glutaraldehyde reacts with amino groups of amino acid residues such as lysine [27]. Interestingly, the CRL is found to contain a significant number of amino groups containing amino acid residues [26] in the active site. Glutardehyde might have reacted with them, resulting in the reduction of activity of the cross-linked enzyme [27,28]. Perhaps in the presence of BSA, which has a very high number of lysine residues on the surface, glutaraldehyde reacts with them shielding CRL.

3.1.3. Effect of CRL: PEI ratio

Trials were performed to screen two precipitants (ammonium sulphate and acetone) and one co-aggregator (PEI), for the preparation of CLEA from free-CRL. Precipitation with ammonium sulphate and acetone exhibited low activity recovery (data not shown), which was disagreeing with the results obtained with *Pseudomonas* sp. lipase by Zhao et al. [29]. Hence, experiments with precipitants were discontinued any further. However, co-aggregation with PEI, gave the highest activity recovery and aggregate yield. Further, CRL: PEI ratio of 1:1 showed the highest activity recovery and aggregate yield (Fig. 2C). The increase in quantity of PEI for cross-linking resulted in the decreased activity recovery. These results substantiate with the previous findings by Vaidya et al. [19] where 1:1 ratio of aminoacyclase co-aggregated with PEI showed pronounced activity recovery. A similar trend was observed during the development of CLEA from *Geotrichum* sp. lipase [30]. PEI is a water soluble, cationic polymer possessing high density of ionized amino groups. Perhaps, this high density of cationic groups in PEI may permit the strong ionic exchange between PEI and anionic groups on CRL and BSA aggregates and thus stabilize the protein. Simultaneously, it also participated in the cross-linking reaction with glutaraldehyde resulting in the increased extent of cross-linking [19,31].

3.1.4. Effect of amount of glutaraldehyde

It is already well established that the extent of cross-linking depends upon both glutaraldehyde concentration and cross-linking time [14]. Moreover, these two factors also depend upon the availability of free amino groups of lysine residues on the enzyme and availability of co-aggregators. Thus, it becomes necessary to determine optimum concentration of glutaraldehyde for CRL in the presence of co-aggregators (BSA and PEI). As is evident from Fig. 2D, the activity recovery and the aggregate yields of the enzyme increased with the increase in the amount of glutaraldehyde. Use of 340 μL of glutaraldehyde for cross-linking gave the highest activity recovery and aggregate yield. To get an insight on the enzyme immobilization, lipase activity of the supernatant (after separation of cross-linked enzyme aggregate) was determined. Significant lipase activity was detected in the supernatant when the amount of glutaraldehyde used was less than the optimum (340 μL), signifying insufficient cross-linking at lower amounts of cross-linker. Also, negligible enzyme activity was found when glutaraldehyde used was higher than 340 μL. Moreover, activity recovery also reduced drastically, which may be due to the reduction in the flexibility of the enzyme required for its activity due to excessive cross-linking [32,33].

