Real-time assessment and characterization of immobilized lipase onto a natural matrix and qualitative reaction kinetic studies using swept-source optical coherence tomography

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Abstract

The current study presents a method based on Optical Coherence Tomography (OCT) for non-destructive, real-time analysis and portrayal of immobilization efficacy for lipase on a natural matrix namely, eggshell. Subsequently, qualitative biochemical reaction kinetics of immobilized lipase was also studied. Successful immobilization of lipase on eggshell was confirmed by the presence of a clear peak in 'A' scan of OCT image. From immobilization kinetics it is clearly observed that the thickness of the highest peak of the A-scan increases significantly and peak intensity saturated after 90 min of incubation. Hydrolysis of oil using immobilized lipase indicated that the release of free fatty acids increased up to 8 h during reaction and the result was in accordance with the 'B' scan data of the OCT system. Changes in scattering coefficient-based analysis were performed with respect to incubation time to showcase the immobilization process and hydrolysis reaction of lipase. Scanning electron microscope analysis with smoother surface indicated presence of lipase on eggshell matrices, with no further change after oil hydrolysis.

Keywords Lipase · Eggshell · Enzyme-immobilization · Swept-source optical coherence tomography (SSOCT) · Reaction kinetics · Scattering coefficients

Introduction

Triacylglycerol lipase (EC: 3.1.1.3) is one of the most important enzymes that catalyses oil into free fatty acid (FFA) and glycerol (Ribeiro et al. 2011). Industrial-scale production of lipase is primarily restricted to the sources such as bacteria, fungi or animal pancreas (Zhang et al. 2012; Yang et al. 2009; Kumari et al. 2009); whereas plant-based lipase (primarily from oilseeds, rice, wheat, maize etc.) are gaining importance nowadays because of their availability and higher yield (Seth et al. 2014). Lipases are mostly used in biodiesel production, pharmacy industry, cosmetics manufacturing, paper and pulp industry etc. (Paul et al. 2015; Chattopadhyay et al. 2011; Jaeger et al. 1999).

Several researchers reported that using immobilized lipase is the better alternative compared to free one due to its enhanced stability, reusability and cost-effectiveness (Yilmaz et al. 2011; Hernandez et al. 2011). Lipase immobilized on eggshell reported to be superior compared to other matrices, and its reusability, stability, and specificity were proved to be a better alternative than other matrices. It was already established that the immobilization of lipase on eggshell is purely an adsorption process without the formation of any covalent interactions (Chattopadhyay and Sen 2012).

In general, the microscopic technique like SEM (scanning electron microscopy) was used for surface characterization. SEM can easily observe the qualitative immobilization as a morphological change due to the absorption of lipase on the eggshell surface, which is a disparaging technique and requires critical effort in sample preparation whereas the optical coherence tomography (OCT) imaging technique gives the quantitative, real-time and non-destructive way to characterize the immobilization of lipase.

In this study, the physical characterization of lipase immobilization on eggshell was performed using newly developed non-destructive and non-contact Swept-source

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optical coherence tomography (SSOCT) system. The system is used to real-time monitoring and analysis of immobilization kinetics and oil hydrolysis. The present study will help to improve the specificity of the immobilized enzyme and help researchers to develop a new enzyme-based system for various industrial applications.

Description of the SSOCT system

The SSOCT system is built on Mach Zehnder interferometer as shown in the schematic diagram (Fig. 1). A broadband sweep source laser (AXSUN Tech, USA) having a central wavelength of 1060 nm (FWHM bandwidth of 110 nm) with a sweeping rate of 100 kHz used as a scanning light source. The source beam splits into two equal parts, through fiber-based 50:50 optical couplers. Here, fiber coupler C1 acts like an optical circulator and part of source light feedback to balance photodetector for coherence detection. Forward traveling light further splits into two parts between reference arm and sample arm, through another 50:50 fiber coupler C2. The reference arm consists of a collimator, a lens and a gold-coated mirror. On the other hand, the sample arm has a collimator, X–Y scanner (Cambridge Technologies) and an objective lens. The light beam scan over sample surface and backscattered light detected using confocal gating technique to achieve high noise immunity. An interference signal detected by a balanced photodetector having a clock speed of 400 MHz, through C3 fiber coupler. A data acquisition card (AlzarTech ATS9350, having 12-bit resolution and the sampling rate of 500 MS/s) used to acquire the data from the balanced photodetector also receives an A-line trigger and

k-clock signal from laser source and store in a workstation for further post-processing. A NI PCIe-6363 DAQ card is used as a function generator to control the scanner. Further processing of acquired signal performed using a custom-built LabVIEW post-processing program, which was discussed in our earlier work (Mohan et al. 2019; Mishra et al. 2019).

Materials and methods

Materials

Lipase from wheat germ was procured from Sigma-Aldrich (Cat no: L3001). The lyophilized powder was kept at 4 °C. Eggshells were collected from locality of BIT Mesra, Ranchi, India. The Bradford protein estimation kit purchased from Bangalore Genei Pvt. Ltd. (Bangalore, India). Tributyrin was obtained from Himedia (Mumbai, India). All other chemicals were procured from SRL (Mumbai, India) and were analytical grade.

Determination of enzyme activity

At first, enzyme solution of 10 mg/mL concentration was prepared by dissolving lyophilized lipase powder into phosphate buffer (50 mM, pH 7.4). Enzyme activity was determined by the method described earlier (Chattopadhyay and Sen, 2012). In brief, an emulsion of polyvinyl alcohol (2% w/v) and tributyrin (9:1) was prepared. The reaction mixture comprising 1 mL emulsion, 0.8 mL

Fig. 1 Schematic representation of swept-source optical coherence tomography system. *SS-laser* sweep source laser (central wavelength 1060 nm), *BPD* balance photodetector, *C1*, *C2*, *C3* 50:50 fiber cou-

pler, *CO* collimator, *L1* 50 mm Lens, *L2* 30 mm objective lens, *DAQ* data acquisition card

phosphate buffer (50 mM, pH 7.4) and 0.2 mL free enzyme solution (containing 2 mg enzyme) was then prepared and incubated at 37 °C for 1 h. The reaction was terminated by adding chilled acetone-ethanol (1:1) mixture and then titrated with 0.1 N sodium hydroxide solution using phenolphthalein as an indicator. Total protein was measured using the Bradford protein estimation method with bovine serum albumin (BSA) as the standard. Specific activity was expressed in terms of the unit of enzyme per mg protein.

Immobilization of lipase on eggshell

Details of lipase immobilization on eggshells were described elsewhere (Vemuri et al. 1998; Chattopadhyay and Sen 2012). At first, the eggshells were boiled in a 0.1% SDS solution for 15 min. Then the shells were washed with distilled water followed by acetone subsequently dried at 60 °C for 5 h. The dried eggshells were then crushed and sizes of 5 mm × 5 mm were selected for further studies. Lipase solution was mixed with eggshells and incubated at 37 °C for varying amounts of time (30–150 min). After incubation, all of the conical flasks containing the mixture were kept at 4 °C for 5–10 h. The resulting immobilized enzymes were then washed with phosphate buffer to remove any unbound enzyme.

Monitoring of changes in surface characteristics of the immobilized enzyme after oil hydrolysis using SSOCT

Immobilized eggshell was added into a mixer of 10 mL olive oil and 10 mL distilled water and was kept at 37 °C for 10 h in a shaking incubator. After each 2 h interval, immobilized eggshell was recovered, washed with phosphate buffer and kept at the refrigerator for 5 h. The change in surface characteristics of the immobilized eggshells due to reaction was analyzed using OCT. OCT signal (A-scan) was acquired over a length of 6 mm on the sample surface.

Table 1 SSOCT system and scanning protocol

Parameters	Values
Number of points per A-scan	2048
Number of A-scan per B-scan	2000
Scanning length	6 mm
Total number of B-scan	100
Axial resolution	~ 14 µm
Transverse resolution	~ 5 µm
Axial imaging range	~ 2.5 mm

The scanning protocol given in Table 1. The OCT images were obtained by averaging the 100 B-scan using custom-made software. A custom-made program was also built to extract the scattering coefficient as well as depth-dependent A-scan profile at a reason of interest (ROI).

