Chapter 2

Enhanced activity and stability of *Pseudomonas cepacia* lipase via physical adsorption on β-cyclodextrin based polyurethane polymers
2.1 INTRODUCTION

Since past few decades, enzyme catalysis has emerged as an attractive tool for the synthesis of valuable commodities with wide industrial application in the production of pharmaceuticals, foods, flavors, fragrances, textiles, cosmetics, polymers, and biofuels. Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are of significant interest due to their high chemo-, regio- and stereo-selectivity (Hasan et al., 2006; Tan et al., 2010). However, the utility of lipases in non-aqueous reaction medium remains a challenge since enzymes are generally sensitive, unstable and insoluble in organic solvents (Klibanov, 2001). As a result, lipase immobilization on solid supports is an area of continued research interest (Dhake et al., 2011; Hanefeld et al, 2009; Lozano et al., 2010). Most of the detail issues on lipase immobilization and importance of immobilization has been discussed previous chapter.

Several solid supports such as chitosan, β-glucan, bentonite, diatomaceous earths, silica aerogels, mesoporous silica have been reported for lipase immobilization (Hanefeld et al, 2009; Wang et al., 2011). However, there is no single universal support that exists as an ideal platform for enzyme immobilization. Thus, there is continued interest in immobilization studies such as adsorption, encapsulation, ionic interaction, covalent cross-linking (Miletic et al., 2012). Adsorption is relatively facile and widely employed among the various immobilization methods since it helps to retain the optimal level of enzyme activity (Hartmann et al., 2007). This is partly because noncovalent enzyme-sorbent interactions such as van der Waals, dipolar, H-bonding, and the hydrophobic effect contributes to the stability of the immobilized material (Park et al., 2009; Salis et al., 2010; Talbert et al., 2012).

Cyclodextrins (CDs) are structurally well-defined macrocyclic host compounds which possess a toroidal conformation in both the solid state and in solution form (Figure 2.1). They are generally produced by partial degradation of starch followed by the enzymatic coupling of the glucose units into crystalline, homogeneous toroidal structures of different molecular size. Three of the most widely characterized cyclodextrins are the alpha-, beta- and gamma-cyclodextrins that have been shown to contain 6 (cyclohexamylose), 7 (cycloheptamylose) and 8 (cyclooctamylose) glucose units respectively. These cyclic, chiral, torus shaped macromolecules contain the D
(+)-glucose residues bonded through α-(1-4) glycosidic linkage. The arrangement of glucose units in the three different forms of cyclodextrin are shown in Figure 2.1. The upper aperture of the torus-shaped cyclodextrin molecule has a larger circumference than the base aperture (Bender, 1978).

![Figure 2.1](image)

**Figure 2.1** Molecular structures of alpha (α), beta (β), and gamma (γ) form of cyclodextrins (Bender, 1978).

Among the various CDs (i.e. α, β, γ form), β-cyclodextrin (β-CD) has interesting amphiphilic properties arising from the numerous hydroxyl groups in the annular region of the macrocycle and apolar groups within the interior of the macromolecule (Bender, 1978; Szejtli, 1998a, 1998b, 2004). Addition of β-CD in reaction mixture has been reported for increased initial reaction rates and lipase stability (Ghanem, 2003). Recently, β-CD based copolymers containing hexamethylene diisocyanate (HDI) as a crosslinker have been investigated as support for lipase immobilization and were found to retain the catalytic activity (Figure 2.2) (Ozmen et al., 2009a, b). Moreover,
Boscolo and coworkers (2010) have reported the preparation of β-CD based nanosponges using carbonate crosslinkers. The foregoing studies illustrate that polymeric supports containing β-CD have promising potential as immobilization supports in enzyme catalysis and there is need to carry out further studies.

![Diagram](image)

**Figure 2.2** Our approach over the reported β-CD based lipase immobilization.

Recently, Mohamed et al. (2011a,b) prepared a series of novel copolymer materials containing β-CD with various crosslinkers such as 4, 4’-dicyclohexylmethane diisocyanate (CDI) and 4, 4’-diphenylmethane diisocyanate (MDI). The objective of work discussed in this chapter; was to investigate the influence of β-CD polyurethane copolymer supports bearing an alicyclic or aromatic
crosslinker on immobilization of PCL. The linkers chosen have similar structure but vary in their hydrogen deficiency (i.e. alicyclic vs. aromatic) as shown in Scheme 2.1. The differences in crosslinker material were anticipated to show differences in catalytic activity and stability of immobilized lipase. FT-IR (ATR), Raman Spectroscopy and PXRD were used to characterize the immobilized PCL. Further, the effect of pH, temperature, and solvent on lipase activity was also evaluated in later section of this chapter.

