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HPMC-PVA Film Immobilized *Rhizopus oryzae* Lipase as a Biocatalyst for Transesterification Reaction

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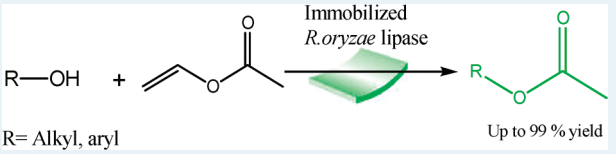
ABSTRACT: The film prepared using a blend of hydroxypropyl methyl cellulose (HPMC) and polyvinyl alcohol (PVA) was investigated for immobilization of *Rhizopus oryzae* lipase. These immobilized lipase films were characterized using a combination of techniques like SEM, TGA, FT-IR, and Karl Fischer titration analysis. The biocatalyst was subjected to transesterification reaction of benzyl alcohol with vinyl acetate and was optimized for various reaction parameters such as effect of support, molar ratio, solvent, concentration of biocatalyst, time and temperature. Furthermore, the developed methodology was then effectively applied to various alcohols for synthesis of industrially important acetates providing good to excellent yields of desired products. Interestingly, the experimental results demonstrated catalytic activity of immobilized lipase to be 4-fold greater than that of free lipase for transesterification reaction. The immobilized biocatalyst was effectively recycled for four consecutive cycles and exhibited remarkable stability for a period 90 days.



KEYWORDS: *Rhizopus oryzae* lipase, immobilization, acetate synthesis, transesterification, polymers, biocatalysis

1. INTRODUCTION

Enzymes are well-known as nature's catalyst, performing various biological processes about which we can just wonder and that are difficult to study in laboratory. However, in past few decades, numerous efforts have been made to mimic various biochemical reactions, such as hydrolysis, esterification, transesterification, amidation, oxidation−reduction reaction, methyl-like group transfer reaction, in the laboratory using enzymes as a biocatalyst for synthesis of industrially important compounds.[1] Until now, very few enzymes are well studied, among which lipase has attracted an enormous attention finding a great application in pharmaceuticals, cosmetics, food, flavor, and fragrances industries.[2−4] Indeed, the main concern related to application of enzymes is that they are sensitive, unstable, and poorly soluble, and thus, they must be used in aqueous medium, which limits their application as biocatalysts. Various efforts are sought to make them stable and increase their activity, and a graceful solution to this problem is found to be immobilization using support matrices.[5] Immobilization technique offers a range of advantages, like increased synthetic activity, ease of separation from a reaction mixture, and improved stability by preventing enzyme from thermal or solvent denaturation during repeated use in continuous processes.[6] A variety of natural supports, such as cellulose fibers,[7] chitosan,[8] smectite nanoclays,[9] β-glucan,[10] toyonite,[11] diatomaceous earth,[12] silica aerogels,[13] and mesoporous silica, such as MCM 41, SBA 15, and many more, are reported for lipase immobilization.[14]

Recently, Gao et al. developed a new methodology for immobilization of *Candida rugosa* lipase by cross-linking method using SBA-15 with chitosan and gluteraldehyde.[15] Also, Hara et al. reported the CLEA method for immobilization of *Burkholderia cepacia* (BCL) lipase using bovine serum albumin as protecting reagent in the presence of dextrin.[16] However, despite of many reported methods, the immobilization procedure still needs to be developed to enhance the synthetic activity of lipases so as to make them the most versatile biocatalyst. In context, entrapment of lipases into a film proves to be a simple and efficient method of immobilization as they provide a high surface area for interaction of enzyme with substrate followed by ease of separation and greater enzyme stability with less chances of leaching and circumvents the use of chemical reagents. The Dalla-Vecchia et al.[17] reported immobilization of *R. oryzae* lipase using blend of carboxymethyl cellulose (CMC) and polyvinyl alcohol (PVA) for esterification reaction and found improved catalytic activity but however to best of our knowledge use of blend of HPMC-PVA for *R. oryzae* lipase immobilization and its application for industrially important acetate synthesis has not yet been studied.