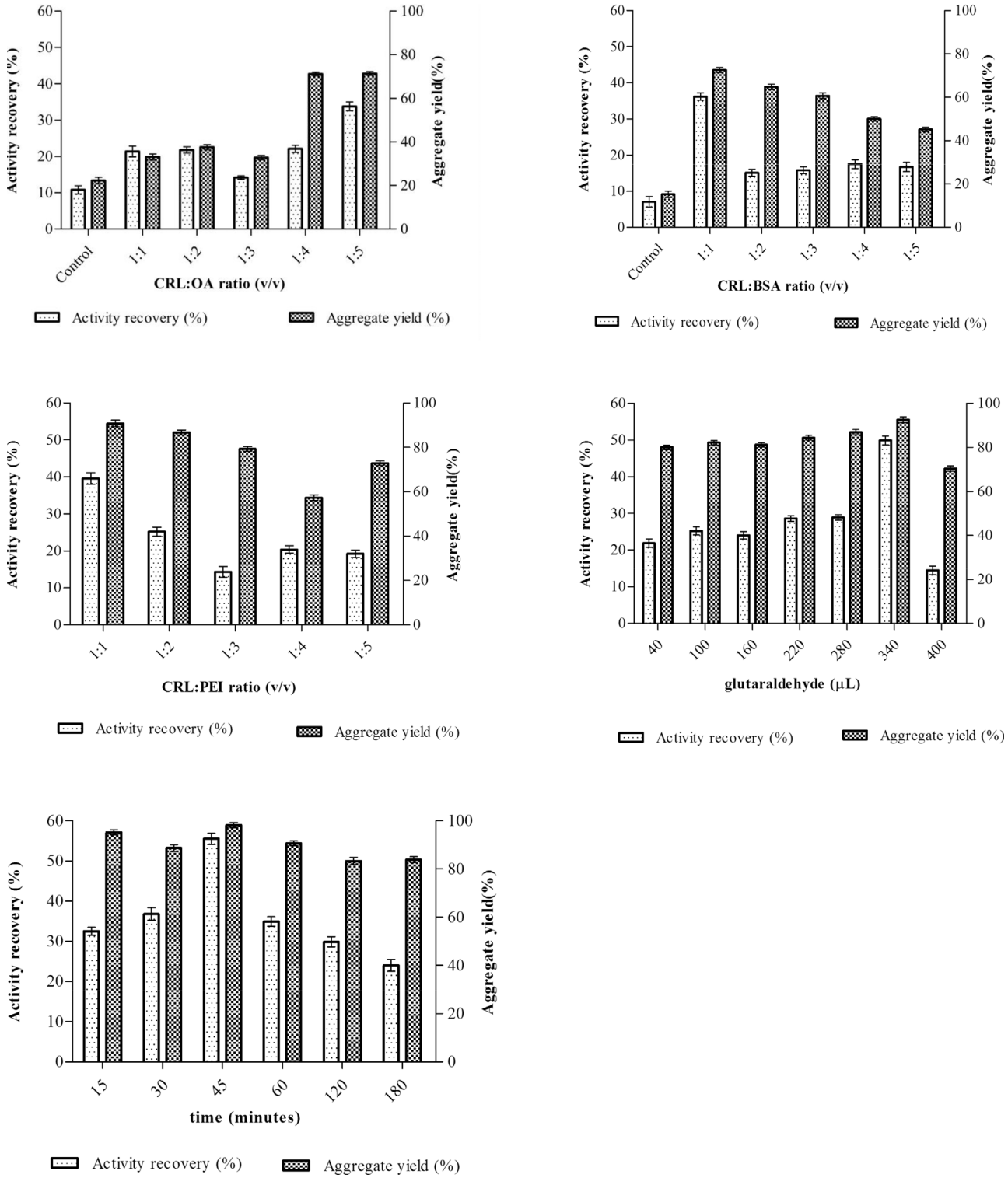


Fig. 2. Optimization of process parameters on the extent of cross-linking of CRL. (A). Effect of CRL:OA ratio; (B) Effect of CRL:BSA ratio; (C) Effect of CRL:PEI ratio; (D) Effect of amount of glutaraldehyde; (E) Effect of cross-linking time.

These results seem to be in agreement with the previous findings of Kartal et al. [34] and Zhou et al. [5].

3.1.5. Effect of time on cross-linking

Since cross-linking is a time dependent reaction, the time required to achieve maximum activity recovery of the enzyme during the process becomes vital. The experiments were performed with the optimized amounts of PEI and glutaraldehyde. It should be noted that the initial cross-linking experiments performed till this stage was for 30 min. Now, the effect of cross-linking time was studied between 15 and 180 min (Fig. 2E). As can be seen from the graph, the maximum activity recovery was achieved in 45 min of cross-linking beyond which there was a gradual reduction in the values. These findings seem to be in consonance with the previous reports by Vaidya et al. [19] and Talekar et al. [35] which stated that the increase in the cross-linking time resulted in the increase in the activity recovery of the obtained CLEA until

