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Lipase catalyzed esterification of Docosahexaenoic acid (DHA) with immobilized *Pseudomonas cepacia* and *Thermomyces lanuginosus*

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The selective behaviour of lauryl alcohol to catalyze esterification for the synthesis of tuna free fatty acids (TFFAs) using immobilized *Pseudomonas cepacia* (PCL) and *Thermomyces lanuginosus* lipases (TLL) have been investigated. The characterization of both immobilized PCL and TLL and their support immobead-150 is carried out using particle size analyzer, BET method and FT-IR. The suitable reaction conditions have been determined as lauryl alcohol to TFFAs ratio 4:1 (wt/wt), *p*H 8.0, buffer to TFFAs ratio 1:1 (wt/wt), temperature 50°C and agitation speed 800 rpm. Reusability of both the immobilized lipases for esterification has also been carried out and the activity was stabilized at 60.9% and 47.6% after 5 cycles of repeated use of immobilized PCL and TLL, respectively. Maximum 93.8 wt% Docosahexaenoic acid is recovered in esters with immobilized PCL after 16 h.

Keywords: DHA, Esterification, Lauryl alcohol, Lipases, Tuna free fatty acids

The potential of DHA rich food have been widely accepted around the world in terms of its ability to control and prevent different chronic disorders[1-8]. Due to their wide applications, it is crucial to extract highly pure form of DHA cost effectively[9,10]. As reported by various researchers, the major steps involved in the extraction of DHA are hydrolysis and esterification using selective and non-selective applications of different lipase[11-17]. For the esterification of free fatty acids, DHA can be separated either in fatty acids or in esters, depending upon the type of lipase used. For example, lipases from *Candida rugosa*[18,19], *Rhizopus oryzae*[20,21] and *Rhizopus delemar*[22] have shown less selectivity for the DHA during esterification; hence DHA remains in fatty acid phase, whereas lipases from *Candida antarctica* lipase-B, and *Aspergillus niger* etc. have selectivity for DHA esterification[23,24]. Therefore, the selection of suitable lipase is important in both methods of esterification reaction. Furthermore, type of alcohol can also equally affect the extent of reaction and lipase selectivity. The association of lipase selectivity with type of alcohols used in the esterification reaction has been observed by different groups. Shimada *et al*.[22] have noted that the presence of long chain fatty alcohol enhances esterification of fatty acids. In 1998, Shimada *et al*.[25] have reported that lipase can recognize long chain fatty alcohol as a substrate more easily than other alcohols. Again in 2001, Shimada *et al*.[26] found that *Candida antarctica* lipase-B (CAL-B) can also recognize polyunsaturated fatty acids and short chain alcohols as substrate. Relative selectivity and discriminative power of lipases among the series of fatty acids have been characterized in terms of specific alcohol acceptors used in the studies reported by Chang *et al*.[27]. Later, the conversion of oleic acid was correlated with type of alcohol and after 1 h, 100% conversion of oleic acid was observed with 1-butanol and pentanol.

Therefore in the present work, the selective behavior of immobilized PCL and TLL lipases have been studied for a range of fatty acids with various alcohols to improve the separation of DHA. Simultaneously, study for activation energy and reusability of lipases have also been performed for the esterification of DHA selectively in iso-octane medium.

Experimental Section

Materials and methods

Substrate and lipases

The tuna free fatty acids (TFFAs) extracted from tuna fish oil by hydrolysis with immobilized *Candida antarctica* lipase-B (CAL-B) as reported by

Sharma *et al.*[23]. Hence, TFFAs were utilized as a substrate for the selective esterification reaction. For the selective esterification studies, *Pseudomonas cepacia* lipase (Lot No.: 54327; activity ≥900 U/g), *Thermomyces lanuginosus* lipase (Lot No.: 76546; activity ≥3000 U/g), *Rhizopus oryzae* lipase (Lot No.: 89445; activity ≥300 U/g[a]) immobilized on immobead-150 and *Mucor miehei* (Lot No.: 62350; activity ≥30 U/g[b]) immobilized on ion-exchange were purchased from Sigma-Aldrich, Saskatoon (SK), Canada. Immobead-150 (94129) was also purchased from Sigma-Aldrich, Canada to compare their physiochemical properties with the immobilized lipases used in the present work.

Standards of Tridocosahexaenoin (T-310), Didocosahexaenoin (D-311), Monodocosahexaenoin (M-314) and Trieicosahexaenoin (T-325) were purchased from Nu-Check-Prep., Elysian, MN for the analysis and standardization of HPLC with ELSD detector.

Experimental Procedure

The separation of DHA from other fatty acids has been conducted by the selective esterification of tuna free fatty acids (TFFAs) with immobilized PCL and TLL enzymes. The optimization study for esterification reaction was carried out with immobilized PCL for various alcohols (ethanol, 2-propanol, butanol, hexanol, heptanol and lauryl alcohol), alcohol amount of 0.5-3.5 g, *p*H (4-9), buffer amount of 0-1.75 g, speed of agitation of 200-1000 rpm, and temperature of 20-60°C. The optimization experiments were performed in 20 ml screw-capped glass vials (VWR, Canada make) with 0.25 g of tuna free fatty acids as substrate, 1:1 (wt/wt) iso-octane solvent and 250 U (0.27 g) of immobilized PCL for selective esterification of fatty acids in 6 h. The activation energy for the reaction was calculated for both immobilized PCL and TLL at determined optimized parameters by plotting a curve between ln k vs. 1/T as shown in equation (1):

$$\ln k = -\frac{E}{R}\left[\frac{1}{T}\right] + \ln A \quad \ldots (1)$$

where k is the rate constant, A_o is the pre-exponential function, T is temperature in Kelvin, E is the activation energy measured in (kJ/mol) and R is the gas constant. The reaction was stopped by filtering out the lipase from the reaction mixture after every sample collection which was diluted with 3 mL of iso-octane for the GC injection to analyze the results.