Synthesis of acetates by enzymatic route would offer them a green label of "natural compounds" as the process is environmentally benign. Acetate ester compounds have a great application mainly in food and cosmetic industries due to their characteristic fragrance and flavor. These esters can be directly obtained with extraction from plant materials, but the low quantities of product obtained and, thus, the high cost would make this technique inadequate for industrial applications. Also, the developed chemical procedure suffers from several drawbacks with a main concern of purity and thus invites the development of enzymatic methods.[18] Considering the above issues, in present paper, we

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report immobilization of commercially available *R. oryzae* lipase on a film prepared using a blend of HPMC-PVA for the first time. The main idea behind using biodegradable HPMC-PVA as immobilization support was to develop an environmentally benign immobilization protocol which evades the use of harmful chemical reagents. The cellulose derivative, that is, HPMC[19] has appreciable properties, like emulsifier, adhesive, elasticity, and thickening, while PVA[20] bears high tensile strength, flexibility, excellent film forming, emulsifying, and adhesive properties with resistance to organic solvents. These physicochemical properties of HPMC and PVA would help to prepare a good support in a form of film to immobilize the enzyme. Furthermore, immobilization of lipase on such large surface area of films would encourage the interfacial activation and thus would provide better accessibility of lipase for enzymatic reaction. Besides, the various other biocatalyst film containing microcrystalline cellulose (MCC), CMC, PVA, CMC-PVA blend, and MCC-PVA blend (1:1 w/w) were comparatively investigated for immobilization of *R. oryzae* lipase. Further, we demonstrated the application of immobilized biocatalyst for synthesis of various important acetates using vinyl acetate as acyl donor providing remarkable yields of desired products.

2. EXPERIMENTAL SECTION

2.1. Material. The *R. oryzae* lipase (light brown powder, ≥ 30 000 U/g) and PVA were purchased from Sigma-Aldrich Ltd. The HPMC (5, 15, and 50 cps), MCC, CMC and all other chemicals were purchased from S. D. Fine Chemicals Ltd. with their highest purity available. The bovine serum albumin (BSA) was purchased from Hi Media, India.

2.2. Immobilization of Lipase. The immobilization of *R. oryzae* lipase was carried out as described elsewhere with a slight modification.[17] The 500 mg of HPMC, CMC, MCC, PVA, HPMC-PVA (1:1), CMC-PVA (1:1), MCC-PVA (1:1) blend were dissolved in 25 mL of deionized water at room temperature with continuous stirring until complete dissolution. Lipase 100 mg was dissolved in 1−2 mL deionized water and was added to the solution of polymer formed. The solution was gently stirred for about 60 min and then was slowly poured into a Teflon petridish and was allowed to dry at 45−47 °C for 24−48 h, which was then cut off into several small sections of 2−3 mm^2. This developed biocatalyst films were then well characterized with the mentioned techniques and was subsequently subjected for transesterification reaction. The protein content of immobilized lipase was determined in triplicate by Lowry method using bovine serum albumin as a standard.[21]

2.3. Characterization of Free and Immobilized Lipase. The scanning electron microscopy (SEM) analysis (FEI, Quanta 200) was carried out to study the surface morphology. The representative film samples were placed on carbon stub and the images were recorded at 5−15 kV using LFD detector under low vacuum. The Thermo gravimetric analysis (TGA) was carried out using Q series 600 analyzer. About 8−10 mg of samples were placed in ceramic crucible and the analysis was programmed from 30 to 600 °C with 10 °C min^{-1} rise in temperature, under 99.99% pure nitrogen atmosphere with flow of 100 mL min^{-1}. The reference run was carried out with an empty sample crucible pan and the results were recorded accordingly. The various immobilized film biocatalyst and free lipase were investigated for their native confirmation using FT-IR analysis (Perkin-Elmer, Spectrum 100). The water content of 100 mg immobilized biocatalyst films were determined by Karl Fischer titration analysis (784 KFP Titrino).

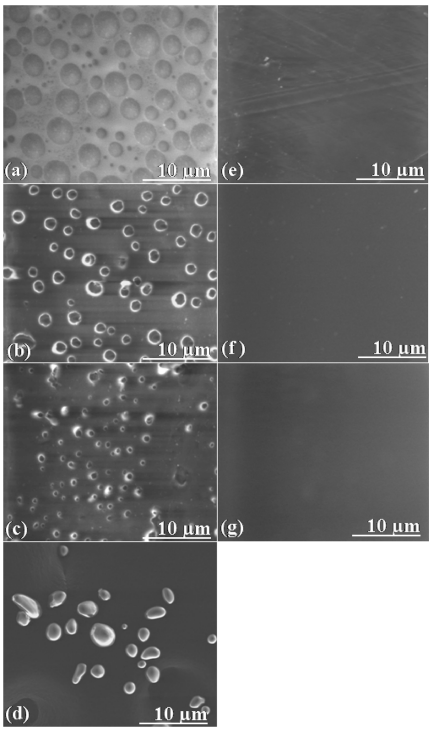

Figure 1. SEM images of: (a) HPMC- PVA-Lip, (b) HPMC-Lip, (c) PVA-Lip, (d) free *R. oryzae* lipase, (e) HPMC-PVA-control, (f) HPMC-control, and (g) PVA-control.

