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Immobilization of *Candida cylindracea* lipase on poly lactic acid, polyvinyl alcohol and chitosan based ternary blend film: Characterization, activity, stability and its application for *N-acylation* reactions

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ABSTRACT

The ecofriendly ternary blend polymer film was prepared from the chitosan (CH), polylactic acid (PLA) and polyvinyl alcohol (PVA). Immobilization of *Candida cylindracea* lipase (CCL) was carried out on ternary blend polymer via entrapment methodology. The ternary blend polymer and immobilized biocatalyst were characterized by using N_2 adsorption–desorption isotherm, SEM, FTIR, DSC, and (%) water content analysis through Karl Fischer technique. Biocatalyst was then subjected for the determination of practical immobilization yield, protein loading and specific activity. Immobilized biocatalyst was further applied for the determination of biocatalytic activity for *N*-acylation reactions. Various reaction parameters were studied such as effect of immobilization support (ratio of PLA:PVA:CH), molar ratio (dibutylamine:vinyl acetate), solvent, biocatalyst loading, time, temperature, and orbital speed rotation. The developed protocol was then applied for the *N*-acylation reactions to synthesize several industrially important acetamides with excellent yields. Interestingly, immobilized lipase showed fivefold higher catalytic activity and better thermal stability than the crude extract lipase CCL. Furthermore various kinetic and thermodynamic parameters were studied and the biocatalyst was efficiently recycled for four successive reuses. It is noteworthy to mention that immobilized biocatalyst was stable for period of 300 days.

1. Introduction

Enzymes are nature's catalyst having excellent catalytic features. Today, almost more than 4000 enzymes are known and out of these nearly 200 are used for the mercantile purpose [1]. Commercially, lipases (triacylglycerol hydrolases; E.C. 3.1.1.3) are extensively used enzymes for several applications such as organic synthesis, pharmaceuticals, detergent, food, bakery, beverages, cosmetics, leather, paper industries, waste treatment etc. [1,2]. Enzymes possess excellent features such as stereo-, regio-, chemo-, enantio-selectivity, operation at mild reaction conditions, which make them more ecofriendly and advantageous than the conventional chemical catalysis [2]. Beyond unquestionable advantages, still there are many practical difficulties such as instability, isolation, separation, sensitivity to organic solvents and reaction conditions which limit their use as biocatalyst [3–5]. Various methodologies such as physical adsorption, covalent—ionic bond, entrapment, cross linked enzyme aggregates (CLEAs) formation was employed for successful immobilization to overcome above difficulties [3–6]. Several advanced immobilization techniques are proposed to enhance the activity, stability, yield, recyclability and organic solvent tolerance capacity [3–6]. However, still there is a great scope to develop the new environmentally benign protocols for enzyme immobilization. Hence, current study reports the development of biocompatible, ecofriendly suitable immobilization support via entrapment methodology. As entrapment is a simple method of immobilization which is generally free from the use of cross linking reagent [7]. Entrapment methodology have the advantages like reduction in mechanical sheer and hydrophobic solvent effect and thus leads to enhance catalytic activity and stability of lipase in organic media along with operational stability [1,3–7]. Entrapment of the enzymes within the membrane matrix can be incorporated during the membrane manufacturing [7].

Ecofriendly polymers have gained a special importance for enzyme immobilization, drug delivery systems and tissue engineering due to their biodegradable nature [8,9]. Various biodegradable polymers such as cellulose, PVA, CH, starch, gelatine, carboxymethyl cellulose (CMC), hydroxylpropyl methylcellulose

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(HPMC) were used for enzyme immobilization in the form of film, membrane or beads and these polymers can be easily recovered and reused from the reaction mixture [5,10–16]. Recently, Adriano et al. [15] and Chang and Juang [17] utilized CH for the chymotrypsin and β-glucosidase immobilization respectively. Chronopoulou et al. [18] used the polylactic acid nanoparticles for the immobilization of *Candida rugosa* lipase. Dong et al. [19] applied the organobentonite for immobilization of the porcine pancreatic lipase. Hilal et al. [20] used the cellulose immobilized lipase membrane for the enzymatic esterification while Dhake et al. [21] used hydroxylpropyl methylcellulose-PVA immobilized lipase for acetate synthesis. Several techniques such as structure modification, enzyme and genetic engineering were developed to improve the enzyme stability and activity [1,3–7]. Furthermore, various immobilization protocols were developed [3–7,10–21] which showed the improvement in the pH, thermal and operational stability of enzyme. Besides these immobilization protocols, considering limitations of the traditional supports in the enzyme immobilization, many efforts have been made to develop an ideal ecofriendly immobilization support to prepare the robust biocatalyst.

In present protocol a polymeric film was prepared by using ternary blend of PLA, PVA, and CH which is biodegradable. The prepared film has good biocompatibility and stability as compared to the binary blend [22]. PLA is a biodegradable, biocompatible, ecofriendly biopolymer produced from starch like materials [23]. PVA is an excellent compatibilizing polymer for preparing miscible blend with PLA and CH. PVA has impressive features like excellent film formation, high interfacial adhesion, flexibility, and emulsification, high tensile strength, non-toxic nature, biodegradable, stabilization of blend and essentially resistant to organic solvent which makes them more ideal for lipase immobilization [21,22]. CH always has good attention for the enzyme immobilization because of several characteristics such as inertness to chemical, lack of toxicity and its biodegradable nature [10–14]. In addition to this, free hydroxyl and amino groups in CH is expected to offer the higher degree of immobilization. Recently, Grande and Carvalho [22] synthesized a ternary blend film of PLA-PVA-CH and the outcome invites the researcher for application in enzyme immobilization.

Formation of the C—N bond has great importance in the natural as well as synthetic organic chemistry. It is a backbone of the life beginning bio-molecules such as amino acids, DNA, RNA, proteins, micro cycles, glycopeptides, amino glycosides, and alkaloids as well as in the synthesis of many active pharmaceutical intermediates (API) [24]. However its formation is a challenging task in organic synthesis. The American Chemical Society Green Chemistry Institute of Pharmaceutical Roundtable (ACS GCIPR) was established to promote green chemistry; engineering and technology in the area of worldwide pharmaceuticals [25]. The ACS GCIPR concluded that C—N bond formation was one of the most exploiting key steps for production of many pharmaceutical intermediates [25]. More recently in a survey of pharmaceutical industries, Carey et al. [26] found that 15 (12%) of acylation reactions while 84 (66%) *N*-acylation reactions were involved in the synthesis of 128 drug intermediates indicating the importance of *N*-acylation reactions [26]. In multistep synthesis of a drug molecule, acylation is a basic elementary step for protection of reactive amine which blocks nucleophilic as well as basic character of amines and thus avoids the interference in multistep synthesis [24]. Basically, *N*-acylation synthesis involves harsh chemicals such as the acid/thionyl/oxalyl chloride or by using coupling agents such as 1-hydroxybenzatriazole, 1-hydroxyl-7-azabenzotriazole, carbonyldiimidazole, dicyclohexylcarbodiimide [24,25]. Each of these above methods has several drawbacks such as production of toxic and harmful side products, shock sensitivity, poor atom efficiency, non-ecofriendly methodology [24–26].

In continuation to our research interest in amidation reactions [27–31] and bearing in mind the goal of non-toxicity, low cost, recovery, reusability of the catalyst, high substrate specificity and smooth reaction conditions; we herein report an effort towards development of green protocol. Certain enzymes such as amidase and protease, etc. are reported for the amide bond formation reaction. However, they have a major drawback as they hydrolyze the amide bond readily which lowers the yield and activity in the organic solvent [32]. Lipase has numerous benefits in comparison with other enzymes such as greater tolerance to organic solvents, high stability, easy viability, wide substrate array [25,32]. Taking the advantage of this, we were interested in developing amidation and *N*-acylation (C—N bond formation) reactions; which need to be explore more for comprehensive research than the conventional transesterification reactions. Acetamides are the biologically active molecules and are widely used in biomedical applications, enzyme inhibitor and herbicides along with the pharmacy intermediate [31]. To best of our knowledge acylation of amine via immobilized lipase on biocompatible and biodegradable ternary blended thin film has not yet reported. Considering this issue, we have studied the synthesis of acetamides using vinyl acetate as an acyl donor with lipase CCL immobilized on a ternary blend biopolymer (PLA—PVA—CH) as a biocatalyst. Also a mechanistic pathway was elucidated for the lipase catalyzed acetamide synthesis reactions. Furthermore we characterize the immobilized lipase, examine kinetic-thermodynamic parameters, leaching, activity, stability and recyclability.

2. Experimental

2.1. Materials

Mucor javanicus lipase (AmanoM, lipase MJL, white powder, ≥10,000 U/g), was kindly gifted by Amano Enzymes (Nagoya, Japan) while *Candida cylindracea* lipase (lipase CCL, yellow-brown powder, ≥2000 U/g), *C. rugosa* lipase (lipase CRL, light brown powder, ≥2000 U/g), PVA (Mw. 9000–10,000), CH (Brookfield viscosity > 200.0 cps) and *p*-NPP were purchased from Sigma–Aldrich Ltd., India. Bovine serum albumin (BSA) and Folin-Ciocalteu reagent was purchased from Hi Media Ltd., India. PLA was synthesized in the lab by the Marques et al. procedure [33]. Other all chemicals of analytical grade were purchased from Hi media (I) Pvt. Ltd. and S.D. Fine Chemicals Ltd.