The extraction of DHA rich lauryl ester and the release of DHA rich free fatty acids have been carried out after the selective esterification. Then, reusability study was also conducted up to five repeated uses of immobilized PCL and TLL at optimized reaction conditions. According to equation (2), the activity retention is defined as the %conversion achieved after reuse of immobilized enzyme with respect to % conversion achieved after first round of its use. The activity retention can also be expressed as ratio's of %conversion as given in equation (2):

$$\%\ \text{Activity retention for (n+1) cycle} = \frac{\%\ \text{Conversion after (n+1) time use of immobilized enzyme}}{\%\ \text{Conversion after one time use of immobilized enzyme}} \times 100 \quad \ldots (2)$$

All the results were repeated in 3 sets of experiments for performing the reproducibility analysis. This process was performed with a set of saponification and solvent extraction method, a standard procedure reported by Wanasundara and Shahidi[28] and Senanayake and Shahidi[29] with slight modifications.

Analysis

The activity of lipase has been expressed as μmoles of fatty acid esters formed per mg of immobilized lipase per min. After esterification of fatty acids, the mixture containing fatty acid esters and free fatty acids have been analyzed by HPLC (Agilent 1200 Series model) with electron light scattering detector, and an Agilent based PL gel organic GPC column 100A°, 7.5 × 300 mm 5μm column (p/n PL 1110-6520) protected with guard column and equipped with Chemstation for LC3D was used. The area percentage of ester peak was taken as the percentage of esterification (%E) and the area percentage representing FFAs accounts for the wt% of FFA in the mixture. The experiments were performed in repeated sets and a variation in TFFAs ester formation <±5% was observed. An Agilent make gas chromatography system (model 7890A) has been used, equipped with flame ionization detector (FID, 260°C) and capillary column DB-23 (dimensions: 60 m length, 0.25 mm ID, 0.25 μm film) for analysis of free fatty acids in methyl ester form (FAMEs). The initial composition of TFFAs was analyzed by converting the free fatty acids into methyl ester form with BF_3/methanol

solution under inert N_2 purging atmosphere. Throughout the experimentation, the GC was operated at constant conditions (Carrier: hydrogen gas with flow rate 20 cm/min & 23.148 psi pressure; Oven: 140 to 240°C at 4°C/min. and Injection: 1μL sample, 260°C & split: 20:1). The surface properties of a sample are estimated based upon the amount of nitrogen gas absorbed in relationship with its pressure, at the boiling temperature of liquid nitrogen under normal atmospheric pressure. The specific surface area, pore volume, and average pore diameter for immobilized lipases and their support materials immobead-150 and ion-exchange resin, were measured by N_2-physisorption at 77 K using a Micro metrics ASAP 2000. Approximately 0.2-0.3 g of sample was used for each analysis. The moisture and other adsorbed gases present in the sample were removed before analysis by degassing the sample at 60°C for 1 h under 66.7 Pa (500 mmHg). The sample was then evacuated at 2.67 Pa (20 mmHg) before N_2 adsorption[30,31]. The particle size distribution for both the materials was determined with Master sizer long bench particle size analyzer supplied by Malvern instruments, Canada. The instrument was provided with minimum He-Ne laser (633 nm wavelength), 18 beam diameter and 42 element composite solid state detector arrays. For this analysis, the particle size analyzer was fitted with lens in the size range of 300RF (Reserve Fourier) for measuring particles of size 0.05-900 μm. The percentage volume of particles was recorded with respect to particle diameter in μm. The FT-IR spectra for both, the immobilized lipases and the support materials, were obtained by KBr pelleting method using Perkin Elmer, FT-IR spectrum GX in the IR range of 400-4000 cm^{-1} (Ref. 32,33).

Reaction scheme

The overall reaction scheme for the esterification of total free fatty acids which were extracted from the hydrolysis of tuna fish oil, with lauryl alcohol in presence of immobilized PCL and TLL has been given in Reaction Scheme 1 to form DHA rich lauryl esters selectively. The reaction scheme explains the formation of lauryl ester by the breakdown of lipase-substrate complex in the presence of stoichiometric amount of alcohol.

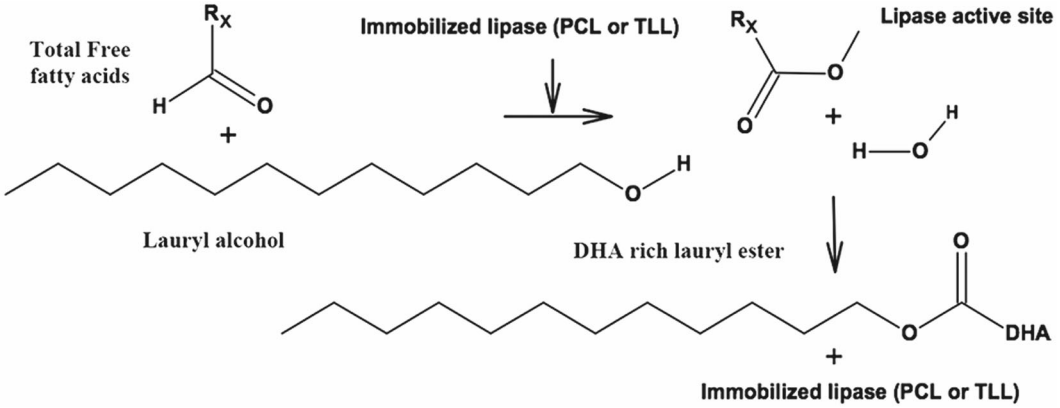
Results and Discussion

Characterization of immobilized lipases

The immobilized lipases such as PCL and TLL along with their support material immobead-150 were characterized for their physiochemical properties using various standard analytical instrumental methods such as Brunauer Emmett Teller (BET) and particle size distribution for surface properties and Fourier Transform-Infrared Spectroscopy (FT-IR) for the presence of functional groups.

