2.1. Introduction

Non-aqueous enzymology nowadays has been of wide research interest due to its vast applications in the biotechnology based industries. The use of enzymes in organic solvents presents several advantages like solubility of non-polar substrates, increased conformational rigidity, enhanced thermostability and altered substrate specificity. But there always exists the risk of enzyme denaturation in presence of organic solvents. Several strategies like solvent and protein engineering (Svendsen et al, 2000), chemical and surface modification of lipase (Klibanov et al, 1993; Basri et al, 1995; Noda et al, 1997; Okahata et al, 1995), use of biphasic and reversed micellar systems (Wu et al, 2001), enzyme immobilization and enzyme bioimprinting (Palomo et al, 2002; Mingarro et al, 1995) have been employed to enhance lipase performance in organic solvents.

2.1.1 Modification of lipase using surfactants

Bio-imprinting has been emerged as a novel approach for enhancing catalytic activity of lipases in non-aqueous media. This approach exploits the conformational change in lipase which gets activated at lipid-water interface, resulting in increased substrate accessibility to the active site. As the lipase gets activated at lipid-water interface, this is also termed as “Interfacial activation-based molecular bioimprinting” (IAMI) of lipolytic enzymes (Mingarro et al, 1995). To imprint lipase at lipid-water interface, various surfactants (Liu et al, 2000), detergents (Fernandez-Lorente et al, 2006), crown ethers (Van et al, 1998), lyoprotectants (Klibanov et al, 1993), amphiphiles (Mingarro et al, 1995) are being employed. Coating the enzymes with surfactants is advantageous due to its ease in preparation procedure and good solubility in a wide range of organic solvents (Goto et al, 1995; 1996). Therefore, surfactant coated enzymes have been regarded as very competitive and promising biocatalysts in organic media in comparison to enzymes modified with polyethylene glycols (PEG) (Klibanov et al, 1993) or entrapped in reverse micelles (Wu et al, 2001). The improved performance of lipase depends not only on the nature of lipase but also on the type of the interface used as an additive. In aqueous homogeneous solutions, *Candida rugosa* lipase exists in equilibrium between two conformational states (Tilbeurgh et al, 1992; Tilbeurgh et al, 1993; Derewenda et al, 1992; Derewenda et al, 1994): a “closed” (inactive) one, in which the catalytic triad in the
active site is covered by a helical “lid” (flap) and an “open” (active) one, in which the lid has been displaced, adopting a totally different conformation and exposing the catalytic residues. Very interestingly, this lid displacement and other entailed conformational changes ultimately result in both providing space in the active site for lipid docking and shaping the catalytic machinery (“induced fit”) (Derewenda et al, 1992; Derewenda et al, 1994; Grochulski et al, 1993; Mingarro et al, 1995, Mancheno et al, 2003).

2.1.2 Immobilization of lipase in microemulsion based organogels

Many researchers have successfully designed a number of immobilization techniques, taking into account its processibility and feasibility, such as the immobilization of the catalyst onto a porous support by adsorption or deposition, entrapment in a gel matrix, or covalently attaching the enzyme to an immobilization carrier (Dyal et al, 2003; Gitlesen, et al, 1997; Persson et al, 2000; Reetz et al, 1996), which have proved to be useful techniques for improving enzymatic activity. Amongst the various methodologies studied for effective biotransformation in organic solvent media, the use of reverse micelles or water-in-oil microemulsion is an attractive approach (Rees et al, 1991). The enzymes in a nearly anhydrous environment can exhibit exciting features, such as increased conformational rigidity, enhanced stability, altered substrate specificity, and more favorable thermodynamic equilibria. The enzyme molecules are very sensitive to the organic solvent involved, which results in low catalytic activity. To overcome this problem, embedding of enzymes in reverse micelles is in use (Chang et al, 1999). The advantage of using this type of system, apart from dispersibility of the enzyme at the molecular level, is that it is capable of solubilizing a wide range of polar and apolar substrates (Soni and Madamwar et al, 2000). However, the recovery of product and regeneration of enzyme is challenging. To make enzyme recovery more easy, the use of gelled microemulsion systems, especially that of gelatin and Aerosol-OT w/o microemulsion systems, have been employed. The formation of microemulsion-based organogels was first demonstrated by Haering and Luisi et al. in 1986. However, Jenta et al (1997) were the first to report the use of micro emulsion based organogels as a matrix for lipase immobilization.
2.1.3 Structure of Microemulsion based organogels (MBGs)

Microemulsions are characteristically thermodynamically stable and optically transparent. A well-defined and highly ordered structure formed when the surfactant + water volume-fraction is less than about 0.2, where the water is present in a dispersed form (tiny droplets). Each droplet is coated with a lose-packed surface monolayer of surfactant molecules, oriented such that the surfactant head-groups are hydrated at the surface of water-droplet, with the apolar tails protruding out into, and solvated by, the oil. The stability of microemulsions is attributed to the presence of the interfacial surfactant layer, which prevents a thermodynamically unfavorable direct oil and water.

Microemulsion based organogels are formed by solubilizing gelatin in w/o microemulsions to give transparent gels whose rigidity is generally determined by the amount of gelatin added. The structural characterization of MBGs has been attempted using a wide variety of techniques, including conductivity measurements and diffusion of radioactive tracers in MBGs (Quellet et al, 1991) in addition to a preliminary analysis of small-angle neutron and X-ray scattering data (Atkinson et al, 1989). These MBGs are extensive ion-conducting channels and a mobile oil phase (Atkinson et al, 1991; Atkinson et al, 1995). A small angle neutron studies indicate that the aqueous content of the gels consists of a rigid network of gelatin/water rods stabilized by a monolayer of surfactant coexisting with microemulsion droplets.