Analysis of scattering coefficients related to immobilization and hydrolysis reaction

Scattering coefficient calculation was performed based on Beer-lambert's law, detailed was discussed in our previous work (Mohan et al. 2019; Mishra et al. 2019). Mismatch of refractive indices is directly correlated with changes in scattering coefficients. Intensity of scattered light represents the presence of the scattered substance inside the sample.

The equation used for determining the scattering coefficient is given by:

$$I(z) = I_0 e^{-2(\mu_a + \mu_s)z}$$

where I_0 is the incident light, μ_a is the absorption coefficient and μ_s is the scattering coefficient and z denotes the depth from the surface of the tissue. The absorption coefficient of most of the biological tissues is negligible in NIR region, hence the scattering coefficient (μ_s) is the dominating factor and the above eqn. can be re-written as:

$$\log\left(\frac{I}{I_0}\right) = -2\mu_s z$$

Here in this study, the presence of the immobilized enzymes on the eggshell membrane layer imparts a high amount of scattering and changes in refractive indices. The occupancy of the immobilized enzyme over the eggshell can be stated by evaluating the scattering coefficient through OCT image processing.

Rate of oil hydrolysis

Immobilized eggshell was added into a mixer of 100 mL olive oil and 50 mL phosphate buffer and was kept at 37 °C for 10 h in a shaking incubator. The sample was withdrawn in 2 h intervals, and the production of free fatty acid (acid value) was measured by the titration method (Chattopadhyay and Sen 2013). The acid value was calculated from the following equation:

$$AV = \frac{56.1 \times N \times V}{m}$$

Here, N is the normality of the ethanolic KOH (0.1 N); V is the volume need to titrate, and m is the mass of the sample (2 g). The FFA was measured by titration with 0.1 N ethanolic KOH using Phenolphthalein (1% v/v) as an indicator

to detect the endpoint. The acid value was expressed as mg of KOH required to neutralize FFA present per gram of oil.

Scanning electron microscopy (SEM)

To confirm the change in surface characteristics, all samples (eggshell, eggshell with the membrane, immobilized enzyme, and immobilized enzyme after reaction) were lyophilized and analyzed in a JEOL JSM6390LV SEM (JEOL, Japan). The samples were coated with a thin layer of platinum using a spray gun and then observed at 20 kV with 250X resolution.

Results and discussion

Activity of enzyme

From enzyme activity assay, the specific activity of free lipase was calculated as 6.77 ± 0.3 unit/mg protein. One unit is defined as the amount of enzyme that can hydrolyze 1.0 micro-equivalent of fatty acid from triglyceride in 1 h at

pH 7.4 at 37 °C. The manufacturer mentioned lipase activity as 5–15 unit/mg protein. This indicates no loss of enzyme activity during transportation and storage.

OCT analysis of lipase immobilization process on eggshell

The OCT system was used to obtain real-time cross-sectional images for eggshells. Immobilization of the lipase can be easily distinguished from these images. The Fig. 2 shows analysis of OCT image for lipase immobilization process. The control sample was taken without immobilization of lipase (Fig. 2a). A-line scan was performed over the region of interest (ROI) marked by the red dotted line. The cross-sectional view of ROI shown in Fig. 2b as an averaged OCT B-scan image. In the B-scan image, a ROI selected (yellow dotted line) for the depth-dependent cross-sectional graph (A-scan) shown in Fig. 2c. Similarly, Fig. 2d–f) shows the real-time image, B-scan image and A-scan plot of lipase immobilized eggshell, respectively.

Comparing control and lipase immobilized sample, we can clearly distinguish the changes with respect to B-scan

Fig. 2 Immobilization process of lipase characterized by Optical Coherence Tomography (OCT); **a** the picture of the bare eggshell with red line shows the reason of interest (ROI) for scanning; **b** averaged B-scan over the reason of interest (ROI); **c** normalized A-scan at reason of interest (ROI) shown in **b**; **d** the picture of lipase immobilized over eggshell; **e** averaged B-scan image of enzyme immobilized egg-shell over reason of interest (ROI); **f** normalized A-scan profile with two different peaks indicating immobilization of enzyme (Red arrow). Scale: 500 μ m

and A-scan. Due to immobilization, the scattering of the scanning beam increases which leads to a higher depth of view. Also, two different layers can be seen; clearly, those are eggshell membrane layer and core eggshell layer. The A-scan has shown in Fig. 2f clearly indicates the immobilization of lipase (red arrow). The appearance of first peak corresponds to adsorption of lipase on matrix. The amount of lipase immobilized with respect to incubation time is discussed in the following section under the study of reaction kinetics with SSOCT.