BET analysis

The characterization of surface properties for both lipases and their support material have been performed with BET method and results are reported in Table 1. The immobilization of PCL and TLL on inert immobead-150 retains high activity and stability of these lipases. Their wide pores and large internal volume correspond to their activity and the ability to influence rate of reaction. The covalent bonding of immobead-150 with their respective lipases allow changes in the lipase conformation from close/inactive to open/active and provides the opening of the hydrophobic active sites towards the organic phase at the interface to minimize the dissolution of lipase in aqueous phase. Therefore, these lipases show better binding of substrate to the available enzymes active sites[34,35].



Reaction Scheme 1 — Esterification of free fatty acids with immobilized PCL or TLL in presence of lauryl alcohol

Table 1 — Characterization of immobilized lipases with BET method

S.No.	BET characteristics	Immobilized lipases		
		PCL	TLL	Immobead-150
1	BET surface area, S_g (m^2/g)	205.1±0.9	144.4±0.5	228.8 ±0.5
2	Pore diameter (A°)	100.1	98.1	89.2
3	Single point pore volume, V_v (cc/g)	0.56	0.35	0.5
4	Bulk density, ρ(g/cm^3)	1.8	2.9	2
5	Average pore dia., $D_p = 4. V_v/S_g$ (cm)	99.5	96.9	91.4
6	Porosity, $\varepsilon = V_v.\rho$	1.01	1.02	1

Particle size distribution analysis

The particle size distribution analysis was performed for immobilized lipases and their support as particle size has important role for explaining immobilization and enzyme activity[36]. The particle size distribution of immobead-150 was found in the range of 0-50 µm particle diameter. The trend in the variation of percentage volume with respect to particle diameter (µm) for immobilized PCL, TLL and immobead-150 has been compared in Figure 1. It was found that immobilized PCL and TLL enzymes show single narrow peak with average particle size of 300 and 500 µm respectively for a volume of 21-27% particles according to the Fig. 1. The support material immobead-150 has non-uniform distribution of particles in the wide range of 100-900 µm.

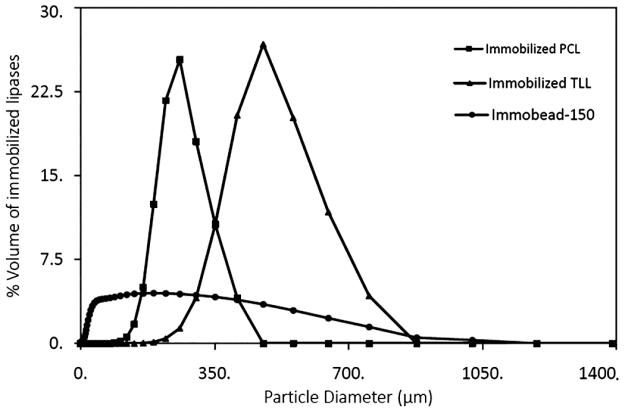


Fig. 1 — Particle size distribution for immobilized lipases and their support

FT-IR analysis

The FT-IR spectra of immobilized lipases and their support material were recorded separately as shown in Fig. 2. According to the spectra shown, the broad peak in the range of 3600-3200 cm^{-1} represents the free hydroxyl group of carboxylic acid. The strong sharp peak at 1700 cm^{-1} corresponds to carbonyl group of the carboxylic acid functional group. The medium peak at 2900 cm^{-1} is the characteristic peak for stretching vibration while strong peak at 1500 cm^{-1} is for the bending vibration of free amine present in the enzyme. According to the Figure, the small peaks ranging from 1200-600 cm^{-1} indicate the finger print region characteristic for immobilization support material immobead-150.

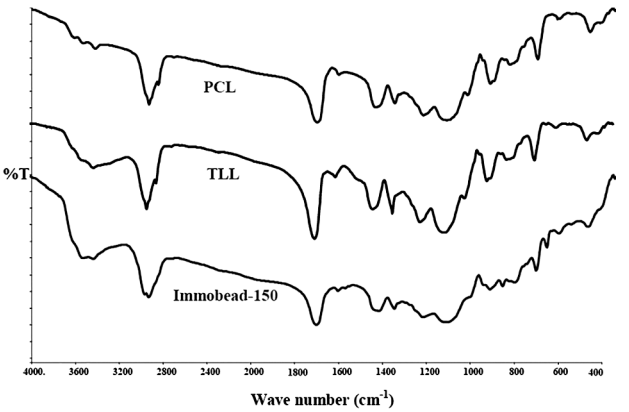


Fig. 2 — FT-IR spectrum of immobilized lipases and support

Parameter optimization

The optimization study of various reaction conditions have been carried out to maximize the production of DHA rich ester. The parameters optimized were type of lipases, type of alcohol, alcohol amount, *p*H, buffer amount, temperature and speed of agitation for the selective esterification of tuna free fatty acids with immobilized lipase and iso-octane solvent. The details of the studies and findings are given below.

Effect of different type of immobilized lipases

Four different immobilized lipases have been investigated for their ability to selectively perform the esterification of DHA from tuna free fatty acids. The screening of immobilized MML, PCL, TLL and ROL was performed at reaction conditions such as *p*H 7.5 and temperature 40°C using 1:1 (wt/wt) solvent to oil ratio, 2.0 g of butanol with 600 rpm speed of agitation. The 250 units (U) of immobilized PCL, TLL, ROL and MML enzymes corresponding to 0.27, 0.04, 0.68 and 2.3 g, were taken for the study respectively. The trend of variation for tuna free fatty

acid ester formation with various immobilized lipases is shown in Fig. 3 at time t = 0 and after esterification reaction performed for 6 and 24 h. According to the results reported, maximum DHA esters of 40.7 and 18.9 wt% were formed with immobilized PCL and TLL respectively than other lipases used in 6 h. Therefore, in the present study immobilized PCL has been chosen to increase the esterification of DHA than other fatty acids selectively. Mbatia *et al.*[37] have also reported immobilized PCL and TLL lipases for enrichment of DHA and EPA by selective esterification of free fatty acids or by hydrolysis of ethyl esters of fatty acids from oil.