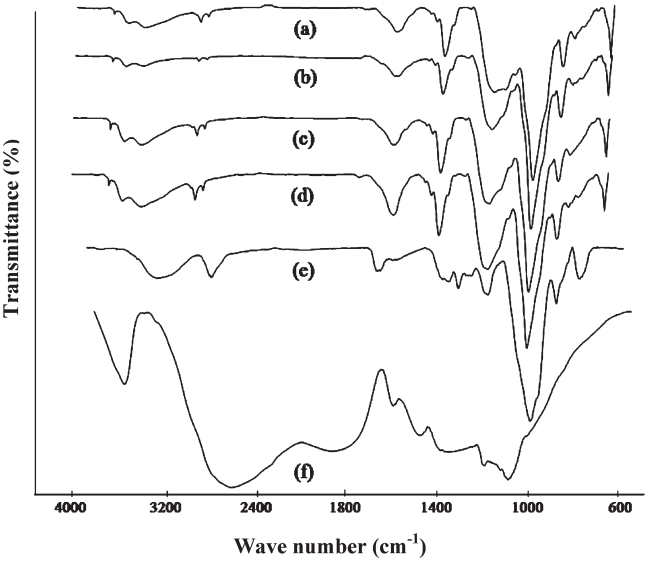

Figure 2. FT-IR spectrum of (a) HPMC- PVA-Lip, (b) HPMC-Lip, (c) PVA-Lip, (d) CMC- PVA Lip, (e) HPMC-PVA-control, and (f) free *R. oryzae* lipase.

2.4. General Experimental Procedure for Acetate Synthesis. The catalytic activity of immobilized lipase was determined from synthesis of benzyl acetate through transesterification reaction of benzyl alcohol with vinyl acetate. In typical experimental procedure, 1 mmol of benzyl alcohol and 3 mL of *n*-hexane was added to 10 mL glass stoppered tube. After gentle stirring, 5 mmol

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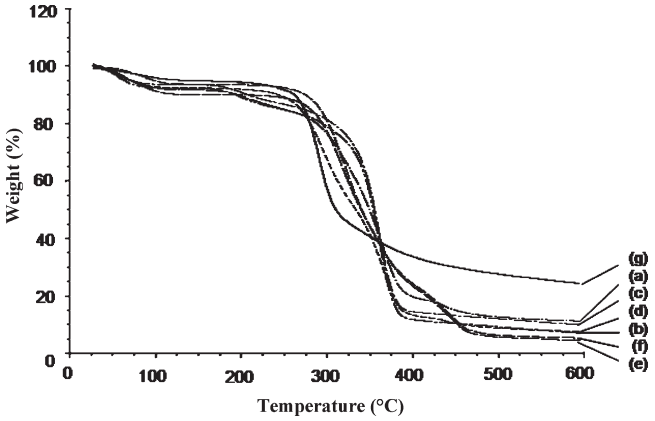


Figure 3. TGA analysis of (a) HPMC-PVA-Lip, (b) HPMC-Lip, (c) PVA-Lip, (d) HPMC-PVA-control, (e) HPMC- control, (f) PVA- control, and (g) free *R. oryzae* lipase.

Table 1. Determination of Water Content by Karl Fischer Method

entry	sample	water content (% w/w)
1	HPMC-PVA-Lip	8.66
2	HPMC-Lip	3.64
3	PVA-Lip	3.91
4	HPMC-PVA-control	8.45
5	HPMC-control	2.18
6	PVA-control	1.26
7	lipase *R. oryzae*	0.72

of vinyl acetate was added. To this, 50 mg of immobilized lipase film or 10 mg of free *R.oryzae* lipase was added to initiate the reaction. The reaction was then placed in an orbital shaker at 45 °C with agitation speed of 180 rpm for 12 h or as specified. The progress of the reaction was monitored with GC analysis. After completion of reaction, the reaction mixture was carefully filtered and the film was washed 2−3 times with *n*-hexane to remove any traces of product if remained adhered to the film. The reaction mixture was analyzed using a gas chromatography (Perkin-Elmer, Clarus 400) equipped with a flame ionization detector (FID) and an capillary column (Elite-1, 30 m × 0.32 mm × 0.25 μm). The column temperature was kept at 80 °C for 3 min and then raised to 250 °C for 30 min with a rise of 10 °C min^{-1}. The temperature of the injector and detector was maintained at 200 and 260 °C, respectively. The reaction mixture was then evaporated under vacuum with very low pressure. The residue obtained was purified with column chromatography (silica gel, mesh size 60−120) using pet ether: ethyl acetate (97: 3) as eluent to afford pure products. All the products are well-known[22,23] and were compared with authentic samples. The products are well characterized by ^{1}H and ^{13}C NMR spectra recorded on NMR spectrometer (Varian-300) using TMS as internal standard and by GC−MS (Shimadzu QP 2010) analysis.