2.1.4 Reusability of surfactant coated lipase immobilized in microemulsion based organogels

There are sparse reports on reusability of surfactant coated lipases (Fernandez-Lorente et al, 2006). Water is recognized as playing a dual role in lipase catalyzed esterification reactions (Jenta et al, 1997). It is required in very small amounts to preserve the catalytic conformation of the enzyme in organic solvents, probably by adhering to the enzyme surface in form of a thin layer and acting as a protective sheath (Abramowicz et al, 1989; Barzana et al, 1989). Alternately, the presence of excess water can adversely affect the thermodynamic equilibrium of the ester synthesis by favoring the hydrolytic reaction (Jenta et al, 1997). It should be noted that the optimum thermodynamic water activity varies from 0.3 to 1.0 for lipases from different microbial sources, which also depends on the type of immobilization matrix used in a given solvent (Gandhi et al, 2000; Noor et al,
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This phenomenon poses the major limiting factor in repeated use of lipase immobilized in MBGs for ester synthesis, as the water produced during the esterification reaction accumulates in the gel. With an increasing amount of water in the gel, the rate of the esterification reaction decreases with time. Thus, if the water formed during the esterification reaction can be selectively removed in a continuous manner as it is produced, or at regular intervals, it should be possible to use the enzyme for more cycles of esterification. There are very meagre reports on the reusability of microemulsion-based organogel systems employing lipases for condensation reactions (Jenta et al., 1997; Soni and Madamwar, 2000). The selective removal of water using molecular sieves has been demonstrated by several workers, in order to maintain the equilibrium toward synthesis; however, it is extremely difficult to remove water continuously using a molecular sieve and also poses difficulty in scale up of the process (Akoh et al., 1992; Berger et al., 1992). Jenta et al. successfully demonstrated the use of a liquid dessicant for selective extraction of water from organogels at regular intervals during repeated cycles of ester synthesis (Jenta et al., 1997).

2.1.5 Synthesis of ethyl isovalerate ester using a combined approach of surfactant coated lipase immobilized in microemulsion based organogels

Recent trends in consumer preference toward “natural” products indicate that biocatalysts have an advantage over their chemical counterparts, as products obtained using biocatalysts may be labeled “natural”. It was also observed that fatty acid esters synthesized using enzymes often have better odor and flavor characteristics in comparison to similar esters produced by chemical means (Rizzi et al., 1992). There are reports of esterification of short-chain alcohols with long-chain fatty acids (Chowdary et al., 2003; Garcia et al., 1999; Wu et al., 2002; Heish et al., 2006) and short-chain acids, among which acetic, propionic and butyric acid esters have been thoroughly studied (Welsh et al., 1990; Langrand et al., 1990) but sparse information is available on esters of valeric acid and isovaleric acid (Chowdary et al., 2003; Gillies et al., 1987). It has been reported that during enzymatic synthesis of ethyl esters, due to the polar nature of ethanol, enzyme inactivation occurs causing lower yields of esters. The maximum yield of ethyl isovalerate using *Rhizomucor miehei* lipase as a catalyst was reported to be 0.487 mol/L at a substrate concentration of 1 mol/L under optimized conditions (Chowdary et
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al, 2003). Gillies et al. (1987) reported the synthesis of various ethyl esters using Candida cylindracea (now known as Candida rugosa) lipase. Although yields of ethyl propionate, butyrate and hexanoate were considerable, the yields reported for ethyl isovalerate was significantly lower (0.0054 mol/L ester at 0.18 mol/L substrate).

This chapter describes the use of a novel approach by combining the use of surfactant coated lipase and immobilization in microemulsion based organogels (MBGs) to enhance performance and reusability of lipase with the improved synthesis of ethyl isovalerate catalyzed by CTAB coated lipase immobilized in AOT microemulsion based organogels. Various combinations of surfactants used for coating the lipase and surfactants used for preparation of MBGs were tried for esterification reaction and the best combination was then further exploited and compared with that of native lipase under similar conditions. We have also focused on the operational reusability of surfactant coated Candida rugosa lipase immobilized in microemulsion based organogels by providing pretreatments and intermittent treatments to the gels for the removal of the accumulated water and monitoring them for the production of ethyl isovalerate.

2.1.6 pH Memory

Most of the enzymes have the ability to “remember” the pH of the aqueous solution in which they are suspended, which is termed as “enzymes pH memory”. Zaks and Klibanov (1988) for the first time demonstrated the concept of pH memory and concluded that the activity of enzyme is in relation to the different buffer solutions from which the enzyme is prepared. The protonation state is very important for the enzymes both in aqueous as well as non-aqueous environment for its efficient functioning. Ren et al. (2008) demonstrated the effect of pH memory of immobilized lipase for catalyzing (±) menthol resolution in the ionic liquid and yielded a better conversion and enantioselectivity at pH 5.0. The pH memory phenomenon has also been demonstrated in case of immobilized enzymes. Thus, carrying out immobilization of enzyme at its optimal pH value will benefit in its activity in non aqueous systems. Moreover, the pH memory has also been used to methylate α-amino groups to determine the free/blacked amino terminus of a protein (Vakos et al, 2000). Interestingly, it has been demonstrated that pH memory is dependent on volatility of buffers used to maintain pH of aqueous solutions.
before dehydration. This is because volatile ions would be removed during dehydration, thereby altering the protonation state of the protein and loss of pH memory (Zacharis et al, 1999). To avoid this use of partly volatile buffers has been recommended which would restore the ionization state of the protein.

