3.1. Introduction:

New paradigms are shrinking our world. Innovations at the intersection of medicine, biotechnology, engineering, physical sciences and information technology are spurring new directions in research and development, commercialization and technology transfer. One such fascinating area is nanotechnology which is currently witnessing impressive advances in aspects such as synthesis of nanoparticles, understanding their fundamental physical and chemical properties and organization of nanoscale matter using weak non covalent interactions. Nanomaterials contain crystalline, quasicrystalline, or amorphous phases; which can be metals, ceramics, or composites with rather unique and improved mechanical, electronic, magnetic and optical properties, than normal, coarse grained polycrystalline materials. Due to their ultrafine sizes, and high surface area, these nanoparticles possess the ability to overcome conventional restrictions of phase equilibria and kinetics. The presence of large fraction of atoms at the surfaces and grain boundaries of these materials confer novel properties. Nanoparticles range from 1 to 100 nm in size.

3.1.1 Methods to synthesize nanoparticles

Mainly three methods are used widely for synthesis of nanoparticles:

a) Vapor Condensation: This approach is used to make metallic and metal oxides ceramic nanoparticles. It involves evaporation of a solid metal followed by rapid condensation to form nanosized clusters that settle in the form of a powder. Inert gases are used to avoid oxidation when creating metal nanoparticles, whereas a reactive oxygen atmosphere is used to produce metal oxide ceramic nanoparticles. Final particle size is controlled by variation of parameters such as temperature, gas environment and evaporation rate.

b) Chemical Synthesis: Most widely used chemical synthesis technique consists of growing nanoparticles in a liquid medium composed of various reactants. This is typified by the sol-gel approach and is also used to create quantum dots. Chemical techniques are generally better than vapor condensation techniques for controlling the final shape of the particles. Approaches are generally low cost and high volume but contamination from the precursor chemicals can be a problem.
c) **Solid-state processes:** Grinding or milling can be used to create nanoparticles. The milling material, milling time and atmospheric medium affect resultant nanoparticles properties.

Also interestingly, variety of nanoparticles can be synthesized biologically when the bacteria are challenged with metals naturally or by providing artificial conditions. For example, magnetotactic bacteria synthesize intracellular magnetic or greigite nanocrystallites (Blakemore RP, 1982). Similarly, certain yeasts when challenged with toxic metals such as cadmium, synthesize intracellular CdS nanocrystallites as a mechanism of detoxification (Dameron et al., 1989). Bacteria involved in silver leaching have been reported to accumulate silver sulphide within their membrane (Pooley FD, 1982) and natural biofilms of sulfate reducing bacteria were shown to deposit nanocrystalline sphalerite, i.e ZnS (Labrenz et al., 2000). Kowshik et al. (2003) demonstrated synthesis of silver nanoparticles extracellularly by silver tolerant yeast species when the cells were challenged with soluble silver in logarithmic growth phase. Nanoparticle synthesis by biological route has gained more importance due to the particle size uniformity and their environment friendly nature.

### 3.1.2 Functionalization of the nanoparticles for application

Coating and chemically modifying a variety of nanoparticles is common practice and an area where new, valuable, innovations are likely to be seen. The basic applications of surface functionalization range from altering the wetting or adhesion characteristics and improving the nanoparticles dispersion in matrices to enhancing the catalytic properties and ordering the interfacial region (Marie-Isabelle Book ed.). The creation of specific surface sites on nanoparticles for selective molecular attachment is considered a promising approach for their applications in nanofabrication, nanopatterning, self-assembly, nanosensors, bioprobes, drug delivery, pigments, photocatalysis, LEDs etc.

The functionalization of nanostructures results in improved dispersibility in liquid and solid media. As a consequence of organic functional layers, dispersions of nanoparticles in aqueous and organic solvents can be stabilized for extended periods of time. This is only possible as the agglomeration is prevented almost completely due to the surface modification.

To overcome the agglomeration problem and enhance their chemical and physical stability, an efficient strategy was developed for the preparation of MNP/polymer
composites in which silver nanoparticles coated with 4-mercaptomethyl styrene act as cross linkers in suspension polymerization (Farah et al, 2008).

Also Shaw et al. (2006) demonstrated that esterase from P. putida was able to be immobilized to APES coated magnetic nanoparticles via glutaraldehyde coupling reaction. It was also reported that carboxymethylated chitosan was covalently bound on the surface of magnetic iron oxide nanoparticles in the presence of the conjugating agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. Such surface modification could improve the colloidal stability of MNPs in aqueous suspension and yield new magnetic nano adsorbent for protein adsorption and purification (Liang et al, 2007).

3.1.3 Nanoparticles as immobilization support

Currently the focus in enzyme immobilization technology is shifting towards use of nanosize materials as supports particularly due to their higher specific surface area. Consequently, variety of nanosupports have been used for enzyme immobilization such as silica nanotubes, phospholipid bilayers, self assembled monolayers, Langmuir Blodgett films, within polymer matrixes, magnetite, silver and gold nanoparticles (Phadtare et al, 2002).