2.4.1. Spectral Data of Selected Products

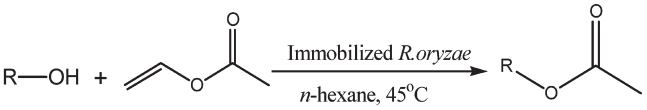
2.4.1.1. Cinnamyl Acetate (Table 4, Entry 7). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 2.1 (s, 3 H, $\underline{CH_3}$-CO), 4.72 (dd, 2 H, J = 6.23, 1.1 Hz, O-$\underline{CH_2}$-CH), 6.28 (td,1 H, J = 15.76, 6.23 Hz, CH_2-$\underline{CH}$=CH), 6.65 (d, 1 H, J = 15.76 Hz, CH=$\underline{CH}$-Ph), 7.2−7.4 (m, 5 H, Ar) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 21.0 (CH_3), 65.1 (O−CH_2), 123.2 (CH_2-$\underline{CH}$=CH), 126.7 (2xCH, Ar), 128.1 (CH, Ar), 128.7 (2xCH, Ar), 134.25 (CH=$\underline{CH}$-Ph), 136.26 (Cq, Ar), 170 (CH_3-$\underline{CO}$-OCH_2) ppm. MS (70 eV, EI) m/z (%): 176 (M^+), 134 (40), 115 (80), 105 (35), 92 (35), 77 (25), 43 (100).

2.4.1.2. 4-Methoxy Benzylalcohol Acetate (Table 4, Entry 8). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 2.07 (s, 3 H, CO-$\underline{CH_3}$), 3.80 (s, 3 H, OCH_3), 5.03 (s, 2 H, Ph-$\underline{CH_2}$-O), 6.87−6.90 (dd, 2 H, J = 6.5 Hz, Ar), 7.28−7.31 (dd, 2 H, J = 6.5 Hz, Ar) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 20.7 (CH_3), 54.9 (OCH_3), 65.9 (Ph-$\underline{CH_2}$-O), 113.7 (2xCH, Ar), 127.9 (2 × CH, Ar), 129.9 (CH, Ar), 159.5 (CH, Ar), 170.6 (CH_3-$\underline{CO}$-OCH_2) ppm. MS (70 eV, EI) m/z (%): 180 (M^+), 138 (25), 121 (100), 109 (15), 91 (35), 77 (30), 43 (25).

2.4.1.3. 2-Phenoxy Ethanol Acetate (Table 4, Entry 9). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 2.08 (s, 3 H, CO-$\underline{CH_3}$), 4.14−4.17 (t, 2 H, J = 4.7 Hz, Ar-O$\underline{CH_2}$), 4.39−4.42 (t, 2 H, J = 4.7 Hz, CO-O$\underline{CH_2}$), 6.89−6.98 (m, 3 H, Ar), 7.25−7.31 (m, 2 H, Ar) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 20.9 (CH_3), 62.9 (CO-O$\underline{CH_2}$), 65.9 (Ar−O$\underline{CH_2}$), 114.7 (2 × CH, Ar), 121.2 (CH, Ar), 129.6 (2 × CH, Ar), 158.6 (CH, Ar), 170.9 (CH_3-$\underline{CO}$-OCH_2) ppm. MS (70 eV, EI) m/z (%): 180 (M^+), 94 (20), 87 (100), 77 (20), 65 (10), 51 (15), 43 (95).