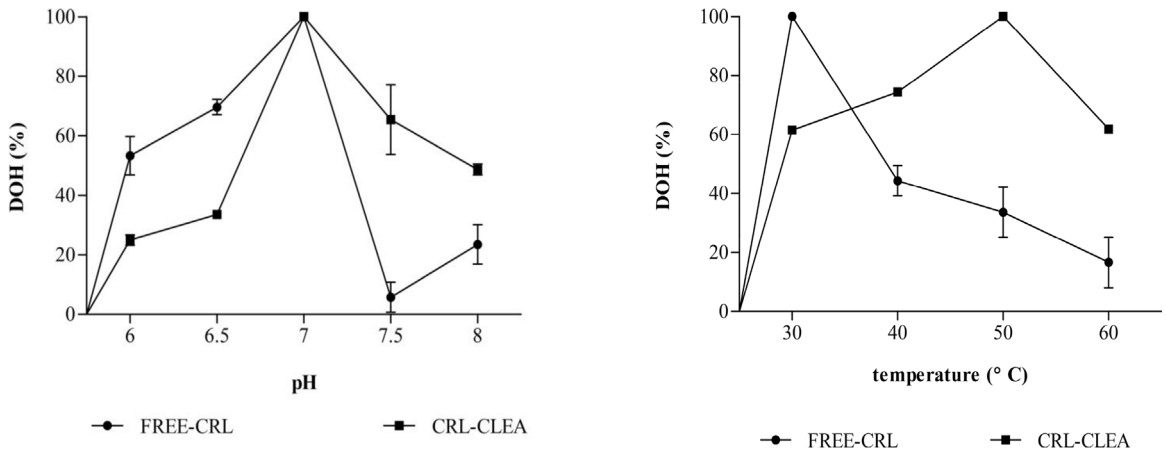


Fig. 3. Influence of (A) pH and (B) temperature on DOH of free-CRL and CRL-CLEA. In case of influence of pH, the DOH corresponds to 30.94% for CRL-CLEA and 3.35% for free-CRL was taken as 100%. In case of influence of temperature, the DOH corresponds to 32.87% for CRL-CLEA and 3.35% for free-CRL was taken as 100%.

a particular point. After 45 min, there was a reduction in the activity recovery due to the loss of flexibility of the enzyme because of intensive cross-linking by the increased duration of contact of glutaraldehyde with CRL [14].

3.2. Hydrolysis of sardine oil using free-CRL and CRL-CLEA

The refined Indian Sardine oil having 17.91% (w/w) of *n*-3 PUFA (Table 1) was taken for enzymatic hydrolysis to enhance *n*-3 PUFA content. Both free-CRL and CRL-CLEA prepared under optimized condition, was used and compared. Important process parameters were studied for both the enzymes independently, and the DOH was evaluated to find the optimal conditions.

3.2.1. Effect of pH and temperature on hydrolytic activity of the enzymes

The results of the effect of pH on hydrolysis of oil using free-CRL and CRL-CLEA are shown in Fig. 3(A). The optimum pH was found with 6 mg/mL of free-CRL and CRL-CLEA at 1:1 oil to water ratio at 30 °C. The optimum pH for both free-CRL and CRL-CLEA was found to be 7, which is conflicting with the published reports [33,35] which in general state that immobilization by cross-linking leads to the shifting of pH to a higher range due to the intense cross-linking of the amino groups of surface amino acid residues on the enzyme. However, in the present case, the optimum pH remained the same in spite of the cross-linking with glutaraldehyde. Perhaps this could be due to the presence of co-aggregates, BSA and PEI. It is known that shift in optimum pH is the result of the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site, which was caused by the newly formed interactions between basic residues of enzyme and glutaraldehyde during cross-linking [36]. It is not unreasonable to say that participation of BSA and PEI in cross-linking reaction with glutaraldehyde and their presence might have influenced the microenvironment of the enzyme thereby negating the effect of side chain ionization due to pH.

The thermal stability of the free-CRL and CRL-CLEA were assessed by incubating both the enzymes in buffer (pH 7) at different temperatures. The profiles of DOH of free-CRL and CRL-CLEA at different temperatures were represented in Fig. 3(B). At 40 °C, the free-CRL retained only 43.1% of activity, while CRL-CLEA retained 74.35% of its initial activity. Similarly, at 60 °C, free-CRL could retain only 16.92%, whereas CRL-CLEA retained 61.88% of activity. This data proves that cross-linking with BSA and PEI conferred good thermal stability to the enzyme. These results are in accordance with the findings of Yan et al. [30] and Vaidya et al. [19] which proves the efficiency of PEI as a precipitating agent.