Scheme 2.1 Structure of diisocyanate crosslinker monomers (i) CDI (ii) MDI.

### 2.2 MATERIALS AND METHODS

#### 2.2.1 Enzyme and chemicals

p-nitrophenylpalmitate (p-NPP), sodium hydroxide (NaOH), sodium carbonate (Na₂CO₃), potassium dihydrogen phosphate (KH₂PO₄), potassium hydrogen phosphate (K₂HPO₄), β-cyclodextrin (β-CD), phenolphthalein, organic solvents (i.e. n-hexane, toluene, acetone), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, Canada. The following diisocyanate crosslinkers were used in this work: methylenediphenyl diisocyanate (MDI; Sigma-Aldrich, Canada), and dicyclohexylmethane-4,4’-disiisocyanate (CDI; Sigma-Aldrich, Canada). The n-butanol was purchased from EMD, USA, while octanoic acid from Eastman Organics, USA and ethanol (anhydrous) from Commercial Alcohols, Ontario, Canada. The lipase PCL (with activity ≥ 23,000 U/g) was kindly donated by Amano lipase (Elgin, IL) USA. Bradford dye reagent was purchased from Bio-Rad, Canada. Triton X-100 was obtained from Terochem Laboratories Ltd, Canada. Deionized water (Millipore) was used during the whole study.

The β-CD based polyurethane polymers were synthesized using procedure reported in previous work (Mohamed et al., 2011). For brevity, the nomenclature
adopted for the crosslinked copolymers is described according to the type of crosslinker used and the comonomer mole ratio (β-CD:crosslinker agent). For example, the 1:3 β-CD: CDI or 1:3 β-CD: MDI copolymer designation is referred to as CDI-3 and MDI-3 respectively, where the molar quantity of β-CD is unity. Solid state $^{13}$C CP-MAS NMR spectra, IR spectra, TGA, elemental analysis (Perkin Elmer 2400 CHN Elemental Analyzer) were obtained and reported elsewhere (Mohamed et al., 2008, 2010, 2011a,b). Porosimetry (Micromeritics ASAP 2010) was used to estimate the surface area and pore structure characteristics of the sorbent materials (Wilson et al., 2011).

### 2.2.2 Immobilization of lipase PCL on β-CD based copolymer

200 mg of β-CD based copolymer was added to a screw capped vial and incubated with 2 mL of ethanol for a period of 16-18 h. The copolymer was then thoroughly washed (2 mL × 3) with potassium phosphate buffer (0.01 M, pH 7.0, 0.1% triton X-100). Further, 3 mL of lipase solution (range 2-4 mg mL$^{-1}$) was subsequently added to the copolymer and an adsorption study was conducted where samples were incubated at 130 ± 5 rpm for 4-24 h. After adsorption, the copolymer was allowed to settle in order to separate the solid phase copolymer from the supernatant enzyme solution which was then removed by micropipette. The immobilized lipase was washed with potassium phosphate buffer to remove unbound lipase followed by vacuum drying at 35-38°C for 20-24 h, and then stored at 4°C until further use. Immobilization yield and amount of protein loaded g$^{-1}$ of polymer was calculated using the protein concentration estimated by the Bradford method (Bradford, 1976; Shu et al., 2011; Wanget al., 2011).

### 2.2.3 Characterization of PCL

FT-IR spectral analysis was performed on a Bruker FTIR Tensor-27 equipped with an MIR detector. The samples were placed on zinc selenide window attenuated total reflectance (ATR) accessory (Harrick MVP) and measurements in the 400-4000 cm$^{-1}$ spectral region with a standard resolution of 1 cm$^{-1}$ and 64 accumulative scans were recorded. DRIFT spectra of lipase were obtained with a Bio-RAD (FTS-40) using powdered samples with KBr. Raman spectra were obtained with a Renishaw system 2000 with a resolution ($\lambda/2$) of 0.257 μm laser spot size. The spectra were
obtained at an excitation frequency of 514 nm using Argon ion laser at the following conditions: scan range of 3400-100 cm\(^{-1}\), 10 mW power with 100% load, 50 \(\times\) magnification, cosmic ray removal (ON), 30 s detection time with multiple scan acquisitions. Different locations of sample were scanned to ensure reproducible results. PXRD analysis was performed on PANalytical Empyrean powder x-ray diffraction (PXRD) instrument using Cu Ka1 monochromatic radiation. A voltage and current of 45 kV and 40 mA was applied respectively. Samples were mounted in vertical configuration as hexane films and PXRD patterns were collected in continuous mode for 5-40° for 20 angle with scan rate of 25° min\(^{-1}\).