Manohar et al. (2005) reported the pH memory effect on lipase catalyzed esterification of anthranilic acid with methanol in organic solvents. The effect of pH on ionization of lipase determines its conformation. At certain pH condition, the ionic groups around the active centre of the lipase molecule achieve the optimum ionic state for lipase-catalyzed reactions which is essential for its activity and substrate specificity. In traditional aqueous systems, the pH value of lipase catalyzed hydrolysis reaction is adjusted with bulk buffers. In non-aqueous reaction systems, as the water activity has to be maintained very low, it is not possible to control pH by addition of buffers. Furthermore, in non-aqueous system it is very difficult to measure the macro-pH of the bulk reaction phase (solvent). The ionization state of the water molecules forming a monolayer surrounding enzyme also cannot be known. Thus, the optimum pH of the catalytic reactions to be carried out in organic solvents cannot be standardized. In this case, pH memory can be used to determine the optimum pH values for catalysis and hydrolysis reactions.

2.2. Materials and Methods:
2.2.1 Chemicals:
Sodium bis-2-(ethylhexyl) sulfosuccinate (AOT), Tween 80 and 1-hexanol were obtained from Fluka (Steinheim, Germany). Cetyl trimethyl ammonium bromide (CTAB) and Triton X-100 were obtained from Sisco Chem. Industries (India) and Hi-Media (India), respectively. Ethyl isovalerate was purchased from Aldrich. Gelatin from porcine skin (Bloom 300) and Candida rugosa lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3) was supplied by Sigma (Japan), with the total activity of 860 U/mg of solid. All organic solvents (Spectrochem), and chemicals used were of analytical reagent grade.
2.2.2 Preparation of surfactant coated lipase (SCL)

Surfactant coated lipase was prepared according to the method of Goto et al. (1996). In the presence of surfactant, lipase (60 mg/mL) was incubated overnight at 4°C in phosphate buffer (pH 7.2). Surfactant coated lipase was prepared using anionic (AOT), cationic (CTAB) and nonionic (Tween 80) surfactants at 1mM concentration and lipase without any treatment served as a control. Surfactant coated lipase and lipase without any treatment was then further immobilized using microemulsion based organogels.

2.2.3 Preparation of w/o based microemulsion organogels (MBGs)

The general scheme for preparation of MBGs is shown in Fig. 2.1

![Figure 2.1 The general scheme for immobilization of lipase in micro emulsion based organogels](image-url)
2.2.3.1 Anionic MBGs. Thermodynamically stable reverse micellar solution consisting of AOT/buffer/isoctane was prepared by mixing each component in a suitable ratio. Surfactant coated lipase containing MBGs were prepared by introducing a concentrated (60 mg/mL) aqueous enzyme solution (at 25°C) to the above prepared reverse micellar solution. After a brief shake, this solution was then immediately added to a second solution of gelatin in buffer at 55°C. Gelatin obtained from porcine skin was dissolved in phosphate buffer (pH 7.2) at 50°C. The mixture was vigorously shaken and stirred until homogeneous and allowed to cool to room temperature to yield MBG. The Wo value defined as the mole ratio of water to surfactant was found to be 60 in above preparation. The Wo value is a key parameter in the description of microemulsions because it relates both to the size of the microemulsion droplets and to the activity of water (aw) present in the core (Crookes et al, 1995). The gel was then poured into the plastic petriplates and kept overnight for air drying. Next day the dried gel was cut into small pieces and used for esterification reaction.

2.2.3.2 Cationic MBGs. Reverse micellar solution containing CTAB/buffer/isoctane/1-hexanol was prepared. Cationic surfactant usually needs a cosurfactant in order to reside in the organic phase and form w/o microemulsion. Co-surfactants which are mainly long-chain alcohols like 1-hexanol usually participate in the interfacial region of the water-in-oil microemulsion. The Wo value was found to be 5 and Po = 4.2. The value of Po is defined as the mole ratio of co-surfactant to surfactant. The thermodynamically stable mixture containing surfactant coated lipase was then added to the gelatin. The further procedure was similar to the preparation of anionic MBGS.

2.2.3.3 Nonionic MBGs. Reverse micellar solution containing Triton X-100/buffer/isoctane/1-hexanol was prepared having Wo = 5 and Po = 4.2. Remaining procedure remained the same as for preparation of cationic MBGs.

2.2.4 Treatments Given to Microemulsion-based Organogels
The pelleted form of MBGs was subjected to mechanical grinding in the presence of liquid N₂, using a mortar and pestle to obtain granulated MBGs. Both pelleted and granulated forms of MBGs were used for further studies. In the case of pretreated MBGs, the prepared granulated and pelleted MBGs were soaked in 1 M AOT dry reverse
micellar solution for 24 h, and these MBGs were washed several times with hexane and used for the repeated cycles of esterification. The dry reverse micellar solution of AOT was prepared in isooctane. The pretreated MBGs were then used for several cycles without giving any intermittent treatment and monitored for the esterification activity at the end of each cycle. In the case of intermittent treatment, the MBGs were treated with dry AOT reverse micellar solution for 24 h after every 3 consecutive cycles. This experiment was carried out until a significant decrease in the esterification efficiency was observed. This type of treatment was given to both the pelleted and granulated MBGs. For optimization of treatment conditions, MBGs were treated with dry reverse micellar solution of isooctane containing varying concentrations of AOT from 0.1 M to 2 M in isooctane. The time period for treatment also was varied from 3 h to 24 h, using 0.2 M AOT in isooctane.

