Chapter 3

HPMC-PVA film immobilized Rhizopus oryzae lipase as a biocatalyst for transesterification reaction
3.1 INTRODUCTION

Enzymes are principally the versatile class of catalysts. They perform various biological processes about which we can just wonder and are difficult to study in laboratory. The unique potential of enzymes is still very far from being fully explored; indeed estimates generally agree that around 1% of the micro-organisms in the environment have been successfully cultivated and their enzymes been isolated/identified. Even though, huge enzymes yet remain to be discovered, array of reactions that could be catalyzed have been identified and characterized (Davis and Boyer, 2001; Dhake et al., 2009, 2010; Hasan and Shah, 2006). Some of these enzymes are now commercially available for use as biocatalysts to catalyze number of biochemical reactions like hydrolysis, esterification, transesterification, amidation, oxidation-reduction reaction, methyl like group transfer reaction. Numerous efforts are being made to mimic these biochemical reactions in laboratory using enzymes as a biocatalyst for synthesis of industrially important compounds (Davis and Boyer, 2001). Among few enzymes studied, lipase has attracted an enormous attention finding a great application in pharmaceuticals, cosmetics, food, flavor and fragrances industries (Hasan and Shah, 2006).

Indeed, the main concern related to application of enzymes is that they are sensitive, unstable and poorly soluble and thus is a challenge to use them in organic solvents. This in turn limits their application as a biocatalyst. Various efforts have been made to make enzymes more stable, to increase their activity which could be accomplished by immobilization using support matrices (Mateo et al., 2007). Immobilization technique offers an array of advantages like improved synthetic activity, ease of biocatalyst separation from reaction mixture, improved stability by preventing enzyme from thermal or solvent denaturation during repeated use in continuous processes (Hanefeld et al., 2009). Numerous natural supports like cellulose fibers, chitosan, smectite nanoclays, β-glucan, toyonite, diatomaceous earth, silica aerogels, mesoporous silica like MCM 41, SBA 15 and many more are reported for lipase immobilization (Hanefeld et al., 2009; Hartmann and Jung, 2010).

Recently, Gao et al. (2010) reported a new methodology for immobilization of Candida rugosa lipase by cross linking method using SBA-15 with chitosan and
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gluteraldehyde. Moreover, Hara et al. (2009) reported the CLEA method for immobilization of *Burkholderia cepacia* (BCL) lipase using bovine serum albumin (BSA) as protecting reagent in the presence of dextrin. Despite many reported methods, immobilization procedure still needs to be developed to enhance the synthetic activity of lipases to make them the most versatile biocatalyst. In context, entrapment of lipases into a film seems to be simple and efficient method of immobilization; as they provide a high surface area for interaction of enzyme with substrate followed by ease of biocatalyst separation and greater enzyme stability with less chances of leaching. It also circumvents the use of chemical reagents. Dalla-Vecchia et al. (2005) reported immobilization of *R.oryzae* lipase using blend of carboxy-methyl cellulose (CMC) and polyvinyl alcohol (PVA) for esterification reaction and found improved catalytic activity. However to best of our knowledge use of blend of hydroxypropyl methylcellulose (HPMC) and 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 (Figure 3.1). Acetate ester compounds have a great application mainly in food and cosmetic industries due to their characteristic fragrance and flavour. These esters can be directly obtained via extraction from plant materials but due 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 hence invites the development of enzymatic methods (Radnik et al., 2005). In present chapter, the immobilization of commercially available *R.oryzae* lipase (ROL) on a film prepared using a blend of HPMC-PVA is discussed.
Figure 3.1 Graphical representation of application of several immobilized lipases for ester synthesis via esterification or transesterification reactions (Bartling et al., 2001; Horchani et al., 2010; Yadav and Devendran, 2012; Maugard et al., 2001; Majumder and Gupta, 2010; Kumar and Kanwar, 2011; Zoumpanioti et al., 2010; Lozano et al., 2007).

The central idea behind using biodegradable HPMC-PVA as immobilization support was to develop an environmentally benign immobilization protocol which could evade the use of harmful chemical reagents. Cellulose derivative, HPMC (Imeson, 2010) has appreciable properties like emulsifier, adhesive, elasticity, thickening while PVA (Goodship and Jacobs, 2009) bears high tensile strength, flexibility, excellent film forming, emulsifying and adhesive properties with resistant to organic solvents. These physicochemical properties of HPMC and PVA would enable to prepare a suitable support in a form of film to immobilize the enzyme.
Furthermore, immobilization of lipase on such large surface area of film would encourage the interfacial activation and provide better accessibility of lipase for enzymatic reaction. Besides, the various other biocatalyst film containing microcrystalline cellulose (MCC), CMC, PVA, CMC-PVA blend, MCC-PVA blend (1:1 w/w) were comparatively investigated for immobilization of ROL. Further, the application of immobilized biocatalyst for synthesis of various important acetates using vinyl acetate as acyl donor was demonstrated.