2.2. Immobilization of lipase

Initially ternary blend of polymer was prepared with reported procedure of Grande and Carvalho [22] with slight modification followed by the immobilization of lipase. CH (50 mg) was dissolved in 1% acetic acid solution while PVA (300 mg) was dissolved in deionized water (2%, w/w solution) separately. The PLA (50 mg) was dissolved in chloroform (2%, w/w solution). Each solution was stirred for 30 min at 1200 rpm separately and was filtered by Whatman paper filter. Then CH and PVA solution was mixed in a beaker with PLA solution and stirred for 4 h at 1500–1600 rpm. After 4 h, the crude extract lipase (100 mg; twice the amount of CH) dissolved in 2 mL deionized water; was added to ternary blend and moderately stirred for 1 h at 150–180 rpm. Finally this immobilized enzyme into ternary blend was carefully poured in Teflon Petri-dish, followed by drying at 40–45 °C for 40–48 h. A thin film of immobilized lipase polymer formed was cut off into small pieces of 2–3 mm^2 and stored in plastic container at 4–8 °C until use. Theoretically, 100 mg of crude extract CCL was loaded on the 400 mg of support. The practical protein content was calculated by Folin and Lowry assay. The prepared immobilized biocatalyst was characterized by various techniques such as thermal analysis, % water content, surface morphology study, I.R. and further exploited for *N*-acylation reactions (Fig. 1).

2.2.1. Immobilization of lipase using cross linking agent glutaraldehyde

Process for the immobilization of lipase using cross linking agent glutaraldehyde was same as described above in Section 2.2. The only minor change was made as after addition of crude extract lipase; the 0.5% of glutaraldehyde solution (25%) was added to ternary blend and moderately stirred for 1 h at 150–180 rpm. Rest of the immobilization process followed was same as indicated in the above Section 2.2.

2.3. Lipase activity assay

Lipase activity of crude extract and immobilized lipase was determined in triplicate by hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) at 410 nm wavelength using

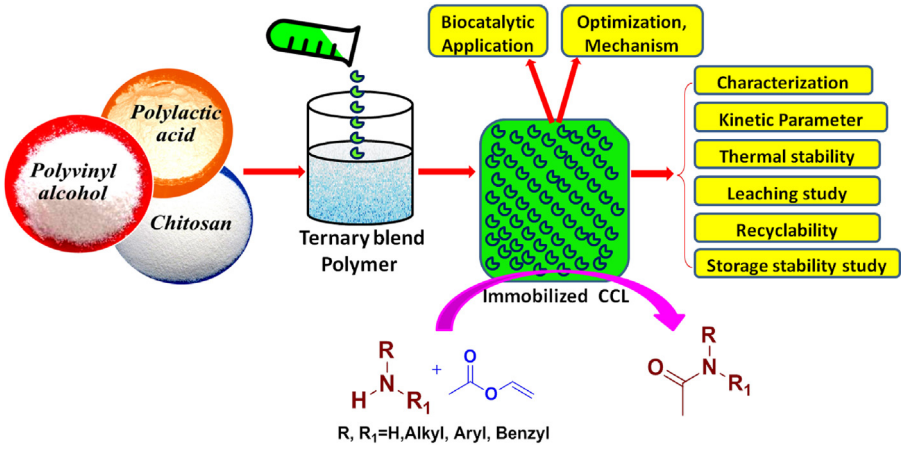


Fig. 1. Immobilization of CCL on compatible ternary blend polymer film: Characterization, activity, stability and its application for *N*-acylation reaction.

the reported procedure with little modification [34]. The samples were centrifuged for 10 min at 15,000 rpm before taking the absorbance at 410 nm. Lipase activity unit (IU) was defined as micromoles of *p*-NPP released min^{-1} by mg^{-1} of lipase under given condition [34]. The immobilization yield was calculated using reported procedure by the Karra-Châabouni et al. [35] and Vaidya and Singhal [36].

2.4. Protein content assay

Folin and Lowry method was used to determine the protein content of crude extract as well as immobilized lipase in triplicate at 660 nm wavelength [35,37]. Bovine serum albumin protein was used as a standard reference. The protein loading and specific activity of immobilized lipase was calculated by reported method [20,35–37].

2.5. Determination of kinetic constant

The maximum reaction velocity (V_{max}) and Michaelis–Menten constant (K_m) for the crude extract and immobilized lipase was determined by plotting Lineweaver–Burk plot for *p*-NPP activity assay in the range of substrate concentration 10–35 mM.

2.6. Characterization of immobilized lipase

Scanning electron microscopy (SEM) analysis (FEI, Quanta 200) was performed to study the surface morphology. The SEM images were recorded at 20 kV using LFD detector under low vacuum by placing sample film on carbon stub. DSC analysis was carried out by Shimadzu DSC-60 H to investigate the glass transition (T_g), melting (T_m) and crystallization (T_c) temperature. About 4–6 mg sample was placed in an alumina pan along with empty alumina pan as a reference. The analysis program was 20 °C/min rise from 30 to 220 °C, followed by cooling rate 8 °C/min, with carrier gas nitrogen at flow rate of 25 cm^3/min. The FT-IR (ATR) analysis (JASO-FTIR-400 model) was carried out to compare parent conformation of immobilized lipase with crude extract lipase. Karl Fischer titration analysis was done to determine the % water content of immobilized biocatalyst films (784 KFP Titrino). The N_2 adsorption–desorption isotherm at the −196 °C was carried out to determine the effect of immobilization on the pore size and surface area of immobilized lipase by the Micrometritics ASAP-2020 analyzer. The samples were degassed at 60 °C for 6 h. The BET surface area and pore volume was determined under the given condition.

2.7. General experimental procedure for N-acylation reaction

The catalytic activity of immobilized lipases was studied by a model reaction of dibutylamine with vinyl acetate in *n*-hexane. The 1 mmol of dibutylamine and 3 mL of *n*-hexane were taken into 10 mL glass stoppered tube followed by the addition of 4 mmol of vinyl acetate. On mild stirring, 50 mg of the immobilized lipase film or 10 mg of crude extract lipase was added as a biocatalyst to start the reaction. Teflon sealed reaction vial was then kept in an orbital shaker at 55 °C with rotation speed of 150 rpm for 12 h. After completion, the reaction mixture was filtered through Whatman filter paper and biocatalyst was washed thrice with *n*-hexane to eliminate likely product or reactant attached to the film. The reaction progress was determined by gas chromatography (Perkin Elmer, Clarus 400 model) equipped with a capillary column (Elite-1, 30 m × 0.32 mm × 0.25 μm) and flame ionizing detector. In GC analysis program, the oven temperature was maintained at 80 °C for 3 min with a rise of 10 °C min^{-1} up to 250 °C and was further continued up to 250 °C for 30 min. The injector and detector temperature was maintained 220 and 260 °C, respectively. All synthesized acetamides are well reported in the literature [32,38–45] and were confirmed by GCMS analysis (Shimadzu QP 2010).

2.8. Reusability study

Reusability of crude extract and immobilized lipase were checked for given model *N*-acylation reaction up to four consecutive cycles for 12 h. On completion of each cycle, the filtered immobilized film CCL or crude extract lipase powder was rinsed with the *n*-hexane (3 times) followed by drying for 12–15 h at 40–45 °C and then stored in airtight plastic container at 3–6 °C till use for next recycle. In a similar way the recyclability of the cross linked immobilized lipase PLA:PVA:CH:CCL (1:6:1:2)-Glu (0.5%) was studied.

2.9. Enzyme leakage study

Immobilized lipase PLA: PVA: CH: CCL (1:6:1:2) was subjected to the leakage study as described by Ozyilmaz and Gezer [46] spectrophotometrically at 280 nm in triplicate. To obtain standard curve, the crude extract CCL concentration was varied from 0.5 to 4.0 mg mL^{-1}. Immobilized lipase (50 mg) was added to *n*-hexane (3 mL) and rotated on orbital shaker at 150 rpm for 3–24 h. In a similar way the leaching of the cross linked immobilized lipase PLA:PVA:CH:CCL (1:6:1:2)-Glu (0.5%) was studied. The absorbance of decanted *n*-hexane was measured at 280 nm from each set at 3, 6, 12, 18 and 24 h using pure *n*-hexane as a blank.

2.10. Determination of thermal stability

The thermal stability study was carried out for the crude extract lipase and immobilized lipase by measuring the residual activities after incubation in the temperature range of 30–70 °C for 2 h in *n*-hexane at 150 rpm by rotating in on orbital shaker. For this the residual activity for control lipase (immobilized and crude extract) was taken as the 100% which was incubated at 45 °C in *n*-hexane at 150 rpm by rotating in orbital shaker.

In the second experiment crude extract and immobilized lipases were incubated for different time intervals at 50 °C and 150 rpm in orbital shaker. In addition to this various kinetic and thermodynamic parameters such as energy of activation (E_a) in the temperature range of 30–45 °C, half life period ($t_{1/2}$) and the deactivation rate constant (k_d) were also determined for crude extract and immobilized lipase by using Dave and Madamwar method [10,47].