2.5. Reusability and Storage Stability Study. The reusability of the immobilized lipase for four consecutive recycles was studied by transesterification reaction for benzyl acetate synthesis following the above-mentioned procedure in section 2.4. In addition, at the end of each recycle the filtered immobilized lipase was

Scheme 1. Immobilized *R. oryzae* Lipase Catalyzed Transesterification Reaction



R= Alkyl, aryl

Table 2. Screening of Various Immobilized Biocatalyst for Acetate Synthesis[a]

		yield (%)[b]						
entry	biocatalyst	1 h	3 h	6 h	9 h	12 h	18 h	24 h
1	free *R.oryzae* lipase	13	20	21	23	23	24	26
2	CMC-PVA-Lip	18	28	31	34	36	51	61
3	HPMC (5 cps)-PVA-Lip	50	86	88	91	99	99	99
4	HPMC (15 cps)-PVA-Lip	33	82	85	90	99	99	99
5	HPMC (50 cps)-PVA-Lip	23	61	87	91	99	99	99
6	MCC-PVA-Lip	15	35	48	56	70	81	94
7	HPMC (5cps)-Lip	42	64	71	90	94	99	99
8	PVA-Lip	38	57	68	88	91	99	99
9[c]	blank	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]
10[d]	HPMC (5cps)-control	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]
11[d]	PVA-control	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]
12[d]	HPMC (5 cps)-PVA-control	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]	nr[e]

[a] Reaction conditions: benzyl alcohol (1 mmol), vinyl acetate (5 mmol), *n*-hexane (3 mL), *R. oryzae* lipase (10 mg)/immobilized lipase (50 mg), temperature (45 °C), agitation speed (180 rpm). [b] Yields based on GC analysis. [c] Without support and lipase. [d] Support/blend without lipase. [e] nr = no reaction.

Table 3. Optimization of Reaction Parameters for Enzymatic Transesterification Reaction[a]

entry	solvent	molar ratio[b]	temperature (°C)	enzyme loading (mg)	yield (%)[c]
			influence of molar ratio		
1	*n*-hexane	1:1	45	50	34
2	*n*-hexane	1:3	45	50	52
3	*n*-hexane	1:5	45	50	99
4	*n*-hexane	1:6	45	50	99
5	*n*-hexane	1:7	45	50	99
			influence of solvent		
6	1,4 dioxane	1:5	45	50	49
7	acetone	1:5	45	50	32
8	THF	1:5	45	50	52
9	diethyl ether	1:5	45	50	91
10	di-isopropyl ether	1:5	45	50	54
11	chloroform	1:5	45	50	71
12	toulene	1:5	45	50	97
			influence of temperature		
13	*n*-hexane	1:5	25	50	84
14	*n*-hexane	1:5	35	50	88
15	*n*-hexane	1:5	55	50	99
16	*n*-hexane	1:5	65	50	99
			influence of catalyst loading		
17	*n*-hexane	1:5	45	10	51
18	*n*-hexane	1:5	45	30	72
19	*n*-hexane	1:5	45	70	99

[a] Reaction conditions: Biocatalyst (HPMC-PVA-Lip), solvent (3 mL), time (12 h), agitation speed (180 rpm). [b] Benzyl alcohol/vinyl acetate. [c] Yields based on GC analysis.

collected carefully, dried at 45−48 °C for 10−12 h and was then used for next recycle.

The free and immobilized lipases were stored in small plastic containers at 6−8 °C for a period of 90 days. The storage stability was investigated with a regular interval of 15 days for free and immobilized lipase for transesterification reaction to yield benzyl acetate using above-mentioned experimental procedure (section 2.4).

2.6. Enzyme Leakage Study. The leaching of *R. oryzae* lipase from immobilization support was studied by the spectrophotometric method (in triplicate) as described by Ozyilmaz et al.[24] The standard curve of *R. oryzae* lipase concentration ranging from 0.5 to 4 mg mL^{-1} was prepared and absorbance of the solution was measured at 280 nm. The immobilized biocatalyst film (50 mg) was added to 3 mL of *n*-hexane and was stirred on orbital shaker at 45 °C with agitation speed of 180 rpm for 48 h. The absorbance of decant filtrate was spectrophotometrically measured at 280 nm after a regular interval of 6 h using pure *n*-hexane as a blank. Then, further the enzyme leakage (%) was calculated as described by Ozyilmaz et al.[24]

3. RESULTS AND DISCUSSION

3.1. Characterization of Immobilized Lipase Films. The surface morphology of immobilized lipase was determined using SEM analysis as shown in Figure 1. The SEM images reveals the even distribution of *R. oryzae* lipase as a small globules in the film (Figure 1a−c), whereas the image of supports without lipase shows absence of globular structures with plane background (Figure 1e−g). The obtained images were compared with an SEM image of a commercially available lipase (Figure 1d) under same instrumental conditions signifying the entrapment of lipase into the film.