### 3.2 EXPERIMENTAL SECTION

#### 3.2.1 Enzyme and chemicals

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

#### 3.2.2 Immobilization of lipase

Immobilization of *R. oryzae* lipase was carried out as described elsewhere with a slight modification as show in fig. 3.2 (Dalla-Vecchia et al., 2005). 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 till complete dissolution. 100 mg of lipase was dissolved in 1-2 mL deionized water and was further added to the solution of polymer formed. The solution was gently stirred for about 60 min and was gently poured into a teflon petridish. It was further allowed to dry at 45-47°C for 24-48 h, which was then cut off into several small sections of 2-3 mm². 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 (Lowry et al., 1951).
3.2.3 Characterization of free and immobilized lipase

Scanning electron microscopy (SEM) analysis (FEI, Quanta 200) was carried out in order 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. 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°C 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 results were recorded accordingly. Several immobilized film biocatalyst and free ROL were investigated for their native conformation 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).

3.2.4 General experimental procedure for acetate synthesis

The synthetic activity of immobilized lipase was determined from transesterification reaction of benzyl alcohol with vinyl acetate to synthesize of benzyl
acetate as a desired product. 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 of vinyl acetate was added. To this, 50 mg of immobilized lipase film or 10 mg of free lipase was added to initiate the reaction. The reaction vial was then placed in an orbital shaker at 45°C with agitation speed of 160 rpm for 12 h or as specified. Progress of the reaction was monitored with gas chromatography (GC) analysis. On 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 GC (Perkin Elmer, Clarus 400) equipped with a flame ionization detector (FID) and a capillary column (Elite-1, 30 m x 0.32 mm x 0.25 µm). 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\(^{-1}\). Temperature of the injector and detector was maintained at 200°C and 260°C respectively. Furthermore, the reaction mixture was evaporated under vacuum with very low pressure. The residue obtained was purified using 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 (Shirai et al., 2005; Firouzabadi et al., 2008) and were compared with authentic samples. The products were 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.

3.2.5 Reusability and storage stability study

The reusability of immobilized lipase for four consecutive recycles was studied by transesterification reaction for benzyl acetate synthesis following the above mentioned procedure in section 3.2.4. In addition, at the end of each recycle the filtered immobilized lipase was collected carefully and further dried at 45-48°C for 10-12 h before subjected to the next recycle.

The free and immobilized lipases were stored in small plastic containers at 6-8 °C for a period of 90 days. 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 3.2.4).
3.2.6 Enzyme leakage study

Leaching of ROL from immobilization support was studied by the spectrophotometric method (in triplicate) as described by Ozyilmaz et al. (2010). The standard curve of lipase concentration ranging from 0.5 to 4 mg mL\(^{-1}\) was prepared and absorbance of the solution was measured at 280 nm. 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. Further, the enzyme leakage (%) was calculated as described by Ozyilmaz et al. (2010).

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of immobilized lipase films

Surface morphology of immobilized lipase was determined using SEM analysis as represented in Figure 3.3. SEM images reveal the even distribution of lipase as a small globules in the film (Figure 3.3a-c) while the image of supports without lipase shows absence of globular structures with occurrence of plane background (Figure 3.3e-g). The obtained images were compared with an SEM image of a commercially available \(R.\) oryzae lipase (Figure 3.3d) under same instrumental conditions signifying the entrapment of lipase into the film.
Figure 3.3 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.

FTIR absorption spectrum of lipase generally shows three major bands caused by peptide group vibrations in the range of 1800-1300 cm\(^{-1}\) (Natalello et al., 2005; Ramani et al., 2010). 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-C\(_{\alpha}\) 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 3.4). However, the amide I band
observed mostly in CRL and acidic lipase of *Pseudomonas sps.* was not observed in case of *R. oryzae* (Natalello et al., 2005; Ramani et al., 2010). Furthermore, it is observed that these amide regions are absent in the FT-IR spectrum of HPMC-PVA control (Figure 3.4e) addressing strong presence of amide bonds due to lipase only.

**Figure 3.4** 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.

Turner et al. (2000) studied the effect of temperature in which enzymes maintain their catalytic activity. It has been reported that temperature above 200°C is required to remove the tightly bound water from proteins. Similar observation was made for TGA analysis (Figure 3.5) of immobilized lipases, where immobilization has also increased the thermal stability of lipase as compared with free lipase.
Figure 3.5 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 3.1** Determination of water content by Karl Fischer method.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sample</th>
<th>Water content (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPMC-PVA-Lip</td>
<td>8.66</td>
</tr>
<tr>
<td>2</td>
<td>HPMC-Lip</td>
<td>3.64</td>
</tr>
<tr>
<td>3</td>
<td>PVA-Lip</td>
<td>3.91</td>
</tr>
<tr>
<td>4</td>
<td>HPMC-PVA-control</td>
<td>8.45</td>
</tr>
<tr>
<td>5</td>
<td>HPMC-control</td>
<td>2.18</td>
</tr>
<tr>
<td>6</td>
<td>PVA-control</td>
<td>1.26</td>
</tr>
<tr>
<td>7</td>
<td>Lipase <em>R. oryzae</em></td>
<td>0.72</td>
</tr>
</tbody>
</table>

The % water content of free, immobilized lipase *R. oryzae* and controls were determined using Karl Fischer titration method (Table 3.1). The obtained results are in
agreement with the earlier reports for determination of water content of lipase (Turner and Vulfson, 2000; Ma et al., 2002). 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 (Lowry et al., 1951) and was found to be 86-94% for all the immobilization supports with 94% for the HPMC-PVA immobilized lipase considering which 10 mg of commercial ROL was used for transesterification reaction. The % immobilization was achieved very high as the method doesn’t follow any washing procedure which is most commonly followed during the use of cross linking reagent for immobilization purpose.

3.3.2 Application of immobilized HPMC-PVA lipase

In order 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 3.1).

![Scheme 3.1 Immobilized R. oryzae lipase catalyzed transesterification reaction](image)

Various immobilized biocatalyst films were screened for benzyl acetate synthesis (Table 3.2, entries 1-12). It was observed that the free lipase provided a low yield i.e. 26% of desired product in 24 h (Table 3.2, entry 1). The CMC-PVA immobilized lipase was reported for esterification reaction by Dalla-Vecchia et al. (2005) however when it was investigated for present study; the yield obtained for desired acetate was only 36% in 12 h (Table 3.2, 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 3.2, entries 3-5). It was observed that the HPMC-PVA film with low viscosity provided highest initial rate of reaction (Table 3.2, entry 3). On other hand, cellulose like micro crystalline cellulose (MCC) provided 94% yield in 24 h (Table 3.2, entry 6).

Table 3.2 Screening of various immobilized biocatalyst for acetate synthesis.\[a\]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Biocatalyst</th>
<th>Yield (%)[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>1</td>
<td>Free \textit{R. oryzae} lipase</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>CMC-PVA-Lip</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>HPMC (5 cps)-PVA-Lip</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>HPMC (15 cps)-PVA-Lip</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>HPMC (50 cps)-PVA-Lip</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>MCC-PVA-Lip</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>HPMC (5cps)-Lip</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>PVA-Lip</td>
<td>38</td>
</tr>
<tr>
<td>9[c]</td>
<td>Blank</td>
<td>--</td>
</tr>
<tr>
<td>10[d]</td>
<td>HPMC (5cps)-control</td>
<td>--</td>
</tr>
<tr>
<td>11[d]</td>
<td>PVA-Control</td>
<td>--</td>
</tr>
<tr>
<td>12[d]</td>
<td>HPMC (5 cps)-PVA-control</td>
<td>--</td>
</tr>
</tbody>
</table>

\[a\]Reaction conditions: benzyl alcohol (1 mmol), vinyl acetate (5 mmol), \textit{n}-hexane (3 mL), \textit{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.
When only HPMC or PVA were used for immobilization purpose, 94% and 91% yield of desired product (in 12 h) were obtained respectively, reflecting the use of blend has synergistically enhanced the lipase catalytic activity (Table 3.2, entries 7-8). Control experiments were also carried out in absence of lipase keeping other reaction parameters constant, where no yield of desired product as obtained; thus signified that ROL was only responsible for the respective transformation (Table 3.2, entries 9-12).

The model reaction was optimized with respect to molar ratio, solvent, temperature and catalyst loading using HPMC (5cps)-PVA immobilized lipase as a best biocatalyst. Molar ratio has always shown a profound effect on yields of the desired product. The molar ratio of benzyl alcohol: vinyl acetate was varied from 1:1 to 1:7 in order to obtain maximum yield of desired product (Figure 3.6.1). Excellent yield of 99% was obtained for molar ratios of 1:5 within 12 h. Similar observations were reported by Majumder et al. (2006) for Lipozyme catalyzed benzyl acetate synthesis where molar ratio of 1:6 of benzyl alcohol to vinyl acetate was employed.

![Figure 3.6.1 Influence of molar ratio on benzyl acetate synthesis. Reaction conditions: n-hexane (3 mL), immobilized lipase (50 mg), temperature (45°C), agitation speed (180 rpm).](image-url)
It is noteworthy to mention that when vinyl acetate is used as an acyl donor for transesterification reaction, vinyl alcohol is formed as byproduct; being unstable it tautomerizes to form acetaldehyde. This released acetaldehyde reacts with the amine group present on the enzyme surface and forms an imine complex; which in turn is reported to inhibit the activity of lipase like *C. rugosa* lipase (Weber et al., 1997).

Biocatalysis in organic solvents undoubtedly offers numerous advantages with a great impact on the enzyme activity which suggests studying their effect on the transesterification reaction (Figure 3.6.2). A variety of solvents with Log P value ranging from –1.1 to 3.5 were studied (Laane et al., 1987), of which *n*-hexane was found to be the best solvent furnishing 99% yield of desired product. This is because unlike polar solvents; 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.

![Effect of solvents on benzyl acetate synthesis.](image)

**Figure 3.6.2** Effect of solvents on benzyl acetate synthesis.

Reaction conditions: alcohol (1 mmol), vinyl acetate (5 mmol), solvent (3 mL), immobilized lipase (50 mg), temperature (45°C), agitation speed (180 rpm).
Enzymes are found to work optimum at a particular temperature. In present study we found that the synthesis of benzyl acetate was much effective at 45°C providing a maximum yield of 99% (Figure 3.6.3). Fascinatingly, even on further increase in temperature (up to 65°C) the biocatalyst remained stable without any significant loss in activity. The results suggest that the proper immobilization matrix helps to make lipase stable at higher temperature which in turn opens the door for application of this biocatalyst for organic reactions requiring elevated temperature.

![Figure 3.6.3](image)

**Figure 3.6.3** Effect of temperature on benzyl acetate synthesis. Reaction conditions: alcohol (1 mmol), vinyl acetate (5 mmol), n-hexane (3 mL), immobilized lipase (50 mg), agitation speed (180 rpm).

In addition, to determine the optimum concentration of biocatalyst, various amount of catalyst loading ranging from 10-70 mg was studied (Figure 3.6.4). The maximum yield of 99% was obtained with 50 mg of immobilized lipase however; further increase in the catalyst concentration had no significant effect on yield of desired product. Thus, the final 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 and biocatalyst loading: 50 mg.
Figure 3.6.4 Effect of biocatalyst loading on benzyl acetate synthesis.
Reaction conditions: alcohol (1 mmol), vinyl acetate (5 mmol), \( n \)-hexane (3 mL), temperature (45°C), agitation speed (180 rpm).

In order 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 3.3). Interestingly, immobilized lipase deserved a good potential to carry out transesterification with significant yields which would merely be difficult for free \textit{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 3.3, entries 1-2). Branched aliphatic acetate like iso-amyl acetate (pear, banana flavor) which is one of the most important acetate finding a large demand in foods and flavor industry was synthesized with 99% yield in 24 h whereas, 2-ethyl-hexan-1-ol acetate was synthesized with 92% yield in 24 h (Table 3.3, entries 3-4). The alicyclic alcohol like cyclopentanol reacted with vinyl acetate providing 61% yield whereas for cyclohexanol the yield decreased to 32% in 48 h (Table 3.3, entries 5-6). Cinnamyl alcohol also reacted effectively providing excellent yield of industrially important cinnamyl acetate (Strawberry flavor) in 24 h (Table 3.3, entry 7) (Kraft and Swift 2005; Rowe, 2005).
Table 3.3 Immobilized lipase catalyzed acetate synthesis.[a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Alcohol</th>
<th>Acetate</th>
<th>Time (h)</th>
<th>Yield (%)&lt;sup&gt;[b]&lt;/sup&gt;</th>
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<td>1</td>
<td><img src="image" alt="C8 alcohol" /></td>
<td><img src="image" alt="Acetate" /></td>
<td>24</td>
<td>99</td>
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<tr>
<td>2</td>
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<td><img src="image" alt="Acetate" /></td>
<td>48</td>
<td>96</td>
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<td>24</td>
<td>99</td>
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<tr>
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<td>61</td>
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<td>32</td>
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<td>24</td>
<td>99</td>
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</table>

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- 98 -
Apart from benzyl alcohol (Table 3.3, entry 8), the substituted benzyl alcohol like 4-methoxy benzyl alcohol provided 99% yield of desired product in 48 h (Table 3.3, entry 9). The 2-phenoxy-derivatives like 2-phenoxy ethanol and 2-phenoxy benzyl alcohol also reacted efficiently providing an excellent yield of 99% in 24 h (Table 3.3, entries 10-11). Diol like 1,4 butane diol was also studied for the developed protocol and was found to furnish a di-acetate product with 99% yield in 24 h (Table 3.3, entry 12). Substituted phenol such as 4-methyl-phenol provided a low yield of 41% in 48 h whereas 2-napthol was not a compatible substrate for the present protocol as the reaction was too sluggish providing poor yield of 11% in 48 h (Table 3.3, entries 13-14).

Furthermore, to make the process more economical it is necessary to study the recyclability of immobilized lipase. The recyclability study of HPMC-PVA-Lip, HMPC-Lip, PVA-Lip was carried out; of which HMPC-PVA-Lip was most effectively recycled for consecutive four cycles (Figure 3.7). There was no significant decrease in yield during the first recycle however the yield declined up to 68% for the fourth cycle. This 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

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<th></th>
<th><img src="image1" alt="Chemical Structure" /></th>
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<td><img src="image10" alt="Chemical Structure" /></td>
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</table>

[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.
enzyme leakage study was carried out as described by Ozyilmaz et al. (2010) 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. Thus we believe the decrease in lipase activity was because of deactivation due to extended exposure to biocatalyst to alcohols and not due to desorption of lipase from the immobilization support (Hanefeld et al., 2009). The similar observation was made by Lozano et al. (2007) for synthesis of citronellol acetate using CaL-B where a continuous decrease in activity was observed as the operation cycles increased.

![Graph showing yield (%) as a function of recycle number](image)

**Figure 3.7** Reusability study of immobilized lipase for benzyl acetate synthesis. Reaction conditions: alcohol (1 mmol), vinyl acetate (5 mmol), \( n \)-hexane (3 mL), immobilized lipase (50 mg), temperature (45°C), agitation speed (180 rpm).

Storage stability study of free and immobilized lipase (HPMC-PVA-Lip) was investigated. Immobilized lipase was found to be appreciably stable for a period of 90 days retaining its original catalytic activity (Figure 3.8). 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. Dalla-Vecchia et al.
(2005) 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.

**Figure 3.8** Storage stability study of lipase.
Reaction conditions: alcohol (1 mmol), vinyl acetate (5 mmol), n-hexane (3 mL), immobilized lipase (50 mg), temperature (45°C), agitation speed (180 rpm).

### 3.4 SPECTRAL DATA OF SYNTHESIZED ACETATES

#### 3.4.1 n-butyl acetate (Table 3.3, entry 1)
MS (70 eV, EI) m/z (%): 117 (M⁺ +1, 0.4), 101(2), 87 (3), 73 (16), 61 (13), 56 (42), 43 (100).

#### 3.4.2 n-octyl acetate (Table 3.3, entry 2)
MS (70 eV, EI) m/z (%): 173 (M⁺ +1, 0.1), 129 (2), 111 (2), 83 (24), 74 (14), 70 (76), 57 (51), 55 (38), 43 (100).

#### 3.4.3 Iso amyl acetate (Table 3.3, entry 3)
MS (70 eV, EI) m/z (%): 131 (M⁺ +1, 0.1), 115 (5), 101 (2), 87 (9), 73 (10), 70 (53), 61 (12), 55 (40), 43 (100).
3.4.4 2-ethyl-hexan-1-ol acetate (Table 3.3, entry 4)
MS (70 eV, EI) m/z (%): 173 (M⁺+1, 0.1), 112 (9), 83 (20), 74 (14), 70 (71), 57 (40), 55 (33), 43 (100).

3.4.5 Cyclopentyl acetate (Table 3.3, entry 5)
MS (70 eV, EI) m/z (%): 129 (M⁺+1, 0.1), 100 (2), 85 (9), 71 (6), 68 (34), 58 (16), 43 (100).