Further, thermal deactivation kinetics of the free-CRL and CRL-CLEA was studied. The deactivation energy of the prepared CRL and free-CRL was found to be 80.97 KJ/mol.K and 55.93 KJ/mol.K, respectively (data not shown) indicating 1.45 fold increase in deactivation energy after the modification of CRL. From this it can be inferred that, more number of stabilizing linkages are formed between the enzyme molecules due to which increased amounts of energy must be put into the system to disrupt these linkages. These results are in agreement with the findings of Vaidya et al. [19], who experimented with L-aminoacylase.

3.2.2. Effect of enzyme load on DOH

A reaction mixture containing 1 g of refined sardine oil, 1 g of water and various concentrations of free-CRL/CRL-CLEA was stirred at 30 °C/50 °C for 15 min at pH 7.0, to examine the effect of enzyme load on the hydrolysis. Highest DOH was achieved when 6 mg/mL of the free-CRL (200U) and CRL-CLEA (1000U) was added (Fig. 4(A)). It was observed that further addition of lipase did not increase the DOH. It could be reasoned out that beyond 6 mg/mL, the oil-lipase solution formed under these conditions gets saturated with lipase along with the formation of monolayer and accumulated intermediates [37]. As can be seen from Fig. 3(A), although the reduction in the DOH was witnessed by both the forms of lipase at higher enzyme concentrations, the reduction of DOH by CRL-CLEA was not as drastic as the free-CRL. This could be explained by the fact that particles of CRL-CLEA did not agglomerate and hence facilitated the distribution of the lipase in the reaction medium [10].

3.2.3. Effect of amount of water on the hydrolysis reaction

Since lipases are dispersed in water during hydrolysis and water is utilized for hydrolysis reaction, it becomes essential to study the effect of water on hydrolysis. From Fig. 4(B), it is evident that the DOH was highest at the oil to water ratio of 1:1 (w/w) for both free-CRL and CRL-CLEA. Further increase in water to oil ratio, progressively decreased the DOH. This could be due to the increased micelle formation as water content increases. As it is evident from various studies, lipase positions itself at the oil-water interface to catalyze hydrolysis. With the increase in the number of micelles and micelle volume, increased number of enzyme molecules is taken away from the oil-water interface due to which a drastic reduction in DOH is observed.

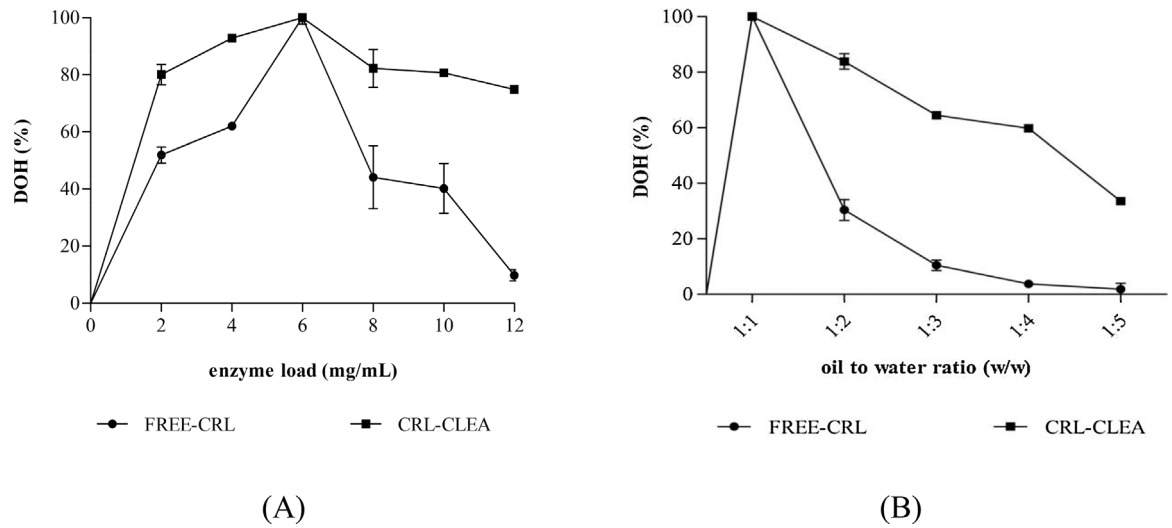
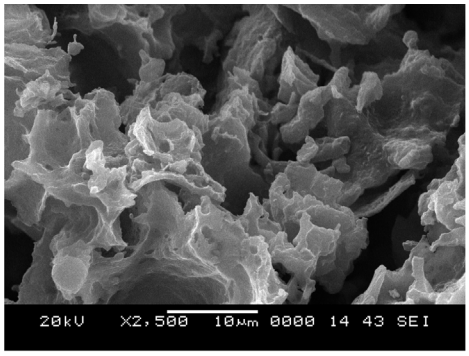