2.2.4 Determination of lipase activity (hydrolytic assay)

To 80 µL of 20 mM \(p\)-NPP stock solution (prepared in isopropyl alcohol), 80 µL of test samples (or 10 mg of immobilized lipase) was added and the volume was make up to 3 ml by adding potassium phosphate buffer (0.01M, pH 7.0, 0.1% triton X-100). The assay was carried out at 25°C for 15 min of incubation. The reaction was terminated by adding 1 mL of 0.1 M Na\(_2\)CO\(_3\) solution and further centrifuged at 1500 rpm for 2-3 min. Absorbance was then recorded at 400 nm at 25°C using a double beam spectrophotometer (Varian CARY 100). Blank and control experiments (without lipase, heat inactivated lipase) were also carried out during the study. Lipase activity (IU) was expressed as micromoles of \(p\)-nitrophenol (\(p\)-NP) released min\(^{-1}\) mg\(^{-1}\) of enzyme under mentioned assay conditions. The lipase activity assay was performed in triplicate and mean values are reported herein. The amount of protein present was determined in triplicate with the Bradford assay using a Biorad dye reagent and bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.2.5 Determination of esterification (synthetic) activity of PCL

The esterification reaction was performed in screw-capped glass vials at 45°C with molar ratio of 1:2 of octanoic acid (0.5 mM) : butanol (1 mM), immobilized PCL lipase (100 mg), and \(n\)-hexane (4 mL) for 1-24 h under stirring. 500 µL aliquots were removed and added to 2 mL ethanol (as a quenching agent) followed by titration against 0.01 M NaOH using phenolphthalein as an acid-base indicator to determine the residual octanoic acid. The esterification activity was determined according to the method reported by Pan et al. (2010).

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2.2.6 Effect of pH, temperature and solvent on free and immobilized lipase stability

The effect of pH on free and immobilized lipase stability was investigated by incubating samples at different pH values ranging from 5-9 for 60 min at 25°C. The phosphate buffer (0.01 M, 0.1% triton X-100) was in the pH range of 5-8 using K$_2$HPO$_4$/KH$_2$PO$_4$ while the buffer at pH 9 utilized NaHCO$_3$/Na$_2$CO$_3$. The temperature stability effect was studied by incubating free and immobilized lipase at different temperatures from 25 to 65°C for 60 min. The effect of polar and nonpolar organic solvents was studied by incubation of immobilized lipase in 1 mL of each respective solvent at 25°C for 60 min. The hydrolytic assay was performed (in triplicate) to determine the effect of pH, temperature and solvent on lipase activity.

2.2.7 Leaching study

Leaching of immobilized biocatalyst was investigated in both aqueous and organic solvents. 50 mg of immobilized lipase was added to 1 mL of phosphate buffer at pH 7 and shaken at 130 rpm for 30 min. It was then centrifuged followed by drying at 35-38°C under vacuum for 20-24 h and finally its hydrolytic activity was measured. The difference in hydrolytic activity before and after leaching enables the estimation of leaching (%) of lipase from support. For organic solvents, 100 mg of immobilized lipase was added to 4 mL of n-hexane and was agitated on shaker at 130 rpm for 24 h. It was then centrifuged, dried at 35-38°C for 4-6 h under vacuum and then the hydrolytic activity was determined to estimate the leaching, as described above in section 2.2.4.

2.2.8 Recyclability and storage stability study

The esterification of octanoic acid with n-butanol was used to study the recyclability of immobilized lipase, as described in section 2.2.5. The immobilized lipase was thoroughly washed with n-hexane (3 × 3 mL) to remove traces of reactant or product followed by drying under vacuum at 35-38°C for 4-6 h before reuse in the next recycle.

Moreover, free and immobilized lipases were stored at 4°C for 30 days and the stability study was performed at 10 day intervals using the hydrolytic assay as described in section 2.2.4.
2.3 RESULT AND DISCUSSION

2.3.1 Characterization of immobilized PCL

β-CD copolymer materials were prepared at 1:3 β-CD:diisocyanate ratios where three mole equivalents of diisocyanate were reacted per mole of β-CD (Schemes 2.1-2.2). The CDI-3 and MDI-3 copolymers were prepared at these monomer ratios to ensure that the copolymers were insoluble in water and to retain favorable accessibility of the inclusion sites of β-CD (Mohamed et al., 2011). The structural characterization (e.g., IR, NMR, TGA, and porosimetry) of the copolymer materials containing β-CD are in good agreement with previously reported results and strongly support the identity and porous nature (Scheme 2.2 and Table 2.1) of the sorbent materials used in this study (Mohamed et al., 2008, 2010, 2011a, b).