3.4.6 Cyclohexyl acetate (Table 3.3, entry 6)
MS (70 eV, EI) m/z (%): 142 (M⁺, 0.1), 100 (4), 82 (68), 67 (61), 54 (19), 43 (100).

3.4.7 Cinnamyl acetate (Table 3.3, entry 7).
MS (70 eV, EI) m/z (%): 176 (M⁺, 24), 133 (37), 115 (88), 105 (33), 103 (10), 92 (29), 77 (20), 55 (12), 43 (100).

3.4.8 Benzyl acetate (Table 3.3, entry 8)
MS (70 eV, EI) m/z (%): 150 (M⁺, 33), 108 (100), 91 (64), 89 (18), 79 (35), 65 (18), 43 (49).

3.4.9 4-Methoxy benzyl acetate (Table 3.3, entry 9)
IR: ν̃ = 2957, 2838, 1738, 1242, 1032.
1H NMR (300 MHz, CDCl₃, 25°C, TMS): δ 7.28-7.31 (dd, J = 6.5 Hz, 2H), 6.87-6.90 (dd, J = 6.5 Hz, 2H), 5.03 (s, 2H), 3.80 (s, 3H), 2.07 (s, 3H) ppm.
13C NMR (75 MHz, CDCl₃, 25°C): δ 170.65, 159.46, 129.92, 127.92, 113.72, 65.86, 54.95, 20.89 ppm.
MS (70 eV, EI) m/z (%): 180 (M⁺, 48), 138 (25), 121 (100), 109 (10), 91 (29), 77 (22), 43 (21).

3.4.10 2-Phenoxy ethyl acetate (Table 3.3, entry 10).
IR: ν̃ = 2954, 2878, 1740, 1226, 1056.
1H NMR (300 MHz, CDCl₃, 25°C, TMS): δ 7.25-7.31 (m, 2H), 6.89-6.98 (m, 3H), 4.39-4.42 (t, J = 4.7 Hz, 2H), 4.14-4.17 (t, J = 4.7 Hz, 2H), 2.08 (s, 3H) ppm.
13C NMR (75 MHz, CDCl₃, 25°C): δ 170.97, 158.57, 129.60, 121.24, 114.69, 65.89, 62.90, 20.89 ppm.
MS (70 eV, EI) m/z (%): 180 (M⁺, 4), 94 (18), 87 (100), 77 (20), 65 (8), 51 (10), 43 (96).
3.4.11 2-Phenoxy benzyl acetate (Table 3.3, entry 11).
MS (70 eV, EI) m/z (%): 242 (M+ , 62), 200 (100), 181 (11), 165 (4), 153 (13), 128 (5), 115 (10), 107 (24), 89 (33), 77 (22), 65 (10), 51 (16), 43 (35).

3.4.12 1, 4 butane diol diacetate (Table 3.3, entry 12)
MS (70 eV, EI) m/z (%): 175 (M+ +1, 0.1), 114 (3), 103 (2), 86 (3), 71 (13), 61 (10), 54 (32), 43 (100).

3.4.13 p-Cresyl acetate (Table 3.3, entry 13)
MS (70 eV, EI) m/z (%): 150 (M+ , 13), 108 (100), 90 (7), 77 (14), 65 (2), 43 (19).

3.4.14 2-Napthyl acetate (Table 3.3, entry 14)
MS (70 eV, EI) m/z (%): 186 (M+ , 13), 144 (100), 115 (37), 89 (10), 65 (4), 43 (16).

Figure 3.9 GCMS spectra of 4-Methoxy Benzyl acetate (Table 3.3, entry 9).
Figure 3.10 $^1$H spectrum of 4-Methoxy Benzyl acetate (Table 3.3, entry 9).

Figure 3.11 $^{13}$C spectrum of 4-Methoxy Benzyl acetate (Table 3.3, entry 9).
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Figure 3.12 GCMS spectrum of 2-Phenoxy ethyl acetate (Table 3.3, entry 10).

Figure 3.13 $^1$H spectrum of 2-Phenoxy ethyl acetate (Table 3.3, entry 10).
3.5 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.

✓ Several industrially important acetates were successfully synthesized using the immobilized lipase, appealing the application of developed protocol for industrial application.

✓ Immobilized lipase 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.

Figure 3.14 $^{13}$C spectrum of 2-Phenoxyl ethyl acetate (Table 3.3, entry 10).