Silica nanoparticles represent an important class of nanosupports, widely investigated for immobilization of enzymes and proteins. Silica nanoparticles are easy to prepare and have been used as carrier for immobilization of several enzymes and proteins including Mucor javanicus lipase, glucose oxidase, catalase, peroxidase, cytochrome C, lysozyme, etc. either by adsorption or by covalent attachment using appropriate functional reagents (Wang et al, 2005; Sun et al, 2006; Moon et al, 2006).

Amongst several supports, magnetic nanoparticles has also received considerable attention for enzyme immobilization due to their higher surface area, lower mass transfer resistance, less fouling and ease of separation of immobilized enzyme from reaction mixture by application of a magnetic field (Halling et al, 1980). Magnetite (Fe₃O₄) is one of the widely employed magnetic materials owing to its biocompatibility, low toxicity and superparamagnetic property (Dresco et al, 1999). Magnetite nanoparticles have been used as a support material for covalently attaching several enzymes including yeast alcohol dehydrogenase, lipase, glucose oxidase, cholesterol oxidase and bacterial esterase (Dyal et al, 2003; Liao and Chen, 2001; Guo et al, 2003).
In the previous chapter it was observed that encouraging results were obtained using surfactant coated CRL immobilized in AOT-based organogels for synthesis of ethyl isovalerate (Dandavate and Madamwar, 2007). However, in an attempt to develop a more efficient immobilized lipase which facilitates its easy recovery and reuse, we have immobilized CRL by covalent attachment onto the surface of silica nanoparticles and magnetite nanoparticles prepared in our laboratory. This chapter deals with immobilization and characterization of CRL on silica and magnetite nanoparticles and its use for synthesis of food esters.

3.2. Materials and Methods:

3.2.1 Chemicals:
Candida rugosa lipase (1140 units/mg) was purchased from Sigma-Aldrich, Germany. Gluteraldehyde was obtained from Loba Chemie. Ammonium hydroxide was obtained from Sisco Laboratories, India. 3-Aminopropyl-trietoxysilan (APTES), ethyl carbodiimide hydrochloride (N-(3-Dimethylaminopropyl)-N) and n-Hexanol were purchased from Fluka, Germany and p-nitrophenol & Triton X-100 from Himedia, India. Tetraethyl orthosilicate (TEOS) and 4-nitrophenyl palmitate (pNPP) were obtained from Merck and Lancaster, France respectively. Ferrous chloride (FeCl₂) was obtained from Loba Chemie. Ferric chloride (FeCl₃) and ammonium hydroxide were obtained from Sisco Laboratories, India. All the substrates for esterification and standard esters were obtained from Merck, Aldrich and Fluka.

3.2.2 Preparation of silica nanoparticles in reverse micelles
Silica nanoparticles were prepared in nonionic water in oil microemulsion according to the method reported by Yang et al. (2004) with modifications. Microemulsion was prepared by mixing 64.82 mL hexane, 15.55 mL n-hexanol, and 15.29 mL Triton X-100 and 2.93 mL milli Q water followed by stirring for 30 min to form reverse micelles. A specified volume (864.3 µL) of TEOS and (518.58 µL) of 28% NH₄OH were further added to the microemulsion and incubated at room temperature for 24 hours. Next day, acetone was added in the mixture followed by centrifugation at 10,000 rpm. The precipitates obtained were then washed with ethanol several times.
3.2.3 Immobilization of CRL on silica nanoparticles
Silica nanoparticles were suspended in toluene containing APTES and subsequently refluxed in a Dean-Stark apparatus for 4 hours in order to carry out the amino-functionalization. The amino-functionalized silica nanoparticles (20 mg) were then treated with 1.5 mL of 0.5% glutaraldehyde for 20 minutes under shaking conditions (200 rpm) followed by excessive washing with the phosphate buffer (0.05 M, pH 7.2), in which the mixture was subjected to sonication for 10 min. The glutaraldehyde-treated silica nanoparticles (50–500 mg) were then suspended in 5 mL of lipase solution (5 mg/mL) for 12 h at 37°C. To cap unreacted aldehyde groups, the sample was washed with Tris–Cl buffer (pH 7.8) once and incubated in the same buffer overnight (during which unreacted glutaraldehyde groups became bound with Tris ions). After the capping process, the lipase-bound silica nanoparticles were washed twice with Tris–Cl buffer and five times with phosphate buffer (0.05 M, pH 7.2). The immobilized enzyme thus obtained was then used for activity measurements and characterization.