2.2.5 Reaction condition

The reaction mixture comprised 20 mL of hexane containing equimolar concentration (100mM) of isovaleric acid and ethanol added to the prepared enzyme immobilized in microemulsion based organogels. The reaction was carried out for the production of ethyl isovalerate ester in 250mL glass stoppered iodine flask kept on orbital shaker at 37 °C at 150 rpm. The samples were withdrawn at regular interval of 24 h and analyzed for accumulation of ethyl isovalerate. For reusability studies, the samples were withdrawn at regular interval of 72 h and analyzed for accumulation of ethyl isovalerate. Total of 3 samples were withdrawn from each cycle at 72 h, 144 h, and 216 h, respectively. After the completion of each cycle, the solvent containing product and unused substrates was completely removed and the MBGs were washed twice with hexane. The washed MBGs were then incubated with fresh reaction mixture for a subsequent cycle of ester synthesis.

2.2.6 Analytical Methods

After initiation of esterification, 500 μL sample from the reaction mixture was withdrawn periodically, and 5 μL was immediately analyzed by gas chromatography (Sigma, Baroda, India) equipped with a flame ionization detector and 3M stainless steel column containing 15% DEGA. Nitrogen served as a carrier gas at a flow rate of 30 mL/min and
the column temperature ranging from 70 to 190°C (programmed to increase the temperature at a rate of 15°C/min). The temperature of injector and detector was 250°C. Ester identification was done by comparing the retention time and peak area of the sample with standard. For reusability studies, the samples were analyzed using Capillary gas chromatography. (Perkin Elmer, Model Clarus 500, Germany) equipped with a flame ionization detector and 30 meter Rtx-R-20 (Cross bond 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 from 40 to 280°C. The temperature was programmed to increase from 40 to 210°C at the rate of 6°C/min and from 210 to 280°C at the rate of 15°C/min. The temperature of the injector was 250°C and that of the detector was 280°C.

2.2.7 Scanning Electron Microscope (SEM) Analysis
Freeze-dried samples before and after esterification reaction were analyzed for the changes occurring in the gel, using Philips XL 30 ESEM (Environmental Scanning Electron Microscope) having a gaseous secondary electron detector. Samples were mounted on aluminum stubs and placed in the chamber.

2.2.8 Determination of Water Content in Microemulsion based Organogels
This was done by two different methods, viz., solid-state 1H-NMR and Karl-Fischer titration. For solid-state NMR, the samples were mounted into a zirconium rotor and analyzed using a 300 MHz Bruker spectrophotometer at 25°C. Mobile H$_2$O is assumed to be contributing to the sharp resonance over a broad gel background. The relative ratios of resonance of mobile water protons to that of bound protons of gel were obtained by relative integrations of peaks after deconvolutions. The water extracted from the MBGs in AOT/isooctane solution was determined by the Karl-Fischer method using Hydranal-E reagent by volumetric titration.

2.2.9 Preparation of Candida rugosa lipase for pH memory
Lipase (40 mg) from Candida rugosa was dissolved in various buffers, pH ranging from pH- 4-9. Different buffers were used i.e. pH 4 and 5 (Acetate buffer), pH 6,7,8 (Sodium
phosphate buffer) and pH 9 (Glycine-NaOH buffer). All the buffers were of same ionic strength i.e. 0.1M. All the samples were then subjected to lyophilization. Stock lyophilized lipase was weighed and redissolved to 10 mg/mL in 0.1M Sodium phosphate buffer of pH 7.2.

2.3. Results and discussion

2.3.1 Ethyl isovalerate synthesis using different surfactant coated lipases immobilized in organogels

Lipase from *C. rugosa* was coated with three different surfactants viz. CTAB (cationic), AOT (anionic) and Tween 80 (nonionic). The lipase coated with different surfactants were then immobilized in MBGs and investigated for their application in synthesis of ethyl isovalerate. It should be noted here that there was no loss of enzyme during immobilization in MBGs and the same amount of enzyme was loaded in different MBGs prepared in this study. CTAB coated lipase immobilized in AOT based organogels (Fig. 2.2) gave the highest yield of ethyl isovalerate with 82% esterification (0.082 mol/L of ethyl isovalerate) in comparison to AOT or Tween 80 coated lipases. The uncoated lipase immobilized in AOT based organogels exhibited significantly lower activity in comparison to SCL. The activity and stability displayed by the SCL is intrinsically connected to the type and structure of coating surfactant (Thakar and Madamwar, 2005) and in this case it would also depend on the nature of the surfactant used for preparation of MBGs.

There are numerous reports on the use of SCL for synthesis of esters, which invariably demonstrates that lipase coated with nonionic surfactants work better than cationic or anionic surfactants (Thakar and Madamwar, 2005; Liu et al, 2000). In our study Tween 80 coated lipase immobilized in AOT based organogels exhibited poor esterification (Fig. 2.2) probably because coating with nonionic surfactant would result in poor immobilization and distribution of enzyme in MBGs. The lower esterification of AOT coated lipase in AOT based organogels can be attributed to either inactivation of enzyme by AOT and/or in this case may be due to its poor entrapment in MBGs.
Fig. 2.2 Synthesis of ethyl isovalerate using cationic, anionic and nonionic surfactant coated lipase immobilized in AOT based organogels. The % esterification was estimated using CTAB (■) coated lipase, Tween 80 (▲) coated lipase, AOT (♦) coated lipase and uncoated lipase (●).