The FTIR absorption spectrum of lipase generally shows three major bands caused by peptide group vibrations in the range of 1800−1300 cm^{-1}. Free and immobilized lipase illustrates a characteristic band of amide II with the maximum of 1590 cm^{-1} due to N−H bending with contribution of C−N stretching vibrations. The presence of amide III band present at maximum of 1451 cm^{-1} is due to N−H bending with C−Cα and C−N stretching vibrations. These significant bands were observed in the free lipase as well as immobilized lipase emphasizing presence of lipase in its native conformation (Figure 2). However, the amide I band observed mostly in *C. rugosa* and acidic lipase of *Pseudomonas sps.* was not observed in case of *R.oryzae*.[25,26] Furthermore, it is observed that these amide regions are absent in the FT-IR spectrum of HPMC-PVA control (Figure 2e) addressing strong presence of amide bonds due to lipase only.

Turner et al.[27] studied the effect of temperature for which enzymes maintain their catalytic activity. It showed that temperature above 200 °C is required to remove the tightly bound water from proteins. The similar observation was made for TGA analysis (Figure 3) of immobilized lipases, where immobilization has also increased the thermal stability of lipase as compared with free lipase.

The % water content of free, immobilized lipase *R. oryzae* and controls were determined using Karl Fischer titration method (Table 1). The obtained results are in agreement with the earlier

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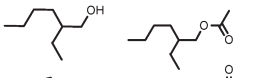
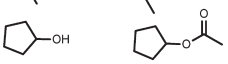
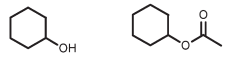
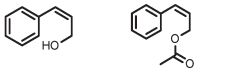
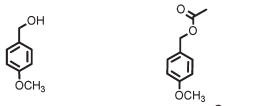
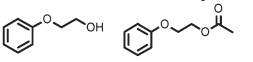
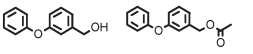
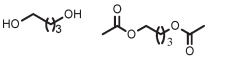
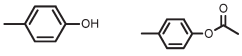
Table 4. Immobilized Lipase Catalyzed Acetate Synthesis[a]

Entry	Alcohol	Acetate	Time (h)	Yield (%)[b]
1			24	99
2			48	96
3			24	99
4			24	92
5			48	61
6			48	32
7			24	99
8			48	99
9			24	99
10			24	99
11			24	99
12			48	41
13			48	11

[a] Reaction conditions: alcohol (1 mmol), vinyl acetate (5 mmol), *n*-hexane (3 mL), immobilized lipase (50 mg), temperature (45 °C), agitation speed (180 rpm). [b] Yields based on GC analysis.

reports for determination of water content of lipase.[27,28] The water content of free lipase was 0.72% while for the best immobilized lipase HPMC-PVA was 8.66%.

The immobilization % efficiency was determined from the protein content by Lowry method[21] and was found to be 86−94% for all the immobilization supports with 94% for the HPMC-PVA immobilized lipase considering that 10 mg of free lipase was used for transesterification reaction. The percent immobilization achieved was very high because the method does not follow any washing procedure, which is most commonly followed during the use of cross-linking reagent for immobilization purpose.

3.2. Application of Immobilized HPMC-PVA Lipase. To study the catalytic behavior of immobilized lipase, the transesterification reaction of benzyl alcohol with vinyl acetate as an acyl donor was selected as a model reaction (Scheme 1).

Various immobilized biocatalyst films were screened for benzyl acetate synthesis (Table 2, entries 1−12). It was observed that the free *R. oryzae* lipase provided a low yield, that is, 26 % of desired product in 24 h (Table 2, entry 1). The CMC-PVA immobilized lipase was used for esterification reaction by Dalla-Vecchia et al.[17] however when it was investigated for transesterification reaction the yield obtained for desired acetate was 36% in 12 h (Table 2, entry 2). As compared to CMC-PVA, using novel HPMC-PVA blend immobilized lipase 99% yield of benzyl acetate was obtained in 12 h. (Table 2, entries 3−5). It was observed that the HPMC-PVA film with low viscosity provided highest initial rate of reaction (Table 2, entry 3). On other hand, cellulose like micro crystalline cellulose (MCC) provided 94% yield in 24 h (Table 2, entry 6). When only HPMC or PVA were used for immobilization purpose, 94% and 91% yield of desired product were obtained respectively, reflecting the use of blend synergistically enhances the lipase catalytic activity (Table 2, entries 7−8). The control experiments were carried out in absence of lipase keeping other reaction parameters constant, where no yield of desired product was obtained signifying that only *R. oryzae* lipase is responsible for the respective transformation (Table 2, entries 9−12).