3.2.4 Preparation of magnetic nanoparticles
Magnetic nanoparticles (Fe₃O₄) were prepared by chemical co-precipitation of Fe²⁺ and Fe³⁺ ions in a solution of ammonium hydroxide under hydrothermal conditions (Huang et al, 2003; Halling and Dunnill, 1980). Fe²⁺ and Fe³⁺ (molar ratio 1:2) were dissolved in milli Q water (to get final concentration of 0.25M) and chemically precipitated at room temperature (25°C) by adding NH₄OH solution (30%) to get a pH 10. The precipitates were heated at 80°C for 35 min under continuous mixing and washed 4 times in milli Q water followed by several washes in ethanol. During washing, the magnetic nanoparticles were separated from the supernatant using a magnetic separator of strength greater than 20 megaersted (MOe). The particles were finally dried in a vacuum oven at 70°C. The dried particles exhibited a strong magnetic attraction.
3.2.5 Immobilization of CRL on magnetic nanoparticles

Magnetic nanoparticles produced (50-250mg) were suspended in 1mL of phosphate buffer (0.05M, pH 7.2). To this 0.5 mL of carbodiimide solution (0.025g/mL in 0.05M phosphate buffer, pH 7.2) was added and then kept in sonicator bath for 10 min. Then 2.5 mL of lipase (5 mg/mL) was added and the reaction mixture was further sonicated for 30 min. at 4°C. Lipase bound magnetic nanoparticles were recovered from reaction mixture using a strong magnetic separator. The precipitates were washed with 0.05M phosphate buffer pH 7.2 and then with 0.1M Tris.Cl, pH 8 and then used for activity measurements and characterization. In order to determine the binding efficiency, the protein was estimated in the supernatant (unbound lipase) and the percentage binding was determined considering the initial protein as 100%.

3.2.6 Characterization of Silica and Magnetite nanoparticles

The size and morphology of silica and magnetite nanoparticles were determined by Transmission Electron Microscopy (Philips Tecnai 20, USA). The sample for analysis was prepared by placing a drop of silica nanoparticles dispersed in absolute ethanol solution onto a formavar coated copper grid and evaporated in air at room temperature.

The FTIR analysis of functionalized nanoparticles, free lipase and lipase bound silica and magnetite nanoparticles was done in order to confirm binding of lipase on nanoparticles. The percentage of protein bound was determined by estimating the protein in the unreacted fraction. X-ray diffraction was used to determine crystal structure of magnetic nanoparticles before and upon lipase immobilization using X’Pert Philips X-ray Diffractometer using Cu-Kα radiation (λ = 0.154 nm). The surface area of magnetite nanoparticles was determined from nitrogen adsorption-desorption isotherm using BET method (Brunauer et al, 1938). The thermal gravimetric analysis and differential scanning calorimetry of magnetite and enzyme bound magnetite nanoparticles were done using PerkinElmer Pyris 1 TGA and PerkinElmer Pyris 1 DSC, respectively.

3.2.7 Kinetic Parameters

The kinetic parameters for both free and immobilized lipase were determined by measuring the initial rate of pNPP hydrolysis (100 μM to 9 mM) using 0.1 mg of
lipase. The Km and Vmax values were obtained from Lineweaver–Burk plots. The influence of the immobilization of the lipase on the activation energy for the lipase-catalyzed hydrolytic reaction of pNPP ester was determined from the Arrhenius plots obtained for the enzyme over a temperature range of 25 - 40°C. All the experiments were performed independently in triplicate, and the mean values were obtained to plot the graphs. The standard error from the mean in all cases was found to be lower than 5% of the mean values.

### 3.2.8 Synthesis of esters using lipase bound silica and magnetite nanoparticles

A typical reaction mixture consisted of 20 mL of anhydrous hexane with equimolar concentrations (100 mM) of isovaleric acid and ethanol. The reaction was initiated by adding 40 mg of lipase-bound silica nanoparticles (≈16 mg lipase), 40 mg of lipase-bound magnetite nanoparticles (≈ 4.16 mg) or 16 mg of the free lipase into the reaction mixture in 250 mL glass-stoppered flasks. The reaction mixture was kept on an orbital shaker at 37°C at 150 rpm for ethyl isovalerate production. The samples were withdrawn at regular intervals of 24 h and analyzed for the accumulation of ethyl isovalerate. In order to determine the effect of the fatty acid chain length of esterification activity of enzyme, the esterification of ethanol with butyric acid, caprylic acid and acetic acid were also investigated using immobilized and free enzyme. The water activity was not controlled during the course of the reaction. The esterification reaction of different esters was carried out as described above for ethyl isovalerate, except that different fatty acids were used in place of isovalerate in the reaction mixture.

