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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

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