Fig. 2.3 Synthesis of ethyl isovalerate using cationic, anionic and nonionic surfactant coated lipase immobilized in CTAB based organogels. The % esterification was estimated using CTAB (■) coated lipase, Tween 80 (▲) coated lipase, AOT (♦) coated lipase and uncoated lipase (●).
The influence of the nature of surfactant used to prepare MBGs on esterification activity of SCL was investigated by immobilizing SCL in CTAB based and Triton X-100 based organogels. In both the cases CTAB coated lipase immobilized in MBGs exhibited higher rate of ethyl isovalerate synthesis in comparison to AOT or Tween 80 coated lipase (Fig.2.3 & 2.4). Furthermore SCL immobilized in Triton X-100 based organogels exhibited comparatively lower esterification activity than SCL immobilized in CTAB based organogels, which may be because of poor stability of Triton X-100 organogels. The Triton X-100 organogels were very fragile and got disintegrated during the course of reaction.

![Graph showing esterification activity](image)

**Fig. 2.4. Synthesis of ethyl isovalerate using cationic, anionic and nonionic surfactant coated lipase immobilized in Triton X-100 based organogels. The % esterification was estimated using CTAB (■) coated lipase, Tween 80 (▲) coated lipase, AOT (●) coated lipase and uncoated lipase (♦)**

Lipases have a tendency to form strong biomolecular aggregates which in presence of detergents get easily disrupted into monomolecular forms (Pernas et al, 2001; Palomo et al, 2003; Wilson et al, 2006). Furthermore, biomolecular and monomolecular forms of lipase display differences in terms of specific activity, stability and enantioselectivity (Palomo et al, 2003; Wilson et al, 2006). Surfactants are known to increase the activity of lipases and are proposed to act by causing disaggregation of biomolecular forms of enzyme, promoting interfacial adsorption and shifting the lipase conformational...
equilibrium towards the open form (Palomo et al, 2003). According to this hypothesis, surfactants bind to hydrophobic areas surrounding the active site, thereby causing displacement of peptide loop covering the active site and promoting the “interfacial activation of the enzyme” (Derewenda et al, 1992; Mingarro et al, 1995; Fernandez-Lorente et al, 2006). This may be a likely reason for higher esterification activity of CTAB coated lipase in comparison to uncoated lipase immobilized in AOT based organogels. Alternately, Okahata et al (1995) proposed that the hydrophilic head groups of surfactant molecules would interact with the enzyme surface whereas the lipophilic alkyl chains extend away from its surface, thus increasing the solubility of lipase in organic solvents. Considering this model, another likely explanation for the formation of CTAB coated lipase immobilized in AOT based organogels is given as follows: When lipase is coated with CTAB, the hydrophilic cationic head groups would interact non covalently with negatively charged enzyme surface and lipophilic tails extend out of the surface. Further during immobilization of CTAB coated lipase in AOT based organogels, the attractive forces exerted by negatively charged polar groups of AOT and positively charged polar groups of CTAB coated lipase would orient the enzyme molecules at interface in MBGs. In addition, the lipophilic tails of CTAB extending from surface of SCL would get embedded in non polar layer of AOT based emulsion acting as an anchor holding lipase molecules at the interface in MBGs. The interaction between positively charged CTAB and negatively charged AOT around the lipase might also alter the 3-dimensional structure of lipase which in turn influences its activity. This model may also be used to explain why CTAB coated lipase when immobilized in AOT based organogels exhibits maximum esterification efficiency (Fig 2.2, 2.3 & 2.4). Similar observations were made by Noda et al (1997) suggesting that coating of lipase with surfactant molecules exerts significant effect on resultant enzymatic activity.

2.3.2 Influence of temperature on ethyl isovalerate synthesis catalyzed by CTAB coated and uncoated lipase immobilized in AOT based organogels

In order to determine if CTAB coating upon immobilization in AOT based organogels influences catalytic activity of lipase, the activation energy for synthesis of ethyl isovalerate catalyzed by CTAB coated and uncoated enzyme immobilized in AOT based organogels was determined. Figure 2.5 shows that the activation energies within a
temperature range of 25 – 45 °C were found to be 29.8 and 9.15 kJ for uncoated and CTAB coated lipase immobilized in AOT based organogels, respectively. Thus CTAB when coated on lipase upon immobilization in AOT based organogels induces some structural change in the lipase, which probably is responsible of enhancing its esterification activity. It has been suggested that surfactants might shift open/close equilibrium of lipases towards the open conformation, by coating the hydrophobic areas of lipase that surround the active site of the enzyme (Fernandez-Lorente et al, 2006). Such a structural change might account for the decrease in the activation energy of surfactant coated lipase catalyzed esterification reaction (Huang et al, 2003).

2.3.3 Effect of CTAB concentration on the lipase catalyzed esterification
The amount of surfactant used for coating lipase had a profound effect on the catalytic activity of the surfactant coated lipase. As shown in Fig. 2.6, increase in the concentration of CTAB beyond 1mM led to a decrease in the esterification activity. At higher surfactant concentrations the hydrophobic moiety of the free surfactant would form micellar structures with other surfactant molecules and move away from the enzyme surface or might form a bilayer around the enzyme molecule. In either case, a decrease in the enzyme activity would be expected (Hsieh et al, 2006). The decrease in lipase activity upon coating
the enzyme with higher concentrations of surfactants have been reported by various workers (Hsieh et al, 2006; Thakar and Madamwar, 2005; Liu et al, 2000).

