Chapter 6

Summary
and
Conclusions
Enzymes are exquisite biomolecules capable of catalyzing biochemical reactions at a manifold higher rate than the uncatalyzed ones. In addition to biochemical reactions, they have been employed in a wide variety of chemical and biotechnological industrial applications owing to their versatility and modifiable properties. With the recognition of the ability of some enzymes to catalyze reactions which are much unexpected or completely reversal to their natural counterpart reactions (termed as ‘enzyme promiscuity’), there has been an outburst of basic and applied research to explore the potential of such enzymes. Lipases belong to one such class of enzymes, which have been a keen subject of study for both, biologists and chemists. These interesting enzymes by virtue are designed to hydrolyze the ester bonds (in aqueous medium) which are present in fats, into their components resulting in the corresponding acid and alcohols. However, if the water component is replaced by a total or partial non-aqueous medium, a reversal of the hydrolytic reaction i.e. the synthesis of an ester is observed. A number of such ‘reverse’ or ‘unusual’ reactions have been carried out utilizing the promiscuous nature of lipases in non-conventional medium such as organic solvents, ionic liquids, supercritical fluids etc. Compounds linked with value added products such as pharmaceutical intermediates, fine chemicals and foods and beverages are a few examples of resultants of non-aqueous biocatalysis.

It is well known that enzymes lose their activity (totally or at least partially) when they are placed in medium other than aqueous medium. This is because of a certain amount of water which is absolutely necessary to ‘hydrate’ the enzyme molecules leading to a stable and active conformation. This essential water may be stripped off in non-aqueous medium leading to a denatured or inactive enzyme configuration. Hence, an important prerequisite for ascertaining the feasibility of such reactions is to control the water content of such systems in a way that it is sufficient to maintain the enzyme structure integrity and at the same time, small enough to participate as a substrate for the reverse hydrolytic reaction. In addition to maintain the water activity, it is also necessary to engineer enzyme system to suit the non-aqueous medium for optimum activity. Various methods have been studied for this purpose, immobilization being one of the more advantageous and widely studied. Immobilization of enzyme refers to their confinement
within a certain specific area which can be achieved by a number of ways. It not only assists in shielding the enzyme from the unnatural environment, but also makes convenient to be separated from the reaction mixture easily for further reuse. An ideal immobilization system must fulfill certain requirements such as stability in non-aqueous media, inert towards substrates and products, sturdy to withstand high temperatures and protect the enzyme molecules from the deleterious effects of external environment. Microemulsion based organogels (MBGs) are one of the immobilization systems which prove to be perfectly suited for non-aqueous biocatalysis. MBGs are also referred to as ‘double immobilization’ as the enzyme is first entrapped within reverse micelles followed by addition of a solidifying agent. Reverse micelles are relatively ordered structures that consist of a water pool surrounded by a surfactant layer, with the hydrophobic moieties of the surfactant molecules interacting with the bulk hydrophobic solvent. Being dynamic structures, the micelles can exchange their constituents (enzyme, water, substrates and products) between each other and also with the bulk organic solvent. These systems are particularly attractive for biocatalysis as they mimic the natural environment that many enzymes experience within cells. The reversed micellar systems are of much significance for enzymes such lipases, which are activated in the presence of an interface, since this system provides a high interfacial area, with the enzyme anchoring on the aqueous side of the surfactant interface. However, there are a couple of drawbacks of such systems. It is difficult to recover the product from reverse micelles due to the presence of the surfactant and other components of the system, such as protein and water. This problem is successfully solved by ‘gelling’ the whole reverse micelles system by adding gelling agents such as gelatin, agar, hydroxypropyl methyl cellulose (HPMC), κ- carrageenan etc.

In this work, MBGs were prepared using reverse micellar systems comprising AOT, CTAB, Triton-X-100 and Tween-80 with solvents such as n-hexane, n-heptane, isooctane and cyclohexane. Among all the surfactants, AOT (sodium bis-2-(ethylhexyl) sulfosuccinate) was found to be the best suited in combination with gelatin (14%) which yielded porous and translucent stable organogels. Further, all the MBGs containing Candida rugosa lipase (CRL) were applied for synthesis of short chain flavor esters in
organic solvents. It was observed that the enzyme showed preference towards medium chain alcohols over short chain alcohols. Regarding the acid moiety, again, medium to long chain and straight chain acids were preferred to their short chain counterparts. The MBGs showed better performance in terms of ester production when compared to the free enzyme, thus showing the effectiveness of immobilization. In the study regarding synthesis of ethyl valerate, the optimum values of immobilization and reaction parameters were observed to be AOT as surfactant, Wo value of 60, valeric acid to ethanol concentration ratio (1:1.6), 40°C and pH 7. Inhibition was observed at higher concentrations of ethanol. The organogels exhibited appreciable stability at 50-70 °C after incubation of 10h which approved the strong protection offered by the gelatin network. A decrease in activity following 3-4 esterification cycles was observed which was contributed to excess water (by-product of esterification reaction) accumulation within the MBGs. The water was successfully extracted using the dehydrant solution on 1M AOT/isooctane. Karl-Fischer titration and TGA of the organogels confirmed the presence of excess water within the used MBGs and its extraction in the AOT/isooctane treated MBGs.

In the next study regarding synthesis of pentyl valerate, the statistical method of response surface methodology (RSM) conjugated with Box Behnken Design (BBD) was employed with five variables viz. enzyme concentration, initial water content (Wo), solvent used for MBG preparation, substrate ratio and time, and response as pentyl valerate synthesis. Though there are numerous reports available regarding ester synthesis using lipase immobilized in MBGs, the effects are mostly studied at individual levels. Therefore, this study was attempted to scrutinize the collective effects of selected process parameters on final ester yields. As individual entities, substrate concentration, Wo and enzyme concentration showed significant effect on esterification, which is a known fact. The study of interactions revealed that if higher concentration of enzyme was used, high yields of 80-90% ester could be obtained at medium levels of Wo and substrate concentration values. It was inferred from the study that substrate concentration played a very important role and that at high pentanol concentration, esterification was observed to increase substantially. In all the interactions it was observed that high turnover (80-90%)
could be obtained keeping most of the parameters at medium levels but to obtain 100% yield, the levels had to be increased to the highest coded values. A positive correlation was observed between enzyme concentration and Wo value which showed that lower enzyme concentration preferred low Wo values and higher enzyme concentration required higher Wo values for higher response. The time period could be reduced by increasing substrate and enzyme concentration to achieve high yields. Hence, this study depicted that in case of MBG systems, high yields in the range of 80-90% ester could be obtained in a relatively shorter span of time if the pairs of parameters showing effective interactions are set at appropriately high values (in the levels coded in the study) or vice-versa. Effect of solvent used as reaction medium revealed that when compared to straight chain alkanes, cyclic alkanes assisted in higher conversion. A comparison between straight chain and cyclic forms of pentane, hexane and octane showed that highest esterification was seen in cyclooctane followed by n-octane and cyclohexane. This was perhaps due to the structure of the cyclic alkanes, which could move across the organogel pores at a faster rate when compared to straight or branched chain alkanes.

With the progress of nanotechnology, rapid growth in the area of nanobiotechnology research has followed. ‘Nanobiocatalysis’ is one typical example. Nanomaterials have been studied as enzyme support systems since they exhibit large surface area resulted in improved enzyme loading, which in turn show increased apparent enzyme activity per unit mass or volume compared to that of enzyme systems immobilized onto conventional materials. Of particular interest are carbon nanotubes whose single walled and multiwalled forms are most commonly used for enzyme immobilization. In this work, we attempted to conjugate the two lipases viz Candida rugosa lipase and Candida antarctica lipase B (CALB) onto MWCNTs using different approaches. In the first approach, adsorption onto the nanotubes was performed. These MWCNTs were acid purified and aminated using APTES prior to adsorption. This method assisted in demonstrating the preferred surface group (carboxylic or amine) for conjugation by CRL. Further, as lipases are surface active enzymes, three types of phases i.e. aqueous, non-aqueous and surfactant stabilized interface (reverse micelles) was used for enzyme immobilization. The study showed that in both the cases (carboxylated and aminated nanotubes), reverse
micelles assisted in high enzyme loadings when compared to the other two phases. Also, in case of carboxylated NTs, non-aqueous medium showed high enzyme loadings whereas in aminated NTs, aqueous phase showed high enzyme loadings. These enzyme conjugates prepared using reverse micelles showed exceptionally high performance when applied for synthesis of pentyl valerate compared to their counterparts prepared in aqueous and non-aqueous medium. A possible reason for this could be a strong bond formed by a self catalytic mechanism of lipases at the interfaces which was also confirmed by FTIR analysis. High enzyme loading but low activities in case of attachment using non-aqueous medium may be due to unspecific hydrophobic interactions resulting in a wrong orientation or participation of active site residues in binding, thus leading to lowered activities. The conjugates were reused for 20 cycles wherein the ones prepared using reverse micelles again showed retention of high activity even at the 20th cycle.

For any biocatalytic process to be applied for practical purposes, the most important prerequisite that needs to be met is high reusability in addition to its tolerance to solvents and temperature. Regarding this aspect, in the last part of the study, CALB was attached covalently to carboxylated MWCNTs using the zero length cross linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). It is well known that after covalent immobilization, many a time residues of enzyme molecules interact with CNT surfaces leading to distortion of enzyme structure. Hence, to study this effect, another cross linker, APTES followed by SAA (succinic acid anhydride) were employed. The APTES molecule provides an additional spacer from the nanotube surface while SAA converts the amine terminal of APTES to carboxyl to facilitate conjugation via EDC. Results of enzyme loadings on the two types of functionalized NTs showed that higher amount was attached to APTES and SAA functionalized NTs as compared to direct attachment onto carboxylated NTs using EDC which may be due to reduced steric hindrance as APTES would provide additional spatial area for the enzyme molecule. For the ester synthesis, it was observed that as a consequence of higher enzyme loadings, APTES-SAA conjugated CALB showed higher turnover than that of direct EDC conjugation. Both the enzyme conjugates showed good stability in the range of 20-60°C in terms of ester
synthesis. Appreciable production of pentyl valerate was carried out various organic solvents and highest yields were obtained in cyclic alkanes. The nanobioconjugates were subjected to reusability studies for 50 cycles and brilliant performance by EDC conjugated preparation was obtained retaining activity as high as 85% at the 50th cycle. In a nutshell, microemulsion based organogels and carbon nanotubes were studied as supports for lipase immobilization. Retention of enzyme activity of CRL within MBGs at high temperatures assures their protective nature as well as diffusion of substrates and products across the gel. Easy separation and recyclability of MBGs makes them a friendly tool of lipase immobilization. In addition, treatment using AOT/isooctane proves to be a simple, yet very effective method of extraction of excess accumulated water within MBGs. Adsorption of CRL onto carboxylated NTs using reverse micelles as attachment medium shows the potential of its inherent property to get activated and catalyze bond formation at interface. Also, this method paves way of lipase conjugation without using additional chemical cross linker. CALB conjugated to carbon nanotubes were highly sturdy and catalyzed esterification reaction for 50 consecutive cycles retaining appreciable activities. Hence, this study demonstrates the potential of MBGs and CNTs as excellent supports for lipase immobilization with the ability to assist as robust backbone to tether the enzymes for application in non-aqueous biocatalysis.