Using HPMC (5cps)-PVA immobilized lipase film, the model reaction was optimized with respect to molar ratio, solvent, temperature, and catalyst loading. Molar ratio has always shown a profound effect on yields of the desired product. In order to obtain maximum yield of desired product, molar ratio of benzyl alcohol: vinyl acetate was varied from 1:1 to 1:7 (Table 3, entries 1−5), providing excellent yield (99%) for molar ratio of 1:5 within 12 h (Table 3, entry 3). The similar observations were obtained by Majumder et al.[29] for lipozyme-catalyzed benzyl acetate synthesis, where the molar ratio of the 1:6 of benzyl alcohol to vinyl acetate was employed. During the transesterification reaction using vinyl acetate, the acetaldehyde released has found to inhibit the catalytic activity of some lipases like *C. rugosa* lipase.[30] Biocatalysis in organic solvents undoubtedly offers numerous advantages with a great impact on the enzyme activity which suggests us to study their effect on the transesterification reaction (Table 3, entries 3, 6−12). A variety of solvents with log P value ranging from −1.1 to 3.5 were studied,[31] of which *n*-hexane was found to be the best solvent furnishing 99% yield of desired product (Table 3, entry 3). This is because the hydrophobic solvents with higher log P values do not have a tendency to strip the tightly bound water molecules from the enzyme surface which are essential for catalytic activity of lipase.

Enzymes are found to work optimum at a particular temperature, where synthesis of benzyl acetate was much effective at 45 °C providing a maximum yield of 99% (Table 3, entry 3). Fascinatingly, even on further increase in temperature (up to 65 °C) the biocatalyst remained stable without any significant loss in activity (Table 3, entry 16). In addition, to determine the optimum concentration of biocatalyst, various amount of catalyst loading ranging from 10 to 70 mg was studied (Table 3, entries 3, 17−19). The maximum yield of 99% was obtained with 50 mg of immobilized lipase (Table 3, entry 3); however, further increase in the catalyst concentration had no significant effect on the yield of desired product. Hence, the optimized reaction conditions for transesterification reaction are molar ratio of benzyl alcohol/vinyl acetate, 1:5; solvent, *n*-hexane; temperature, 45 °C; time, 12 h; biocatalyst loading, 50 mg.

To study the generality and scope of developed biocatalytic protocol, the optimized reaction conditions were then employed for transesterification of various aliphatic and aromatic alcohols with vinyl acetate as an acyl donor (Table 4). Interestingly, the immobilized lipase deserves a good potential to carry transesterification with significant yields which would merely be difficult for free *R. oryzae* lipase. Acetates like *n*-butyl acetate (found in many fruits like red delicious apples) and *n*- octyl acetate (fruity-orange) were synthesized with 99% and 96% yield, respectively (Table 4, entries 1−2). Branched aliphatic acetate, such as iso-amyl acetate (pear, banana flavor), which is one of the most important acetate finding a large demand in foods and flavor

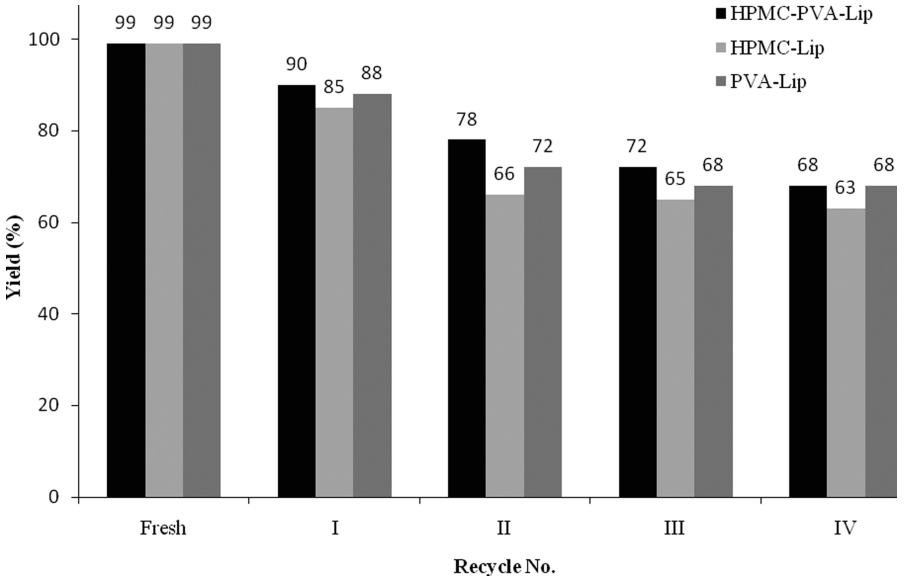


Figure 4. Reusability study of immobilized lipase for benzyl acetate synthesis.