Fig. 4. Influence of (A) enzyme load and (B) amount of water on DOH of free-CRL and CRL-CLEA. In case of influence of enzyme load, the DOH which corresponds to 35.73% for CRL-CLEA and 3.4% for free-CRL was taken as 100%. In case of water quantity in the system, the DOH corresponds to 35.97% for CRL-CLEA and 3.45% for free-CRL was taken as 100%.

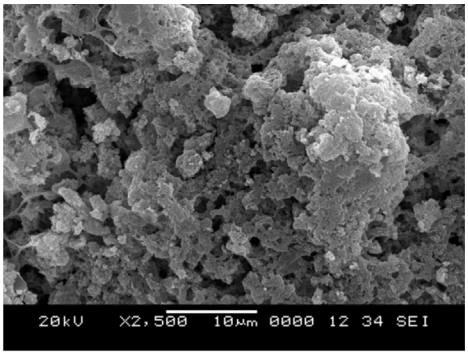
3.3. n-3 PUFA glycerides enrichment using free-CRL and CRL-CLEA

Refined Indian Sardine oil was subjected to hydrolysis for 15 min under optimal conditions (1 g of oil, 1 g of water and 6 mg/mL of both free-CRL/CRL-CLEA incubated at 30 °C/50 °C with a constant stirring at 300 rpm and pH 7.0) and the resulting FFA free oil was assessed for *n*-3 PUFA content. Under optimal conditions, free-CRL which gave 3.45% DOH, showed 1.54 and 1.39-fold enhancement in EPA and DHA, respectively (18.16%w/w EPA and 8.5% w/w DHA). Whereas, CRL-CLEA, exhibited 35.97% DOH, showed 3.87-fold increase in EPA content (45.75% w/w). However, a slight decrease in DHA content (6.1% to 5.04% w/w) was witnessed. This shows CRL-CLEA hydrolyzed ester bonds of small amounts of DHA. These results concur with the observations made by Halldorsson et al. [38] while studying fatty acid selectivity of various lipases.

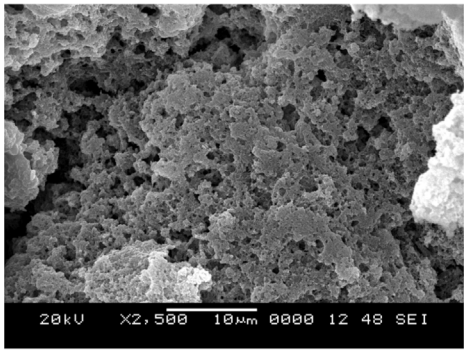
Numerous reports are available describing the application of commercially available lipases to concentrate EPA and DHA from marine oils. They reveal that the commercially available lipases discriminate against *n*-3 PUFA, and that the lipases, which display any significant activity toward *n*-3 fatty acids usually, prefer EPA to DHA as substrate. The reason is discussed to be due to the close proximity of the carbon–carbon double bond to the carboxyl group being located one bond



A



B



C

Fig. 5. Scanning electron micrograph of (A) CRL-CLEA produced from free-CRL (B) CRL-CLEA after five cycles of use (Squeezed CRL-CLEA) and (C) image of CRL-CLEA after seven cycles of use (Squeezed CRL-CLEA).

closer in DHA. This presumably adds strain on the active site of the lipase to accommodate DHA properly [39,40]. Lipase fatty acid selectivity may be due to steric hindrance resulting from the chain length, whereas DHA is 2 carbons longer than EPA. However, this is not true for all lipases, e.g. the bacterial lipases from *Pseudomonas* are known to display higher activity toward DHA than EPA, despite DHA being theoretically the less favourable substrate [41].