Type of alcohol

The formation of DHA esters with selective esterification of TFFAs was studied with various alcohols such as ethanol (CH_3CH_2OH), methanol (CH_3OH), 1-propanol ($CH_3CH_2CH_2OH$), 2-propanol ($CH_3CHOHCH_3$), butanol ($CH_3CH_2CH_2CH_2OH$), hexanol ($CH_3(CH_2)_5OH$), heptanol ($CH_3(CH_2)_6OH$), ocatnol ($CH_3(CH_2)_7OH$), benzyl alcohol ($C_6H_5CH_2OH$), furfuryl alcohol (C_5H_6O) and lauryl alcohol ($C_{12}H_{26}O$). According to the results given in Table 2, although a maximum 48.3% esterification, was observed with butanol which corresponds to only 42.8 wt% DHA. On the other hand, lauryl alcohol (LA) was found to give maximum production of DHA esters i.e. 88.9 wt % (110.9µmoles/ml), corresponding to 33.7% esterification in 6 h. Therefore, lauryl alcohol was chosen to increase the DHA in esters selectively with immobilized PCL in the present study. This indicates that as the LA is a long carbon chain alcohol than other alcohols used in the study, it is able to bind with the long chain fatty acids specially DHA to form its esters. The results clearly indicate that the immobilized PCL can only bind with three types of fatty acids including $C_{18:0}$, $C_{22:6}$ and $C_{24:1}$ in the presence of lauryl alcohol, among which the maximum preference has been seen for DHA ($C_{22:6}$) in esters i.e. 88.9 wt%. Therefore, it is concluded from the present study that both lipase and alcohol are responsible for selectively enhancing the separation of DHA. Triantafyllou *et al.*[38] have used lauryl alcohol for the esterification of ethyl caprate in the presence of di-isopropyl ether with *Candida antarctica* lipase both in free and immobilized form to increase the activity of lipase. Gandhi *et al.*[39] have reported thermo deactivation of lipozyme (immobilized *Mucor miehei*) by exposure to butanol, whereas higher reaction rate was achieved in biphasic system with lauryl alcohol for the esterification of lauric acid.

Effect of alcohol amount

The effect of LA amount on formation of DHA esters from TFFAs has been studied to optimize its amount for the esterification reaction. The study has been conducted at different amounts of LA. The trend of variation in esterification of tuna free fatty acids with immobilized PCL is shown in Fig. 4. The results indicate that maximum 37.4% esterification was achieved with 4.0 g of lauryl alcohol per g of TFFAs, corresponding to 138.5 µmoles of fatty acid esters formed/ml of reaction mixture. The trend in Fig. 4 reflects that the increased amount of LA for

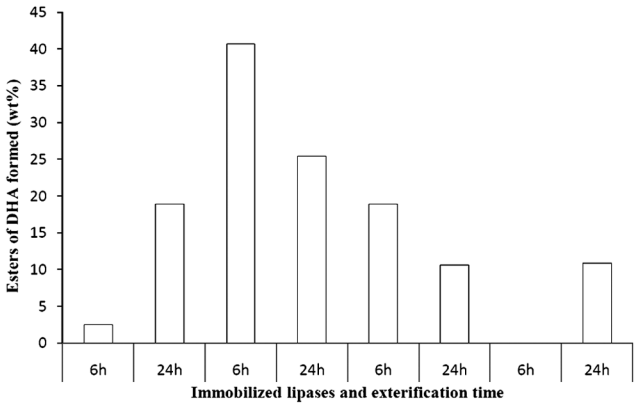


Fig. 3 — Effect of various immobilized lipases for the formation of DHA esters

Table 2 — Effect of different alcohols on activity of immobilized PCL for selective esterification of TFFAs

Type of alcohol	Fatty acid composition in esters (wt%)											%E
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:2	C20:5	C22:6	C24:1	Others	
Initial	0.8	13.8	10.9	15.2	6.4	4.6	6.5	0.6	35.6	1.8	3.8	0.0
Ethanol	10.5	-	33.2	24.4	0.33	14.1	-	0.2	-	8.6	8.7	17.4
2-Propanol	9.1	40.8	-	23.9	-	14.4	-	-	-	5.7	6.1	15.2
Butanol	-	-	-	11.1	1.4	6.2	9.4	-	42.8	2.8	26.3	48.3
Hexanol	-	-	-	-	11.3	-	6.8	0.9	10.7	-	70.3	20.7
Heptanol	-	-	-	-	-	-	-	19.4	4.4	9.4	66.8	22.4
Lauryl alcohol	-	-	-	-	-	3.8	-	-	88.9	7.3	0.0	33.7

esterification reaction was not able to produce higher amount of DHA esters. The possible reason seems to be the substrate inhibition of the reaction at higher amount of alcohol because alcohol is one of the substrate for esterification of fatty acids and according to the stoichiometry of the esterification reaction in presence of LA, equal molar ratio of fatty acids and alcohol is desirable to obtain best results. Syamsul *et al.*[40] have studied esterification at 1:2 molar ratios of palmitic acid to lauryl alcohol with commercial immobilized Novozym 435 *Candida antarctica* lipase-B and obtained a high purity yield (>90%) of lauryl palmitate at their optimized reaction conditions.