Study of reaction kinetics with SSOCT

It is clear from the SSOCT signal analysis (both from B-scan and A-scan) that there is a significant change in peak intensity, supported by the scattering coefficient, on immobilized lipase-eggshell surface before and after reaction. With more reaction time, more amount of light penetration can be observed in SSOCT image. This can be due to the fact that

as time proceeds, more and more triglycerides break due to enzyme action and smaller size molecules (monoglycerides, diglycerides, free fatty acids) are produced, which is responsible for higher light penetration.

The Fig. 3a shows the OCT B-scan image of control sample, i.e. bare eggshell with membrane layer. But it is very difficult to distinguish both the layer separately. After 30 min of incubation, absorption of lipase starts on shell membrane (Fig. 3b), comparing with corresponding A-scan profile (Fig. 4b), and first sharp peak shows the starting of immobilization phase and second peak shows the core eggshell layer. As incubation time increases the absorption of lipase increase, the intensity of OCT signal from membrane layer increases due to higher scattering involved. By observing A-scan profile the thickness of the highest peak of the A-scan increases significantly (Fig. 4b–d). After 90 min of incubation time immobilization reaches its saturation point and there were no such significant changes were observed from both B-scan (Fig. 3d–f) and A-scan (Fig. 4d–f). The

Fig. 3 Optical Coherence Tomography (OCT) images (B-scan) of lipase immobilization kinetics study; **a** bare eggshell as a control sample; After 30 min to 150 min incubation time of lipase adsorp-

tion on egg-shell OCT images were taken at a regular time interval of 30 min shown in images **b–f** respectively; Scale: 500 μ m

Fig. 4 Optical Coherence Tomography (OCT) A-scan plot of the eggshell on regular time interval of 30 min during lipase immobilization kinetics; **a** control, **b** after incubation of 30 min, **c** after incubation of

60 min, **d** after incubation of 90 min, **e** after incubation of 120 min, **f** after incubation of 150 min

above discussed results supported by the scattering coefficient calculation which will be discuss further.

Hydrolysis of oil

The hydrolysis starts from choosing the lipase immobilized eggshell sample as a control (Fig. 5a). SSOCT B-scan

Fig. 5 Swept-source Optical Coherence Tomography (SSOCT) reaction kinetic study for Hydrolysis of oil with different incubation time; **a** control with lipase immobilized over eggshell with membrane

layer; **b** after an incubation time of 2 h; **c** after an incubation time of 4 h; **d** after an incubation time of 6 h; **e** after an incubation time of 8 h; **f** after an incubation time of 24 h; scale: 500 μ m

images show the decrease in the thickness of the membrane layer this is due to the hydrolysis of oil and the removal of lipase slowly from the eggshell membrane. Due to hydrolysis, the absence of scattering media observed over an eggshell membrane which was clearly immobilized lipase in this study. The hydrolysis makes the membrane layer translucent for scanning laser (NIR light). This can be observed in Fig. 5.

It can be noticed from A-scan profile (Fig. 6) that due to hydrolysis reaction, the immobilization of lipase on eggshell discontinued. As the incubation period increases, the hydrolysis reaction also gets saturated. After 8 h. of hydrolysis, no more significant changes were observed in OCT images (both B-scan and A-scan). The above result also supported by scattering coefficient calculations.

A clear correlation between OCT data with laboratory experiments can be observed (Fig. 7). It is evident from the experiment that the amount of free fatty acid increase with time as reaction proceeds and become saturated after

8 h. Hydrolysis of the oil depends on many factors and at particular pH and temperature, and hence the amount of free fatty acid release can become saturated after certain reaction time.