In the current study, use of 200 U of free-CRL resulted in the enhancement of *n*-3 PUFA to 26.67% from the initial 17.91%. On the contrary, Okada and Morrisey [9] had reported *n*-3 PUFA content of 63.86% from an initial 26.86% after 6 h of hydrolysis of Pacific Sardine oil with 250 U of free-CRL. Whereas, treatment with 1000 U CRL-CLEA enhanced the *n*-3 PUFA content to 50.79% in the current study. From this it is evident, enzyme aggregates showed better performance compared to free-CRL. However, the extent of *n*-3 PUFA enrichment obtained in the current study seems to be less compared to the published reports. Yan et al. [30] has reported improved performance of *Geotrichum* lipase after preparing CLEA with PEI. The DOH increased to 42% from the initial 12%. This result seems to substantiate well with the present study. Similar studies conducted by Yan et al. [10] on the enrichment of *n*-3 PUFA using cross-linked imprinted lipase with 960 U, witnessed an enrichment of 41% from 22% *n*-3 PUFA in the original oil after 8 h of hydrolysis. In our study, enrichment of 50.79% (CRL-CLEA hydrolyzed oil) from 17.91% (unhydrolyzed oil) was evinced in 15 min of reaction time. The method adopted in the present work resulted in 2.84 times enhancement of *n*-3 PUFA and thus can be concluded that the suggested method can be used for the application of hydrolysis of sardine oil. From the discussions given above, it is evident that the differences in the DOH and the enrichment in *n*-3 PUFA content vary. These variations depend upon fatty acid content of the oil, which in turn depends on the sources and also the lipases or modifications of the lipases used.

The HPLC analysis of FFA-free (deacidified) hydrolyzed oil obtained after free-CRL and CRL-CLEA treatment, revealed considerable differences in the glyceride profile (Fig. 6). In comparison to unhydrolized oil, hydrolyzed oil contained pronounced quantities of monoglycerides and a drastic reduction in the diglycerides and the triglycerides was evidenced. The HPLC analyses corroborated the findings of GC analyses and shows a more uniform distribution of *n*-3 PUFA in the form of monoglycerdies in the oil. These results bear similarities with the results of Okada and Morrissey [9].

Further profiling of the oil was performed by LC–MS with the purpose of confirming detailed lipid information on the glyceride clusters shown as peaks in the HPLC chromatograms. By extracting the mass of EPA and DHA in association with the various fatty acids based on the GC report obtained, the lipid composition of different hydrolyzed samples was established. It was obtained from the LC–MS reports that free-CRL resulted in a hydrolyzed oil containing large quantities of DHA (Figure.S1) and palmitic acid (PA) (Fig. S2) in the form of monoglycerides. These results concur with the findings of Aziz et al. [42]. LC–MS data also revealed the presence of DHA as diglycerides along with myristic acid (MA) and OA in the glycerol backbone (Fig. S3). Although, EPA was not present as monoglyceride, it was in the form of diglyceride with myristic acid (EPA-MA) and palmitic acid (EPA-PA) (Fig. S4). This could be attributed to the abundance of PA and MA in the sardine oil (Table 1). Likewise, LC–MS data analysis of the hydrolyzed oil using CRL-CLEA revealed the presence of large quantities of monoglycerides of EPA (Fig. S5) and PA (Fig. S6). The following decreasing order of diglycerides was observed in the hydrolyzed oil, EPA–EPA > EPA-PA > EPA-MA > DHA-MA. This result was seen to be in agreement with the GC results which revealed a huge enhancement of EPA (45.75%) by CRL-CLEA.

3.4. Reusability of CRL-CLEA

After every run of hydrolysis, CRL-CLEA was separated from the reaction mixture by centrifugation, washed with distilled water twice, dried at room temperature and used again for the next cycle. As shown in Fig. 7, the% DOH of CRL-CLEA remained consistent in the first two cycles, after which there was a gradual reduction in the ability of CRL-

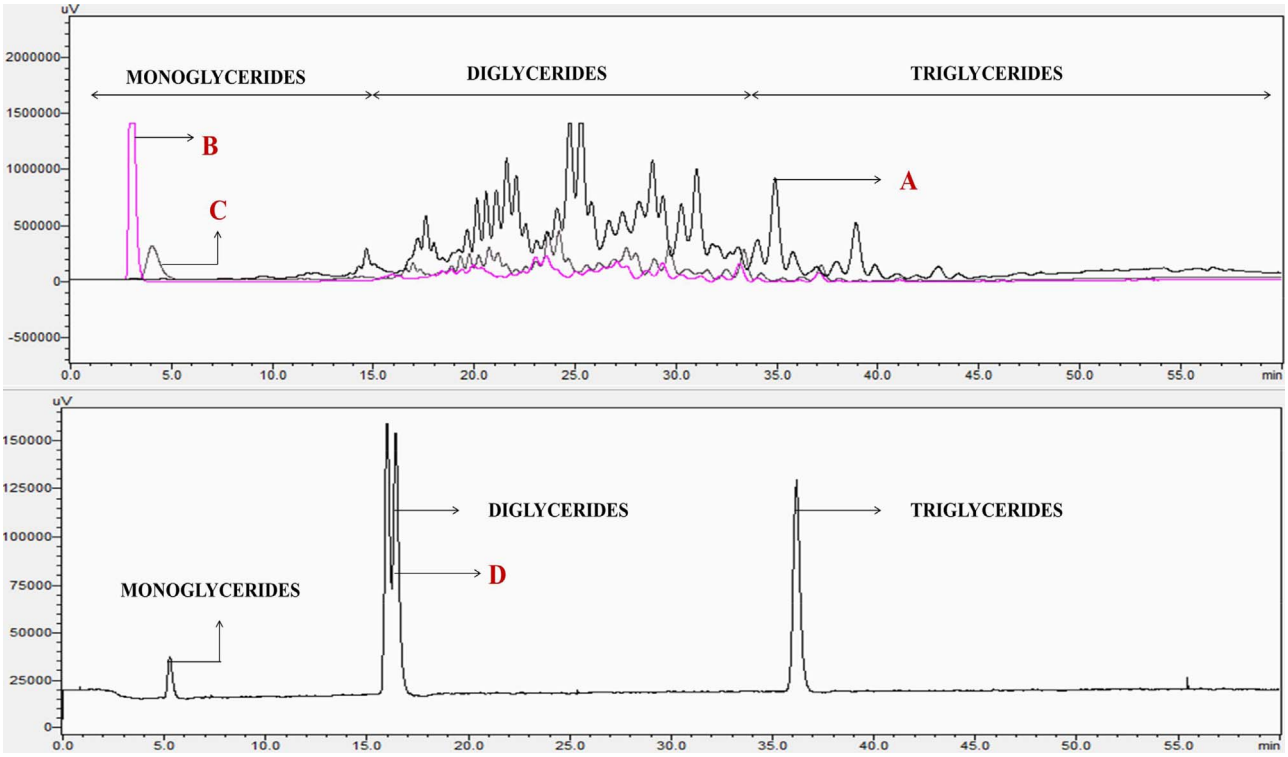


Fig. 6. The comparison of chromatograms of (A) Refined oil, (B) Hydrolyzed oil using CRL-CLEA, (C) Hydrolyzed oil using free-CRL and (D) HPLC glyceride standards using ELSD. Mobile phase: acetonitrile/2- propanol (60:40,v/v), flow rate: 0.5 mL/min, Column: RP-C18.