Effect of pH

The nature of acidic and basic environment in terms of *p*H (4.0 to 9.0) was evaluated for esterification reaction to produce DHA rich esters using immobilized PCL enzyme. The trend of variation in esterification of tuna free fatty acids with immobilized PCL is shown in Fig. 5. According to the results obtained, the maximum 35.6 % esterification of fatty acids is at *p*H 8.0 i.e. 131.9 µmoles of fatty acid esters formed in 6 h and 93.5% of maximum enzyme activity was noted at *p*H 7.0. It has been seen from Fig. 5, that the immobilized PCL is able to reflect optimum activity for esterification reaction in the basic (*p*H >7.0) conditions than the acidic (below *p*H < 7.0). The increase in *p*H from 4.0 to 8.0 shows an increasing trend in the enzyme activity but a sharp reduction in activity was observed at *p*H 9.0. The possible reason for this behavior of immobilized PCL is due to the presence of net positive charge on amino acid residues present in active sites of lipase at *p*H 8.0. Hence, positively charged active sites provide more binding affinity for negatively charged fatty acids residues to carry out esterification reaction optimally in basic biphasic solvent environment. Zhang *et al.*[41] have reported that the highest esterification of L-ascorbic acid occurred at neutral *p*H in case of lipase from *Thermomyces lanuginosus* immobilized on silica gel. Dosanjh and Kaur [42] have compared the physiochemical properties of *Bacillus* lipase in free and immobilized form and reported *p*H 8.0 as optimum condition for esterification.

Effect of buffer amount

The important aspect of an enzyme catalyzed esterification reaction in biphasic environment is to maintain the constant *p*H conditions as confirmed in the previous section throughout the course of reaction for achieving optimum enzyme activity because enzymes are very fragile in nature and their protein folding structure can be easily degraded upon exposure to variable adverse *p*H conditions. Therefore, it is crucial to maintain stable *p*H environment using appropriate amount of buffer which prevents the change in *p*H of a reaction mixture. The effect of buffer amount has been studied to maintain *p*H 8.0 in the present case and the trend of variation in esterification of tuna free fatty acids with immobilized PCL is shown in Fig. 6. Results show that the optimized amount of buffer was 1.0 g per g of TFFAs to produce 152.4 µmoles of fatty acid esters, which led to 36.6% esterification in 6 h whereas 124.8 µmoles of fatty acid esters was formed with 2.0 g of buffer per g of TFFAs. This reflects that esterification reaction can be optimally carried out with 1.0-2.0 g of buffer per g of TFFAs as not much variation in the amount of fatty acid esters product was obtained within this range. Various studies have shown that lipases need a small amount of water for esterification reactions to retain their active three-dimensional conformational state,

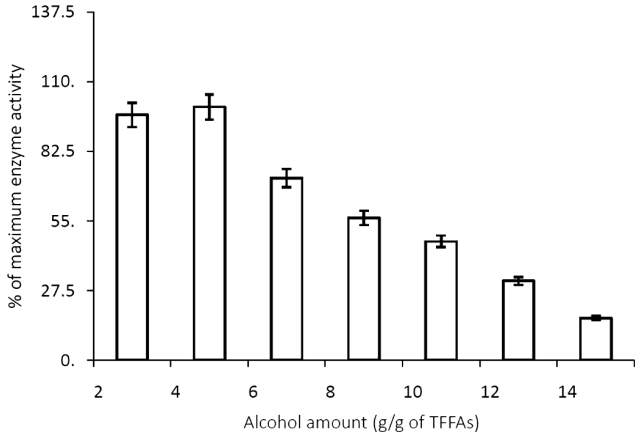


Fig. 4 — Effect of alcohol amount on activity of immobilized PCL

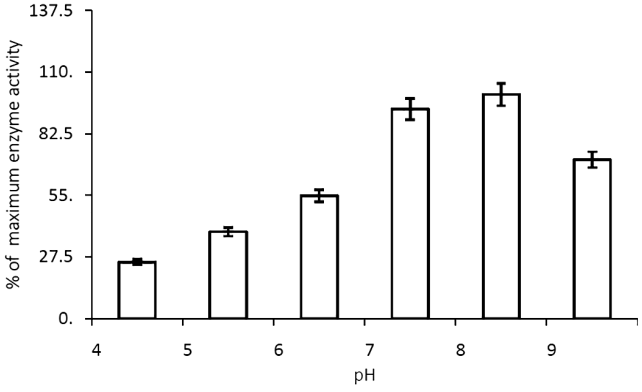


Fig. 5 — Effect of *p*H on activity of immobilized PCL

even when enzyme is covalently bound to support. Water can also limit the solubility of substrate around the enzyme even in the form of buffer[43,44].

Effect of temperature

In order to account the structural integrity and stability of an immobilized lipase with respect to temperature variation in the reaction conditions[45], the effect of temperature change on the esterification of free fatty acids into ester form was studied with immobilized PCL. From this study, it was concluded that maximum activity was observed at 50°C in terms of maximum 36.8% esterification corresponding to 153.3 µmoles fatty acid esters formed per mL of reaction mixture which was reduced to 96.7 µmoles/mL at 60°C in 6 h. The trend of variation in esterification of tuna free fatty acids with immobilized PCL is shown in Fig. 7. This reflects that under the present reaction conditions, the immobilized PCL remained stable only up to 50°C and then, its denaturation started causing drastic reduction in activity at higher temperature. The conversion decreased after 60°C probably caused by the vibration and movement of the enzyme molecule, which would affect the hydrogen bonds and other bonds in the lipase structure [46]. Mbatia *et al.*[37] have also used 50°C to carry out esterification reaction of PUFA extracted from Nile perch fish oil using immobilized PCL and TLL lipases.