Scheme 2.2 Synthesis of urethane copolymer supports containing β-Cyclodextrin (where, m=7, n=14, DMA=dimethylacetamide). Note that only a small two-dimensional section of three-dimensional copolymer framework is shown for clarity purposes.

It is worthwhile noting that the textural properties of each copolymer vary according to the nature of the cross linker unit and has been attributed to differences in the swelling characteristics respectively (Table 2.1). The differences in surface area from nitrogen porosimetry (~1 m²/g) and dye adsorption method illustrate contributions arising from swelling in the anhydrous and hydrated states (Mohamed et al., 2011b).
Table 2.1 Textural properties of copolymers materials obtained from sorption of p-nitrophenol (PNP) at 295 K and pH 4.60 in aqueous solution (Wilson et al., 2011).

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>SA (m²/g)</th>
<th>V_{ads} (m³/g)</th>
<th>Q_{m} (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDI-3</td>
<td>620</td>
<td>33.9</td>
<td>1.69</td>
</tr>
<tr>
<td>MDI-3</td>
<td>449</td>
<td>24.6</td>
<td>1.42</td>
</tr>
<tr>
<td>GAC</td>
<td>641</td>
<td>35.1</td>
<td>2.03</td>
</tr>
</tbody>
</table>

[a] Surface area (SA; m²/g) = (Q_{m} × N × σ) / Y; where Y=1 and σ= 52.5 Å²

[b] V_{ads} (m³/g) as in eqn (4) where Y=1 and V_{PNP} = 0.64 nm × 0.43 nm × 0.33 nm = 9.08 × 10⁻²⁶ m³ (Maffei et al., 2006)

[c] Monolayer sorption capacity (Q_{m}; mmol/g) of PNP

Note: The average pore diameter for CDI-3 and MDI-2 is estimated to be less than 20 nm according to the BJH adsorption isotherm using N₂ as the backfill gas.

FTIR analysis is a powerful tool used to observe the conformation of lipase via amide signature bands. Lipase has three main amide bands while in certain cases fourth amide region has been reported (Barth, 2007; Dhake et al., 2011). The amide I band arises due to C-O stretching vibration at 1645 cm⁻¹ whereas the amide II band is due to N-H bending and C-N stretching vibrations ~1583 cm⁻¹. The amide III band is observed ~1434 cm⁻¹ because of N-H bending, C-C and C-N stretching vibrations; while amide IV band is observed at ~556 cm⁻¹ (Misiūnas, 2008). The amide I band can be used to elucidate the secondary structure of lipase. In this study, the amide I band was observed at ~1641 cm⁻¹ for PCL and ~1643 cm⁻¹ for CDI-3-PCL as shown in Figure 2.3. Moreover, Figure 2.4 illustrates the DRIFT spectra for the amide I band observed at ~1653 cm⁻¹ (i.e. for PCL) and ~1626 cm⁻¹ (i.e. for CDI-3-PCL). Hence, the presence of amide I band observed in this study represents the native conformation of lipase after immobilization (Barth, 2007; Misiunas, 2008).
Figure 2.3.1 FT-IR (ATR) spectra of free and polymer bound lipase in powder form (i) PCL (ii) CDI-3-PCL (iii) CDI-3.

Figure 2.3.2 FT-IR (ATR) spectra of free and polymer bound lipase in powder form (i) PCL (ii) MDI-3-PCL (iii) MDI-3.

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Figure 2.4.1 DRIFT analysis of lipase PCL and copolymer in powder form (i) PCL (ii) CDI-3 (iii) CDI-3-PCL.

Figure 2.4.2 DRIFT analysis of lipase PCL and copolymer in powder form (i) MDI-3 (ii) MDI-3-PCL (iii) PCL.
Since immobilization of lipase on β-CD based copolymer was done via physical adsorption, the conformation of lipase was expected to be retained. It is noteworthy to mention that the vibrational bands for the urethane bonds of copolymer overlap with those of the lipase amide group bands. The overlap is more readily observed in the case of MDI-3-PCL where the lipase amide regions are obscured as shown in Figure 2.3.2 and 2.4.2.