### 3.2.9 Reusability of immobilized lipase

For reusability studies, the esterification reaction was carried out as described above for ethyl isovalerate, and upon completion of one cycle, the immobilized enzyme was recovered by centrifugation at 10,000 × g for 20 min. The immobilized enzyme was then washed with anhydrous hexane once and used for the next cycle of esterification. This procedure was repeated for several cycles.
3.2.10 Analytical procedures

3.2.10.1 Detection and quantification of esters

After the initiation of esterification, a 500 mL sample was periodically withdrawn from the reaction mixture and immediately analyzed by a gas chromatograph (PerkinElmer, Model Clarus 500, Germany) equipped with a flame ionization detector and a 30 mRtx-R-20 (Crossbond 80% dimethyl-20% diphenyl polysiloxane) capillary column. Nitrogen served as a carrier gas at a split flow rate of 90 mL/min, and the column temperature range was from 40 to 280°C. The temperature was programmed to increase from 40 to 210°C at a rate of 6°C/min and from 210 to 280°C at a rate of 15°C/min. The injector and detector temperatures were 250 and 280°C, respectively. Ester identification and quantification was done by comparing the retention time and peak area of the sample with a standard. Pure ethyl isovalerate was used as an internal and external standard.

3.2.10.2 Determination of lipase activity

The activity of free and bound lipase was determined colorimetrically using 4-nitrophenyl palmitate (pNPP) as a substrate at 37°C (Wrinkler et al, 1979; Gupta et al, 2002). The substrate solution was prepared by dissolving 37.7 mg of pNPP in 10 mL of propan-2-ol, which was then mixed with a solution consisting of 0.207 g of bile salts and 0.1 g of gum Arabic in 90 mL of 0.05 M phosphate buffer (pH 7.2) prepared separately. To this substrate solution, Triton X-100 was added to a final concentration of 0.4% (v/v). The lipase assay was carried out by incubating 100 mL of lipase-bound nanoparticles (2.5 mg/mL) or free lipase (1 mg/mL) with 3.4 mL of substrate solution in a test tube at 37°C for 10 min. The increase in p-nitrophenol concentration was measured at 410 nm using a Hewlett-Packard UV-Visible diode array spectrophotometer (HP-8452). One unit of lipase activity was defined as the amount of enzyme required to release 1 mmole of pNP/mL/min at 37°C and pH 7.2.
3.3. Results and Discussion

3.3.1 Properties of Silica nanoparticles
Reverse micellar systems have been widely used for the synthesis of uniformly sized nanoparticles of various types. In this study, we synthesized fine, spherical and monodispersed silica nanoparticles using non-ionic surfactant-based reverse micelles. The size of the silica nanoparticles was determined by TEM and found to be $100 \pm 20$ nm (Fig. 3.1).

![Fig. 3.1: TEM image of silica nanoparticles](image)

3.3.2 Binding of lipase on to silica nanoparticles
The covalent binding of lipase on the silica nanoparticles was demonstrated with FTIR analysis. Fig. 3.2 shows the characteristic peaks due to SiO2 vibrations (540, 1087 and 1627 cm$^{-1}$), and shifts in these peaks were observed in the spectra of enzyme-bound silica nanoparticles. The characteristic bands of protein at 1120, 1640 and 2920 cm$^{-1}$ were observed in the spectra of pure lipase and lipase-bound silica nanoparticles, which clearly demonstrated the binding of lipase to the surface of the silica nanoparticles.

The binding efficiency was investigated by increasing the amount of silica nanoparticles at a fixed concentration of lipase and it is seen from the Figure 3.3 that with increasing amount of nanoparticles, the amount of bound protein increased, and above 60 mg of silica nanoparticles/mL, the efficiency of binding was 100%.

Thus, a weight ratio of lipase to silica of 0.4 mg/mg was required for the complete binding of lipase on the silica nanoparticles.
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Fig. 3.2. FTIR spectra of silica nanoparticles with and without bound lipase and pure lipase. (A, Silica nanoparticles; B, Silica nanoparticles + glutaraldehyde + lipase; C, lipase)

Fig. 3.3: Lipase binding efficiency of glutaraldehyde activated silica nanoparticles
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The specific activity of the immobilized lipase was found to be 1.433 units per mg of silica nanoparticles. The loading capacity of the lipase was found to be much higher than that reported by Moon et al. (2006).

### 3.3.3 Kinetic parameters

The values of $K_m$ and $V_{max}$ for free and silica immobilized lipase were estimated from the double reciprocal plots of the initial rates of pNPP hydrolysis. The $K_m$ of immobilized enzyme (0.3 mM) was 4-fold lower than that of free lipase (1.43 mM) while $V_{max}$ of the immobilized lipase (212 mmoles/mg/min) was 8-fold higher than that of free lipase (26.17 mmoles/mg/min). Thus, immobilized lipase-catalyzed pNPP hydrolysis had a 38-fold higher efficiency in comparison to the free lipase. These changes in the kinetic parameters suggest that covalent binding of lipase onto the glutaraldehyde-activated silica nanoparticles resulted in an increased affinity for the substrate and improved accessibility of the active site. Most likely, the covalent coupling of a lipase molecule onto a silica nanoparticle leads to a favored orientation of the enzyme on the surface of the carrier.