Effect of agitation speed

The effect of agitation speed was studied for esterification reaction catalyzed by immobilized PCL enzyme to reduce the influence of bulk mass transfer resistances. For a reaction catalyzed in the presence of immobilized enzyme, it is significant to eliminate the effect of mass transfer resistances by providing desired contact between substrate and immobilized enzyme through optimum speed of agitation. The results indicate that a speed of 800 rpm was sufficient to maximize formation of fatty acid esters i.e. 159.6 µmoles/mL corresponding to 38.3% esterification in 6 h. The trend of variation in esterification of tuna free fatty acids with immobilized PCL is shown in Fig. 8. The increasing order of product formation was observed with increase in speed up to 800 rpm, due to reduction in the resistances generated by mass transfer. On the other hand, a sharp reduction in enzyme activity was found when further speed was increased from 800 to 1000 rpm due to the denaturation of immobilized PCL in terms of structure and activity.

Activation energy for esterification of TFFAs

The determination of activation energy for selective esterification of DHA with immobilized PCL and TLL was studied using Arrhenius plot as described in equation (1). The percentage conversion of tuna free fatty acids into esters of lauryl alcohol

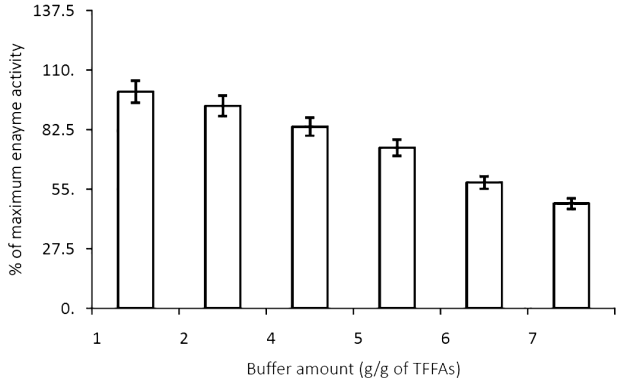


Fig. 6 — Effect of buffer amount on activity of immobilized PCL

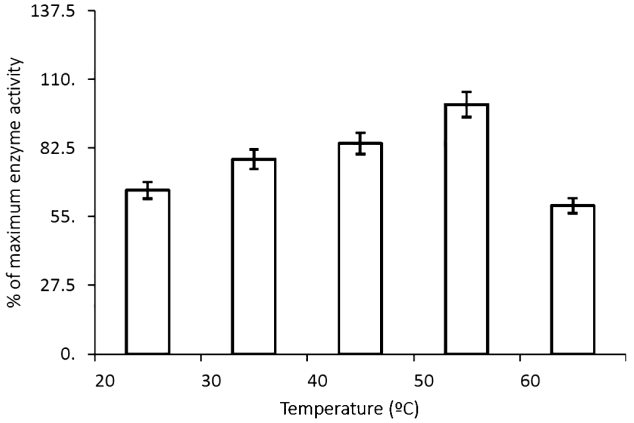


Fig. 7 — Effect of temperature on activity of immobilized PCL

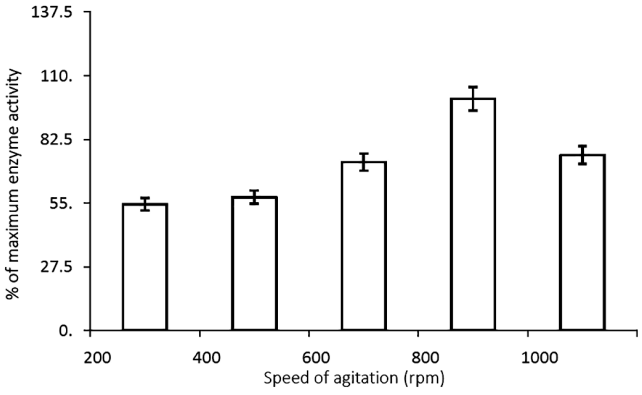


Fig. 8 — Effect of speed of agitation on activity of immobilized PCL

Table 3 — Effect of temperature on rate constant (k) with immobilized PCL and TLL

Temperature T (ºK)	Rate constant k (μmoles of DHA esters formed per ml)		1/T (ºK^{-1})	ln k	
	Immobilized PCL	Immobilized TLL		Immobilized PCL	Immobilized TLL
303	0.3674	0.2991	0.0033	-1.0	-1.35
313	0.44	0.3576	0.0032	-0.82	-1.03
323	0.6389	0.3762	0.0031	-0.45	-0.75

[Reaction Conditions: 0.5 g TFFAs, 0.5 g phosphate buffer of *p*H 8.0, 50°C, 800 rpm, 2.0 g lauryl alcohol, 1.0 g of iso-octane, immobilized PCL 0.54 and TLL 0.08 g and reaction time 6 h]

was plotted with time (up to 6 h) at varying temperatures such as 30, 40 and 50°C for immobilized PCL and TLL respectively.

The rate of the reaction increases with the temperature and facilitates more collision between reactants and enzyme. Therefore, reactants bind and product releases easily from the enzyme and this causes the reduction in the activation energy of the enzymatic system. The linearized form of Arrhenius law was used to calculate the value of activation energy. Therefore, ln k was plotted with respect to the reciprocal of temperature as given in Fig. 9 with data given in Table 3. The Arrhenius plot indicates that the slope of the linear equation is equivalent to the value of (-E/R) and intercept gives the pre-exponential factor (A_0). Hence, the activation energy (E) equivalent to 22.4 ($E_{imm.,PCL}$ and 24.9 ($E_{imm.,TLL}$) kJ/mol were determined from Figure 9 for immobilized PCL and TLL enzymes, respectively. The magnitude of activation energy (E) is a mathematical measure to represent the ease of reaction in presence of catalyst. The summary of results for activation energy study is given in Table 4.