2.11. Effect of the organic solvent stability

The effect of various organic solvents on the stability of PLA:PVA:CH:CCL (1:6:1:2) and cross linked PLA:PVA:CH:CCL:GLU (1:6:1:2:0.5%) immobilized lipase was determined. The immobilized lipase was placed in orbital shaker at speed of 150 rpm for incubation in various organic solvents for given time interval at 50 °C. After incubation the solvent was removed by the filtration and immobilized lipase was dried. This immobilized lipase was then assayed for *p*-NPP lipase activity under standard conditions. The residual activity of the lipase was defined as the ratio of the activity of solvent incubated immobilized lipase to the activity of the non-incubated immobilized lipase.

2.12. Activity and storage stability study

Immobilized lipase polymer was stored in airtight plastic container for a period of 300 days at 4–6 °C. For given shelf life period; the % yield for *N*-acylation

Table 1
Determination of (%) immobilization yield, (%) protein loading and specific activity.

No.	Crude extract or immobilized enzyme	Lipase activity		C: % immobilization yield	Protein content		F: % protein loading	G: specific activity
		A: Crude extract lipase	B:immobilized lipase		D: crude extract lipase	E: immobilized lipase		
1	Crude extract MJL	30.39	–	–	275.38	–	–	0.110
2	Crude extract CRL	40.30	–	–	226.53	–	–	0.177
3	Crude extract CCL	44.68	–	–	230.76	–	–	0.193
4	PLA:PVA:CH:MJL (1:6:1:2)	30.39	27.55	90.65	275.38	259.23	94.13	0.106
5	PLA:PVA:CH:CRL (1:6:1:2)	40.30	38.03	94.37	226.53	217.69	96.10	0.174
6	PLA:PVA:CH:CCL (1:6:1:2)	44.68	42.47	95.05	230.76	222.12	96.25	0.191
7	PLA:PVA:CH:CCL (1:6:1:1)	44.68	23.54	52.69	230.76	124.77	54.07	0.188
8	PLA:PVA:CH:CCL (1:4:1:2)	44.68	36.47	81.62	230.76	220.04	95.35	0.165
9	PLA:PVA:CH CCL (1:8:1:2)	44.68	43.26	96.82	230.76	224.30	97.20	0.192
10	CH:CCL (4:1)	44.68	35.49	79.43	230.76	198.76	86.13	0.178
11	PVA:CCL (4:1)	44.68	33.79	75.63	230.76	191.15	82.83	0.176
12	PLA:CCL (4:1)	44.68	34.38	76.95	230.76	189.69	82.20	0.181
13	CH:PVA:PLA:(1:6:1) control	0	0	0	0	0	0	0

A: Crude extract lipase activity (U); B: Immobilized lipase activity (U/g of support); C: % Immobilization yield (B/A × 100); D: Amount of protein content of crude extract lipase (mg/g); E: Amount of protein content of immobilized lipase (mg/g of support); F: % Protein loading for immobilization (E/D × 100); G: Specific activity (A/D for crude extract enzyme and B/E for immobilized enzyme).

reaction, immobilization yield, protein loading and specific activity were calculated as described in above section at regular interval of 30 days [34–37].

3. Result and discussion

3.1. Lipase activity assay

Activity for crude extract as well as immobilized lipase was determined and it was found as: lipase CCL > lipase CRL > lipase MJL. Immobilization yield was found to be 95.05%, 94.37% and 90.65% for lipase CCL, CRL and MJL, respectively (Table 1, entries 1–6). High immobilization yield assures the immobilization of lipase on polymeric support [20,36]. The immobilization yield of several support compositions was studied and it was found that maximum immobilization yield for the best suitable support PLA:PVA:CH:CCL (1:6:1:2) biocatalyst was 95.05% (Table 1, entry 6).

3.2. Protein activity assay

The protein content for crude extract and immobilized lipase was determined and it was found as: lipase MJL > lipase CCL > lipase CRL. While protein loading was found to be 94.13%, 96.10% and 96.25% for lipase MJL, CRL and CCL, respectively (Table 1, entries 1–6). For various immobilized lipases support the protein loading was calculated and found to be 82–97% (Table 1, entries 4–13). The maximum protein loading for best suitable support PLA:PVA:CH:CCL (1:6:1:2) biocatalyst was 96.25% (Table 1, entry 6).

The present methodology does not have any rinsing or decant step which results into the high protein loading and immobilization yield [21]. We found slight increment in lipase activity, the protein content, protein loading and immobilization yield for the immobilized PLA:PVA:CH:CCL (1:8:1:2) when compared to the PLA:PVA:CH:CCL (1:6:1:2) lipase, but still we chose the PLA:PVA:CH:CCL (1:6:1:2) lipase for the further study as we required a lowest PVA content to produce a robust biocatalyst (economically more attractive).

3.3. Kinetic parameter study

Effect of kinetic parameter (V_{max} and K_m) was determined for the crude extract and immobilized lipase for the *p*-NPP activity assay at various substrate concentrations from 10 to 35 mM by Lineweaver–Burk plot. V_{max} indicates the maximum possible velocity for the corresponding transformation while the K_m indicates the enzyme substrate affinity [10,35]. V_{max} for the crude extract and immobilized enzyme was 0.032 and 0.0345, respectively, which indicates the slightly greater value of V_{max} for immobilized enzyme. If the V_{max} values considered as the hydrolytic activity of crude extract lipase under substrate non-limited condition, then the maximum activity yield of immobilized lipase was found to be 108% [10]. While the K_m value for the crude extract and immobilized enzyme was 10.57 and 16.59 mM, respectively. The higher K_m value showed that the immobilized enzyme has the lesser affinity towards the substrate than the crude extract enzyme which might be contributed for the reason of (i) poor substrate diffusion rate for enzyme accessibility (ii) steric hindrance created by the active site of support or (iii) the loss of enzyme flexibility at the time of immobilization [10,35].

3.4. Characterization of immobilized lipase film

3.4.1. Surface morphology study (SEM)

SEM analysis of PLA:PVA:CH (1:6:1) control and immobilized CCL were carried out to study surface morphology (Fig. 2). The control ternary blend film of PLA:PVA:CH (1:6:1) showed a clear plane surface with very few globules of PLA (Fig. 2A) [22]; While that of immobilized lipase film (CH–PVA–PLA–CCL) showed an even mosaic surface (Fig. 2B), indicating the immobilization of lipase on the support [21]. Thus surface morphological study demonstrated the immobilization of lipase CCL on biocompatible PLA, PVA, CH based ternary blend film [21,22].

3.4.2. FTIR analysis

FTIR spectroscopy is the one of the most vital technique to examine the conformation of lipase after immobilization (Fig. 3). The FTIR analysis of lipase majorly consists of the three characteristics amide I, II, III bands in between spectral region of 1700–1300 cm^{-1} [17,48]. Amide I band at 1600–1700 cm^{-1} is a characteristic feature of the C—O stretching vibrations. Amide II band is observed at 1500–1600 cm^{-1} because of N—H bending and C—N stretching vibrations. The amide III band is attributed at 1300–1450 cm^{-1} owing to, C—C, C—N stretching and N-H bending vibrations [48]. In the present work, the amide I, II, III region for crude extract lipase was observed at 1665, 1554 and 1415 cm^{-1}, respectively (Fig. 3i); while for immobilized lipase it was at 1643, 1570 and 1425 cm^{-1}, respectively (Fig. 3h). Thus the wave number values of the amide

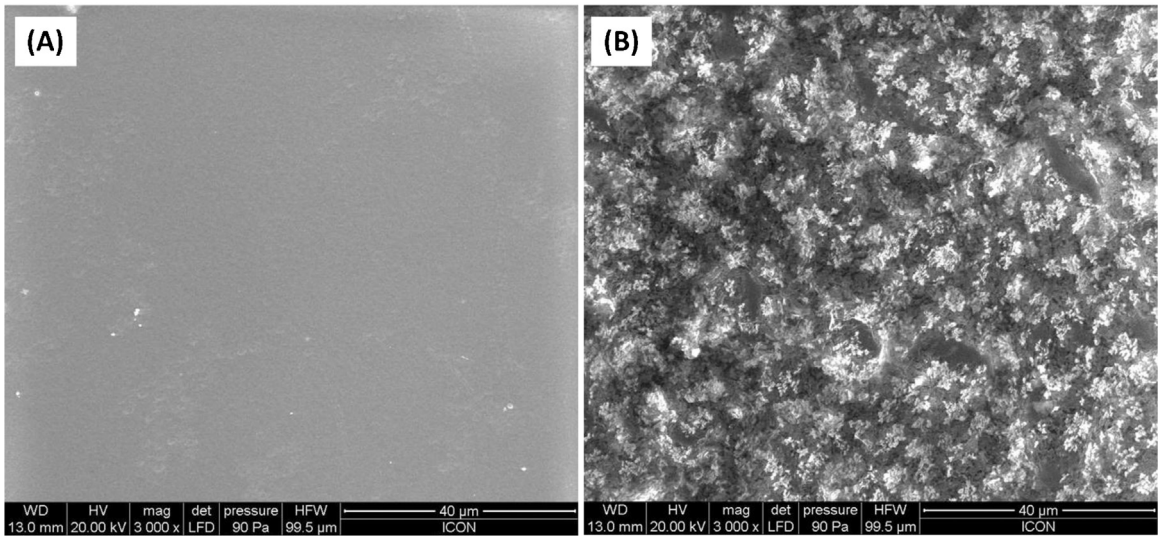


Fig. 2. SEM images of: (A) PLA:PVA:CH (1:6:1) control, (B) PLA:PVA:CH:CCL (1:6:1:2) immobilized biocatalyst.