![Graph showing the effect of CTAB concentration on the lipase catalyzed esterification. The % esterification was estimated using 1mM (♦), 5mM (■), 10 mM (▲), 20mM (×) of CTAB concentration.]

Fig. 2.6 Effect of CTAB concentration on the lipase catalyzed esterification. The % esterification was estimated using 1mM (♦), 5mM (■), 10 mM (▲), 20mM (×) of CTAB concentration

2.3.4 Reusability of CTAB coated lipase immobilized in AOT based MBGs

In order to exploit lipases effectively under very low-water conditions the use of immobilization technology is of key importance, as it offers the benefits of the reuse of enzyme and easy separation of products from enzyme as well as the potential to run a continuous process. During esterification reaction between acid and alcohol, however, an equimolar amount of water is formed, which has a disadvantageous effect on the reaction rate and enzyme activity. Therefore, it is necessary to remove the excess of water generated during the reaction.

The yield of ethyl isovalerate was not significantly affected up to 5 runs, but it declined to almost 40% after the 5th run (Fig. 2.7). This may be due to accumulation of water, a byproduct of esterification to the level that it would alter the thermodynamic equilibrium of the reaction favoring hydrolytic activity of lipase (Dandavate and Madamwar, 2007; Dudal et al, 1995; Gandhi et al, 2000). In order to solve this problem, after 5 runs the MBGs were
treated with 1M AOT in isoctane for 24 hours. Then these pretreated MBGs were used for esterification. If no treatment was given the activity kept decreasing whereas in AOT treated gels, the esterification activity could be stabilized to about 60% for next two cycles. The dry solution of AOT in isoctane probably causes selective removal of water from MBGs resulting in recovery of lost esterification activity (Jenta et al, 1997). However, complete removal of accumulated water does not seem to occur by this treatment as a result only partial recovery of esterification activity could be achieved. The similar phenomena was reported by Jenta et al. who employed granulated MBGs and demonstrated the recovery of lost esterification activity of lipase immobilized in MBGs upon treatment with dry solution of AOT in n-heptane. This raises the possibility for the reuse of MBG immobilized lipase several times with AOT treatment. Treatment with silica for water absorption was not as efficient as the treatment with dry reverse micellar solution. This observation encouraged us to investigate the reusability of surfactant coated lipase immobilized in MBGs by removal of excess water using intermittent treatment with a dry reverse micellar solution of 1 M AOT/isoctane.

![Fig. 2.7 Reusability of CTAB coated lipase immobilized in AOT based MBGs. Untreated MBGs (■), AOT Pretreated MBGs (♦)](image-url)
2.3.5 Repeated Use of Lipase Immobilized in MBGs With and Without Pretreatment

It has been reported earlier that lipase immobilized in MBGs with the lowest R value exhibited maximum esterification activity, which is commonly observed for synthetic activities of enzyme in microemulsion media (Bianucci et al, 1990; Verhaert et al, 1991). However, it is not possible to synthesize stable MBGs with a gelatin concentration of 14% (w/v) having a R value lower than 60, but it has been shown earlier that the R value can be reduced below 60 by extraction of water from preformed MBGs using a dry reverse micellar solution as a liquid dessicant (Jenta et al, 1997). Thus, the freshly prepared MBGs (pellet form) were pretreated with 1 M AOT/isooctane solution for 24 h and then monitored for the repeated runs of esterification reaction. Fig. 2.8A and 2.8B show that pretreated MBGs exhibited slightly higher esterification activity, with enhanced reaction rate, up to 5 cycles in comparison with untreated MBGs. The initial rate of ethyl isovalerate synthesis using pretreated MBGs was found to be ca. 1.15-fold higher than for untreated MBGs. The esterification efficiency of untreated MBGs started decreasing after five cycles and was completely lost up to 8 cycles. However, in contrast to untreated MBGs, the esterification efficiency of pretreated MBGs declined or decreased slowly after 5 cycles, with about 50% reduction in esterification activity at the end of the 12th cycle. The amount of water extracted during pretreatment of MBGs with dry reverse micellar solution was monitored by Karl-Fischer titration and the R values of MBGs were calculated. The R value of freshly prepared MBGs was reduced from ca. 60 to 47.5 after pretreatment with 1 M AOT/isooctane solution for 24 h. The lower R value of pretreated MBGs could be accounted for by the 1.15-fold higher initial rate of ethyl isovalerate synthesis during the first cycle of esterification over that exhibited by untreated MBGs. Moreover, the lower initial R value enhanced the reusability of pretreated MBGs, with 40% loss of initial esterification activity after 11 cycles at which the R value was found to be ca. 89.7. The free water content of MBGs was also monitored in situ using solid-state 1H-NMR by determining the ratio for protons of free water to bound protons of the gel. This ratio was found to increase from 0.27 for pretreated gel to 0.53 for MBGs after 11 cycles of reuse. We monitored the changes in physical appearance of the gel (initial and after 10 cycles) using SEM. The SEM (Fig. 2.9A and 2.9B) images demonstrate that there was considerable swelling in the gel after
10 cycles, as can be seen by the increase in pore size in comparison with the initial gel. This swelling of the gel could be attributed to the accumulated water produced during ester synthesis. It has been reported earlier that with an increase in water concentration, coexisting w/o microemulsion droplets will be expected to increase in size (Atkinson P, 1991; Hedstrom et al, 2001). The decrease in esterification activity with increasing water concentration in MBGs may be due to several reasons. One of the reasons may be that increased water may alter the hydrophobicity of MBGs, thereby altering the partitioning of substrate and products and thus affecting the condensation activity. Another reason may be that the water produced in the esterification reaction hydrates the enzyme excessively, which can lead to lower reaction rates and/or unfavorable equilibrium (Dudal et al, 1995; Svensson et al, 1994). The increasing water content is also expected to progressively favor the hydrolysis reaction of synthesized ester (Gandhi et al, 2000). Thus, a decrease in activity upon repeated use of MBGs could be co-related to the increased concentration of free water, making it essential to remove excess water in order to enhance reusability (Bianucci et al, 1990; Okamoto et al, 1993; Verhaert et al, 1991).