In the present case, the amount of activation energy required by immobilized PCL and TLL enzymes ($E_{imm.,PCL}<E_{imm.,TLL}$) indicates that the esterification with immobilized PCL is comparatively more favorable than with immobilized TLL. For this system, to the best of our knowledge, no study has been reported earlier for estimation of activation energy. However, there are some studies reported on determination of activation energy for lipase catalyzed reactions such as alcoholysis of methyl propionate and 1-propanol with immobilized *Candida antarctica* lipase B in an organic liquid medium[47], esterification of lauric acid and 1-propanol with immobilized *Rhizomucor miehei* lipase[48] and esterification of lauric acid and lauryl alcohol with immobilized Porcine pancreatic lipase[49].

Table 4 — Activation energy (E) for selective esterification of TFFAs with immobilized PCL and TLL

Lipase	Activation energy, E (kJ/mol)	Pre-exponential factor, A_0
Immobilized PCL	22.8	31.12 x 10^2
Immobilized TLL	24.9	52.01 x 10^2

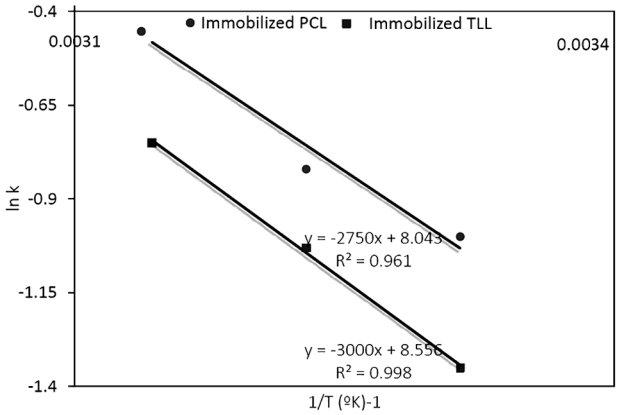


Fig. 9 — Arrhenius plot for selective esterification of TFFAs with immobilized PCL and TLL enzymes

Reusability study with immobilized PCL and TLL for esterification of TFFAs

In the enzymatic treatment processes, the high cost of the enzyme is a crucial parameter that should be controlled to make the process economically feasible and therefore, the cost of the process can be reduced by repeated use of the enzyme, even at comparatively low activity.

The reusability study for immobilized PCL and TLL enzymes were performed at optimized reaction conditions with 0.5 g of tuna free fatty acids and 0.53 and 0.08 g of immobilized PCL and TLL respectively. The reusability experiments were conducted up to five runs and reaction mixture was placed in the orbital shaker at 800 rpm for 6 h. After every run, the immobilized lipases were isolated by filtration, washed three times with iso-octane solvent (10 mL), distilled water (10 ml) and dried at room temperature. The results for reusability study are given in Table 5.

Table 5 — Reusability of immobilized PCL and TLL for esterification of TFFAs

	%E		Fatty acids in FFAs (wt%)		Fatty acid esters formed (μmoles/ml)		% Activity retention of immobilized lipases	
	PCL	TLL	PCL	TLL	PCL	TLL	PCL	TLL
1	47.4	39.7	52.6	60.3	197.5	165.4	100	100
2	43.6	35.2	56.4	64.8	181.7	146.7	91.9	88.7
3	38.9	28.5	61.1	71.5	162.1	118.8	82.1	71.8
4	29.2	19.8	70.8	80.2	121.7	82.5	61.6	49.8
5	28.9	18.9	71.1	81.1	120.4	78.8	60.9	47.6

[Reaction Conditions: 0.5 g TFFAs, 0.5 g phosphate buffer of *p*H 8.0, 50°C, 800 rpm, 2.0 g lauryl alcohol, 1.0 g iso-octane, immobilized PCL 250 U (0.53 g) and immobilized TLL 250 U (0.08 g) and reaction time 6 h]

The trend in the variation of percentage activity retention of immobilized PCL and TLL is shown in Fig. 10 for the esterification of tuna free fatty acids. The esterification of tuna free fatty acids with the repeated use of lipases was found to decrease from 43.6 to 38.9% and 35.2 to 28.5% after second and third runs for immobilized PCL and TLL, respectively (Table 5). Additionally, activity retention was calculated for repeated runs of immobilized PCL and TLL, which indicates that activity of lipase, has been reduced to 29.2 and 19.8% after fourth recycling. It indicates that immobilized lipases retains ability to react with the substrate even up to four repeated runs but the activity was found to decrease drastically. The reduction in activity is a clear indication of partial blockage of active sites present in lipase causing lesser conversion. To confirm this, the BET analysis was performed for the recycled immobilized PCL and TLL and compared with the fresh immobilized lipases. The results indicate that after fourth recycle of the immobilized PCL and TLL, the BET surface area was reduced to 115.4 and 69.7 m^2/g in comparison with 225.1 and 144.4 m^2/g for fresh immobilized PCL and TLL respectively.

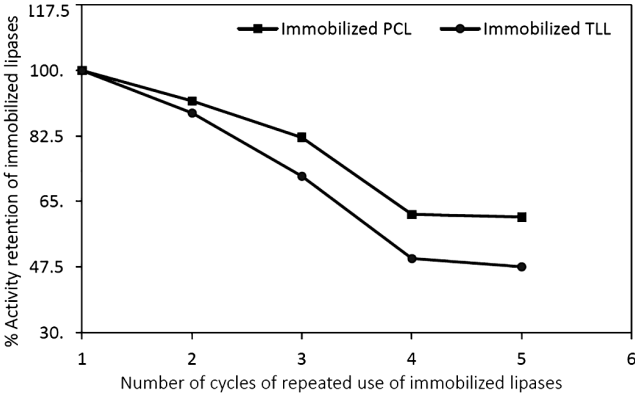


Fig. 10 — Reusability study with immobilized PCL and TLL for esterification of TFFAs with lauryl alcohol