industry was synthesized with 99% yield in 24 h, whereas 2-ethylhexan-1-ol acetate was synthesized with 92% yield in 24 h (Table 4, entries 3−4). The alicyclic alcohol, such as cyclopentanol, reacted with vinyl acetate providing 61% yield, whereas for cyclohexanol the yield decreased to 32% in 48 h (Table 4, entries 5−6). Furthermore, cinnamyl alcohol reacted effectively providing excellent yield of industrially important cinnamyl acetate (strawberry flavor) in 24 h (Table 4, entry 7).[32,33] Substituted benzyl alcohol, such as 4-methoxyl benzyl alcohol, provided 99% yield of desired product in 48 h (Table 4, entry 8). The 2-phenoxy-derivatives, such as 2-phenoxy ethanol and 2-phenoxy benzyl alcohol, also reacted efficiently providing an excellent yield of 99% in 24 h (Table 4, entries 9−10). The diol, such as 1,4 butane diol, was also studied for this developed protocol and was found to furnish an diacetate product with 99% yield in 24 h (Table 4, entry 11). The substituted phenol, such as 4-methylphenol, provided a low yield of 41% in 48 h, whereas 2-napthol was not compatible substrate for the present protocol as the reaction was too sluggish providing poor yield of 11% in 48 h (Table 4, entries 12−13).

Furthermore, to make the process more economical it is necessary to study the recyclability of immobilized lipase. We carried out recyclability study of HPMC-PVA-Lip, HMPC-Lip, and PVA-Lip of which HMPC-PVA-Lip was most effectively recycled for consecutive four cycles (Figure 4). There was no significant decrease in yield during the first recycle however; yield declined up to 68% for the fourth cycle. The decrease in yield is believed owing to deactivation of lipase or possibility of desorption of lipase from the support as cycles are increased. Considering which enzyme leakage study was carried out as described by Ozyilmaz et al.[24] and it was observed that there was no significant leaching (below 1%) of lipase from the immobilization support even after a period of 48 h. In addition, it is known that enzymes are naturally insoluble in organic solvents and thus we believe the decrease in lipase activity was because of deactivation due to extended exposure of biocatalyst to alcohols and not due to desorption of lipase from the immobilization support.[6] The same observation was made by Lozano et al. for synthesis of citronellol

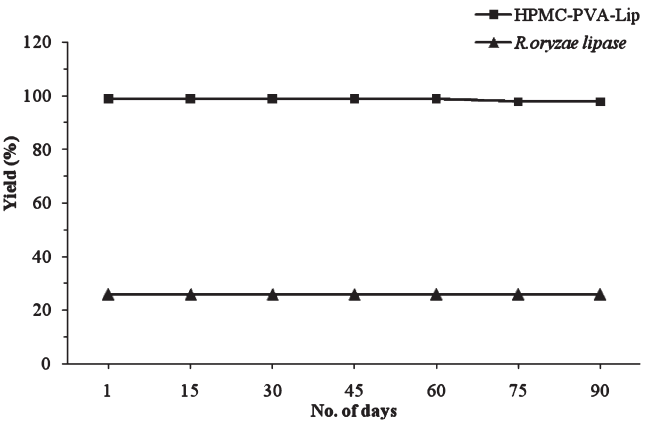


Figure 5. Storage stability study of lipase.

acetate using CaL-B where a continuous decrease in activity was observed as the operation cycles increased.[34]

The storage stability study of free lipase and HPMC-PVA-Lip was investigated and the immobilized lipase was found to be appreciably stable for a period of 90 days retaining its original catalytic activity (Figure 5). The immobilized enzyme provided excellent yield up to 98% of desired product whereas commercially available lipase provided 26% yield after a considerable period of 90 days. The Dalla-Vecchia et al[17] studied storage stability of CMC-PVA lipase for a period of 80 days and observed a considerable decrease in the esterification of lauric acid with *n*-pentanol after 10 days.

CONCLUSION

In conclusion, immobilization of *R. oryzae* lipase using environmentally benign and biodegradable HPMC-PVA polymer has significantly enhanced the catalytic activity thus making them an eligible biocatalyst for transesterification reaction. The several industrially important acetates were successfully synthesized using the immobilized lipase, appealing the application of developed protocol for industrial application. The immobilized lipase

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was effectively recycled for four consecutive cycles providing good yields of desired product. The immobilized lipase was remarkably stable for a storage period of 90 days which highlights its appreciable shelf life.

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