I, II, III region for the crude extract CCL and immobilized CCL lipase are closely related. The above results assure that the parent conformation of lipase was preserved after immobilization [48]. The wave number values of CH, PVA, and PLA are in good agreement with the earlier literature [22]. Also appearance of similar bands at the end of fourth recycle revealed no change in the native conformation of lipase after four successive reuses (Fig. 3j).

3.4.3. DSC analysis

Furthermore, DSC analysis provided the T_g, T_m, T_c values of CH:PVA:PLA (1:6:1) control at 76.80, 171, 128 °C respectively. While T_g, T_m, T_c for immobilized lipase CH:PVA:PLA:CCL (1:6:1:2) was observed at 71.9, 174, 116 °C respectively. Thus decrease in T_g value for immobilized lipase was attributed due to the protein degradation [49]. Increase in T_m value for immobilized lipase is due to the high molecular weight of enzyme immobilized on the control support. Reduction in T_c value of immobilized lipase was indicating the decrease in the crystallization temperature because of change in physical properties of control polymer support after immobilization. The endothermic peaks are responsible for the degradation of protein [49] which is attributed at 73.6 °C for crude extract lipase CCL (please refer supporting information for various DSC graphs).

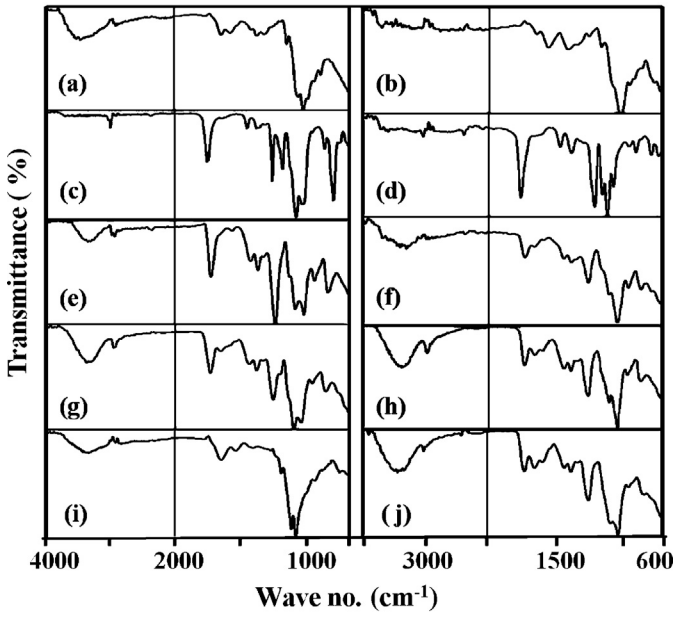


Fig. 3. FT-IR Spectrum of: (a) CH control, (b) CH-CCL, (c) PVA control, (d) PVA-CCL, (e) PLA control, (f) PLA-CCL, (g)PLA:PVA:CH (1:6:1) control, (h) PLA:PVA:CH:CCL (1:6:1:2), (i) crude extract CCL, (j) PLA:PVA:CH:CCL (1:6:1:2) after 4 recycle.

3.4.4. (%) water content analysis

Karl Fischer titration method was used to determine the % water content (Table 2) for various immobilized support and crude extract lipase CCL. The water content for PLA–PVA–CH (1:6:1) control was 8.18% and for the best immobilized lipase support PLA–PVA–CH–CCL (1:6:1:2) it was 8.82% while for crude extract lipase it was 0.91% (Table 2, entries 1, 5 and 9). The PLA–PVA–CH-CCL (1:4:1:2) and PLA–PVA–CH–CCL (1:8:1:2) having water content 8.66% and 8.90%, respectively (Table 2, entries 10 and 11). Furthermore cross linked immobilized lipase PLA:PVA:CH:CCL:GLU (1:6:1:2:0.5%) having the water content 8.87% (Table 2, entry 12). This presence of trace amount of water is very essential to maintain the biocatalytic activity of enzyme [21].

3.4.5. N_2 adsorption–desorption isotherm

N_2 adsorption–desorption isotherm were studied for the control support and immobilized lipase (of various lipase loading). The control support PLA:PVA:CH (1:6:1) have the BET surface area 0.8047 m^2/g and total volume in pore 0.00710 cm^3/g (Table 3, entry 1). While the immobilized lipase PLA:PVA:CH:CCL (1:6:1:2) has the BET surface area 0.5828 m^2/g and total volume in pore

Table 2
Determination of (%) water content by Karl Fischer method.

Entry	Sample	Water content (%, w/w)
1	PLA:PVA:CH (1:6:1) control	8.18
2	CH-control	2.78
3	PLA-control	1.97
4	PVA-control	3.40
5	PLA:PVA:CH:CCL (1:6:1:2)	8.82
6	CH:CCL (4:1)	5.04
7	PLA:CCL (4:1)	3.38
8	PVA:CCL (4:1)	3.40
9	Crude extract CCL	0.91
10	PLA:PVA:CH:CCL (1:4:1:2)	8.66
11	PLA:PVA:CH:CCL (1:8:1:2)	8.90
12	PLA:PVA:CH:CCL:GLU (1:6:1:2:0.5%)	8.87

Table 3
N_2 adsorption–desorption isotherm.

Entry no.	Sample	Lipase loading (mg)	BET surface area (m^2/g)	Pore volume (cm^3/g)	Pore area (m^2/g)
1	PLA:PVA:CH (1:6:1)	000	0.8047	0.00710	1.368
2	PLA:PVA:CH:CCL (1:6:1:2)	100	0.5828	0.00184	0.128
3	PLA:PVA:CH:CCL (1:6:1:3)	150	0.4373	0.00107	0.064

Table 4
Screening of lipases for dibutyl acetamide synthesis[a].

Entry	Lipase source	Yield (%)[b]				
		1 h	3 h	6 h	9 h	12 h
1	Crude extract CRL	2	4	7	10	14
2	Crude extract CCL	3	6	10	13	18
3	Crude extract MJL	nr	2	5	09	12
4	PLA:PVA:CH:CRL (1:6:1:2)	12	20	47	67	80
5	PLA:PVA:CH:CCL (1:6:1:2)	14	33	59	86	99
6	PLA:PVA:CH:MJL (1:6:1:2)	2	8	19	28	40

[a] *Reaction conditions*: dibutylamine (1 mmol), vinyl acetate (4 mmol), *n*-hexane (3 mL), crude extract lipase (10 mg)/immobilized PLA:PVA:CH:lipase (1:6:1:2) – (50 mg), temperature (55 °C), agitation speed 150 rpm.
[b] Yields based on GC analysis; nr indicates "no reaction" means reaction was performed but did not get any yield (No Yield).

Table 5
Screening of various support composition of immobilized biocatalyst for acetamide synthesis[a].

Entry	Biocatalyst	Yield (%)[b]				
		1 h	3 h	6 h	9 h	12 h
1	PLA:PVA:CH:CCL (1:6:1:1)	10	19	39	61	78
2	PLA:PVA:CH:CCL (1:6:1:2)	14	33	59	86	99
3	PLA:PVA:CH:CCL (1:6:1:3)	15	39	60	94	99
4	CH:CCL (4:1)	8	16	34	57	70
5	PLA:CCL (4:1)	3	7	15	19	25
6	PVA:CCL (4:1)	11	22	45	68	85
7	PLA:PVA:CH (1:6:1) control	nr	nr	nr	nr	nr
8[c]	CH-control	nr	nr	nr	nr	nr
9[c]	PLA-Control	nr	nr	nr	nr	nr
10[c]	PVA-control	nr	nr	nr	nr	nr
11[d]	Blank	nr	nr	nr	nr	nr

[a] Reaction conditions: dibutylamine (1 mmol), vinyl acetate (4 mmol), *n*-hexane (3 mL),immobilized CCL biocatalyst (50 mg), temperature (55 °C), agitation speed 150 rpm.
[b] Yield based on GC analysis.
[c] Support without lipase.
[d] Without support and without lipase, nr denotes no reaction.

0.00184 cm^3/g where lipase loading is 100 mg (Table 3, entry 2). When further increase in lipase loading up to 150 mg then PLA:PVA:CH:CCL (1:6:1:3) have the BET surface area 0.4373 m^2/g and total volume in pore 0.00107 cm^3/g (Table 3, entry 3).

Thus after determination of the BET surface area and pore volume we found that the immobilized lipase occupies the lower N_2 volume than the control support itself (Table 3). Thus from the above results it was clear that as the lipase loading increases then there is a decrease in the BET surface area and the pore volume (Table 3, entries 1–3). This showed that the lipase molecules were entrapped inside the polymer matrix which leads to block the pores and hence pore volume decreased after immobilization of the lipase or increase in lipase loading from the 100 to 150 mg (Table 3, entries 1–3)[7,50–52]. Similar types of results were reported by the Pirozzi et al. [50], He et al. [51] and Yunyu et al. [52] where the BET surface area and pore volume were decreased after the immobilization of enzyme by entrapment.