The effect of temperature on the activity of free and silica-bound lipase can be seen in the Arrhenius plots (Fig. 3.4). The free and immobilized lipase exhibited a linear relationship in the temperature range of 25 – 40°C, and the corresponding activation energies were calculated to be 7.8 and 1.25 KJ/mol, respectively.

![Arrhenius plots for initial hydrolysis rate of pNPP catalyzed by free and immobilized lipase.](image_url)

**Fig. 3.4** Arrhenius plots for initial hydrolysis rate of pNPP catalyzed by free and immobilized lipase. Activation energy for free lipase (♦) was calculated to be 7.8 KJ/mol and that of immobilized lipase (■) was 1.25 KJ/mol.
3.3.4 Ester synthesis
The immobilized lipase on silica nanoparticles was used for the synthesis of ethyl isovalerate. Fig. 3.5 shows that immobilized lipase catalyzed the ethyl isovalerate synthesis with a maximum esterification efficiency of 73% after 196 h of incubation, which is 7-fold higher than the free lipase. However, no significant difference was observed in the esterification efficiency of immobilized or free lipase for the synthesis of ethyl caprylate or ethyl butyrate, while immobilized lipase exhibited 1.62-fold higher esterification efficiency for ethyl acetate in comparison to the free enzyme. These results indicate that the covalent binding of lipase on the surface of the silica nanoparticles probably leads to a conformational change as discussed above, which alters the substrate specificity of the enzyme. In our previous studies, we demonstrated that unmodified lipase exhibits significantly lower esterification efficiency for ethyl isovalerate synthesis in comparison to ethyl butyrate, ethyl caprylate and ethyl acetate (Dandavate and Madamwar, 2007; Soni and Madamwar, 2000; Thakar and Madamwar, 2005).

![Fig. 3.5 Ethyl isovalerate synthesis catalyzed by free and lipase immobilized on silica nanoparticles](image)

Furthermore, we have previously shown that the coating of CRL with cationic surfactant results in a modified enzyme that exhibits more than 82% efficiency for the synthesis of ethyl isovalerate (Dandavate and Madamwar, 2007). A similar alteration
of substrate specificity and catalytic efficiency of the lipase upon immobilization or chemical modification has been reported by several authors (Basri et al, 1995; Noda et al, 1997; Okahata et al, 1995). It should be noted that the esterification efficiency of lipase covalently attached to silica nanoparticles is slightly lower (0.18-fold) than the surfactant-coated lipase immobilized in micro emulsion-based organogels reported by us earlier (Dandavate and Madamwar, 2007). Nevertheless, the process for ethyl isovalerate synthesis described in the present study (0.073 mol/L at a substrate concentration of 0.1 mol/L and E/S ratio of 8 g/mol) is significantly more efficient than that reported for lipase from Mucor miehei immobilized on macroporous weak anionic resin beads (0.487 mol ethyl isovalerate/L at a substrate concentration of 1 mol/L and E/S ratio of 48.41 g/mol) considering the enzyme to substrate ratio in both cases (Chowdary and Prapulla, 2003). In the case of covalent attachment of the enzyme on the carrier surface, some inactivation of the enzyme is expected to occur; however, it may be compensated for by the stability and recoverability of the enzyme that is achieved. The reusability studies showed that immobilized enzyme was stable for up to 8 cycles of esterification, during which no significant decrease in esterification efficiency was noticed. However, during the 9th cycle and onwards, the esterification efficiency of the immobilized enzyme decreased (Fig. 3.6). The lipase immobilized on the silica nanoparticles would be more suitable for commercial applications due to its easy recovery from the reaction system and efficient reuse.

![Fig. 3.6 Repeated cycles of ethyl isovalerate synthesis using lipase immobilized on silica nanoparticles](image)
3.3.5 Properties of magnetite nanoparticles

Fine magnetite nanoparticles with a mean diameter of 10-20 nm were synthesized (Fig. 7)

![Fig. 3.7 TEM image of magnetic nanoparticles](image)

The particle size was evaluated using 5 different TEM images and it can be seen in size distribution histogram that particle size varied from 9 to 22 nm with maximum particles having a size of about 11nm (Fig. 8). The similar size of magnetite nanoparticles with a mean diameter of 12.7 ± 3.1 nm were obtained by Huang et al. (2003) using the same method.

![Figure 3.8 Size distribution of magnetite particles obtained from electron micrographs](image)
The XRD patterns of magnetite particles with and without bound lipase exhibited six characteristic peaks for Fe$_3$O$_4$ ($2\theta = 30.1^\circ$, 35.6$^\circ$, 43.3$^\circ$, 53.6$^\circ$, 57.2$^\circ$ and 62.5$^\circ$) as shown in Figure 3.9. The obtained XRD patterns of unbound and lipase bound magnetite nanoparticles matched with the standard XRD data file of magnetite (JCPDS card no. 019-0629). Further, the XRD data did not reveal any significant difference between unbound and lipase bound magnetite nanoparticles, suggesting that covalent attachment of lipase to the surface of particles did not cause any alteration in its crystal phase.