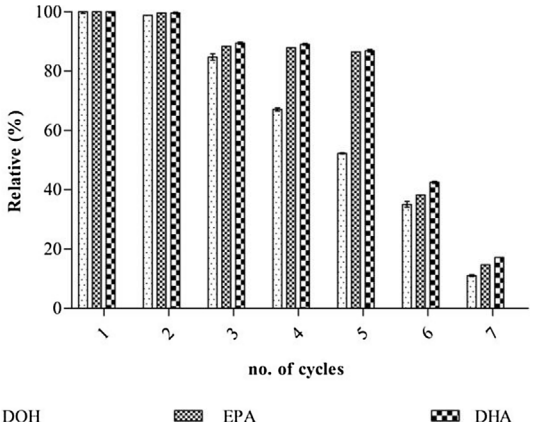


Fig. 7. Reusability of CRL-CLEA. 32.87% DOH, 46.75% EPA and 5.04% DHA achieved in the first run is taken as 100% and the performance of the remaining runs were compared.

CLEA to hydrolyze the oil. 49% of the reduction in DOH was observed from the first cycle to the fourth cycle. Despite the reduction in the hydrolyzing ability of the enzyme over 7 cycles, it was evinced that the EPA and DHA content showed a different pattern of reduction compared to the pattern of hydrolysis. As is clear from the figure, there was a gradual reduction of EPA and DHA from the second cycle to the third which remained almost consistent until the 5th cycle after which there was a sudden reduction as opposed to the trend of DOH. This could imply that the hydrolysis by CRL-CLEA released free forms of fatty acids other than EPA and DHA, which is the most striking result emerging from this data. Similar results were obtained by Xei and Zhang [43,44] and Xei and Wang [45] using CRL enzyme for the interesterification of lard and soybean oil. The lipase was stable with repeated use for four cycles without any severe loss of its activity. It can be deduced from the graph that the reduction in% DOH of CRL-CLEA after the first five cycles may not be due to the leakage of the enzyme. The amount of EPA and DHA in the first five cycles can be a confirmation to the above inference made. SEM images of the fresh CRL-CLEA (Fig. 5A), after the fifth cycle (Fig. 5B) and seventh cycle (Fig. 5C) shows a distinct change in morphology of the CRL-CLEA. The distinct granular structure found in the fresh CRL-CLEA was getting transformed into less-distinct clusters. Perhaps, due to the continuous stirring and repeated washing with water, compaction of CLEA might have occurred [32,46]. Thus, decrease in the performance of the enzyme (DOH) could be because of the squeezing of the enzyme molecules which reduces the accessibility of substrates like oil through the CLEA [33,47]. Interestingly, the residual DOH exhibited by free-CRL was almost nil when used for the second time. Therefore, CRL enzyme subjected to cross-linking was more stable, withstanding various conditions much better than free-CRL.

4. Conclusions

Bioimprinting of commercial free-CRL with oleic acid in the presence of Tween 60 and ethanol, and immobilization with co-aggregates and cross-linking agent was found to have doughty catalytic ability of CRL, even in aqueous environment. This lipase was further employed as a biocatalyst for the hydrolysis of Indian Sardine oil. The prepared imprinted and immobilized lipase were characterized by SEM technique. The characterization results revealed type 2 catorgory of CLEAs. Under optimal conditions of hydrolysis, CRL-CLEA showed 10.4 times higher DOH and 2.83-fold increase in *n*-3 PUFA content as against free-CRL. The free-CRL was not reusable, whereas CRL-CLEA could be reused up to 5 runs without substantial reduction in its performance. Improvement in degree of hydrolysis, thermostability, efficiency of hydrolysis and reusability were achieved due to bioimprinting and subsequent immobilization of CRL in the form of CLEA. Perhaps, it is worth studying the performance of CRL-CLEA in non-aqueous environment for the synthesis of various industry relevant lipophilic esters.

Authors agreement

First author, Ms. S. Charanyaa is the PhD scholar working under second and third authors. All bench scale work has been done by her. Second and corresponding author, Dr. Prasanna Belur has guided the student and compiled the data and prepared the manuscript. Third author has also played a major role in the guidance as well as preparation of the manuscript. All the authors have vouched for the authenticity of results and concur with the submission. We hereby declare that work has not been published elsewhere, either completely, in part, or in another form and the manuscript has not been submitted to another journal and will not be published elsewhere.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.enzmictec.2017.12.003.

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