To further understand the nature of the enzyme-polymer complex, Raman spectra were obtained (Figure 2.5 and 2.6). Both figures illustrate the Raman spectra of free lipase, free polymer, and immobilized lipase in the presence of buffered H\textsubscript{2}O and unbuffered D\textsubscript{2}O. When the spectra were obtained in potassium phosphate buffer at pH 7 (Figure 2.5.1 and 2.6.1); no distinct peaks were observed for both native and immobilized lipase. Recently Misiunas et al. (2008) studied the environmental effects and structural changes in *Thermomyces lanuginosus* lipase at the interface when interacted with liquid crystalline phases containing phytantriol. In addition, aromatic amino acids like Phe, Tyr, Trp, and His in proteins are reported to display enhanced Raman activity in D\textsubscript{2}O relative to H\textsubscript{2}O environments due to isotopic exchange (Takeuchi, 2011). Based on the reported studies, the H\textsubscript{2}O solvent was isotopically replaced by D\textsubscript{2}O at different pH (mainly at pH 3, 7 and 10). As a result, two distinct vibrational bands were observed at 557 cm\textsuperscript{-1} and 1091 cm\textsuperscript{-1}. The amide IV region occurs ~556-558 cm\textsuperscript{-1} and the appearance of a band at ~557 cm\textsuperscript{-1} in present study is believed due to amide IV. The band observed at ~1091 cm\textsuperscript{-1} is due to C-C, C-N, C-O stretching vibrations. Both the peaks can be optimally observed at pH 7 in D\textsubscript{2}O environment for CDI-3-PCL (Figure 2.5.3) while the peak intensity is attenuated in acidic and alkaline pH respectively (Figure 2.5.2 and 2.5.4). The lipase enzyme is more stable at neutral pH. This pH dependence was not observed in the case of MDI-3-PCL and may be related to its reduced swelling behavior relative to CDI-3 due to the presence of aromatic crosslinker units in the copolymer framework. Moreover, the peaks observed for MDI-3-PCL are strongly overlapped with peaks of MDI-3 copolymer, and represents problems with the assignment of respective vibrational bands for the copolymer and lipase (Figure 2.6.2 to 2.6.4).

With respect to CDI-3, the spectra are similar in either solvent system for the unbound polymer (Figure 2.5.1 to 2.5.4 iii). In contrast, significant changes in line

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width and relative intensity are observed for lipase and immobilized lipase in \( \text{H}_2\text{O}/\text{D}_2\text{O} \) systems. In particular, substantial line broadening is observed for C-H, C-O, and amide regions (~500 cm\(^{-1}\)), as shown in Figure 2.5.1 to 2.5.4 i-ii. It should be noted that the spectra are not normalized in Figure 2.5 i-ii and the lipase contribution in Figure 2.5 ii is comparatively small due to the level of adsorption (Table 2.3, ~1.8-2%). It is noteworthy to mention that the spectral lines for lipase undergo substantial broadening from \( \text{H}_2\text{O} \) to \( \text{D}_2\text{O} \) solvent systems. This can be understood due to isotopic exchange (\( \text{H}^+ / \text{D}^+ \)) of acidic sites, changes in solvent polarizability (Avila et al., 2003), hydrophobic effects, and possible aggregation of lipase (Ngarize et al., 2004).

**Figure 2.5.1** Raman spectral analysis in powder form (i) PCL (ii) CDI-3-PCL (iii) CDI-3 (in buffer at pH 7).
Figure 2.5.2 Raman spectral analysis in powder form (i) PCL (ii) CDI-3-PCL (iii) CDI-3 (in D₂O at pH 3).

Figure 2.5.3 Raman spectral analysis in powder form (i) PCL (ii) CDI-3-PCL (iii) CDI-3 (in D₂O at pH 7).
Figure 2.5.4 Raman spectral analysis in powder form (i) PCL (ii) CDI-3-PCL (iii) CDI-3 (in D₂O at pH 10).

Figure 2.6.1 Raman spectral analysis in powder form (i) PCL (ii) MDI-3-PCL (iii) MDI-3 (in buffer at pH 7).
Figure 2.6.2 Raman spectral analysis in powder form (i) PCL (ii) MDI-3-PCL (iii) MDI-3 (in D$_2$O at pH 3).

Figure 2.6.3 Raman spectral analysis in powder form (i) PCL (ii) MDI-3-PCL (iii) MDI-3 (in D$_2$O at pH 7).
Figure 2.6.4 Raman spectral analysis in powder form (i) PCL (ii) MDI-3-PCL (iii) MDI-3 (in D₂O at pH 10).

Another notable feature in Fig. 2.5 and 2.6 (except 2.5.1. and 2.6.1) is the appearance of the band ~2500 cm⁻¹ and this corresponds to the uncoupled OD oscillator bands. The variation in the line width and respective components are related to the nature of hydration (i.e. H-bonded vs. non H-bounded water (Mundy et al., 1973)). The above results provide strong evidence of the change in molecular environment of lipase in its free form compared to its immobilized form on CDI-3 and MDI-3, support the formation of a noncovalent complex (Ngarize et al., 2004).

Figure 2.6 illustrates the Raman spectra of free lipase, free polymer, and immobilized lipase in the presence of buffered H₂O and unbuffered D₂O for MDI-3, analogous to that presented for CDI-3 in Figure 2.5. One notable difference is the appearance of the aromatic C-H band (~3100 cm⁻¹). As described above, changes in the line width and peak intensity were observed for the lipase in its free and immobilized form in H₂O/D₂O systems. The interpretation of such changes is attributed to several effects, as described for Figure 2.5. The band ~2500 cm⁻¹ displays contributions arising from two or more spectral components which coincide with the foregoing argument of bound and unbound water. However, the presence of
aromatic crosslinker units may contribute to a particular type of bound water distinctive of that for typical aliphatic compounds, as evidenced by the water-benzene complex (Suzuki, 1992). As well, the polarizability of an aliphatic vs. aromatic residues (Mohamed et al., 2009) is anticipated to affect the Raman intensity and wave number due to the variable molecular environments.

PXRD spectra were obtained to evaluate the effects of changes to the surface morphology of the copolymers upon adsorption of lipase. The broad diffraction lines for each copolymer framework in presence or absence of lipase supports that the structure of copolymer is amorphous in nature (Figure 2.7). Differences in the PXRD spectra of the copolymer in the presence and absence of lipase are negligible, and may be attributed to the relatively small amounts of adsorbed lipase. Upon immobilization of lipase, a slight shift (2θ ~ 0.3) is observed, compared to the diffraction lines observed for the free copolymer in each case (i.e. CDI-3 and MDI-3) (Guan et al., 2004).

![PXRD spectra](image)

**Figure 2.7** Powder XRD analysis of (i) CDI-3 (ii) CDI-3-PCL (iii) MDI-3 (iv) MDI-3-PCL (v) PCL.
2.3.2 Determination of lipase activity, protein content and biochemical characterization

The hydrolytic activity of free lipase was determined to be 7.1 IU/mg while for the immobilized lipase it was 21.9 and 29.8 IU/mg for CDI-3-PCL and MDI-3-PCL, respectively. The amount of lipase adsorbed onto the copolymer material was determined from the residual activity of lipase. Adsorption of enzyme on β-CD based copolymer was studied for 4-24 h where a relatively higher protein loading was observed after 24 h. The considerable decrease in residual activity was observed at lower concentration of the lipase (2 mg/mL) after 24 h, and thus provided a higher immobilization yield (Table 2.2). The immobilization for CDI-3-PCL was greater (72%) relative to MDI-3-PCL (46%). The relative amount of protein loading was determined by Bradford method according to a reported procedure (Wang et al., 2011). The ~1.2 - 1.8 mg of protein was adsorbed per gram of sorbent according to Table 2.2.

Even though a lower amount of protein was adsorbed on the copolymer materials, higher catalytic (i.e. hydrolytic) activity was observed relative to the free lipase. The observed low loading levels of lipase denotes that adsorption is more uniform across the copolymer surface and is restricted to monolayer deposition. Favorable hydration and enzymatic activity is anticipated for monolayer adsorption relative to multi-layer lipase adsorption. It has been reported that aggregation and multilayer formation results in decreased lipase activity even though the loading level is higher (Al-Duri and Yong, 2000). Moreover, Salis et al. (2010) made a similar observation for lipase immobilization on surfaces of mesoporous materials via physisorption where there was comparatively higher catalytic activity even though the enzyme loading was lower as compared to chemisorption. This is because physical adsorption does not dramatically alter enzyme conformation and thus subsequently higher activity is observed.
**Table 2.2.1** Influence of CDI-3 copolymer: lipase ratio on enzyme loading.

<table>
<thead>
<tr>
<th>Immobilized lipase</th>
<th>Polymer : PCL ratio</th>
<th>Residual activity (%) after 4 h</th>
<th>Residual activity (%) after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDI-3-PCL (4mg/mL)</td>
<td>200:12</td>
<td>93±0.9</td>
<td>59±1.8</td>
</tr>
<tr>
<td>CDI-3-PCL (3mg/mL)</td>
<td>200:9</td>
<td>85±1.9</td>
<td>46±1.6</td>
</tr>
<tr>
<td>CDI-3-PCL (2 mg/mL)</td>
<td>200:6</td>
<td>63±1.8</td>
<td>28±1.4</td>
</tr>
</tbody>
</table>

**Table 2.2.2** Influence of MDI-3 copolymer: lipase ratio on enzyme loading.

<table>
<thead>
<tr>
<th>Immobilized lipase</th>
<th>Polymer : PCL ratio</th>
<th>Residual activity (%) after 4 h</th>
<th>Residual activity (%) after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDI-3-PCL (4mg/mL)</td>
<td>200:12</td>
<td>97±0.8</td>
<td>89±1.6</td>
</tr>
<tr>
<td>MDI-3-PCL (3mg/mL)</td>
<td>200:9</td>
<td>85±1.4</td>
<td>70±1.8</td>
</tr>
<tr>
<td>MDI-3-PCL (2mg/mL)</td>
<td>200:6</td>
<td>79±1.3</td>
<td>54±1.7</td>
</tr>
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</table>
Table 2.3 Enzyme loading and catalytic activity of PCL.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Yield (%) *</th>
<th>Enzyme loading (mg of protein g$^{-1}$ of polymer)</th>
<th>Hydrolytic activity (U mg$^{-1}$ min$^{-1}$)</th>
<th>Esterification activity (mM g$^{-1}$ h$^{-1}$)</th>
<th>Leaching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>--</td>
<td>--</td>
<td>7.1±0.5</td>
<td>3,638</td>
<td>--</td>
</tr>
<tr>
<td>CDI-3-PCL</td>
<td>72±1.4</td>
<td>1.8±0.2</td>
<td>21.9±1.8</td>
<td>4,850</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>MDI-3-PCL</td>
<td>46±1.7</td>
<td>1.2±0.1</td>
<td>29.8±2.0</td>
<td>9,700</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
</tbody>
</table>

*Refers to immobilization yield of lipase onto copolymer.

Scheme 2.3 illustrates the immobilization of lipase for each type of β-CD-copolymers (i.e. MDI-3 and CDI-3). In either case, adsorption of lipase onto the surface of the copolymer involves a range of non-covalent interactions (i.e. H-bonding, dipolar, dispersion, and the hydrophobic effect). However, in the case of MDI-3, the presence of aromatic crosslinkers facilitates the occurrence of π-π interactions between aromatic units of lipase with the MDI crosslinker units. The latter is anticipated to contribute to additional stability of the immobilized lipase. However, it should be noted that the swelling behavior of MDI-3 and CDI-3 differ and this contributes to differences in the effective surface area of the copolymer adsorption sites. The greater catalytic activity of MDI-3 immobilized lipase might be due, in part, to the occurrence of π-π interactions (Sjetil et al., 1996).
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Scheme 2.3 Schematic illustration of lipase immobilization on two types of β-CD-copolymer (MDI-3 and CDI-3) via physisorption. The dashed lines represent noncovalent interactions between the lipase and the support.

The pH, temperature, solvent effects on lipase stability was studied in order to probe changes in the interaction of lipase with surrounding environment. The pH stability study (Figure 2.8) revealed that immobilization shifted the optimum pH values for optimal lipase stability. CDI-3-PCL showed higher stability at pH 6 and MDI-3-PCL at pH 7 while the free lipase was stable at pH 5-7. Overall, the activity of immobilized lipase was greater than the free lipase (~threefold for CDI-3-PCL and ~fourfold for MDI-3-PCL) over wide range of pH. At alkaline conditions pH 8-9, the hydrolytic activity of immobilized lipase was attenuated. This is due to alteration in the microenvironment surrounding the lipase followed by subsequent changes in the interaction like ion-dipole, dipolar, dispersion, and H-bonding between enzyme and support. The effect of temperature on the stability of free and immobilized lipase is
shown in Fig. 2.9. As the temperature is increased from 25 to 65°C, the enzyme-copolymer interaction was modified resulting in a lowering of the hydrolytic activity of the immobilized lipase. However, the hydrolytic activity for immobilized lipase was higher than free lipase at all temperatures studied (25-65°C) which might be due to the favorable adsorption enthalpy between lipase and the copolymer framework.

**Figure 2.8** Effect of pH on stability of free and immobilized lipase.

**Figure 2.9** Effect of temperature on stability of free and immobilized lipase.

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In general, lipase catalyzed reactions include esterification, transesterification and amidation (Hanefeld et al., 2009; Klibanov et al., 2001). The reactants used or products obtained from these lipase-catalyzed reactions are generally insoluble in water (Klibanov et al., 2001). Thus, the use of non-aqueous solvents as a reaction medium for such biocatalytic transformations is of great interest. The effect of solvent stability on the immobilized lipase is illustrated in Figure 2.10. The presence of polar solvents like methanol (Log P value = -0.76; where P is the partition coefficient for n-octanol/water two phase) attenuate the activity of immobilized lipase. In the case of aprotic polar solvents like acetone (Log P value = -0.23), acetonitrile (Log P value = 0.33), the hydrolytic activity was favorably retained (59-83%).

![Figure 2.10 Effect of solvent on the stability of immobilized lipase (relative activity was calculated from the hydrolytic activity of immobilized lipase; the hydrolytic activity of untreated immobilized lipase (control) was considered to be 100%).](image)

In general, polar protic solvents have a tendency to undergo desolvation of the essential water of the active site of the enzyme; thereby attenuating its catalytic activity. Furthermore, organic solvents which may undergo competitive hydration via H-bonding and other dipolar interactions may reveal different solvent effects with the copolymer framework interface which results in variation of the lipase activity. The lipase catalytic activity was significantly retained in apolar solvents like toluene (Log
P value = 2.5) and n-hexane (Log P value = 3.5). Similar observations have been reported for various lipase catalyzed reactions (Dhake et al., 2011; Guncheva et al., 2011).

The leaching of immobilized lipase is an important parameter since it can be used to gauge the relative stability of the interactions between enzyme and the copolymer. Leaching was investigated in both aqueous and non-aqueous reaction media (Table 2.3). Enzymes are known to be relatively more soluble in aqueous media vs. non-aqueous solvents. Thus, a decrease in the lipase activity (~40% and 45%) occurred in aqueous media as the lipase is desorbed from the support upon mechanical agitation for CDI-3-PCL and MDI-3-PCL respectively (Table 2.3). Supporting evidence of the latter is provided by Mitchell et al. (2011). Recently, Zou et al. (2011) studied immobilization of porcine pancreas lipase (PPL) on a series functionalized ionic liquid modified mesoporous silica SBA-15 via adsorption method and they observed ~28-51% leaching. Reduced leaching of lipase in organic solvents is expected even on prolonged agitation and similar results on leaching of immobilized lipase in apolar solvents like n-hexane, were observed herein. After 24 h of incubation in n-hexane, 16% and 12% leaching was observed for CDI-3-PCL and MDI-3-PCL respectively. Differences in leaching of each copolymer observed in various solvents indicate that different types of interaction occur between lipase and each copolymer support.

Use of enzymes at an industrial scale represents limitations, in part, due to the relatively high cost of such biocatalysts. Therefore it becomes important to study the reusability of immobilized lipase materials to make such biocatalytic processes more feasible. To assess the recyclability study, an immobilized lipase was used for synthesis of butyl octanoate, an important model flavor compound (Kraft and Swift, 2005). Fig. 2.11 shows that the immobilized lipase could be efficiently recycled for first two cycles, whereas, it is attenuated to 75-80% of initial activity at the end of the fourth recycle. The decrease in lipase esterification activity might be due to prolonged exposure to octanoic acid. Also, the minor amounts of leaching in organic solvents may attribute to decrease in esterification activity, as described above.
Figure 2.11 Recyclability study of immobilized lipase for esterification reaction. Reaction conditions: octanoic acid (0.5 mmol), n-butanol (1 mmol), n-hexane (4 mL), immobilized lipase (100 mg), temperature (45 °C), time (24 h).

Figure 2.12 Stability of immobilized lipase at 4°C for 30 days.

Half life is another important factor in enzyme activity because immobilized enzymes may be susceptible to decreased activity on prolonged storage. Recently, Ondul et al. (2012) demonstrated that immobilized *Candida antarctica* A lipase...
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retained ~ 60% of initial activity on storage for 28 days at 4°C while the free enzyme lost ~ 65% of its initial activity under similar storage conditions. In this study, free lipase in phosphate buffer solution was noted to be quite stable retaining 90% of its initial activity at 4°C. The immobilized lipase CDI-3-PCL (92%) and MDI-3-PCL (84%) retained much of the initial hydrolytic activity as evidenced in Fig. 2.12. In addition, it should be noted that CDI-3-PCL retained ~100% initial activity for up to 20 days where after it decreased on incubation to 30 days.

2.4 CONCLUSION

✓ Noncovalent immobilization of lipase onto microporous urethane copolymers containing β-CD was achieved in present study.
✓ In all cases, immobilization occurred via physisorption wherein the MDI-3 copolymer presented a more favorable immobilization support with four-fold enhanced hydrolytic activity of immobilized lipase relative to free lipase in aqueous solution.
✓ The difference in lipase activity for each copolymer is attributed to the nature of the crosslinker unit of the copolymer scaffold, resulting in variable solvent accessible surface areas and hydrophobic effects.
✓ The favorable stability of MDI-3-PCL lipase is attributed to the occurrence of additional π-π interactions between the aromatic protein subunits of PCL with the diphenyl crosslinker units of the copolymer.
✓ Immobilized lipase preparations had remarkable catalytic activity over a wide range of pH, temperature and solvents like toluene and n-hexane.
✓ The immobilized lipase was recycled four times retaining 75-80% of its initial activity at the end of fourth recycle and exhibited considerable stability for a period up to 30 days.
✓ Synthetically engineered urethane copolymers, as described herein, offer an opportunity to design immobilized biocatalysts with tunable catalytic activities over a range of conditions.