![XRD patterns of magnetite (A) and lipase bound magnetite (B) nanoparticles](image-url)

**Figure 3.9** XRD patterns of magnetite (A) and lipase bound magnetite (B) nanoparticles
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The size of unbound and lipase bound magnetite nanoparticles was calculated using Debye-Scherrer equation, which was found in agreement with the sizes observed by TEM (Table 3.1). The surface area of magnetite nanoparticles was determined by BET method and was found to be 112.145 m²/g.

Table 3.1: Calculation for size of magnetite nanoparticles (unbound and lipase bound) from XRD data using Debye-Scherrer equation

<table>
<thead>
<tr>
<th>No.</th>
<th>( \beta_2 ) (°)</th>
<th>( \beta_20 ) (radians)</th>
<th>( ^{\circ}2\theta ) (radians)</th>
<th>( \cos \theta ) (radians)</th>
<th>Thickness (Å)</th>
</tr>
</thead>
</table>
| Unbound magnetite nanoparticles
| 1   | 0.8             | 0.0139555       | 30.11977        | 0.96565         | 114.317     |
| 2   | 0.64            | 0.0111644       | 35.51700        | 0.95235         | 144.89      |
| 3   | 0.56            | 0.0097688       | 42.75817        | 0.931188        | 169.35      |
| 4   | 0.56            | 0.0097688       | 54.13451        | 0.89047         | 177.1       |
| 5   | 0.96            | 0.0167466       | 56.99992        | 0.8788          | 104.67      |
| 6   | 0.32            | 0.0055822       | 62.68900        | 0.854055        | 323.149     |
| Lipase bound magnetite nanoparticles
| 1   | 0.96            | 0.0167466       | 30.19117        | 0.96549         | 95.318      |
| 2   | 0.72            | 0.01256         | 35.64954        | 0.951997        | 128.84      |
| 3   | 0.96            | 0.0167466       | 43.33235        | 0.92935         | 99.0247     |
| 4   | 0.96            | 0.0167466       | 53.62231        | 0.89249         | 103.1145    |
| 5   | 0.32            | 0.0055822       | 57.26863        | 0.87769         | 314.44      |
| 6   | 0.4             | 0.0069777       | 62.52424        | 0.854802        | 258.31      |

Thickness (t in angstroms) = \( K \lambda / \beta_2 \cos \theta \)

Value of K was considered as 1 and \( \lambda \) was 1.54056

3.3.6 Immobilization of lipase on magnetite nanoparticles

In our studies we preferred to immobilize lipase on magnetite nanoparticles by covalent attachment since physisorption based immobilization of enzymes is often associated with a problem of enzyme leaching from the surface during course of reaction (Palomo et al, 2002). The lipase upon carbodiimide activation was covalently attached to –NH₂ groups on magnetite nanoparticles (Kouassi et al, 2005; Huang et al, 2003). The immobilization of lipase onto magnetic nanoparticles was monitored by
FTIR analysis. Fig. 3.10 shows the FTIR spectra of naked magnetite nanoparticles, lipase bound to carbodiimide activated magnetite and free lipase. Band at 592.81 cm\(^{-1}\) is due to Fe-O of magnetite. Weak bands at 2923.37 and near 2900 cm\(^{-1}\), which are not present in magnetite, are due to symmetric and asymmetric C-H stretching vibrations of enzyme. The shifting of characteristic band at 1122.18 cm\(^{-1}\) in spectrum of free enzyme to 1062 cm\(^{-1}\) with reduced intensity in spectrum of enzyme bound magnetite nanoparticles can be attributed to the bonding of enzyme onto the nanoparticles. The presence of a characteristic band for proteins (1631.15 cm\(^{-1}\)) in spectra of lipase bound Fe\(_3\)O\(_4\) nanoparticles confirms the binding of enzyme on the nanoparticles. Furthermore, the characteristic band of -NH\(_2\) group at 1593.92 cm\(^{-1}\) observed in magnetite nanoparticles (spectrum A) disappeared in case of lipase bound magnetite nanoparticles (spectrum C), suggesting that binding occurred between amine group on Fe\(_3\)O\(_4\) nanoparticles and the carboxyl group of lipase after being activated by carbodiimide (Huang et al, 2003).

Fig. 3.10 FTIR spectra of magnetic nanoparticles with and without bound lipase.
(A, magnetite nanoparticles; B, free lipase C; magnetite + carbodiimide + lipase)
The differential scanning calorimetric (DSC) analysis of enzyme bound magnetite nanoparticles exhibits an exothermic peak at 155 °C (\(\Delta H = -88.03\)) which also supports cross-linking of enzyme onto carbodiimide activated magnetite nanoparticles (Fig. 3.11).