Analysis of scattering coefficients

When we deal with the interaction of light with any scattering media, then the scattering coefficient calculation plays a vital role. Here, the scattering coefficient calculation was performed over SSOCT B-scan images. The high scattering coefficient indicates the higher scattering media and vice-versa. Analysis of OCT image is well aligned with this statement.

Calculation of scattering coefficient performed over the lipase immobilized sample with different incubation time and plotted with respect to the corresponding incubation period (Fig. 8a). At 0 min i.e. on the control sample, when there was no immobilization happen, the scattering

Fig. 6 A-scan profile of corresponding B-scan (Fig. 5) during the hydrolysis of oil. **a** control with lipase immobilized over eggshell with membrane layer; **b** after incubation time of 2 h; **c** after incuba-

tion time of 4 h; **d** after incubation time of 6 h; **e** after incubation time of 8 h; **f** after incubation time of 24 h

coefficient was lowest as the incubation period increases the scattering coefficient increases which indicated the lipase immobilization. After 90 min of the incubation period, the scattering curve starts saturating which indicates the

immobilization of lipase approaching its saturation level, which is exactly stated earlier from Figs. 3, 4.

Similarly, the hydrolysis of oil mobilizes the lipase present over the eggshell membrane layer. However, the scattering curve (Fig. 8b) gradually decreases up to 8 h. and then it

Fig. 7 Hydrolysis of oil using immobilized lipase

saturates. Which indicated the presence of scattering media was washed out slowly. The absence of lipase over eggshell provides the less scattering results in lower scattering coefficient. The above scattering coefficient result (Fig. 8b) supports the above-stated discussion regarding hydrolysis also OCT results (Figs. 5, 6).

SEM analysis

The comparative surface morphology can be seen from SEM analysis in Fig. 9. SEM of the eggshell indicated a highly porous filamentous structure for the matrix with proteins attached, such as albumin, as was previously reported (Li et al. 2008). The SEM image also indicates cavities between the protein fibers. It is difficult to visualize separate protein molecules in an SEM image. However, surface morphology changed upon immobilization, and the cavities were

occupied by the enzyme molecules, which makes the surface smoother. Higher-resolution data was not shown as very bright spots were generated (resolution 500X or more) that could be due to the charging up of enzyme by the electron beam on the surface. Other researchers also reported similar changes after the adsorption of textile dyes (Tsai et al. 2006) and oxalate oxidase (Pundir et al. 2009) on eggshell. No change in the surface was observed after the hydrolysis reaction as it is expected that by SEM analysis, such small change on the immobilized matrix cannot be determined. The less rigorous change indicates the stability of the immobilized system and its reusability for further reaction.

Conclusion

Change of surface characteristics due to immobilization of lipase on eggshell and during oil hydrolysis was characterized by an indigenously developed SSOC system. The system also analyzed hydrolysis of olive oil using immobilized lipase and it showed a good correlation with the laboratory results. SEM analysis also confirms the SSOC results and similar changes in surface morphology were observed after enzyme immobilization and oil hydrolysis. These results indicate the usefulness of the SSOC system as a better alternative compared to conventional analysis techniques in terms of non-destructiveness and real-time analysis technique with better resolution. The present study will help to characterize the immobilized system in more precision and detail to improve the catalytic activity of the industrial enzyme. The reported method has its immediate or future impact on biosensors.

Fig. 8 Scattering coefficient vs. incubation time plot corresponds to Optical Coherence Tomography (OCT) images; **a** scattering coefficient graph for lipase immobilization with respect to increases in

incubation time; **b** scattering coefficient vs. incubation time graph during the hydrolysis of oil

Fig. 9 SEM analysis of eggshell. **a** Empty eggshell; **b** eggshell with membrane; **c** immobilized lipase on eggshell and membrane; **d** eggshell membrane after oil hydrolysis

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Author contributions MM and ZA are involved in sample preparation, mathematical model building, OCT data analysis and manuscript writing. SC contributed towards the development of immobilized lipase, oil hydrolysis, SEM analysis and manuscript preparation, where RP is involved in overall monitoring and manuscript writing.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

Ethical approval The authors have no infringement of ethical issues.

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