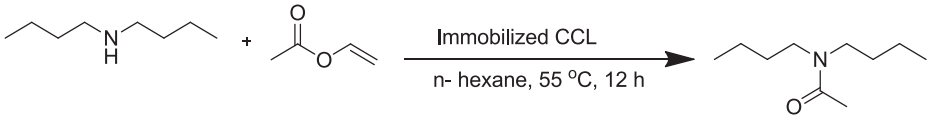
3.5. Application of immobilized CCL for N-acylation reaction

3.5.1. Lipase screening for N-acylation reaction

Initially crude extract and immobilized lipases namely *C. cylindracea* (CCL), *C. rugosa* (CRL), and *M. javanicus* (MJL) were screened to check out the catalytic activity. For this purpose, the reaction of dibutylamine with vinyl acetate as an acyl donor was chosen as a model reaction. Crude extract CRL, CCL and MJL provided only 14%, 18% and 12% yield of desired product (Table 4, entries 1–3) in 12 h. While the immobilized lipase CRL, CCL and MJL provided 80%, 99% and 40% yield of desired product in 12 h (Table 4, entries 4–6). Screening results showed that the catalytic activity of immobilized lipase was remarkably improved by the immobilization when compared with the crude extract lipase [3,4,16] (Table 4, entries 1–6). The lower activity of the crude extract lipase was attributed due to aggregates of lipases formed by strong interaction of hydrophobic pockets surrounding the active site of lipase molecules in open conformation. However, after immobilization on ternary blend polymer films, these aggregates were displaced to monomeric form, yielding good dispersion of enzyme molecules after immobilization step [53]. Thus from the above screening results it was clear that immobilized CCL (PLA:PVA:CH:CCL (1:6:1:2)) was found to be best suitable biocatalyst providing the 99% yield in 12 h as compared to immobilized CRL and MJL for this transformation and selected for further study (Table 4, entries 4–6) (Scheme 1).

3.5.2. Screening of support composition

In addition to this, various support compositions (Table 5, entries 1–6) of immobilized biocatalyst (CCL) films were prepared to find out the best support composition for improvement of immobilization, catalytic activity and economic feasibility. We had selected the suitable PVA content to prepare robust hybrid support for the lipase immobilization as PVA promoted microenvironment which allowed a better diffusion of substrate molecules to the active site of immobilized lipase [22,23,47,53]. Various preparations of immobilized biocatalyst were screened for dibutyl acetamide synthesis; where it was found that PLA:PVA:CH:CCL (1:6:1:2) was the best biocatalyst for given model reaction giving 99% yield in 12 h of the corresponding product as compared to other compositions of supports (Table 5, entries 1–6). When compared to the amount of lipase loading one could states that PLA:PVA:CH:CCL (1:6:1:2) is a better suitable support than PLA:PVA:CH:CCL (1:6:1:3) and hence used for the further study. (Table 5, entries 2 and 3). Various control experiments were carried out to check the effect of support on reaction; however no reaction took place (Table 5, entries 7–11) which confirms that lipase was exclusively responsible for acetamide synthesis.



Scheme 1. Immobilized lipase catalyzed *N-acylation* of dibutylamine.

Table 6
Optimization of reaction parameters for immobilized CCL catalyzed dibutyl acetamide synthesis[a].

Entry	Solvent	Molar ratio[b]	Temp (°C)	Biocatalyst[c] (mg)	Orbital rotation (rpm)	Yield (%)[d]
Influence of molar ratio						
1	*n*-Hexane	1:1	55	50	150	20 (nr)
2	*n*-Hexane	1:2	55	50	150	29 (nr)
3	*n*-Hexane	1:3	55	50	150	63 (10)
4	*n*-Hexane	1:4	55	50	150	99 (18)
5	*n*-Hexane	1:5	55	50	150	99 (24)
Influence of solvent						
6	Dimethyl formamide	1:4	55	50	150	15 (nr)
7	Acetone	1:4	55	50	150	41 (nr)
8	1,4-Dioxane	1:4	55	50	150	38 (03)
9	Carbon tetrachloride	1:4	55	50	150	23 (nr)
10	Tetrahydrofuran	1:4	55	50	150	19 (nr)
11	Diethyl ether	1:4	55	50	150	79 (13)
12	Toluene	1:4	55	50	150	86 (15)
13	Cyclohexane	1:4	55	50	150	99 (17)
14	*n*-Hexane	1:4	55	50	150	99 (18)
Influence of temperature						
15	*n*-Hexane	1:4	25	50	150	20 (nr)
16	*n*-Hexane	1:4	35	50	150	50 (08)
17	*n*-Hexane	1:4	45	50	150	76 (15)
18	*n*-Hexane	1:4	55	50	150	99 (16)
19	*n*-Hexane	1:4	65	50	150	99 (14)
Influence of catalyst loading						
20	*n*-Hexane	1:4	55	10	150	23 (05)
21	*n*-Hexane	1:4	55	30	150	49 (09)
22	*n*-Hexane	1:4	55	50	150	99 (18)
23	*n*-Hexane	1:4	55	70	150	99 (29)
Influence of orbital rotation speed						
24	*n*-Hexane	1:4	55	50	60	45 (08)
25	*n*-Hexane	1:4	55	50	100	71 (12)
26	*n*-Hexane	1:4	55	50	150	99 (18)
27	*n*-Hexane	1:4	55	50	180	99 (21)

[a] *Reaction conditions*: biocatalyst PLA:PVA:CH:CCL (1:6:1:2), solvent (3 mL), time (12 h), agitation speed (150 rpm).
[b] *Molar ratio*: Dibutylamine:vinyl acetate.
[c] Biocatalyst-immobilized PLA:PVA:CH:CCL (1:6:1:2) lipase: 50 mg/crude extract lipase CCL: 10 mg (50 mg immobilized CCL lipase is theoretically equivalent to 10 mg of crude extract CCL lipase by composition).
[d] Yields based on GC analysis and yield obtained by crude extract lipase catalyzed *N*-acylation are indicated in parentheses; nr denotes no reaction.

3.5.3. Optimization of various reaction parameters

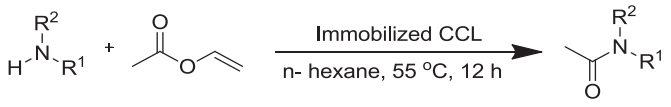
Various reaction parameters were optimized for the model reaction such as molar ratio, solvent, temperature, catalyst loading and orbital rotation speed using immobilized PLA: PVA: CH: CCL (1:6:1:2) film. The molar ratio of dibutylamine: vinyl acetate was varied from 1:1 to 1:5 to obtain the maximum yield of desired product. It was found that increase in molar ratio from 1:1 to 1:4 leads to increase in yield, while further increase in molar ratio to 1:5 did not influence the yield of dibutyl acetamide (Table 6, entries 1–5). Vinyl alcohol is formed as a byproduct when vinyl acetate is used as an acyl donor. The vinyl alcohol being unstable, it tautomerizes in to the acetaldehyde which reacts with the amino group present in the lipase tends to form Schiff's base hence deactivate the lipase catalytic activity [54].

Variety of polar and nonpolar organic solvents having log *P* value from −1.0 to 3.5 were tested for the given model reaction (Table 6, entries 6–14). It was found that yield of the reaction decreases in polar solvents, as compare to nonpolar solvents (Table 6, entries 6–10). This is probably due to removal of an essential amount of trace water from the catalytic site of enzyme which causes deformation of the native lipase in polar solvents and hence reduces the catalytic efficiency, such type of stripping of water from the vicinity of enzyme molecule is not observed in case of the nonpolar solvents [55]. Thus the non-polar solvents such as *n*-hexane and cyclohexane having Log *P* value near about 3.5 were found to be good solvent providing outstanding yield (99% yield) of desired product (Table 6, entries 13 and 14) while solvents like toluene and diethyl ether (Table 6, entries 11 and 12) gave the moderate yield.

In general enzymes are active at optimum temperatures while their stability and catalytic activity reduce at elevated temperature [3–5]. In present optimization study, the immobilized lipase CCL was subjected at temperature range 25–65 °C (Table 6, entries 15–19) for given model reaction. Raising the temperature from 25 to 55 °C leads to increase in yield of desired product, while further increase in the temperature up to 65 °C does not show any change in yield. Hence 55 °C was considered to be the optimum temperature for catalytic activity of immobilized lipase CCL (Table 6, entry 18). Above observations indicated the better thermal stability of immobilized lipase CCL at higher temperatures (from 55 to 65 °C) without any significant loss of catalytic activity as compared to crude extract enzyme (Table 6, entries 18 and 19).

Amount of biocatalyst loading was studied to make the protocol economically feasible. The immobilized lipase loading was varied from 10 to 70 mg for model reaction (Table 6, entries 20–23). 50 mg of immobilized lipase provided 99% yield of the desired product (Table 6, entry 22). However further increase in amount of biocatalyst loading had no more effect on the yield since, the excess amount of biocatalyst promotes the reaction to reach the equilibrium in advance, just not change the balance; indicating that 50 mg of immobilized lipase was sufficient for the corresponding biotransformation (Table 6, entry 22).

Finally, speed of orbital rotation was optimized from 60 to 180 rpm; where yield increased when rotation increased from 60 to



R^1- Alkyl or aryl
R^2- Alkyl, aryl, or H

Scheme 2. Immobilized CCL catalyzed acetamide synthesis.

150 rpm beyond that further increase in rotation speed from 150 to 180 had no effect on the yield indicating that no external diffusion limit was observed by increasing the orbital rotation speed (Table 6, entries 24–27).

Parallel control experiments (in the absence of biocatalyst) were performed (data not shown in the Table 6) under the optimized reaction parameters, where no yield of corresponding product was obtained signifying that reaction was solely catalyzed by lipase. Also the optimized study showed that as compared to crude extract lipase CCL; the catalytic activity of immobilized lipase CCL was significantly increased four to five times (Table 6, entries 1–27, yield indicated in parentheses are obtained by crude extract lipase CCL loading). This was due to the agglomeration of crude extract lipase in the organic solvents which leads to reduce the catalytic activity as compared to the immobilized lipase [53]. Thus, concluding optimized reaction parameters were the molar ratio of dibutylamine:vinyl acetate 1:4, solvent: *n*-hexane, temperature: 55 °C, biocatalyst loading: 50 mg, orbital rotation speed: 150 rpm and time: 12 h.

3.5.4. Application of immobilized lipase CCL for various acetamide synthesis

To study the broad applicability and scope of the developed biocatalytic protocol, the optimized reaction parameters were used for *N*-acylation reaction of various aliphatic, alicyclic, cyclic, aromatic amines with vinyl acetate (Scheme 2). All synthesized acetamides were confirmed by GCMS analysis (please see the supporting information).

Simple secondary aliphatic amines (Table 7, entry 1), cyclic and heterocyclic amines (Table 7, entries 2–4) gave 99% yield of the corresponding acetamide in 12 h. Acetamide of diallyl amine (Table 7, entry 5), 1,2,3,4-tetrahydroisoquinoline (Table 7, entry 6), and *N*-methylbenzylamine (Table 7, entry 7), provided 99% yield in 11, 14 and 13 h, respectively (Table 6, entries 5–7). Primary amines like butyl amine (Table 7, entry 8), heptylamine (Table 7, entry 9) and benzyl amine (Table 7, entry 10) provided 99% yield of the corresponding acetamides in 14 h. Bulky primary amine such as tert-butyl amine (Table 7, entry 11) provided sluggish yield (40%) of the desired acetamide in 30 h. Isopropyl amine (Table 7, entry 12) offered 99% yield of respective acetamide in 16 h. Acetamide of cyclohexylamine (Table 7, entry 13) was synthesized in 15 h with 99% yield. Interestingly, the heterocyclic amine such as 5-methyl furfuryl amine (Table 7, entry 14) provided excellent yield (99%) of the corresponding *N*-acylated product. Attractively, various aromatic amines (Table 7, entries 15–18) and their derivatives give corresponding *N*-acylated product with 99% yield in 26–30 h. The 2-amino-napthol (Table 7, entry 19) and 3-aminopyridine (Table 7, entry 20) were too sluggish to provide lower yield of 20–25% of desired product in 30 h. The lower conversion was found using bulky amines such as tert-butyl amine (Table 7, entry 11) 2-Aminonapthol (Table 7, entry 19) and 3-aminopyridine (Table 7, entry 20).

Lipase shows the serine hydrolases mechanism, involving the nucleophilic attack of the amines on the acyl intermediate; which may be affected by the branching, steric crowding, basicity and nucleophilicity of the amines [25,31]. Secondary amines (Table 7, entries 1–5) showed more reactivity than the primary amines (Table 7, entries 8–10 and 14) probably because of the higher basicity and nucleophilicity. In case of secondary amines; 1,2,3,4-tetrahydroisoquinoline and *N*-methyl benzyl amine (Table 7, entries 6 and 7); the reaction rate was marginally decreased because of the steric hindrance of bulky moieties [25,31]. The steric crowding created due to the branching at the α-carbon atom which greatly affects the nucleophilicity of the tert-butyl amine and hence, it gave low yield of the corresponding product (Table 7, entry 11). Similar type of effect was found for the substrate isopropyl amine and cyclohexylamine, which showed lower reaction rate (Table 7, entries 12 and 13) [25,31,32]. The aromatic amines (Table 7, entries 15–18) such as aniline and its various derivatives were slowly reacted as the nucleophilicity was lowered in the aromatic amines because of the delocalization of lone pair of nitrogen. Furthermore the 2-amino-napthol and lone pair of nitrogen in 3-aminopyridine might be produce the strong spatial hindrance which results into very poor yield (Table 7, entries 19 and 20) [25,31,32].

3.6. Mechanism for the catalytic activity of Lipase CCL for the N-acylation reaction

Most commonly the amidase and the protease are responsible for the hydrolysis of the amide bond and hence causes a lower yield in case of the amide synthesis; however the lipase generally not involves for cleavage of the amide bond [25,32]. The lipases show the serine hydrolases mechanism which is widely accepted mechanism for the hydrolases family [25,32] (Scheme 3). It is a double displacement mechanism in which the tetrahedral intermediate involves while the catalytic activity of the lipase basically involves the catalytic triad of the serine, aspartic acid and the histidine residue [56]. At first the lipase forms the Michaelis–Menten complex with the ester (vinyl acetate) which then transform into the tetrahedral intermediate by the serine hydroxyl attack which results in the discharge of the first product (vinyl alcohol) and leads to form the acyl lipase covalent intermediate. In second step the nucleophilic species (amine) attacks on the electron deficient center of the acyl lipase covalent intermediate and leads to form the second tetrahedral intermediate. This tetrahedral intermediate again breaks up into the product (amide) and lipase. During this process, the abstraction of the proton and hydrogen bonding are incorporated by the triad of the amino acids; while the formation of the negative charge is responsible for the formation of the final corresponding product and lipase. Thus the polarization basically induced in situ by the hydrogen bonding, proton abstraction and the negative charge formation to carry out the corresponding transformation [56] (Scheme 3).

3.7. Recyclability study

Furthermore, to express the economical feasibility of the developed protocol the reusability of immobilized lipase was studied. Reusability of immobilized lipase support with varying enzyme quantity {PLA–PVA–CH–CCL (1:6:1:3), PLA–PVA–CH–CCL (1:6:1:2)} was studied up to four recycle. When compare to the quantity of lipase (CCL) loading on support; one could evaluate that PLA–PVA–CH–CCL (1:6:1:2) was more efficient biocatalyst than PLA–PVA–CH–CCL (1:6:1:3). The immobilized lipase was efficiently reused for first two consecutive recycles without any significant loss in its catalytic activity while the yield reduced up to 69% for the fourth cycle (Fig. 4). At the end of reaction acetaldehyde formed (tautomerized form of vinyl alcohol) is released which is reported to inactivate the lipase catalytic power which may contribute in decreasing yield of the recyclability [21,54]. Also the decrease in yield of the desired product was anticipated due to insignificant leaching of lipase from support and handling loss of biocatalyst

Table 7
Immobilized CCL catalyzed acetamide synthesis[a].

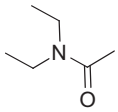
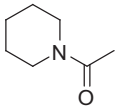
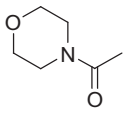
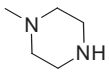
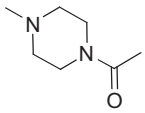
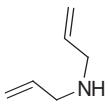
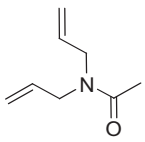
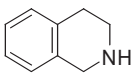
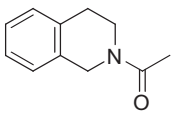
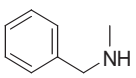
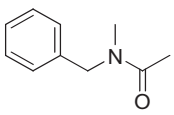
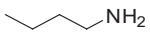
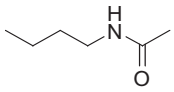
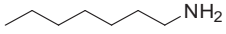
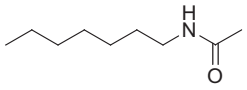
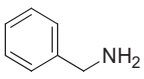
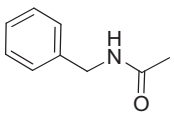
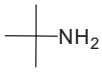
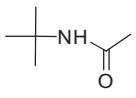
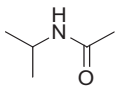
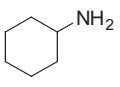
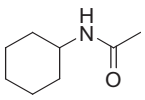
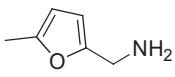
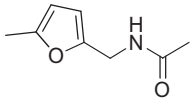
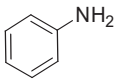
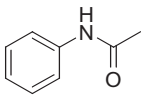
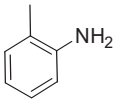
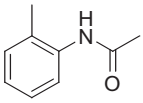
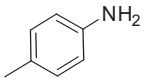
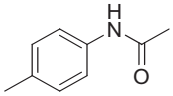
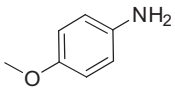
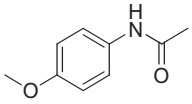
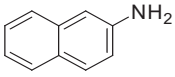
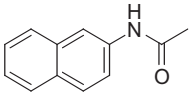
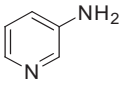
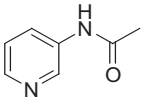
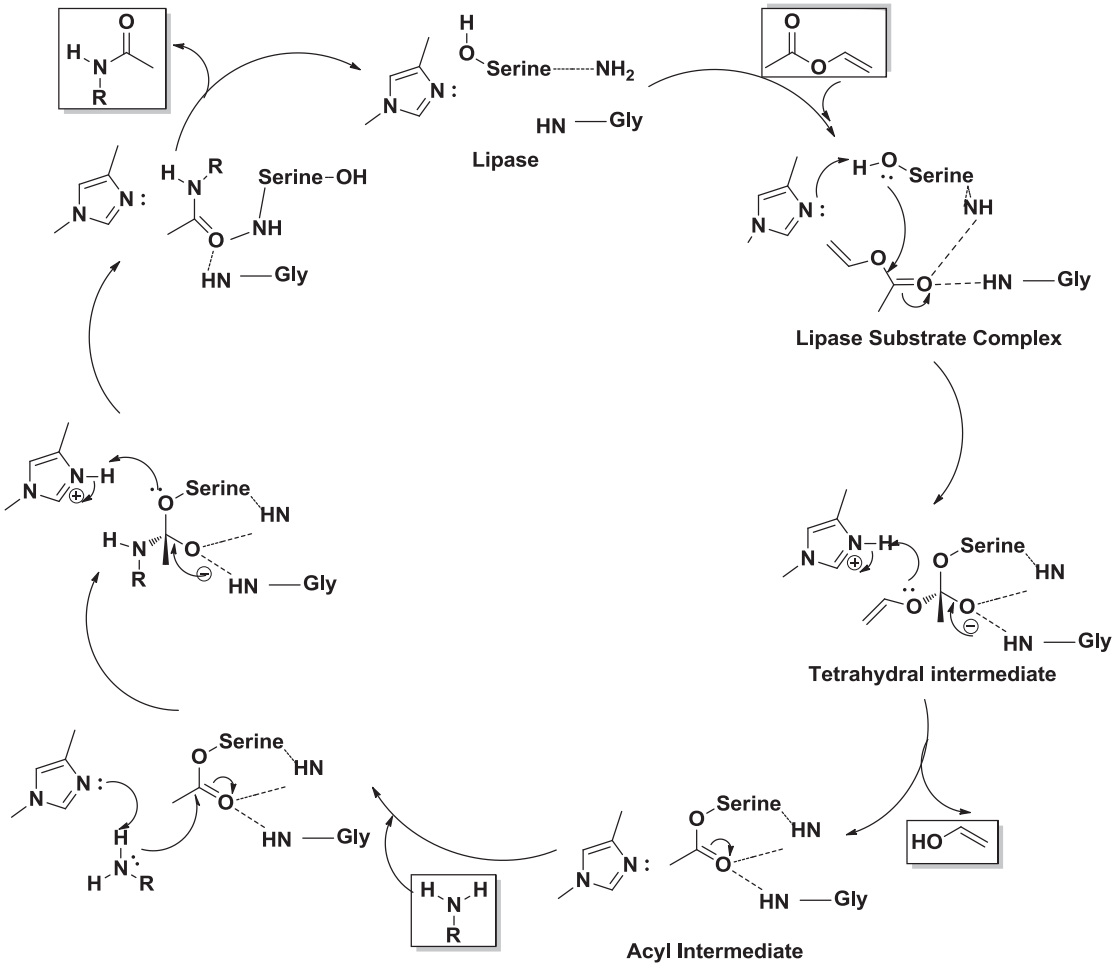
Entry	Amine	Acetamide	Time (h)	Yield (%)[b]
1			12	99 (20)
2			12	99 (18)
3			12	99 (17)
4			12	99 (20)
5			11	99 (23)
6			14	99 (16)
7			13	99 (20)
8			14	99 (15)
9			14	99 (16)
10			14	99 (17)
11			30	40 (nr)
12			16	99 (12)
13			15	99 (14)
14			14	99 (18)
15			28	99 (13)

Table 7 (*Continued*)

Entry	Amine	Acetamide	Time (h)	Yield (%)[b]
16			30	99 (15)
17			26	99 (19)
18			26	99 (17)
19			30	20 (nr)
20			30	25 (nr)

[a] *Reaction conditions*: amine (1 mmol), vinyl acetate (4 mmol), *n*-hexane (3 mL), immobilized CCL (50 mg)/crude extract CCL (10 mg), temperature (55 °C), agitation speed (150 rpm).

[b] Yields based on GC analysis and yield obtained by crude extract lipase catalyzed *N*-acylation are indicated in parentheses; nr denotes no reaction.



Scheme 3. Mechanism for the lipase catalyzed *N*-acylation reaction.

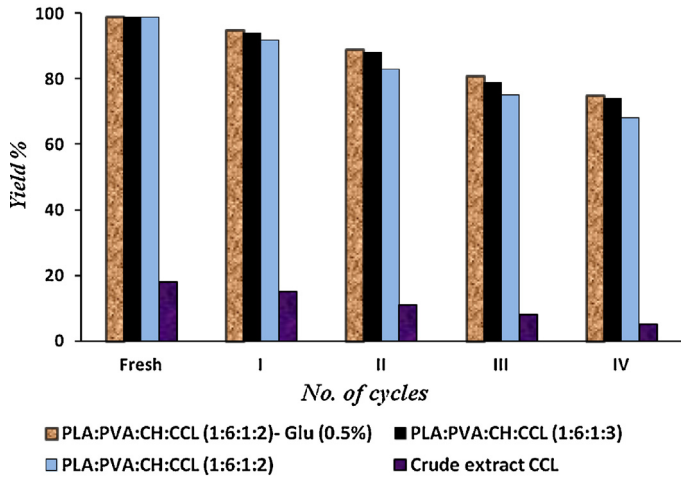


Fig. 4. Recyclability study of immobilized CCL for *N*-acylation of dibutylamine.

during the study [46]. In addition, the FTIR analysis of the fourth recycled immobilized lipase was performed which showed that no significant change in the lipase conformation was observed (Fig. 3j).

However the use of the cross linking agent immobilized lipase PLA:PVA:CH:CCL (1:6:1:2)-Glu (0.5%) showed better results for the recyclability than the PLA:PVA:CH:CCL (1:6:1:2) immobilized lipase. It showed the almost 3–5% increment in the % yield for each recycle (Fig. 4). These results might be attributed due to the low leaching of the lipase from the support after using the cross linking agent glutaraldehyde [3–7].

3.8. Enzyme leaching study

Ozyilmaz and Gezer [46] method was used to determine enzyme leakage (Table 8). The 8% and 13% of lipase leaching was occurred in 12 and 24 h, respectively, from the best suitable immobilization support PLA: PVA: CH: CCL (1:6:1:2) at 150 rpm speed of orbital shaker (Table 8, entries 3 and 5). Leaching for the 3, 6 and 18 h, was 3.59%, 5.94% ad 10.64%, respectively, for the best suitable support (Table 8, entries 1, 2 and 4). While the leaching for cross linked immobilized lipase PLA:PVA:CH:CCL (1:6:1:2)-Glu (0.5%) was found to be less than PLA:PVA:CH:CCL (1:6:1:2). Leaching for the 3, 6, 12, 18 and 24 h was 1.34%, 3.21%, 4.78%, 5.94% and 7.42%, respectively (Table 8, entries 1–5). The % leaching was decreased when cross linking agent was used for the immobilization [17,46].

3.9. Thermal stability study

The thermal stability study is necessary for the application of the enzymes in the process as enzymes are sensitive to thermal deactivation [3,47]. Hence it is necessary to determine the thermal stability as a crucial factor for practical application [35]. As compared with the crude extract lipase, the immobilized lipase exhibited a better thermal stability when studied in the temperature range of 30–70 °C (Fig. 5A). We found the broad optimum temperature range of 40 to 60 °C for the immobilized lipase; while for the crude extract lipase it was 40–45 °C. Thus thermal activity of the immobilized lipase was better conserved for the temperature range of 45–60 °C as compared to crude extract lipase.

The energy of activation was calculated in between the temperature range of 30 to 45 °C. The activation energy for the crude extract and immobilized lipase was 18.9 and 14.2 kJ/Mol respectively. Lower activation energy for the immobilized lipase indicates that low thermal energy access and maximum velocity for the transformation as compared to crude extract lipase [10,47].

After incubation for 135 h at 50 °C, the activity decay rate was more for the crude extract lipases than immobilized lipase (Fig. 5B). The original activity lost rapidly for the crude extract lipase might be due to the protein denaturation in the organic solvent at 50 °C, but for the immobilized lipase residual activity lost was found to be the 72% after incubation of the 135 h at 50 °C. These results suggested that the thermo stability of immobilized lipase increased considerably because of entrapment of lipase within the polymer matrix [3–7,10,47].

The enzyme kinetics showed the first order reaction kinetics from which the half life ($t_{1/2}$) and thermal deactivation rate constant (k_d) were calculated at temperature 50 °C. The crude extract and immobilized lipase loses the 50% of its original activity in 32 h and 95 h respectively. Higher half-life time indicates the greater thermal stability for the immobilized lipase at temperature 50 °C. The deactivation rate constant (k_d) for the crude extract and immobilized lipase was 0.0216 and 0.00729 h^{-1}. Thus the lower thermal deactivation rate constant (k_d) for the immobilized lipase showed the enhanced thermal stability [47]. Thus here we could say that lipase entrapped in the polymer matrix has higher stability for the higher temperature as compared crude extract lipase [3–7,47].

Interestingly when crude extract lipase directly loaded in the organic solvents then it may cause the agglomeration which inhibits the activity of lipase and causes the lowering of activity of crude extract lipase [53]. However the catalytic activity of immobilized lipase was found to be more by four to five folds than the crude extract lipase. These results clearly demonstrated that the enzymes molecules which are entrapped inside the polymer matrix are responsible for the improvement of catalytic activity and thermal stability of the immobilized lipase than the crude extract lipase [3–5,7,47].

Table 8
Enzyme leaching study for PLA:PVA:CH:CCL (1:6:1:2) in *n*-hexane at 150 rpm rotation speed.

No.	Time (h)	Enzyme leaching (%) PLA:PVA:CH:CCL (1:6:1:2)	Enzyme leaching (%) PLA:PVA:CH:CCL (1:6:1:2)-Glu (0.5%)
1	3	3.59	1.34
2	6	5.94	3.21
3	12	7.92	4.78
4	18	10.64	5.98
5	24	13.12	7.42

3.10. Effect of the organic solvent on stability of immobilized lipase

It is well known fact that the biocatalytic activity of enzymes was greatly affected by the nature of organic solvents. As the organic solvent may changes the parent conformation of the lipase by disturbing the hydrogen bonding and hydrophobic interactions, which leads to affect the activity and stability of the enzymes [35,55]. In present study, the stability of immobilized lipase was studied in different organic solvent by measuring the residual activity versus time.

The solvent stability study showed that immobilized lipase have better stability in the non-polar solvents such as *n*-hexane and toluene which gave the almost 94% and 86% of residual activity after the 24 h at 50 °C, respectively. While in case of the polar solvents such as 1,4-dioxane and dichloride methane activity was reduced to almost 47% and 23%, respectively (Fig. 6).

However, cross linked PLA:PVA:CH:CCL:GLU (1:6:1:2:0.5%) immobilized lipase; showed better organic solvents stability than the PLA:PVA:CH:CCL (1:6:1:2) immobilized lipase. The non-polar solvents such as *n*-hexane and toluene showed almost 98% and 89% of residual activity after incubation of 24 h at 50 °C, respectively, for the cross linked PLA:PVA:CH:CCL:GLU (1:6:1:2:0.5%) immobilized

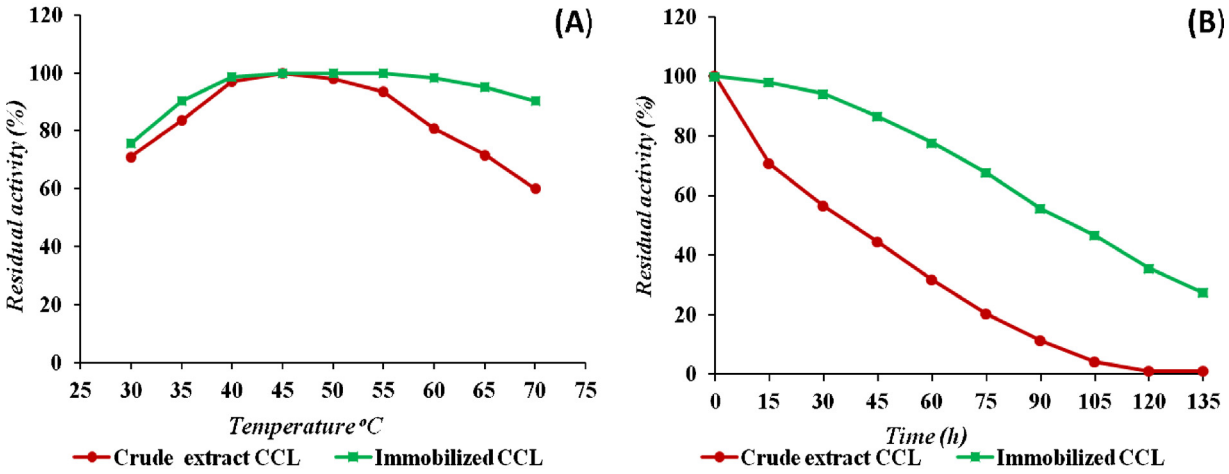


Fig. 5. Thermal stability of crude extract and immobilized lipase CCL in *n*-hexane. (A) At various temperatures and (B) at 50 °C for given time interval.

lipase. While in case of the polar solvents such as 1,4-dioxane and dichloride methane activity was found to be 51% and 35%, respectively, for cross linked immobilized lipase (Fig. 6).

The lower activity of lipase in polar solvents was attributed due to the reason that polar solvent removes the trace amount of water layers present around the enzymes which is essential for the catalytic activity [35]. This stripping of water layer around the enzyme is not possible in case of the non-polar solvents and hence non-polar solvents have good compatibility with enzymes [21,35]. Furthermore cross linked PLA:PVA:CH:CCL:GLU (1:6:1:2:0.5%) immobilized lipase showed the better stability and activity than the PLA:PVA:CH:CCL (1:6:1:2); this might be due to low leaching of lipase from the PLA:PVA:CH:CCL:GLU (1:6:1:2:0.5%) cross linked immobilized lipase support [46]. These results are in good agreement with many reports where non-polar organic solvents showed the better stability for the enzymes [35,46,55].

3.11. Activity and storage stability study

Additionally, the shelf life period of immobilized CH–PVA–PLA–CCL (1:6:1:2) lipase was studied for 300 days. It was noteworthy to mention that there was no significant decrease in immobilization yield, protein content, (%) yield for *N*-acylation reaction and specific activity within the studied range of 300 days. Hence it could be concluded that immobilized lipase was exclusively stable for the period of 300 days (Fig. 7).

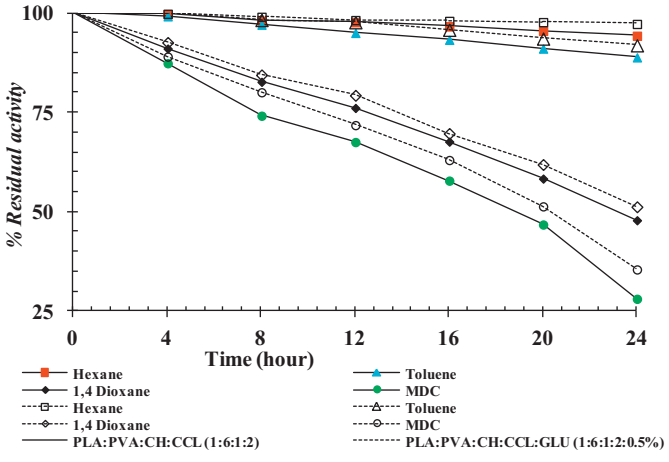


Fig. 6. Effect of the organic solvent on stability of immobilized lipase.

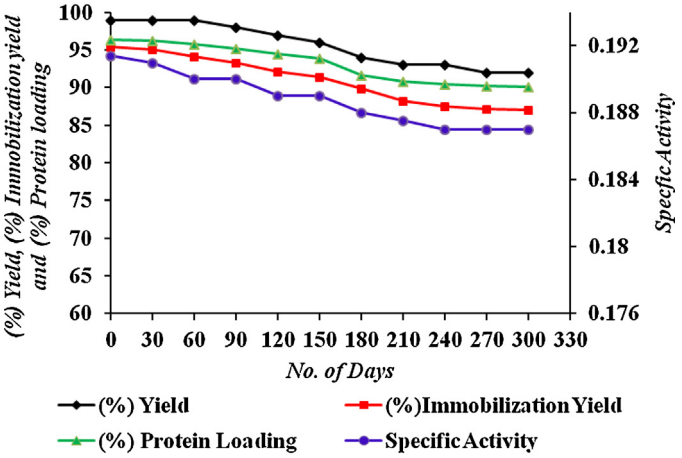


Fig. 7. Activity and storage stability study of immobilized CCL.

4. Conclusion

C. cylindracea lipase was successfully immobilized on environmentally benign and biodegradable ternary blend polymer CH–PVA–PLA (1:6:1) which remarkably improved the catalytic activity (five-fold) of the lipase. The polymer support and immobilized lipase CCL were characterized by the various methods to understand immobilization of enzyme on support as well as the enzyme-support interaction. The immobilization yield, protein content and specific activity was determined for immobilized and crude extract lipase to know the activity of lipase after immobilization. Ternary blend composition was screened to obtain the best compatibility of support for enhancement of lipase activity. Furthermore immobilized biocatalyst was subjected for the practical application in biocatalysis. Hence various reaction parameters were optimized and then immobilized biocatalyst was employed for synthesis of array of acetamides with good to excellent yield. Also immobilized lipase showed better thermal and organic solvent stability as lipase molecule entrapped inside the polymer matrix. It was successfully reused for four consecutive recycles and was remarkably stable for period of 300 days. The present study delivers the preparation of biocompatible polymer and its application for the immobilization of lipase for biocatalysis.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2013.06.009.

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