![Fig 3.11. Differential scanning calorimetric analysis of Lipase bound magnetite nanoparticles](image)

The thermal gravimetric analysis showed that lipase bound magnetite nanoparticles suffered 20% weight loss in a temperature range of 100-450°C which can be attributed to decomposition of enzyme as well as loss of bound water molecules to enzyme. Further increase in temperature up to 900°C did not cause any additional significant weight loss (Fig. 3.12). In contrast unbound magnetite nanoparticles did not exhibit any significant change up to 900°C.

![Fig. 3.12 Thermal gravimetric analysis of magnetite (A) and lipase bound magnetite (B) nanoparticles](image)
3.3.7 Binding efficiency of lipase on magnetite nanoparticles

The binding efficiency of enzyme was investigated for increasing amount of magnetic nanoparticles at fixed concentration of lipase (3.125 mg protein/mL). Figure 3.13 shows that with increasing amount of nanoparticles, the percentage of bound lipase increased and above 30 mg/mL of magnetite particles the efficiency of binding was found to be 100%. Thus a weight ratio of lipase to magnetite of 0.104 was required for complete binding of lipase on to magnetite nanoparticles and specific activity of immobilized lipase was found to be 43.24 units/mg of lipase bound magnetite nanoparticles.

![Figure 3.13 Lipase binding capacity of carbodiimide treated magnetite nanoparticles](image.png)

3.3.8 Kinetic parameters

The maximum specific activities ($V_{\text{max}}$) and Michaelis constants ($K_{m}$) for free and magnetite bound lipase were determined using Lineweaver-Burk plots for lipase catalyzed hydrolysis of $p$NPP. The $K_{m}$ of immobilized enzyme (0.48 mM) was 2.97-fold lower than that of free lipase (1.427 mM) while $V_{\text{max}}$ of immobilized lipase (0.357 mmoles/mg.min) was 13.7-fold higher than that of free lipase (0.026 mmoles/mg.min). The lower $K_{m}$ value indicates the higher affinity of enzyme for the substrate and higher $V_{\text{max}}$ value indicates the higher activity of enzyme. Thus, the immobilized lipase was found to be 40 times more efficient catalyst for $p$NPP hydrolysis in comparison to free lipase as inferred from the ratio of $V_{\text{max}}$ to $K_{m}$ values.
These changes in the kinetic parameters suggest that covalent binding of lipase onto magnetite nanoparticles probably caused favored orientation of enzyme on the surface of nanoparticles, thereby resulting in increased affinity for substrate and improved accessibility of active site (Huang et al, 2003).

The effect of temperature on the activities of free and magnetite bound lipase can be seen in Arrhenius plots (Fig. 3.14). The free and immobilized lipase exhibited linear relationship in the temperature range of 25 to 40°C and corresponding activation energies were found to be ca. 7.8 and 4.1 KJ/mol, respectively. The lower activation energy of immobilized lipase in comparison to free lipase also supports the change in conformation of enzyme leading to requirement of lower energy at the surface of nanoparticles (Kouassi et al, 2005).

The immobilized lipase on magnetite nanoparticles was used for synthesis of ethyl isovalerate. Table 3.2 shows that immobilized lipase exhibited higher esterification efficiency for synthesis of ethyl isovalerate in comparison to free lipase. However, no significant difference was observed in esterification efficiency of immobilized or free lipase for synthesis of ethyl acetate or ethyl butyrate, while free enzyme was found to favor synthesis of ethyl caprylate in comparison to immobilized lipase.
Table 3.2 Esterification efficiency of free and immobilized lipase

<table>
<thead>
<tr>
<th>Ester</th>
<th>% Esterification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>43.6 ± 2.6</td>
</tr>
<tr>
<td>Ethyl butyrate</td>
<td>60 ± 4.3</td>
</tr>
<tr>
<td>Ethyl isovalerate</td>
<td>10.3 ± 0.9</td>
</tr>
<tr>
<td>Ethyl caprylate</td>
<td>65.6 ± 5.7</td>
</tr>
</tbody>
</table>

These results suggest that covalent binding of lipase on the surface of magnetite nanoparticles probably leads to a conformational change in enzyme as discussed above, which might also influence the substrate specificity of the enzyme.

In our previous studies, we have demonstrated that unmodified CRL catalyzed synthesis of ethyl isovalerate with lower efficiency in comparison to ethyl butyrate whereas, modifying CRL with cationic surfactant treatment resulted in altered catalytic efficiency with higher yields for ethyl isovalerate than for ethyl butyrate (Dandavate and Madamwar, 2007; Thakar and Madamwar, 2005). In present study the immobilized lipase on magnetite nanoparticles catalyzed ethyl isovalerate synthesis with maximum esterification efficiency of 55% upon 196 h of incubation which is 5-fold higher than that obtained with free lipase (Fig. 3.15).