It is thus essential to remove the water produced during esterification reactions in order to prevent its adverse effect on condensation activity. This was done by intermittent treatment of pelleted MBGs with AOT/isoctane dry reverse micellar solution after every 3 cycles. Fig. 2.10 shows that, upon intermittent treatment, the esterification activity could be retained (80%) up to 8 cycles over a period of 72 days, after which it reduced to 60% during the next 3 cycles. Thus, a considerable improvement in reusability could be achieved with intermittent treatment. The removal of excess water from MBGs upon treatment with dry reverse micellar solution was demonstrated by solid-state 1H-NMR as well as Karl-Fisher titration for gels obtained after 3 cycles of esterification. The ratio of free water protons to bound protons of gel decreased from 0.47 to 0.39 after treatment of the gel with 1 M AOT/isoctane reverse micellar solution, which indicates removal of free water from the gel.
Fig. 2.8. A. Repeated cycles of ethyl isovalerate synthesis using surfactant-coated lipase immobilized in AOT-based organogels; Δ, Untreated MBGs; , Pretreated MBGs. B. Initial rate of esterification during repeated cycles of ethyl isovalerate synthesis using surfactant-coated lipase immobilized in AOT-based organogels; Δ, Untreated MBGs; , Pretreated MBGs.
The amount of water extracted from the gel in dry reverse micellar solution was monitored using Karl-Fischer titration and used to calculate R values of MBGs. The R value of MBGs increased from 60 (initial) to 82.6 after three cycles of ester synthesis, which upon treatment with dry reverse micellar solution was reduced to 68.6. Thus, by giving intermittent treatment to MBGs with dry reverse micellar solution after every three cycles, accumulation of water to inhibitory concentration was prevented. Jenta et al.

Fig. 2.9 A. SEM image of pretreated gel. B. SEM image of gel after 10 cycles
(1997) found the use of liquid dessicant to be most effective amongst various methods for removal of water from MBGs. They proposed that regeneration of biocatalytic activity upon treatment of gels with dry reverse micellar solution occurs predominantly because of the selective extraction of water from the gel matrix, rather than from the influx of surfactant into the MBG from the reservoir of dry micelles.

![Figure 2.10 Repeated cycles of ethyl isovalerate synthesis using surfactant coated lipase immobilized in AOT based organogels; ▲ Untreated MBGs, ● Intermittently treated MBGs. Arrows indicate the time of treatments](image)

**2.3.6 Optimization of Treatment Conditions for MBGs**

The intermittent treatment of MBGs after every 3 cycles enabled the use of immobilized lipase for up to 9 cycles without significant loss in esterification activity. Thus, further studies were done to optimize the treatment conditions. In all of the previous reports, dry reverse micellar treatment has been done using 1 M AOT in organic solvent (Jenta et al, 1997, Soni and Madamwar, 2000). However, since the higher concentration of AOT may have deleterious effects on enzyme activity, we investigated treatments of MBGs with dry reverse micellar solutions at varying concentrations of AOT/isooctane. It was found that 0.2 M AOT in isoctane was found to be as efficient in the regeneration of esterification activity as 1M AOT in isoctane. Thus, in further experiments, MBGs were treated with reverse micelles containing 0.2 M AOT concentration. Then, in an attempt to
optimize the treatment time with dry reverse micellar solution, MBGs were treated with 0.2 M AOT in isooctane solution for varying time periods. As per earlier reports, the dry reverse micellar treatment was given for 24 h. However, in our studies, we found that a 9-h treatment was enough for effective recovery of enzyme activity (Fig. 2.11).

![Figure 2.11 Treatment of MBGs with 0.2 M AOT in isooctane for varying time periods](image)

2.3.7 Comparison Between Pelleted and Granulated MBGs

The rate of ester synthesis in MBGs also depends on enzyme loading and surface area per unit volume of immobilizate (Hedstrom et al, 2001). It has been very meticulously demonstrated earlier that in the case of pelleted MBGs at higher enzyme loading, because of the diffusional limitation of substrates/products, the surface catalysis would predominate resulting in inefficient use of the enzyme (Jenta et al, 1997). In order to study the same phenomenon, we compared the esterification efficiency and reusability of untreated pelleted and granulated MBGs at equal enzyme loading, where MBGs were intermittently treated with dry reverse micellar solution of 0.2 M AOT in isooctane after every 3 cycles. The total surface area of pelleted and granulated MBGs was found to be $2.8 \times 10^4$ mm$^2$/cm$^3$ and $2 \times 10^5$ mm$^2$/cm$^3$ of the gel, respectively. It was observed that the yield of ethyl isovalerate at equilibrium using granulated MBGs was slightly higher in comparison with pelleted MBGs (Fig. 2.12); however, granulated MBGs exhibited
significantly higher (1.2-fold) initial reaction rates (Fig. 2.13) than pelleted MBGs. Granulation of MBGs results in reduced transport distances in organogels, and a higher surface area to volume ratio, resulting in more efficient use of immobilized enzyme and thus higher initial rates of esterification in comparison with pelleted MBGs (Cao L, 2005). Furthermore, it would lead to more effective extraction of water during treatment with dry reverse micellar solution and, thus, more efficient reusability of enzyme in comparison with pelleted MBGs. The treatment of MBGs with a dry reverse micellar solution of AOT in isooctane leads to selective extraction of free water from the gel, and this approach can be employed for removal of excess water from gels containing immobilized lipase, resulting in an enhanced rate and reusability of enzyme for condensation reactions. The reusability of lipase immobilized in MBGs can be improved by obtaining MBGs with lower R values upon pretreatment and/or intermittent treatment with liquid desiccant at regular intervals during repeated cycles of esterification. The granulated form of MBGs provides a higher surface area and thus exhibits a higher rate of esterification in comparison with the pelleted MBGs.

![Graph](image)

**Fig. 2.12** Comparison of esterification efficiency of pelleted MBGs and granulated MBGs during repeated cycles of ethyl isovalerate synthesis; ■ Pelleted MBGs, ▲ Granulated MBGs
Fig. 2.13 Rate of ethyl isovalerate synthesis during repeated cycles using granulated and pelleted form of surfactant coated lipase immobilized in MBGs; ■ Pelleted MBGs, ▲ Granulated MBGs

We carried out a small experiment wherein lipase from *Candida rugosa* was first suspended in buffers having different pH values i.e. pH 4-9 and then subjected to lyophilization. Thus, the lipase with different “pH memory” in a broad range were prepared for further catalytic reactions.

2.3.8 Effect of pH memory on hydrolytic activity of *Candida rugosa* lipase
The hydrolytic activity of lipase at various pH in aqueous conditions was determined using pNPP hydrolysis assay method (Winkler and Stuckman, 1979).

The optimum pH for hydrolytic activity of CRL lipase (~38 units/mL) was found to be pH 7 (Fig. 2.14). The higher lipase activity at pH 7 may be attributed to some change in the ionization state of the catalytic site leading to change of the secondary and tertiary structure of the lipase molecule.
Figure 2.14 Effect of pH on hydrolytic activity of *Candida rugosa* lipase at 37°C

3.9 Effect of pH memory on esterification activity of *Candida rugosa* lipase under non aqueous system

To study the effect of pH memory on esterification activity of CRL, lipase was dissolved in equal quantity in the buffers of different pH values (pH 4-7). The samples then were lyophilized and used for the synthesis of ethyl isovalerate in organic solvents. Figure 2.15 shows that a strong pH memory effect is seen in terms of ester synthesis. Maximum esterification was observed at pH 6.0. At alkaline pH the synthesis of ethyl isovalerate decreased drastically. Also there was a 2.2 fold decrease in the synthesis of ethyl isovalerate at pH 4.0 and 5.0. The conversion rate was found to be the lowest at basic pH values.

A lower yield of ethyl isovalerate was obtained when CRL without lyophilization in buffer with optimum pH was employed for ethyl isovalerate synthesis. Around 3-fold increase in the % yield of ethyl isovalerate was observed due to pH memory phenomenon. The ionization state of the lipase relies on the pH of the aqueous solution used. Due to the complete removal of water molecules during lyophilization there is a reduction in the dielectric charges resulting in the pairing of the ions. There is exchange of H⁺ ions between the protein and the buffer species finally leading to change in the
conformation state (catalytic activity) of a protein in a dry state, thus exerting pH memory to the protein.

![Graph showing the effect of pH on esterification](image)

**Fig. 2.15** Effect of pH memory of *Candida rugosa* lipase on synthesis of ethyl isovalerate at different pH values (4-9)

The above results indicate that pH optima for ρNPP hydrolysis and ethyl isovalerate synthesis by CRL differ by one unit. Similar observations have been recorded by Buthe et al (2005) in their studies on pH optima in lipase catalyzed esterification. They further reported that esterification activity of lipase increased with the concentration of protonated acid in the reaction system.

### 2.4. Conclusion

Surfactant coated lipase immobilized in MBGs provides a novel approach to improve activity of enzyme. Amongst various combinations of surfactants used for coating lipase and for preparation of micro emulsion based organogels, the CTAB coated lipase immobilized in AOT based MBGs proved to be better system for the synthesis of ethyl isovalerate. The activity of the CTAB coated lipase immobilized in AOT based organogels was found to be eight times higher than that of uncoated lipase immobilized in AOT based organogels. The lower activation energy of CTAB coated lipase immobilized in AOT based organogels in comparison to that of uncoated lipase
immobilized in AOT based organogels depicts some structural change in the CTAB coated lipase. This system permitted the reuse of enzyme up to five cycles of reaction without any significant loss in activity. Further the treatment of MBGs with a dry reverse micellar solution of AOT in isooctane leads to selective extraction of free water from the gel, and this approach can be employed for removal of excess water from gels containing immobilized lipase, resulting in an enhanced rate and reusability of enzyme for condensation reactions. The reusability of lipase immobilized in MBGs can be improved by obtaining MBGs with lower R values upon pretreatment and/or intermittent treatment with liquid desiccant at regular intervals during repeated cycles of esterification. The granulated form of MBGs provides a higher surface area and thus exhibits a higher rate of esterification in comparison with the pelleted MBGs.

Thus, our approach can be considered as a new and a facile method for the efficient synthesis of ethyl isovalerate by using CTAB coated lipase immobilized in AOT based organogels.

2.5. References