Composition of fatty acids in esters after esterification of TFFAs with immobilized PCL

Esterification of tuna free fatty acids with immobilized PCL was carried out up to 56 h. The fatty acid esters of lauryl alcohol obtained after esterification at different time of reaction were separated from the reaction mixture standard procedure reported by Wanasundara and Shahidi[28] and Senanayake and Shahidi[29] with some modifications. The composition of the fatty acid esters obtained after 56 h of esterification reaction of tuna free fatty acids is given in Table 6. From the table, it can be seen that the DHA content of fatty acid esters obtained after esterification was maximum at 93.8 wt% after reaction time of 16 h when the extent of esterification was 32.9%. On the contrary, after 48 h the extent of esterification reached up to 57.5% and at this stage, the DHA content of fatty acid esters was found to be around 70.5 wt%. Higher concentration of DHA (93.8 wt%) in fatty acid esters of lauryl alcohol after the esterification for 16 h compared to that after esterification for 48 h clearly indicates that the immobilized PCL had preference for forming the DHA ester of lauryl alcohol over other fatty acids. Therefore, fatty acid esters formed after 16 h esterification was chosen for the isolation of DHA from the fatty acid esters of lauryl alcohol to study the synthesis of DHA rich glycerides. A batch of 5.0 g tuna free fatty acids was taken for the esterification of fatty acids with immobilized PCL. After esterification for 16 h and separation, the DHA rich (93.8 wt%) fatty acid esters obtained were 1.54 g. The DHA in free fatty acids was recovered and analyzed using HPLC with ELSD detector. The single peak shown in HPLC chromatogram at retention time of 14.6 min. indicates the presence of pure DHA rich FFAs which was isolated successfully. After the chemical breakdown and separation, 1.5 g (30 wt%) of DHA

Table 6 — Composition of fatty acid esters of lauryl alcohol with respect to time for esterification of TFFAs with immobilized PCL

Time (h)	%E	Fatty acids in FFAs (wt%)	Fatty acid composition of esters (wt %)		Composition of FFAs mixture (wt%)		Fatty acid esters formed
			$C_{22:6}$	Other	$C_{22:6}$	Other	(μmoles/mL)
0	0.0	100.0	-	-	35.6	64.4	0
0.1	6.5	93.5	49.6	50.4	34.3	65.7	28.9
0.3	7.7	92.3	54.2	45.8	33.5	66.5	33.7
0.5	10.0	90.0	56.8	43.2	32.3	67.7	44.7
0.8	11.5	88.5	60.9	39.1	31.0	69.0	51.3
1	14.4	85.6	63.7	36.3	28.9	71.1	64.3
2	20.9	79.1	70.4	29.6	24.9	75.1	77.9
4	23.0	77.0	77.5	22.5	18.0	82.0	98.3
6	25.7	74.3	88.6	11.4	7.6	92.4	114.9
8	31.6	68.4	91.6	8.4	5.4	94.6	140.9
16	32.9	67.1	93.8	6.2	7.9	92.1	147.3
20	38.9	61.1	86.8	13.2	14.3	85.7	173.9
24	48.0	52.0	78.5	21.5	14.5	50.7	212.6
32	51.3	48.7	74.7	25.3	15.7	47.0	228.9
40	55.8	44.2	73.3	26.7	19.1	44.5	248.9
48	57.5	42.5	71.9	28.1	19.8	42.7	256.9
56	56.9	43.1	70.5	29.5	18.1	41.9	253.9

[Reaction Conditions: 5.0 g TFFAs, 5.0 g phosphate buffer of pH 8.0, 50ºC, 800 rpm, 10.0 g iso-octane solvent, 20.0 g lauryl alcohol and 5.4 g (250 U) of immobilized PCL]

rich free fatty acids was collected. The DHA recovered was 84.3% with respect to initial DHA content of tuna free fatty acids (35.6 wt%).

Conclusion

The most suitable reaction conditions for selective esterification of TFFAs with immobilized PCL, were established with 1:1 (wt/wt) isooctane and 250 U of enzyme as TFFAs amount: 0.25g, , speed of agitation: 800 rpm, pH 8.0, TFFAs to buffer ratio: 1:1 (wt/wt), and lauryl, TFFAs to temperature: 50°C alcohol ratio: 1:4 (wt/wt) for immobilized PCL.

The activation energies for esterification of TFFAs were found to be 22.8 and 24.9 kJ/mol with immobilized PCL and TLL, respectively, in the temperature range of 30-50°C, indicating that the esterification with immobilized PCL is comparatively more favorable than with immobilized TLL. In 48 h, maximum 57.5% esterification was obtained with immobilized PCL. But maximum DHA content in esters was found to be 93.8 wt% after 16 h at 32.9% esterification. The high concentration of DHA (93.8 wt%) in esters of lauryl alcohol after esterification for 16 h compared to that after 48 h clearly indicated that the immobilized PCL had preference for forming the DHA ester of lauryl alcohol over other fatty acids. Therefore, fatty acid esters formed after esterification for 16 h was chosen for the isolation of DHA from the fatty acids for the further study. The reusability of immobilized PCL and TLL for selective esterification of TFFAs with lauryl alcohol was also studied and activity retentions of 61.6 and 49.8% respectively, were found after four time repeated use of the immobilized lipases.

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Nomenclature

PCL	*Pseudomonascepacia* lipase	TFFAs	Tuna free fatty acids
TLL	*Thermomyceslanugi nosus*lipase	DHA	Docosahexaenoi c acid
MML	*Mucormiehei* lipase	EPA	Ecosahexaenoic acid
ROL	*Rhizopusoryzae* lipase	FAMEs	Fatty acid methyl esters
% E	Percentage esterification	GC	Gas chromatography
HPLC	High performance liquid chromatography	LA	Lauryl alcohol

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