![Graph showing esterification efficiency over time](image)

Fig. 3.15: Ethyl isovalerate synthesis catalyzed by free and magnetite bound lipase.
Although, the esterification efficiency of lipase immobilized on magnetite nanoparticles is lower than observed for surfactant coated lipase immobilized in microemulsion based organogels reported by us earlier (Dandavate and Madamwar, 2007) still, the lipase immobilized on magnetite nanoparticles would be more suitable for commercial applications due to its easy recovery from the reaction system and ease of controlling water activity during its reuse. In case of lipase immobilized in microemulsion based organogels when employed for esterification reaction, water accumulates in the gel during reaction which upon certain concentration alters the equilibrium of lipase catalyzed reaction towards hydrolysis, thus lowering the esterification efficiency during reuse. This accumulated water from the gel then has to be removed using a liquid dessicant in order to restore the esterification activity intermittently (Dandavate and Madamwar, 2007; 2008; Jenta et al, 1997) which is time consuming and cumbersome. Figure 3.16 shows that the lipase attached to magnetite nanoparticles could be reused for 10 cycles of ethyl isovalerate synthesis with about 8.5% decrease in esterification efficiency.

\[ \text{Fig. 3.16: Reusability of magnetite bound lipase for synthesis of ethyl isovalerate} \]

In case of lipase immobilized on magnetite nanoparticles the water produced during esterification reaction will diffuse away from the enzyme in bulk solvent phase and upon completion of one cycle of esterification, the enzyme is to be recovered using magnetic field wherein the question of accumulation of water in the vicinity of immobilized lipase does not arise and thus will be a more efficient system for reuse as well as for development of continuous enzyme bioreactor.
3.4. Conclusion:

Uniform and monodispersed silica nanoparticles were synthesized with a mean diameter of 100 ± 20 nm as analyzed by Transmission Electron Microscopy (TEM). Glutaraldehyde was used as a coupling agent for efficient binding of the lipase onto the silica nanoparticles. For the hydrolysis of \( p \)NPP at pH 7.2, the activation energy within 25–40 °C for free and immobilized lipase was 7.8 and 1.25 KJ/mol, respectively. The \( V_{\text{max}} \) and \( K_{\text{m}} \) of immobilized lipase at 25 °C for \( p \)NPP hydrolysis were found to be 212 mmol/min/mg and 0.3 mM, whereas those for free lipase were 26.17 mmol/min and 1.427 mM, respectively. The lower activation energy of immobilized lipase in comparison to free lipase suggests a change in conformation of the enzyme leading to a requirement for lower energy on the surface of the nanoparticles. A better yield (7 fold higher) of ethyl isovalerate was observed using lipase immobilized onto silica nanoparticles in comparison to free lipase.

Magnetite nanoparticles of uniform size were synthesized by chemical co-precipitation of ferric and ferrous ions under hydrothermal conditions. \textit{Candida rugosa} lipase was immobilized by covalent attachment upon carbodiimide activation to the magnetite nanoparticles, which could be demonstrated by FTIR spectroscopy as well as by differential scanning calorimetry. Kinetics of free and immobilized enzyme suggests that the enzyme undergoes a conformational change during immobilization resulting in lower activation energy needed for hydrolysis of \( p \)NPP. This was further reflected in altered substrate specificity of immobilized lipase which exhibited 5-fold higher esterification activity for ethyl isovalerate synthesis and in comparison to free enzyme whereas no significant difference was observed for synthesis of ethyl butyrate and ethyl acetate. The immobilized lipase exhibited fairly good reusability property with about 8.5 % decrease in esterification efficiency during 10 cycles of reuse. This study demonstrates that lipase immobilized magnetite nanoparticles can be used for synthesis of esters.
Table 3.3 Comparison of silica nanoparticles and magnetite nanoparticles as support for lipase immobilization

<table>
<thead>
<tr>
<th>Size</th>
<th>Ratio of lipase to nanoparticles</th>
<th>Km (mM)</th>
<th>Vmax (μmoles/mg/min)</th>
<th>Activation energy</th>
<th>Ethyl Isovalerate synthesis</th>
<th>Reusability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td></td>
<td>1.43</td>
<td>26.17</td>
<td>7.8</td>
<td>10 %</td>
<td></td>
</tr>
<tr>
<td>Magnetite Nano particles 10-20 nm</td>
<td>0.104 mg/mg</td>
<td>0.48</td>
<td>357</td>
<td>4.1</td>
<td>55 %</td>
<td>10 cycles</td>
</tr>
<tr>
<td>Silica Nano particles 100 ± 20 nm</td>
<td>0.4 mg/mg</td>
<td>0.3</td>
<td>212</td>
<td>1.25</td>
<td>73 %</td>
<td>8 cycles</td>
</tr>
</tbody>
</table>

3.5. References:


