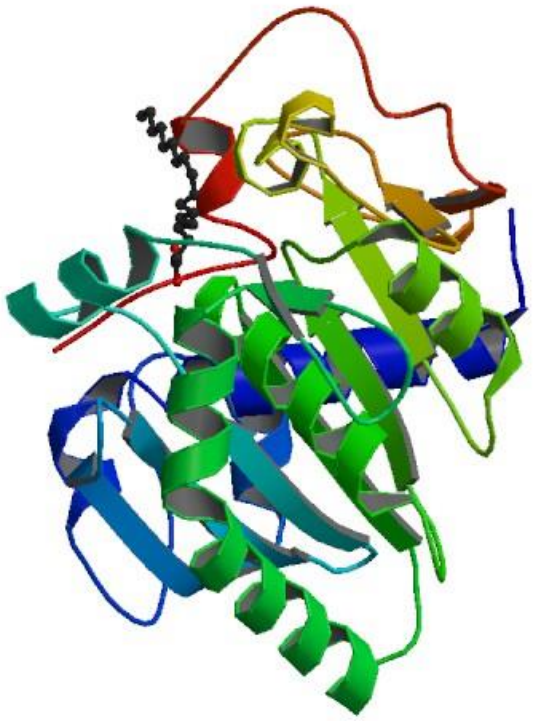
Chapter 3

Synthesis of ZnO Nanoparticles Using Glycine As Template, Their Characterization and Application As Support For Lipase Enzyme Immobilization In Esterification Reaction



Thermomyces lanuginosus lipase

Immobilization of *Thermomyces lanuginosus lipase* on ZnO nanoparticles: mimicking the interfacial environment!

In this study, we propose that enzyme activity on immobilization can be controlled and enhanced by providing the environment mimicking the lipid/water interface.

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3.1 Introduction

With thousands of years of evolution nature has developed molecules called enzymes which can carry out specific biochemical conversions in the constrained environment of cell. Chemically, enzymes are long chain polypeptides folded in such a way that unique reaction sites (pockets) are generated according to predefined genetic program enabling them to act as catalysts. Highly selective and specific reactions occur at these reaction sites usually in a narrow range of temperature and pH in an aqueous medium. From an industrial point of view, enzymes can be manufactured or extracted from the cells and utilized for large scale production of high purity stereoisomers which would be difficult by conventional catalytic processes. However, the major drawbacks are (i) denaturation of enzymes when temperature or pH of the reaction are drastically changed (ii) enzymes function mostly in aqueous medium under homogeneous conditions (iii) enzymes are denatured sometime and reaction sites may be distorted/ blocked at the end of single reaction cycle during recovery process. Such problems can be solved by immobilizing enzyme on a suitable support. By this way the reactions can be heterogenized and the immobilized enzyme can be recovered and recycled for several times maintaining the activity. This makes

overall operation simple, efficient in both aqueous and non-aqueous medium, and of course, economically viable.

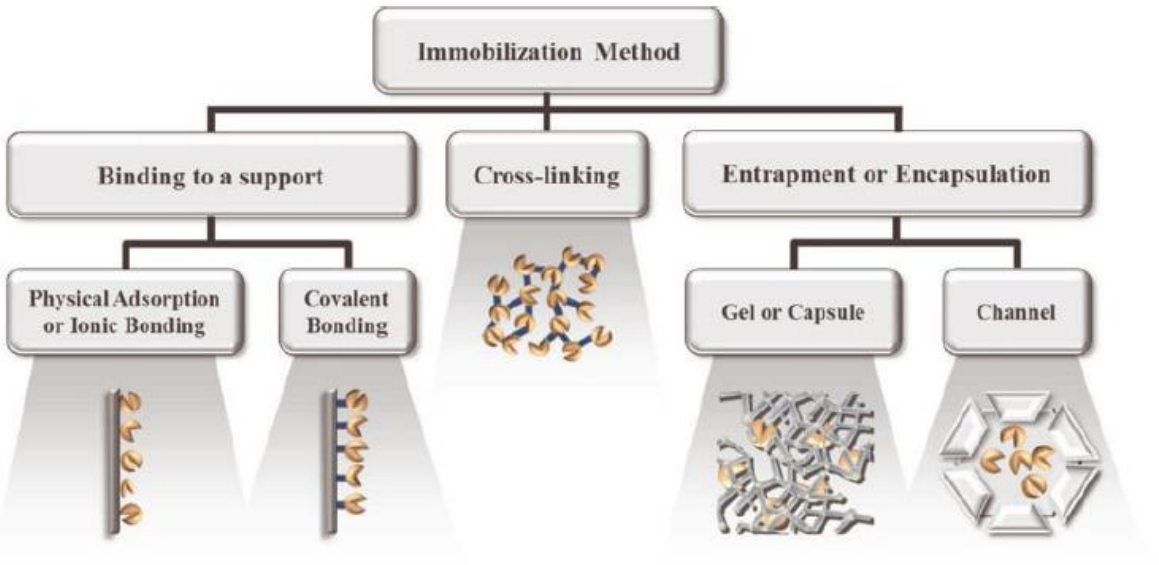


Figure 3.1. Overview of the different enzyme immobilization strategies. (E. T. Hwang M. B. Gu,*Eng. Life Sci.* 2013, No. 1, **49**,13).

Lipase (triacylglycerol ester hydrolase EC 3.1.1.3) is an enzyme which catalyzes the hydrolysis of triacylglycerol to glycerol and fatty acids. It finds wide applications in diverse areas like dairy industry, specialty chemicals, organic synthesis and manufacture of enantiomerically pure pharmaceuticals. Immobilization of lipase is carried out by various techniques[1] such as (i) non-covalent adsorption on robust supports like polymeric beads,[2, 3] films,[4, 5] natural kaolin clay,[6] nanoparticles,[7] etc. (ii) entrapment of enzyme in a polymeric gel or on

membranes by physical adsorption, inclusion or covalent bonding [8-11] (iii) covalent attachment with the supports like polymers[12] or nanoparticles[13] and (iv) cross-linking of an enzyme making it carrier free.[14]

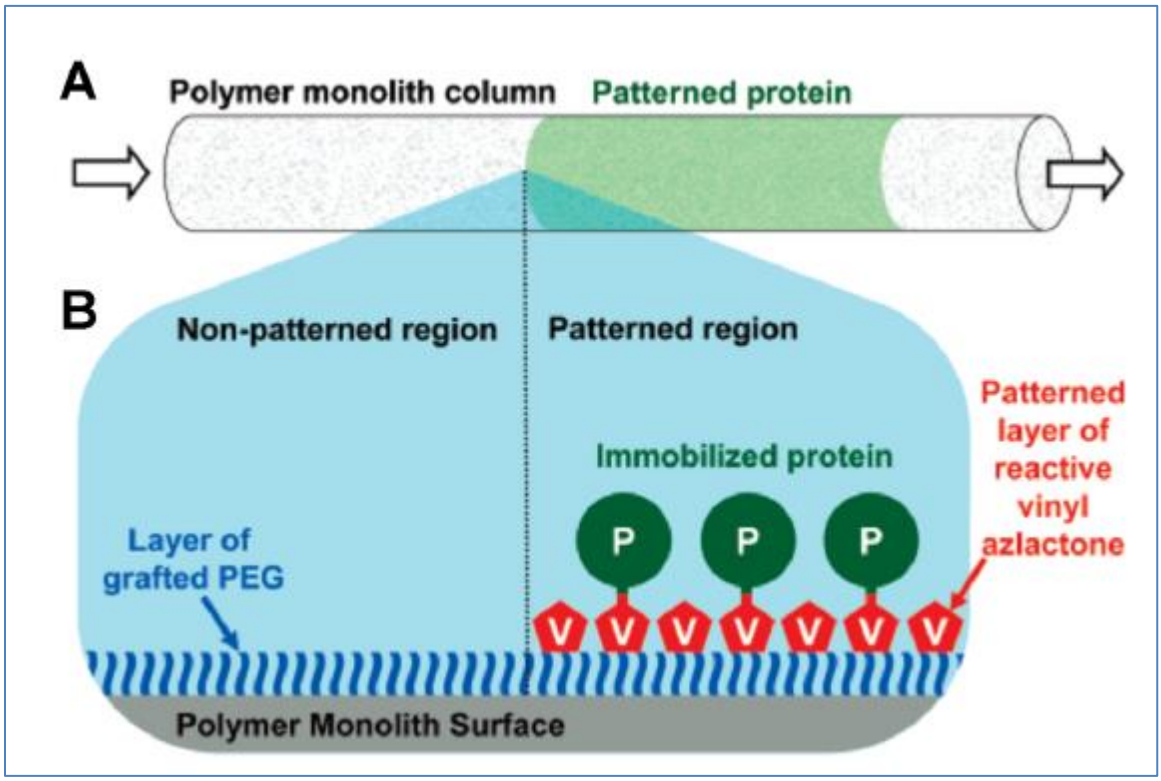


Figure 3.2. **A: Protein is immobilized to the surface of a polymer monolith in patterned regions within a microfluidic channel. B: PEG is grafted to the surface of the polymer monolith to prevent non-specific protein adsorption. Vinyl azlactone is photopatterned onto the PEG surface and activates the surface for protein immobilization.** (F.Jia, B. Narasimhan and S.Mallapragada, *Biotech.Bioengg*, 2014, **111**, 2).

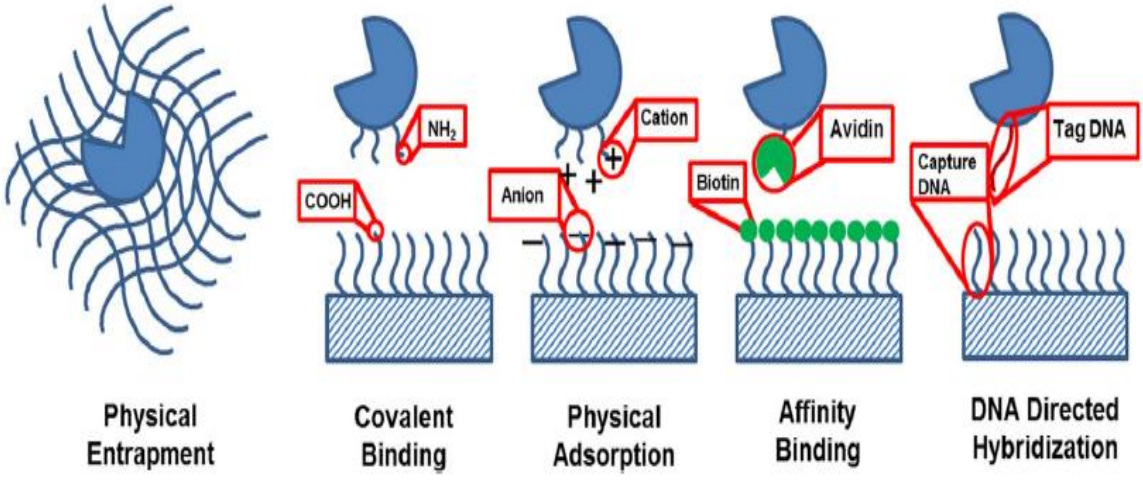


Figure 3.3. **Illustration of representative examples of physical entrapment, covalent binding (amide bond formed by carboxyl and amine groups), physical adsorption (ionic interaction), affinity binding (biotin–streptavidin interaction), and DNA hybridization directed self-assembly of enzymes on carriers.** (F.Jia, B. Narasimhan and S.Mallapragada, *Biotech.Bioengg*,2014, **111**, 2).

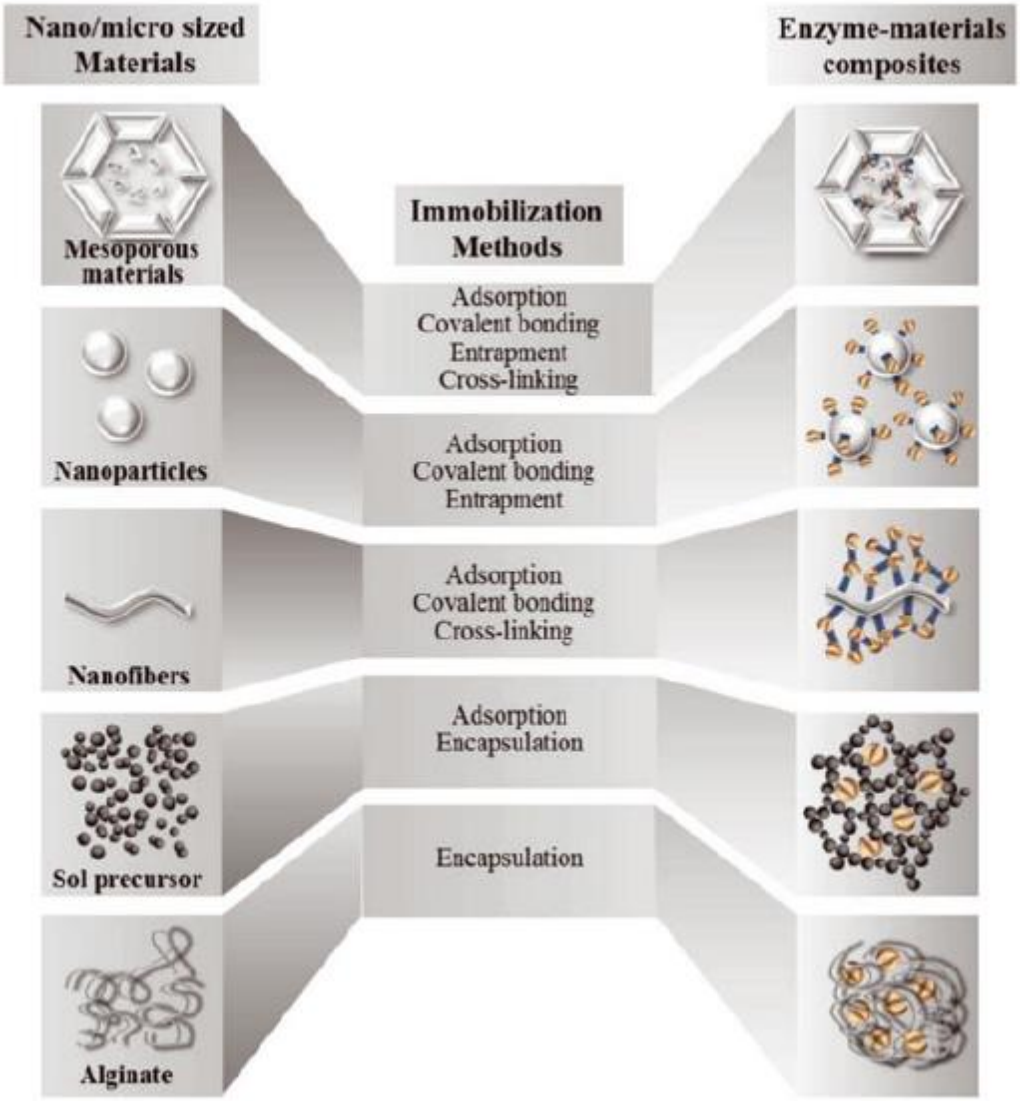


Figure 3.4.Enzyme immobilization using various nano/microsized materials.(E. T. Hwang M. B. Gu,*Eng. Life Sci.* 2013, No. 1, **49**,13).

However, each method has its own drawbacks. For example, non-covalent adsorption of an enzyme may cause multilayers of unfavorable orientations on the support that hamper the activity.[15] Cross-linking may cause inactive enzyme

aggregates, deteriorating the catalytic efficiency of preparation. Physical entrapment in gels or membranes also requires the trapping of enzyme in active orientation in the matrix which is laborious and demands a lot of experimentation. This may restrict the natural movement of the enzyme[16] diminishing the catalytic efficiency. The fact is that there is no universal method available for immobilizing the enzyme without restricting its activity.[17] Suitable selection of a carrier, reaction conditions and enzyme itself are the three major components governing the performance of the developed immobilized enzyme.[18]For example, silicon and its derivatives are used and reported very well in the literature.

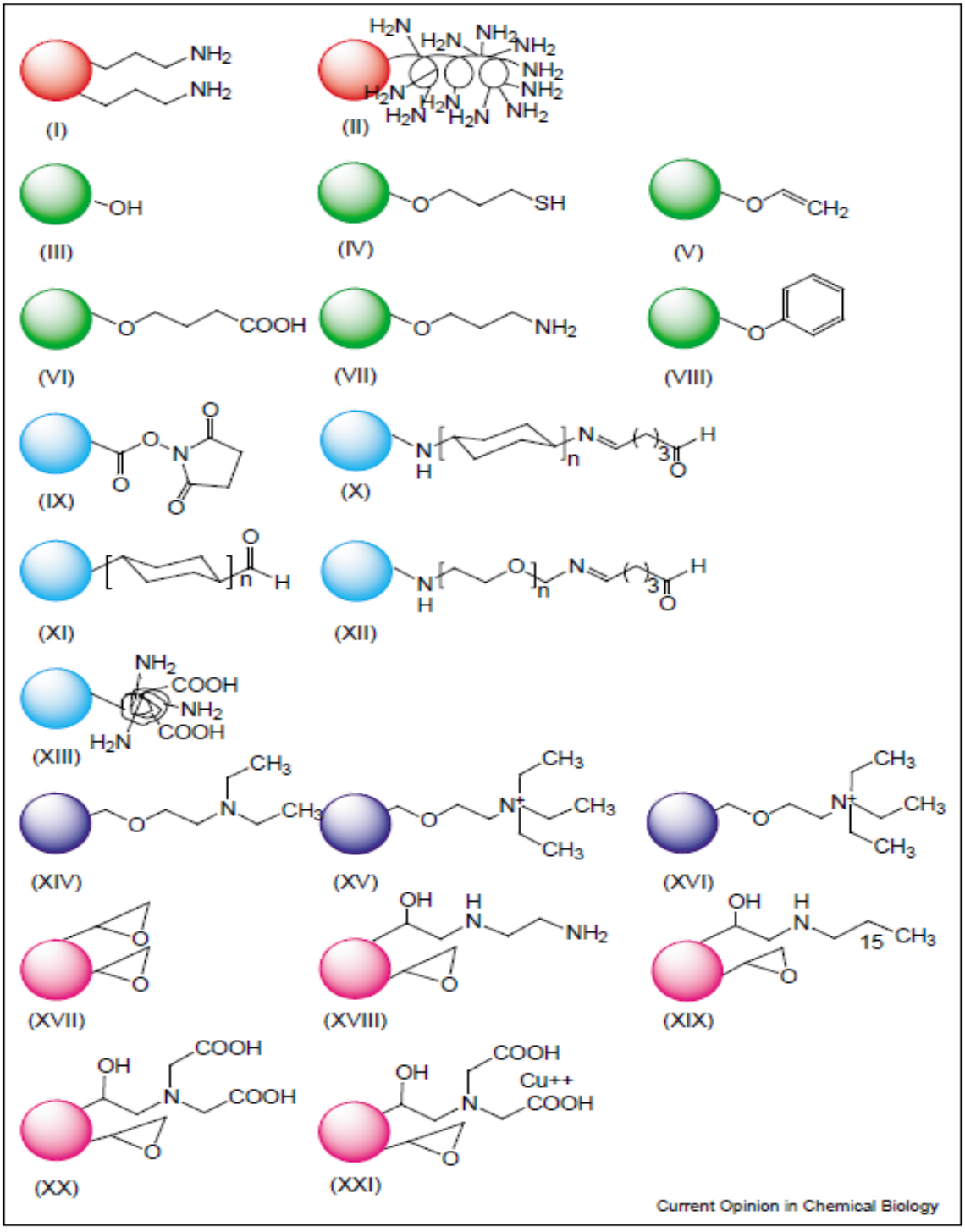


Figure 3.5.Silica and derivatives. (I) Silanized silica and (II) PEI-coated silica; Mesoporous zeolite: (III) SAB-15, (IV) MPTMS, (V) VTES, (VI) TSBN-COOH, (VII) APTES, (VIII) PTMS; hydrophobic carriers: (IX) no spacer, (X) amino dextran as spacer, (XI) aldehyde dextran as spacer, (XII) amino PEG as spacer, (XIII) BSA as spacer; polysaccharide-based carriers: (XIV) DEAE-sephadex, (XV) TEAE-Cellulose, (XVI) Ecetola-cellulose;

functionalized Eupergit C: (XVII) Eupergit C; (XVIII) DAE-Eupergit C, (XIX) dodecyl-Eupergit C, (XX) IDA-Eupergit C, (XXI) Cu++-IDA-Eupergit C.

Proper strategy based on these parameters is required to develop a suitable biocatalyst for a chemical transformation.

ZnO is an ionic solid and environmentally friendly semiconducting material with band gap energy of 3.37 eV at room temperature. It finds wide applications in solar cells,[19] photocatalysis,[20]biosensors,[21] luminescent material [22] etc. Its biocompatible, biodegradable and antimicrobial nature encourage researchers using it as a sensor for biotransformation.[23, 24] In the present strategy ZnO /glycine/glutaraldehyde NPs have been developed as a catalyst akin to α-Al_2O_3, zeolites and cationic clay sheets which could provide an inert support for covalent binding of TLL enzyme and/or it may also enhance the catalytic activity of lipase synergistically by maintaining hydrophilic-hydrophobic balance.

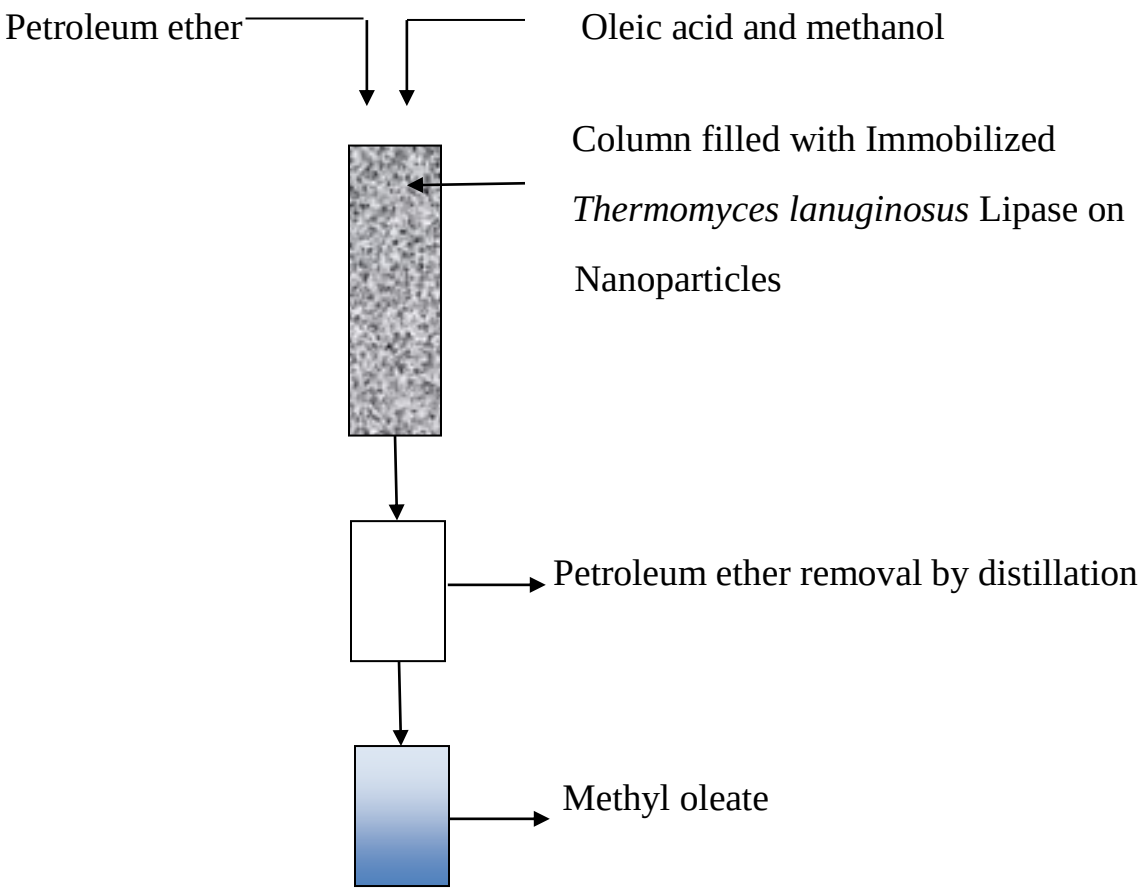
Nowadays, biodiesel which is a mixture of methyl and ethyl ester of fatty acids (mainly palmitic, linolenic, linoleic, and oleic acids) is gaining an importance because of its comparatively clean burning, renewability, biodegradability and superior lubricating properties.[25] Non-edible oils and fats, vegetable oils and their refining by-products are good sources of biodiesel. The amount of free fatty acids (FFA) content and the storage stability are some of the parameters affect the quality of the biodiesel. Oxidation of unsaturated esters in biodiesel occurs by contact with air and other pro-oxidizing conditions during long term storage. It was

observed that the extent of unsaturation in fatty acid molecules (in FFA, mono-, di- or triglycerides) adversely affect the storage stability.[26, 27] Ideally, the FFA content in biodiesel should be less than 1% for superior quality and storage stability.[28] For the purpose, acid or base-catalyzed esterification of FFA is carried out. The major problem associated with base catalysis is that it reacts with FFA instead of catalyzing the reaction and form soap like sticky mass.[29]These decrease the biodiesel yield and creates problem associated to the separation and purification steps due to emulsions and partial dissolution of Fatty Acid Methyl Esters (FAME) into the glycerol phase.[30]Mineral acid (eg. H_2SO_4 or HCl) catalyzed esterification of FFA is also associated with the work-up problems. It is very difficult to tackle the issues like recovery and recycling of the by-products, corrosion and other such environmental problems crop up during the reaction.[31] Solid acid and supported Lewis acid catalysis may be preferred; however, the water produced as by-product severely affects the efficiency (in terms of leaching or solubility) of the catalyst and an environmental problem of discarding the catalyst after several cycles still persists.[32] Catalysis involving the immobilized lipase enzymes for the esterification of FFA may appear a good choice. Careful selection of the support (in term of biodegradability and/or toxicity) for immobilization of the enzyme can minimize the environmental issues. Nanomaterials have high surface area to volume ratio and when coated with catalyst, provide efficient catalysis with minute quantity of

material which can be reused for several cycles and regenerated by immobilizing fresh enzyme on the surface instead of polluting the environment by unrestricted waste disposal.

In the present study, we have attempted the wet chemical synthesis of ZnO NPs using glycine as capping agent to restrict the growth. The synthesized ZnO NPs were used as support to covalently immobilize TLL enzyme. It was observed that the microenvironment in the vicinity of the enzyme in terms of lipophilic-lipophobic balance and flexibility of the adopted conformation as well as porosity of the support directly affect the activity.[18, 33] The coating of zwitterionic glycine molecules functions in two ways. First, they stabilize ZnO NPs by adjusting the surface charge, making the surface hydrophilic. Secondly, they provide sites for glutaraldehyde activation. The glycylimine assembly acts as hydrophobic spacer between ZnO NPs and enzyme. Consequently, the microenvironment in its vicinity is tailored in such a way that the enzyme can be in its high activity state. The activity of the developed immobilized enzyme was evaluated for the esterification of oleic acid with methanol. This process is suitable for industrial continuous process. By making few modifications this process can generate big quantities of esters or biodiesels.

The flow diagram is as follows:



Scheme 3.1. Flow sheet diagram for continuous production of ester from the raw materials through supported TLL enzyme.

3.1 Experimental

3.2.1 Materials

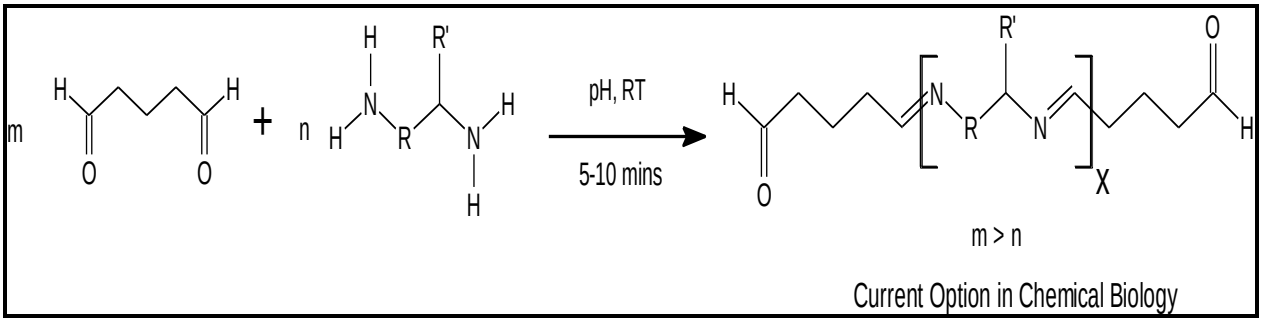
Zinc acetate $Zn(CH_3COO)_2$was obtained from Loba chemicals, India. Potassium Hydroxide (KOH), Glycine (NH_2CH_2COOH), methanol (CH_3OH) were purchased from S.D. Fine Chemicals, Mumbai, India. All the chemicals were AR grade and used without further purification. Freshly prepared aqueous solutions were used for the synthesis of ZnS nanoparticles. Alkaline solution of *Thermomyces Lanuginosus* Lipase (TLL, activity-10^5 U/g) procured from Aumgene Chemicals Limited, India. Glutaraldehyde was purchased from Merck.

3.2.2 Synthesis of ZnO nanoparticles

ZnO NPs were synthesized by a co-precipitation method. Methanolic solutions of zinc acetate (40 mL, 0.03 mmol) and glycine (20 mL, 0.04 mmol) were mixed. To this mixture, alcoholic KOH solution (40 mL, 0.13 mmol) was added drop wise and with constant stirring at 8000 rpm at room temperature. The resulting colloidal solution of ZnO NPs was ripened at 70^oC for three days. The solution was centrifuged, washed several times with distilled water then with absolute alcohol to remove any impurities and dried at 60^oC under vacuum for three days.

3.2.3 Lipase Immobilization

General strategy for the introduction of glutraldehyde functionality as a spacer for enzyme immobilization is reported from the literature below:



Scheme 3.2.Preparation of new cross-linkers with the use of glutaraldehyde and diamino compounds (Current Opinion in Chemical Biology 2005, **217**, 9**).**

Immobilization of lipase on the surface of ZnO NPs was carried out at room temperature. 200 mg of glycine functionalized ZnO NPs were dispersed in 0.5 mL PBS (5mmol) buffer at 7.7 pH. Subsequently, glutaraldehyde (10 µL) was added and the mixture was stirred for about 4-5 h. Then 0.5 mL native *TL* Lipase (1.675 mg/mL, determined from protein assay) aqueous solution prepared in 0.5 mL PBS buffer (pH 7.7) was introduced. The mixture was then gently but thoroughly stirred to ensure complete mixing for further 4 h and then centrifuged at 8000 rpm.The solid collected was washed three times with 60 mL (5 mmol, 20 mL for each washing) PBS solution (Scheme 3.3).

3.2.4 Protein Estimation

Protein estimation was carried out by Lowry's method with Bovine Serum Albumin as standard.[34]It was calculated that on 100 mg of ZnO, 32.6 mg of lipase was loaded.

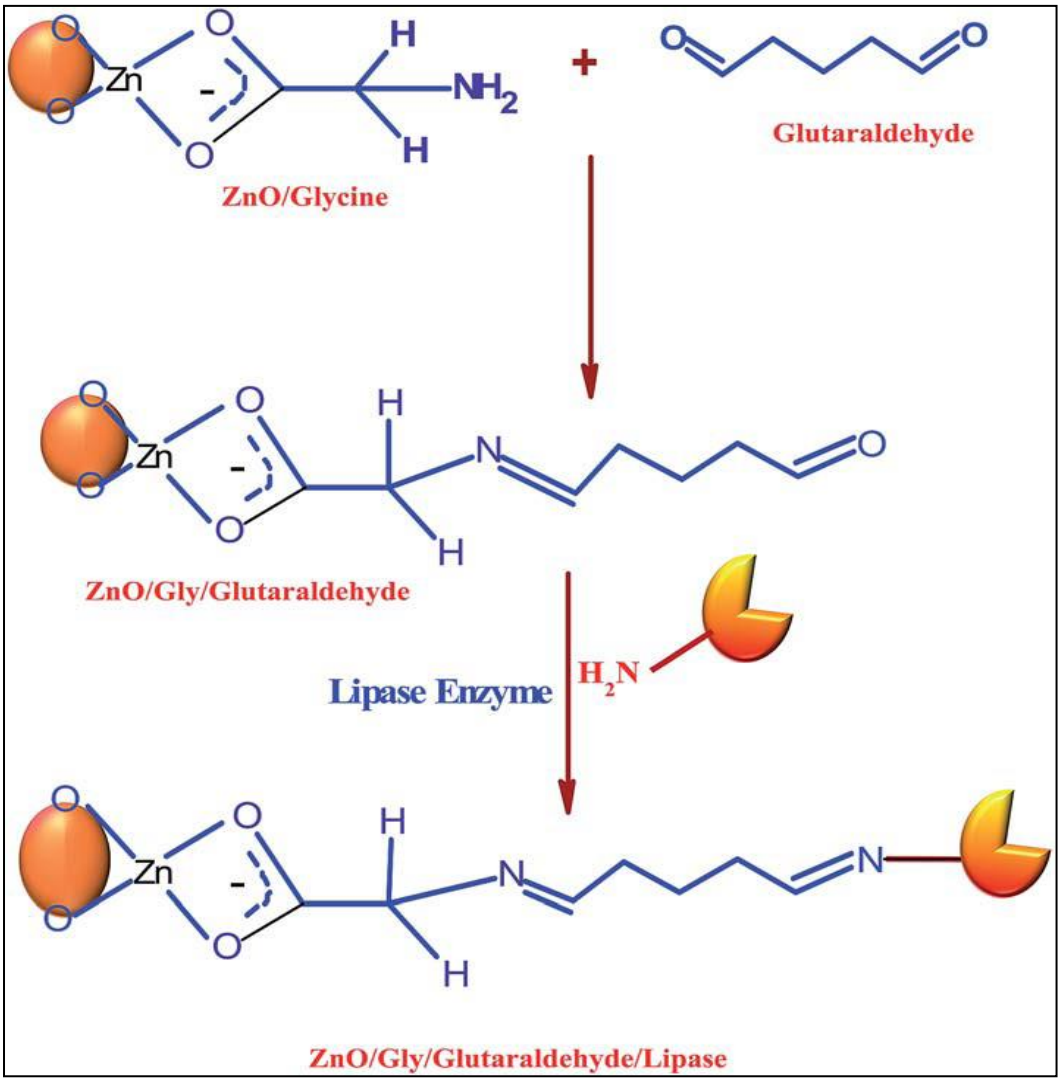
3.2.5 Enzyme Assay. Lipase activity was assayed using p-nitrophenylpalmitate (p-NPP) as substrate.[35]The basis of this assay protocol is the colorimetric estimation of p-NP released as a result of enzymatic hydrolysis of p-NPP at 410 nm. Enzyme solution (0.8375mg/50 μL) was added to 950 μL of the substrate solution consisting mixture of solution A (50 mL, 5.0 mMp-NPP in 2-propanol) and solution B (900 mL, 100 mM potassium phosphate buffer with pH 7.0, 0.4 % Triton X-100 and 0.1 % gum arabic), which were freshly prepared before use. The reaction mixture was incubated at 37°C for 10 min. The reaction was then terminated by adding 1mLethanol. The absorbance of the yellow color product p-nitrophenol (p-NP) was measured in spectrophotometer at 410 nm. Enzyme activity and specific activities were calculated as per the following formula,

$$Lipase\ Activity\ \left(\frac{U}{mL}\right) = \frac{A \times B}{C \times D \times E} \qquad (1)$$

Where, A,B,C,D and E are μmol of p-NPA released, total volume of the reaction mixture, volume used in spectrophotometric determination, volume of enzyme used in assay and time of incubation respectively.One International Unit of lipase

activity was defined as the amount of enzyme catalyzing the release of 1 µmol of p-NP per min from p-NPP under the standard assay conditions.[36]

Specific activity (U/mg) = {Enzyme activity (U/mL)/ Protein content (mg/mL)} (2)



Scheme 3.3.Covalent immobilization of *TL* lipase enzyme on ZnO nanoparticles.

3.2.6 Operational Stability of immobilized lipase

3.2.6.1 Effects of pH on enzyme activity

Lipase activity was measured in different buffers (0.05M) of pH (5 - 12) viz.sodium citrate (pH 4.0–6.0), sodium phosphate (pH 7.0–9.0) and glycine–NaOH (pH 10.0–11.0) at 37 ^{o}C by incubating the free and immobilized lipase with p-NPP (maintaining other conditions same as mentioned above) for 1 h. The lipase activity was measured by the above standard assay method.[35]

3.2.6.2 Effect of Temperature on enzyme activity

The optimum temperature of lipase activity was determined by carrying out enzymatic reactions at different temperature (55, 65, 75, 85 and 95 ^{o}C) at pH 7.4 by incubating the free and immobilized lipase with p-NPP for 1 h. Subsequently, NaOH (1N, 1 mL) was added to stop further hydrolysis and then the lipase activity was determined by above mentioned assay method.[35]

3.2.6.3 Thermal stability of immobilized enzyme

Thermal stability of free and immobilized lipase was studied at 55 ^{o}C, 65 ^{o}C and 75^{o}C. The stability was studied by collecting the aliquot at different time interval and then lipase activity was determined by standard assay method.[35]The stability of the support ZnO/glycine/glutaraldehyde was also studied using differential thermal analysis.

3.2.7 Esterification reaction

Immobilized TLL on ZnO NPs was used as biocatalyst for esterification of oleic acid with methanol. For the reaction, 100 mg of immobilized lipase was dispersed in 5 mL of petroleum ether at room temperature. To this solution, oleic acid (5.95 mmol), methanol (2.97 mmol) and molecular sieves (1.6 g, 3 A^o) were introduced. The temperature of the reaction then gradually rose to 55^{o}C. After about 10 h of reflux, further methanol (2.97 mmol) was added and the reaction was allowed to continue for about 14 h. Progress of the reaction was monitored on TLC plate. The purity of the product, after the completion of reaction, was determined by HPLC For ^{1}H,^{13}C NMR, and FTIR spectral analysis the reaction mixture was passed through column having silica as stationary phase and ethyl acetate:petroleum ether as eluent (Scheme 3.2).

Methyl oleate: ^{1}H NMR: (400 MHz, $CDCl_3$) δ(ppm): 5.37 (m, 2H), 3.69 (s, 3H), 2.33 (2H), 2.19-1.70 (m, 4H), 1.62 (m, 2H), 1.47-1.28 (m, 20H)), 0.88 (t, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 174.31, 129.98, 129.73, 51.42, 34.09, 31.90, 29.76, 29.68, 29.52, 29.32, 29.15, 29.08, 27.21, 27.15, 24.94, 22.68, 14.16.

3.2.8 Characterization of synthesized nanoparticles and esterification products

X-ray powder diffraction (XRD) pattern of the ZnO/Gly NPs and ZnO/gly/glutaraldehyde/Enzyme NPs were obtained from X-ray powder

diffractometer (Bruker D8 Advance) with Cu Kα radiation, λ=0.15418 nm. The nanoparticles were dispersed in water, sonicated for 10 minutes, and then UV-Visible absorption spectra were recorded by means of Perkin-Elmer Lambda 35 UV-Visible spectrophotometer. Photoluminescence (PL) spectra were recorded on a Jasco FP-6300 using xenon lamp as the excitation source at 260 nm. The size and shape of the nanoparticles were studied by means of Transmission Electron Microscopy (TEM, Philips Tecnai 20). A BIC 90 plus (Brookhaven) equipped with 35.0 mW solid state laser operating at 660 nm and an avalanche photodiode detector was used for the measurement of surface charges in term of zeta potential (ξ). All measurements were made at 25^{o}C in deionized water. Differential scanning calorimetric (DSC) analysis of ZnO /glycine/glutaraldehyde was also carried out using Mettler Toledo DSC 822. For the purpose, the material was heated inside a DSC set-up. The heating rate was 10 ^{o}C /min from RT to 500 ^{o}C in N_2 atmosphere. The FTIR (Perkin-Elmer, RX-FTIR) spectra of the samples (biocatalyst and esterification products) were obtained in the range of 400 to 4000 cm^{-1}. The ^{1}H NMR and ^{13}C spectra of the esterification products were recorded on a Bruker Advance 400 MHz spectrometer using TMS as an internal standard in $CDCl_3$. HPLC analysis was carried out by using Shimadzu LC-20AD having C18 column (250 nm x 4.5μ) and methanol:H_2O (80:20 v/v) as mobile phase with a flow rate of 1 mL/min for 30 minutes.

3.3 Results and discussion

3.3.1 Compositional and Morphological studies

The XRD patterns of the as-synthesized glycine functionalized ZnO NPs (ZnO/Gly) are shown in Fig. 3.1 (a). The XRD patterns manifest predominant diffraction peaks at 2θ values 31.7, 34.3, 36.2, 47.4, 56.5, 62.7, 66.3, 67.8, 69.0, 74.4 and 76.8. These peaks are well matched with standard JCPDS card No. 89-1397 corresponding to ZnO with hexagonal wurtzite phase. The particle size was calculated using Debye-Scherrer formula [37] ($D = 0.9\lambda/\beta\cos\theta$) and the FWHM (Full width at Half Maximum) value corresponding to the major plane (101), was 40 nm. On glutaraldehyde activation and covalent attachment of lipase on the ZnO/gly NPs, the XRD peak positions almost remain same with change in intensity. It can be observed (Figure 3.1) that on loading enzyme, the intensity of (002) peak increases indicating preferred unidirectional growth. The compactness and clear resolution of the peaks towards lower angles indicate properly ordered and compact enzyme layer on the surface of NPs. Further, the size and shape of ZnO/Gly NPs were studied by TEM analysis. The TEM image of pristine ZnO/Gly NPs shows almost monodispersed spherical particles with an average size 9 – 16 nm (Figure 3.2 a). On covalent binding of an enzyme, the size and shape of the NPs almost remains same (Figure 3.2 b). The surface morphology of the pristine ZnO/Gly NPs was studied by FEG-SEM analysis (Figure 3.3). It can be seen from

the Figure 3 that the surface of the NPs is highly porous. The pores and cracks are formed due to agglomeration of NPs on the surface, indicating that the surface is capable to show catalytic activity.

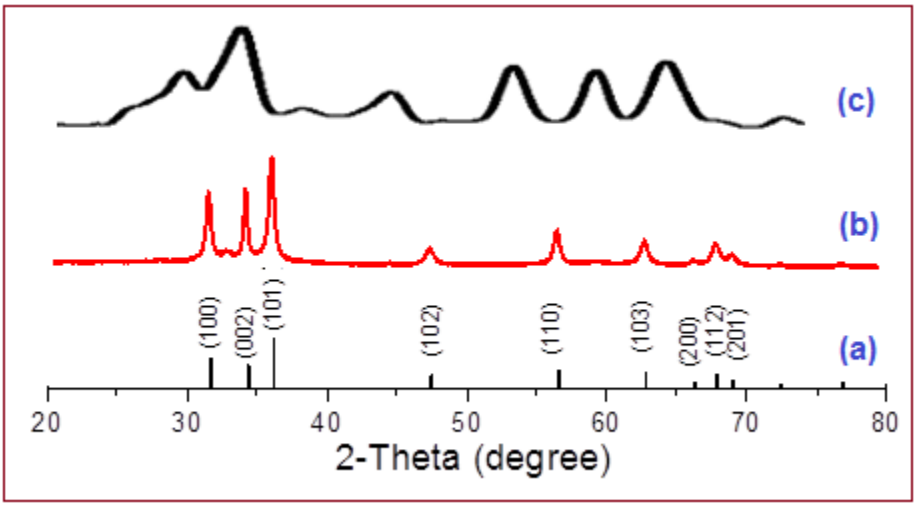
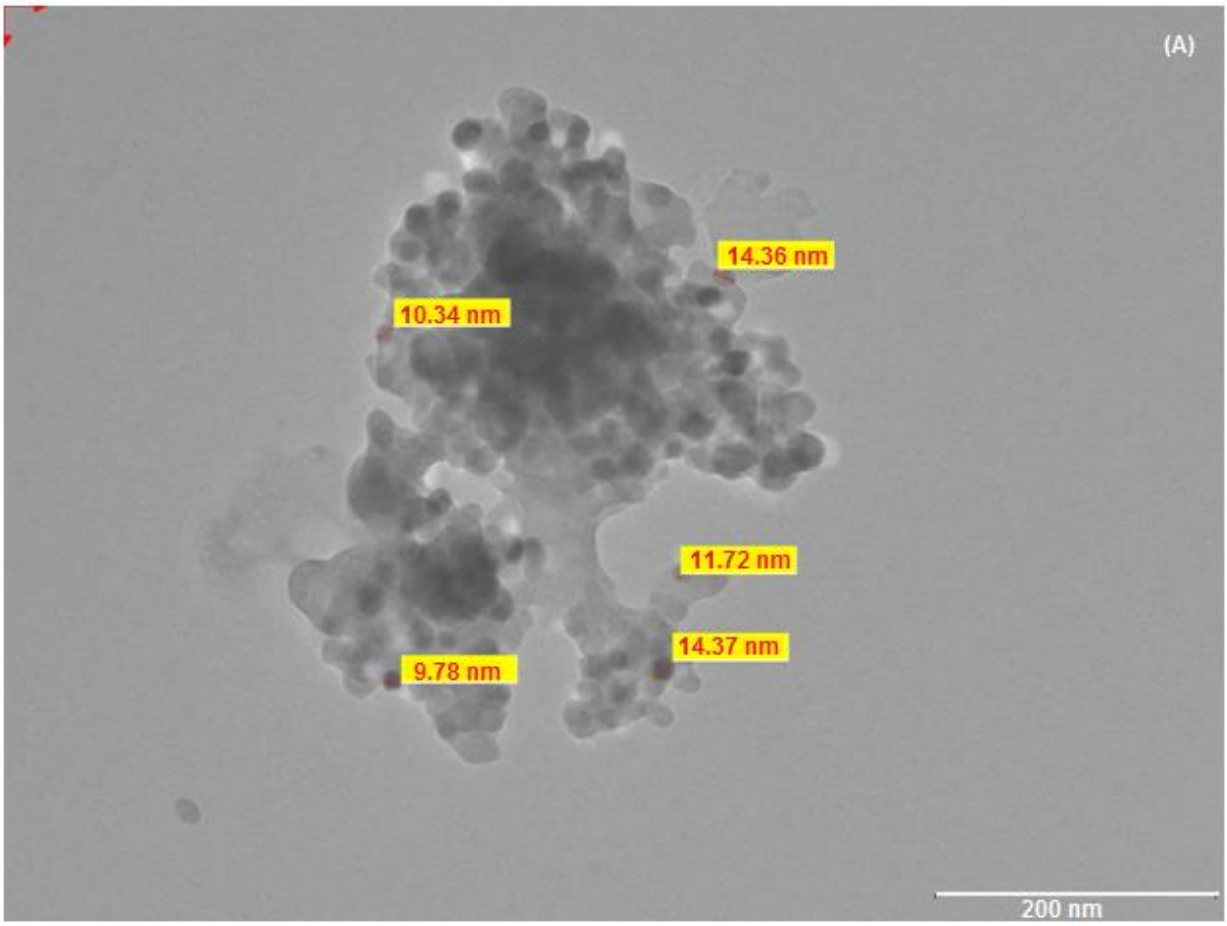


Figure 3.1. XRD patterns of (a) bulk ZnO nanoparticles (JCPDS card No. 89-1397) (b) glycine functionalized ZnO nanoparticles (c) *TL* Lipase enzyme immobilized ZnO nanoparticles.


(A)
14.36 nm
10.34 nm
11.72 nm
14.37 nm
9.78 nm
200 nm

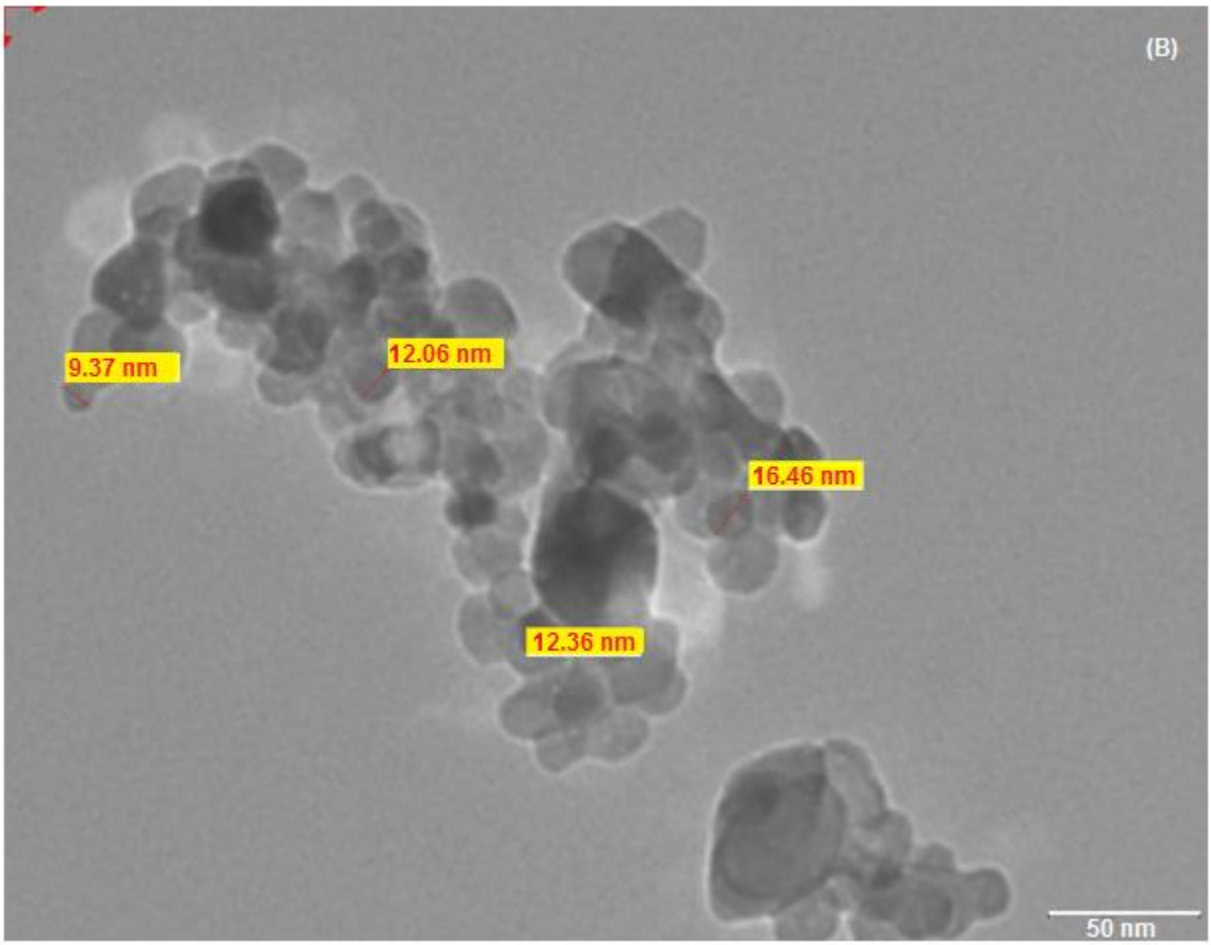


Figure 3.2. TEM images of (A) ZnO/Gly nanoparticles (B) TL enzyme immobilized ZnO/Gly nanoparticles.

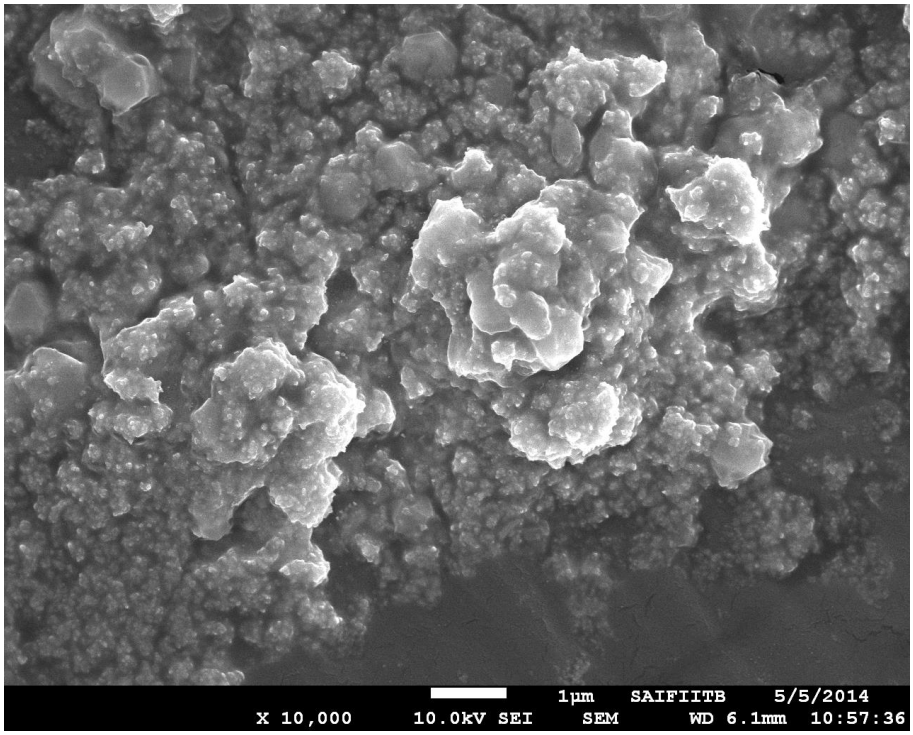


Figure 3.3. FEG – SEM image of glycine functionalized ZnO nanoparticles.

3.3.2 Optical studies. The quality of the pristine ZnO/Gly NPs was further confirmed using optical studies. As can be seen from Figure 3.4 that ZnO/Gly NPs absorb sharply at 371 nm which is blue shifted when compared with bulk ZnO (380 nm). This observation confirms the quantum confinement and nano regime.[38] When suspended in ultrapure water and excited at 200 nm, the ZnO/Gly luminesces at 425 nm. It can be observed from the Figure 3.5 that virgin enzyme fluoresce at 470 nm and on covalent binding with glutaraldehyde on the surface of ZnO/Gly NPs, it is blue shifted to 360 nm. Haldar et al explained the phenomena of blue shifting of $\lambda_{max}$of enzyme on excitation at a suitable wavelength due to unfavorable interactions of dipole moments of polar solvent molecules with

fluorophores of enzyme under excited state.[39] This observation may lead to assume the conformational changes in enzyme and the exposure of catalytic hydrophobic triad (Asp-Hys-Ser) in aqueous medium on covalent binding.

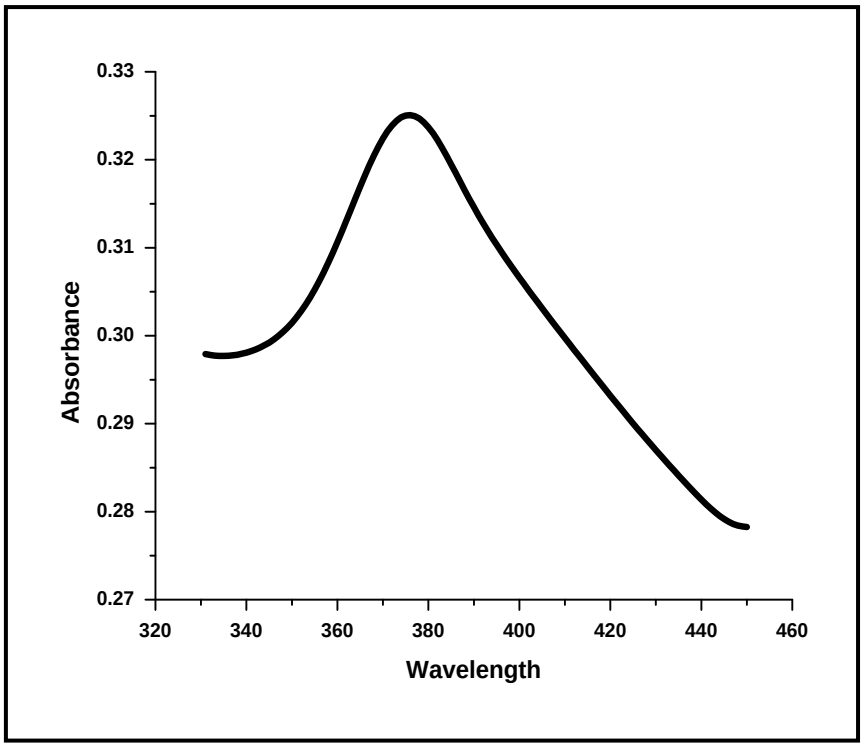
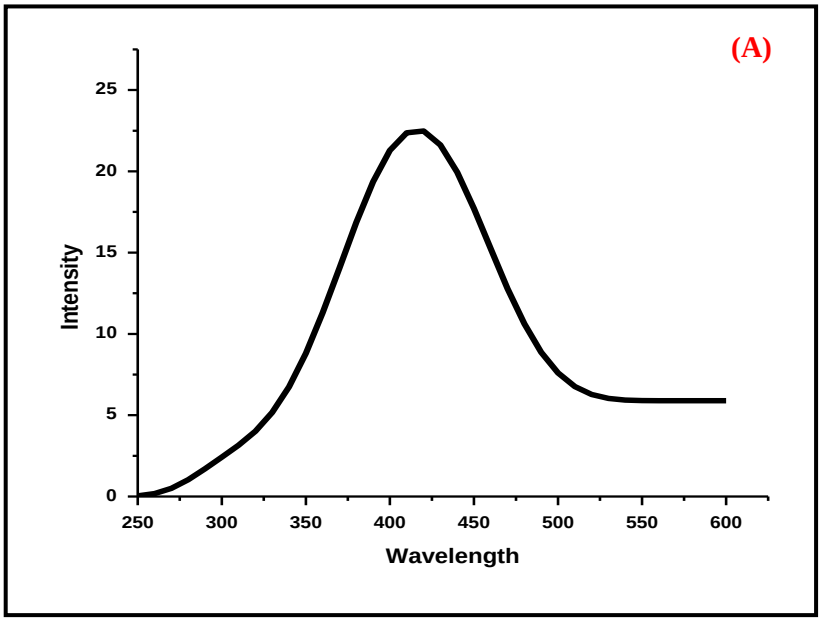


Figure 3.4.UV absorption spectrum of ZnO/Gly nanoparticles.



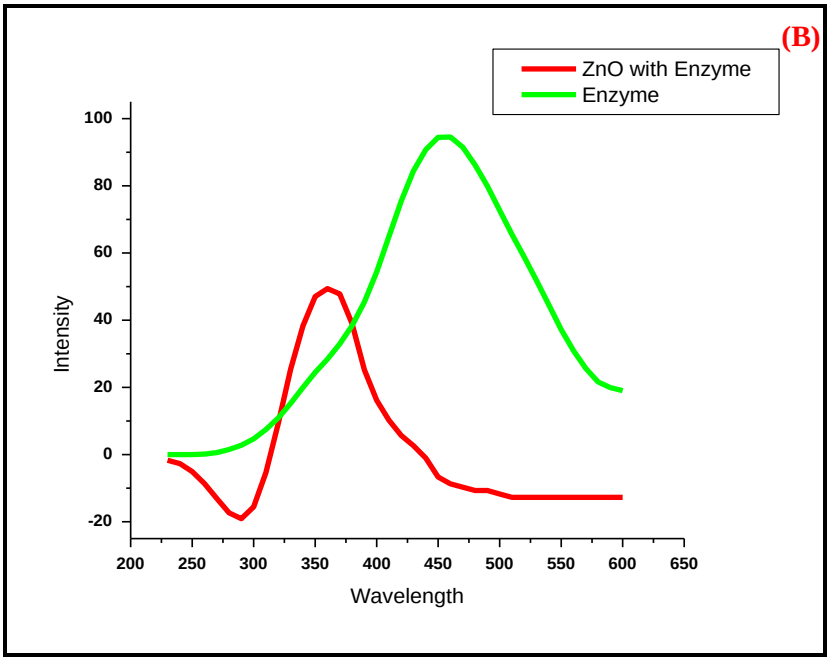


Figure 3.5. Photoluminescence spectra of (A) ZnO/glycine nanoparticles (B) free TL enzyme and TL enzyme immobilized ZnO nanoparticles.

3.3.3 Surface charges. Surface charges on the NPs were studied by zeta potential (ξ) measurement. It gives an idea about the electrostatic charge stabilization during the growth of NPs. From Table 3.1 it can be seen that pristine ZnO/Gly NPs possess negatively charged surface with ξ value -17.15 mV and on glutaraldehyde activation and enzyme binding the surface charge decrease to -6.12 mV. The emergence of charge on the surface is directly influenced by the presence of counter ions in the medium and mode of crystallization. [40] The negative charge on pristine ZnO NPs is due to presence of $^{-}$OH ions in the basic medium. During crystal growth, both $^{-}$OH ions and glycine act as capping agents, however, $^{-}$OH

ions are more prone to be adsorbed on the surface of NPs while glycine makes coordination with the surface Zn^{2+} ions. On glutaraldehyde activation and covalent binding of enzyme, as proposed earlier, the conformational changes in enzyme takes place which increases the hydrophobicity and expel the ^{-}OH ions or polar water molecules present in the vicinity. However, the polar molecules could not be totally expelled due to surface coordinated hydrophilic glycine molecules. This fact is expressed in term of decrease in ξ value to -6.12.

Table 3.1. Surface charge analysis of as-synthesized nanoparticles.

System	**Charge, ξ (in mV)**
ZnO/glycine	-17.15
ZnO/Glycine/Glutaraldehyde/TLL enzyme	-6.12

3.3.4 FTIR Study. To understand the interactions among the ligands and the surface of ZnO NPs, FTIR spectroscopy is one of the best tools. The mode of interaction of carboxylate ions present in an ionic glycine can be confirmed from the vibrational spectroscopy (FTIR). Figure 3.S1 shows FTIR spectra of native enzyme, ZnO/Gly and ZnO/Gly/Glutaraldehyde/Enzyme. In FTIR spectra of native

enzyme (Figure 3.S1 a), the peak near 1632 cm^{-1} is amide I band due to C=O stretching vibrations, the hump near 1510 cm^{-1} is amide II band due to N-H bending/C-H stretching vibration, the amide III band at 1222 cm^{-1} is due to N-H bending vibration. The band at 3430 cm^{-1} is due to N-H bending and at 1404 cm^{-1} is resulted from protein side chain COO^-. [41, 42] Figure 3.6 shows the ways by which carboxylate ion of glycine (at pH 7.7, isoelectric point 6.0) can coordinate to the surface Zn (II) ion either as a unidentate ligand or as a chelating (bidentate fashion) ligand.[43 -46]

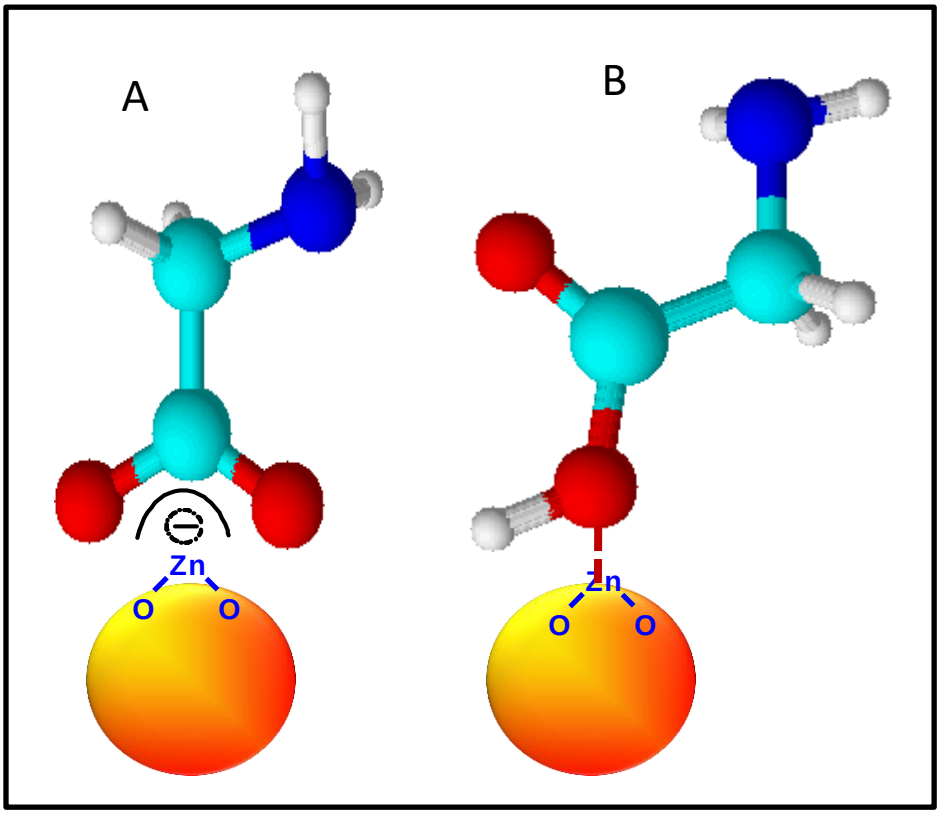


Figure 3.6.Modes of interaction of glycine with the surface of the ZnO nanoparticles. (Orange balls indicate ZnO nanoparticles, light blue for carbon atoms, red for oxygen atoms, white for hydrogen atoms and blue for nitrogen atom).

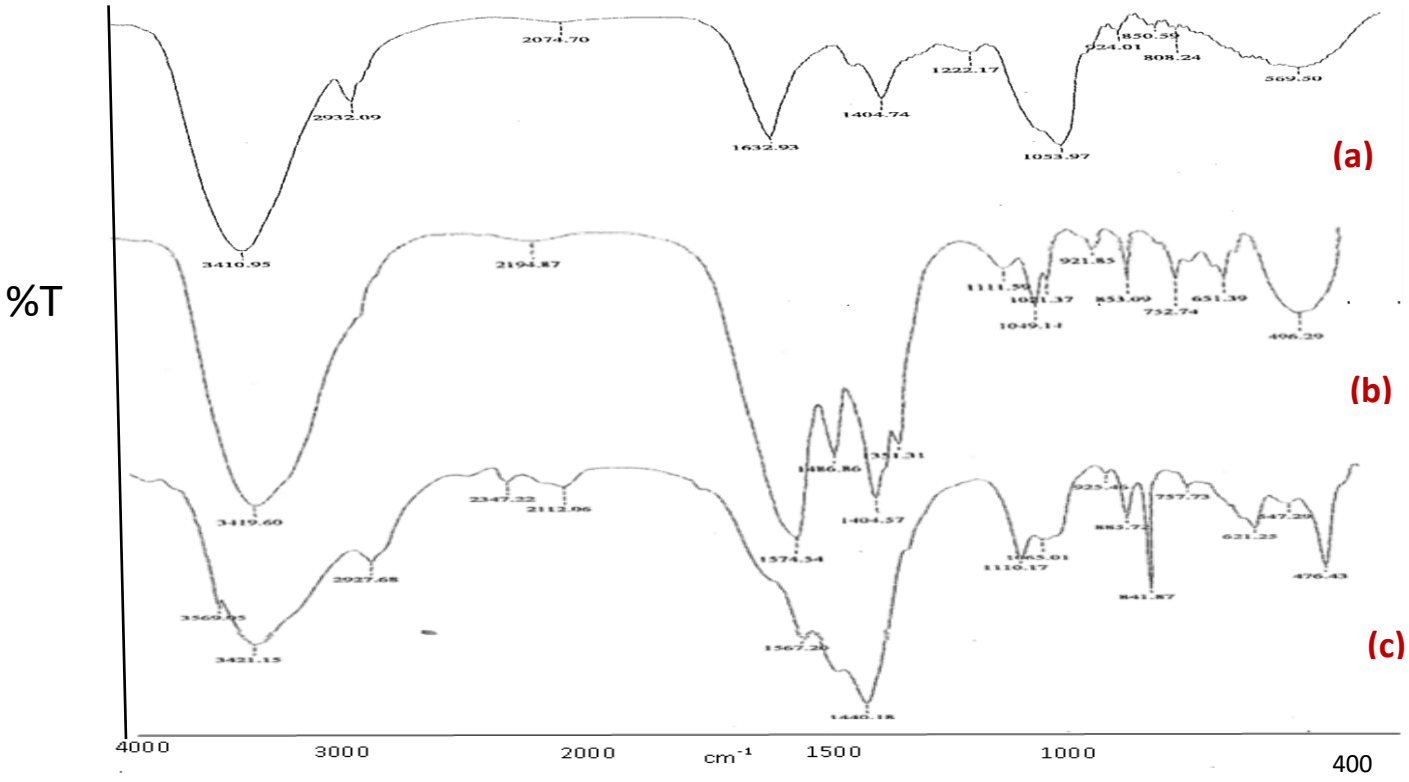


Figure 3.S1 :FTIR spectra of (a) free TLL enzyme (b) ZnO/Glycine nanoparticles (c) ZnO/Glycine/glutaraldehyde/Enzyme nanoparticles.

This can be detected on the basis of COO^- stretching vibration frequencies. On the basis of a normal coordinate treatment two fundamental modes of vibrations of carboxylate ion in free acetate form can be observed: one is COO^- asymmetric stretching mode ν_{as}(-COO-) and the other is symmetric stretching mode ν_s(-COO-). The fundamental frequencies of vibrations for free acetate ion are 1583 and 1422 cm^{-1} for ν_{as}(-COO-) and ν_s(-COO-) respectively. If carboxylate ligand coordinate with surface Zn(II) in bidentate mode then ν_{as}(-COO-) decreases and ν_s(-COO-) increases from the normal modes in free state and vice versa in case of

monodentate mode. [47] On comparing Δ (ν_{as}(-COO-) - ν_{s}(-COO-)) with Δ' (ν'_{as} (-COO-) – ν'_{s}(-COO-)), we found Δ>Δ', (where Δ indicates difference in the absorption bands for free carboxylate ion and Δ' indicates the same for metal bound carboxylate ion) which suggests bidentate coordination of glycine with Zn (II) ion on the surface of ZnO NPs. On glutaraldehyde activation and enzyme covalent binding, the broad amide peak becomes sharp and blue shifted compared to ZnO/glycine due to high energy peptidal N-H stretching vibrations and at 1440 cm^{-1} imine stretching vibrations indicating covalent binding of *TLL* enzyme with glutaraldehyde on the surface of ZnO NPs (Figure 3.S1 C).

It was observed from the previous studies that the catalytic activity of the most of the lipase enzymes is due to Ser -His-Asp/Glu catalytic triad present inside the surface loop having hydrophobic environment. This catalytic triad is not available to the substrates in aqueous medium and under normal conformation of enzyme. However, this hydrophobic catalytic triad can be made available to the substrate by forcing the enzyme to change its conformation. It was proved that at lipid/water interface the microenvironment around the enzyme forces it to change its conformation in such a way that the active sites (triad) expose on the surface and easily available to the substrate.[48 -51] It may be assumed that by proper tuning of the environment around the bound enzyme its suitable conformation can be locked. The lipophilic - lipophobic balance in vicinity of enzyme plays a key role to

achieve the catalytically active conformation. We have designed the enzyme based on the idea that both hydrophilic (in term of zwitterionic glycine around the solid ZnO NPs) and hydrophobic (in term of glutaraldehyde chain) environment mimicking the lipid/water interface available to enzyme which stabilize the catalytically active conformation of enzyme in hydrophobic (organic) medium.

3.3.5 Operational stability of lipase enzyme

3.3.5.1 Effect of pH. Figure 3.7 shows the effect of pH on lipase activity measured by spectrometric method. It can be observed that the immobilized TLL enzyme shows always higher activity than free form at the pH range 4-13. The free enzyme shows very less activity at pH 4.0 which increases with rise in pH. While immobilized lipase manifest constant increase in activity with rise in pH, it can be observed that enzyme activity increases to 21 from 19 U/mL on immobilization at pH 5 while at pH 12 it is increases to 26 from 22.5 U/mL of free counterpart. So it can be concluded that both free and bound enzyme show substantial stability at lower pH and it is increases with pH. Higher enzyme activity can be achieved in basic medium. Ortega et al. also reported enhanced stability of lipase at acidic and alkaline pH upon immobilization.[52]The increase in stability upon immobilization might be due to multipoint covalent linking between lipase and support, which prevents lipase denaturation in acid or alkaline environments.[53, 36]Similar results

were obtained with the immobilized Candida rugosa lipase on chitosan by Hung et al.[54]

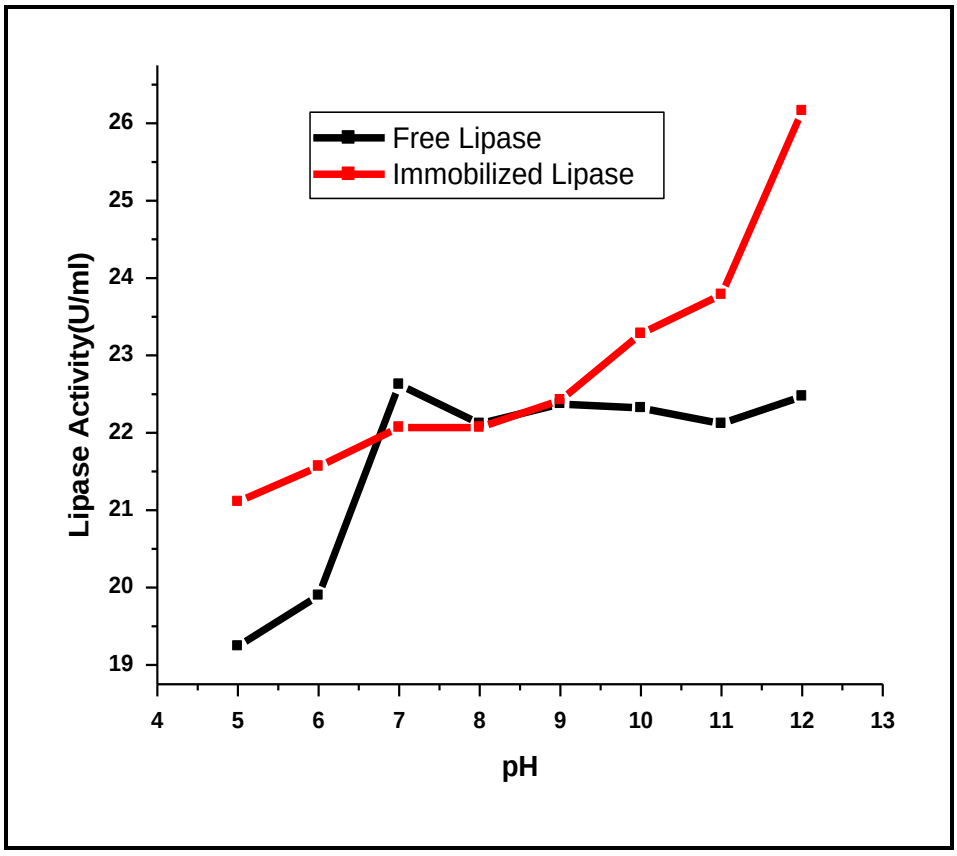


Figure 3.7.Effect of pH on hydrolysis of p-NPP at 40 ^{o}C.

3.3.5.2 Effect of temperature. It is a fact that all enzymes denature and show degradation in activity at higher temperatures. It can be observed from the Figure 3.8 that the activity of lipase increases three folds on immobilization and maximum activity can be achieved at 55 ^{o}C. At 65^{o}C the immobilized enzyme shows almost same activity which would have been shown by free enzyme at 55^{o}C i.e. on immobilization the operational stability and activity of the TLL enzyme can be

extended to 10°C. Industrial point of view, the immobilized enzyme shows moderate activity upto 75°C.It is interesting to note that at 95°C, both free and immobilized enzyme show some activity on hydrolysis of p-NPP. To explain this, a controlled experiment using only glycine functionalized ZnO nanoparticles (without any type of enzymes) was carried out. Similar kind of p-NPP hydrolysis activity was observed in the range of 90-95 °C. Hence, it can be inferred that this 'residual' activity is due to the thermal effect and not to the enzyme activity.

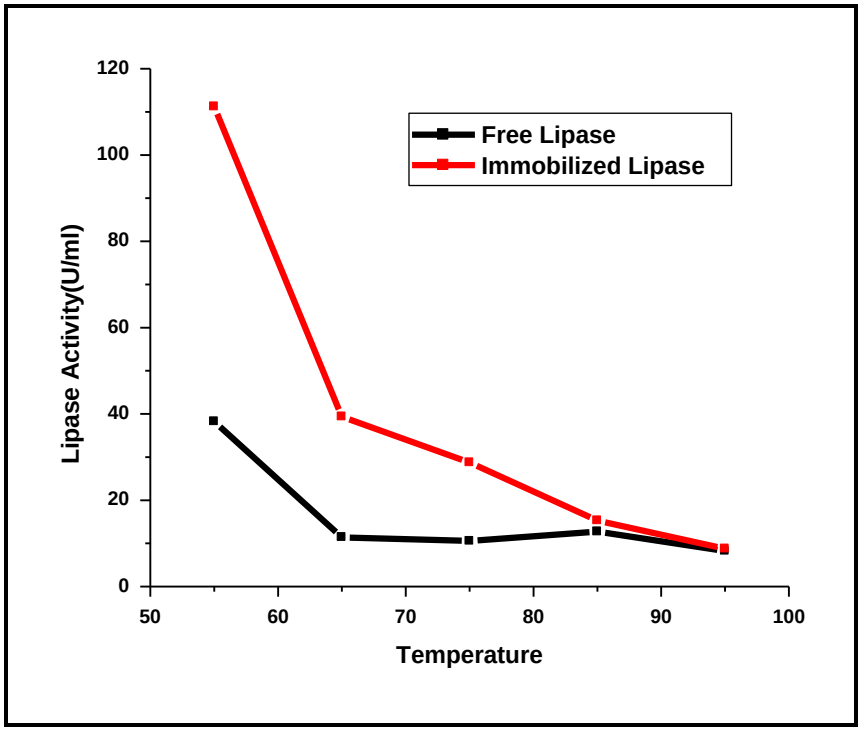
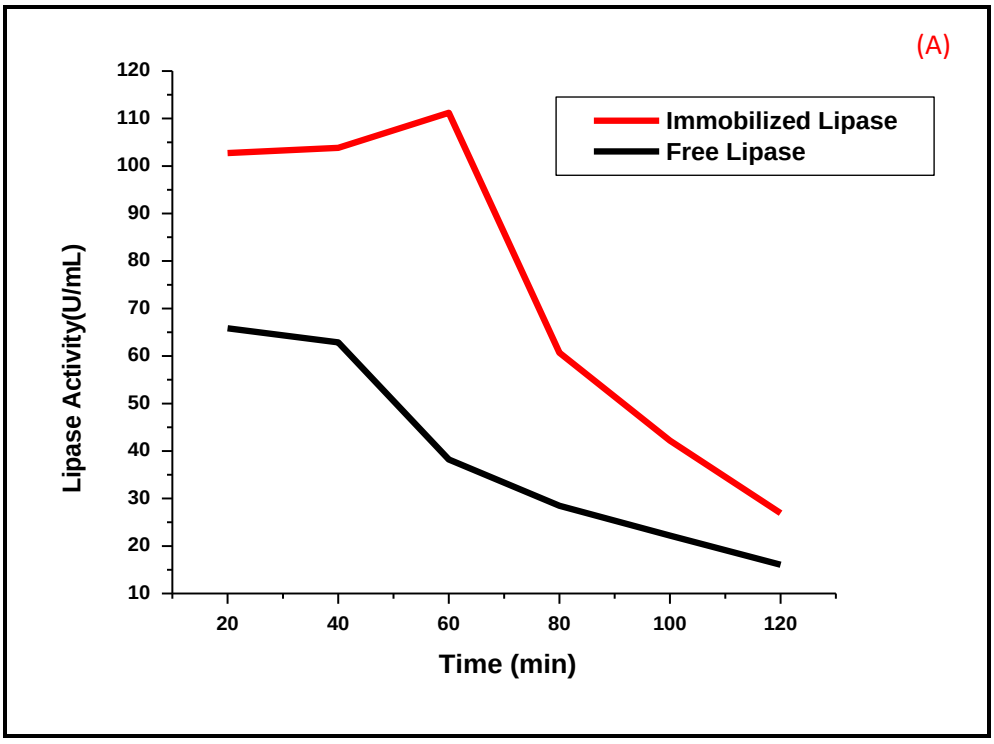
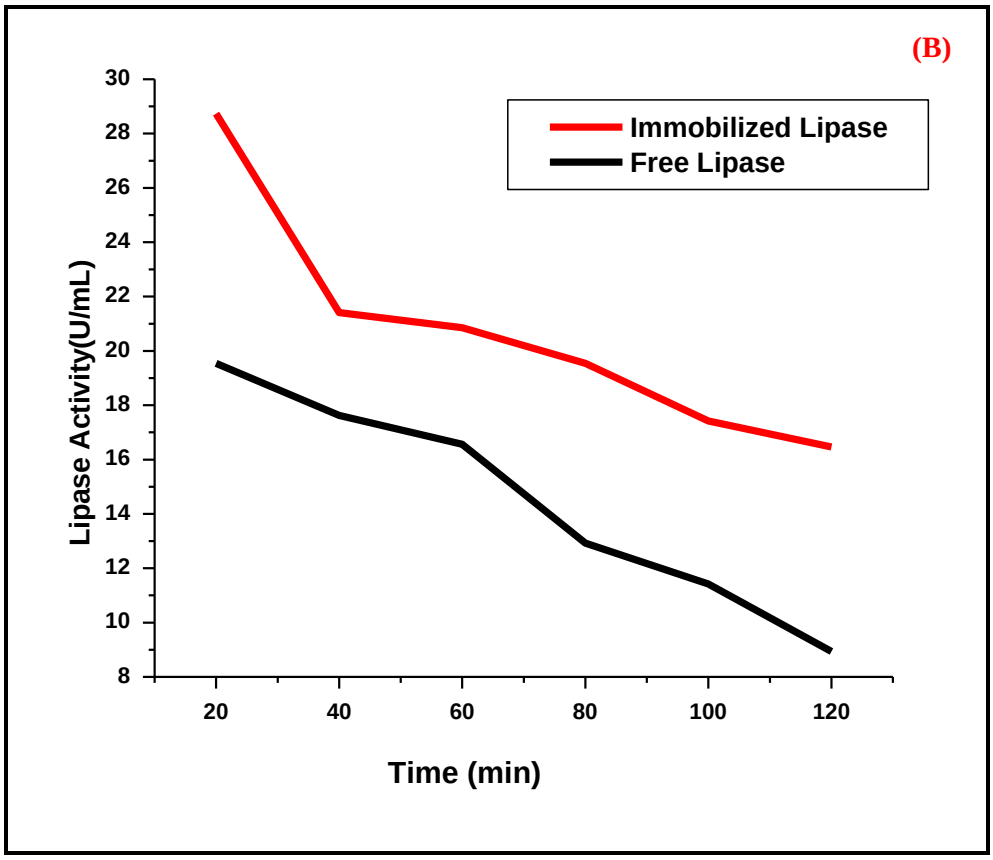


Figure 3.8. Effect of temperature on hydrolysis of p-NPP at 7.4 pH.

3.3.5.3 Thermal stability. Based on temperature studies, we have selected 55 °C, 65 °C and 75°C to monitor the thermal stability of immobilized lipase (Figure 3.9). Both type of enzymes exhibited a similar trend; however, the immobilized lipase is

more stable than that of free one. The half-life of the immobilized lipase is much longer than the free lipase at these three temperatures. This study also supports our argument that the immobilized lipase shows three folds increase in activity than its free counterpart at 55^{o}C.It is obvious that with increase in temperature the efficiency of both the enzymes decreases. We adopted a rational approach to design immobilized lipase catalyst. Firstly, ZnO NPs were functionalized by hydrophilic glycine and then hydrophobic glutaraldehyde activation was carried out before binding the enzyme.




(B)
Immobilized Lipase
Free Lipase
Lipase Activity(U/mL)
Time (min)
30
28
26
24
22
20
18
16
14
12
10
8
20
40
60
80
100
120

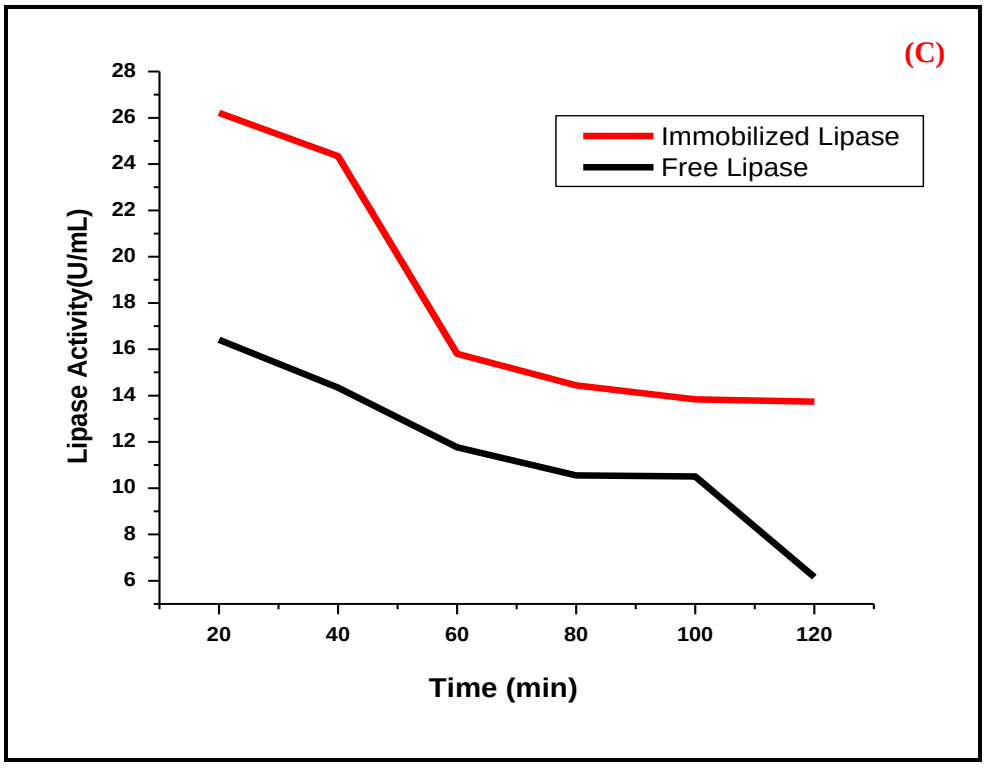

(C)
Immobilized Lipase
Free Lipase
Lipase Activity(U/mL)
Time (min)
28
26
24
22
20
18
16
14
12
10
8
6
20
40
60
80
100
120

Figure 3.9.Thermal stability of *TL* Lipase at (A) 55, (B) 65 and (C) 75 ^{o}C and 7.4 pH.

The stability of resulting assembly (ZnO/gly/glutaraldehyde) was confirmed from thermal analysis (Figure 3.10). Two major weigh losses were observed on TG curve. The first loss (11.0%) occurred at a temperature range between 50 ^{o}C to 110^{o}C accompanied by an endotherm at 104 ^{o}C with a rate of decomposition 0.426 mg/min then the curve becomes almost constant up to 400^{o}C. The horizontal part of the curve between 150 ^{o}C to 400 ^{o}C indicates stability of the support throughout the temperature range. After this, 8.1% weigh loss at 458 ^{o}C can be observed in term of exotherm in DTA curve with maximum rate of decomposition 0.84 mg/min. This may be due to degradation of covalent bonding on the surface of ZnO NPs. This is a straightforward confirmation of presence of hydrophilic/hydrophobic environment on the surface.

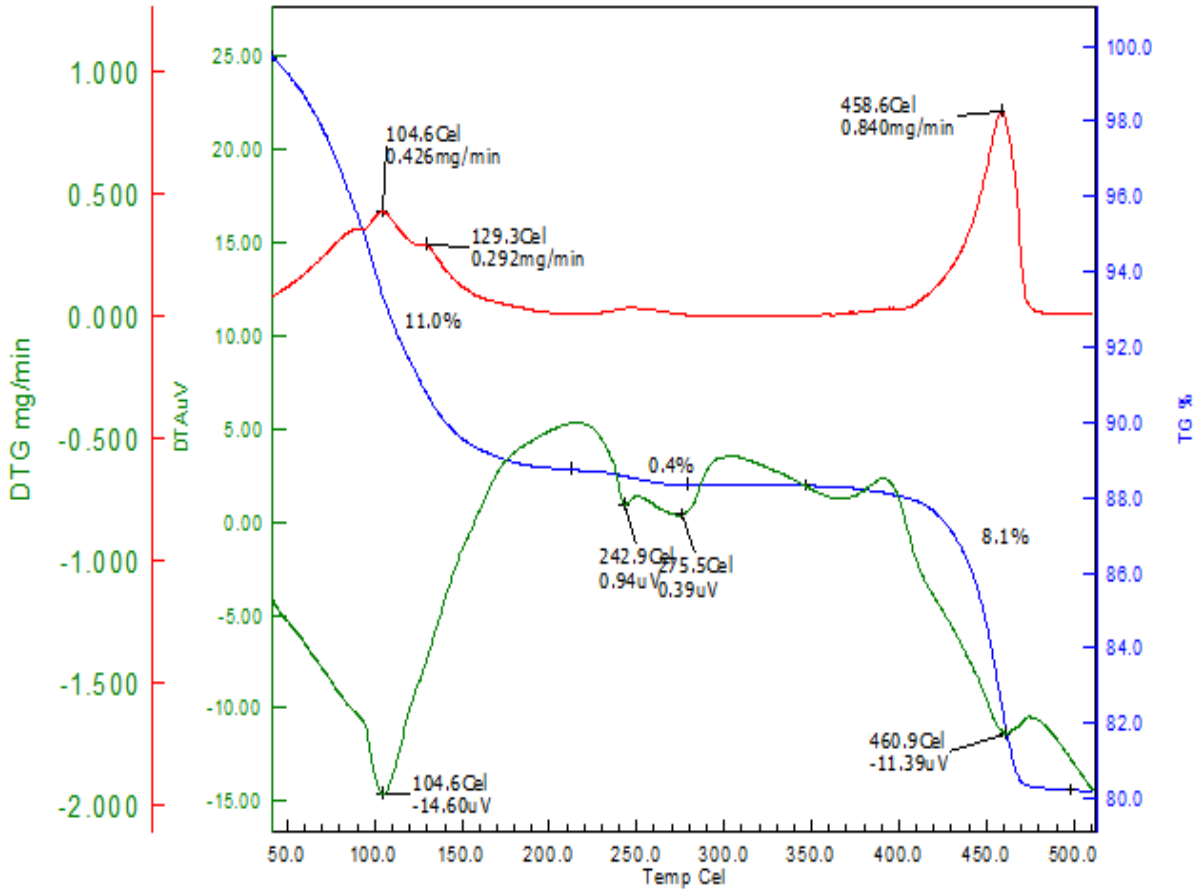
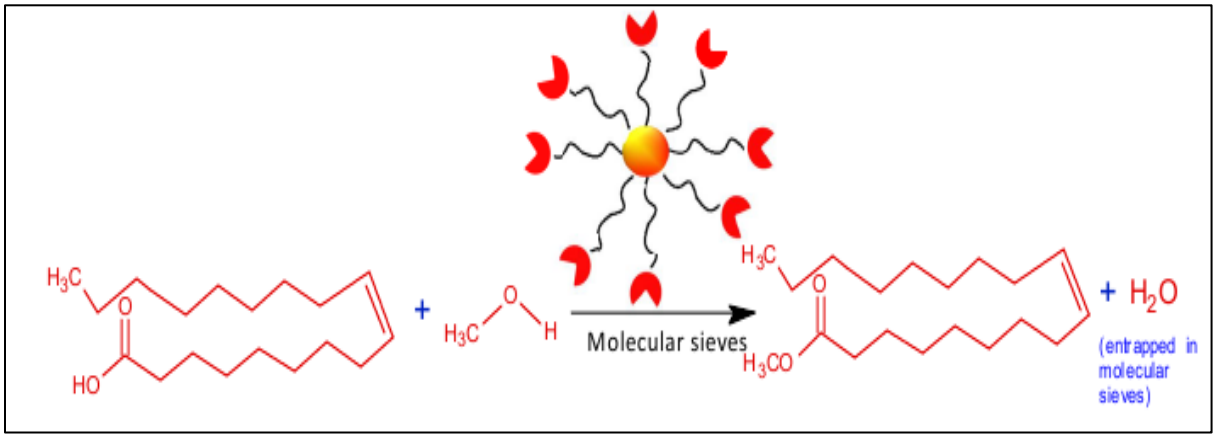


Figure 3.10.Thermal analysis of ZnO/gly/glutaraldehyde system.

3.4 Esterification Reaction. Esterification of long chain fatty acid with alcohol is equilibrium dominated reaction. The rate of reaction becomes slow as it approaches towards the equilibrium and also with the rise in the amount of water as by-product in the reaction mixture. Generally, enzymes are involved to carry out reactions/biotrasformaions in aqueous media. The enzyme activity is greatly hampered by shifting the reaction media from aqueous to non-aqueous. The other factors like resistance of the substrate to diffuse into the immobilized matrix and resistance of the product to diffuse out (diffusional limitations), pH of the medium,

flexibility of the enzyme molecules etc directly affect the efficiency of the enzymatic reactions. [50] The problem of diffusional limitation can be solved by inducing hydrophilic/hydrophobic environment on the surface of the support, the substrate (long chain fatty acid) can be attracted and oriented near the hydrophobic chains of supported matrix such that there would be maximum hydrophobic-hydrophobic interactions before undergoing the reactions in enzymatic lids and the products formed can also be diffused by the same mechanism. The advantage of presence of hydrophilic area near the support is that the by-product water can be collected at the beginning and then entrapped in molecular sieves at certain level. Hence, the equilibrium can be shifted continuously to forward direction by 'pseudo' removal of water from the reaction mixture (Scheme 3.2). Hence, the supported enzyme assembly can be viewed as 'nano reactor' to which the diffusion of reactants and products continuously takes place with instant entrapment of by-product.



Where,

= Glycine/Glutaraldehyde/TL Lipase Enzyme

= ZnO nanoparticles

Scheme 3.2.Esterification of oleic acid (C-18) with methanol in presence of *TL* lipase enzyme immobilized on ZnO nanoparticles.

The maximum esterification yield (90%) is obtained in presence of TLL enzyme (20 mg/mL) with oleic acid (C18) and methanol in the molar ratio of 1:1 in petroleum ether as solvent at 40 ^{o}C. On HPLC analysis, the purity of the product was found to be 84% for the first cycle and 82.5 and 81% for the next two consecutive cycles (Figure 3.S3).

3.5 Leaching study. The stability and reusability of the immobilized enzyme is very important. The leaching study of immobilized enzyme was carried out using n-hexane as washing medium.[55] The recycled immobilized TLL enzyme was used 4 consecutive cycles using 20 mL n-hexane for washing each cycle exhibiting

almost same activity as in the first cycle, however, after 4th cycle there was fall in the activity and become almost 80% compared to the first cycle.

3.6. Conclusion

From this study it can be concluded that (1) microenvironment surround the immobilized enzyme plays an important role for activity and favorable enzymatic conformation can be obtained by mimicking the environment existing at lipid/water interface (2) diffusion limitation which is the major factor to restrict the enzyme activity can be controlled by prior designing of the biocatalyst (3) maximum catalytic activity and reusability can be achieved for several cycles by proper covalent binding of the biocatalyst with the solid support and (4) immobilized lipase enzymes can serve as a 'green catalyst' for the production of biodiesel. This study may provide a direction to future research to achieve maximum catalytic activity from the enzyme by changing its microenvironment eg. using 'spacer' molecules having different chain length or different amino acid molecules as capping agent around the support altogether with optical activity from the metal oxide support which also may be useful in the area of developing bio-sensors for enzymatic reactions.

Advances in our research on the esterification reaction through the comparison with the results reported in the previous literatures.

Notes

There are many advantages of using immobilized *Thermomyces lanuginosus* lipase on ZnO nanoparticles for the production of esters of oleic acid. As mentioned, esterification of oleic acid is a model experiment for the production of biodiesel by esterification of fatty acids.

Esterification of fatty can be carried out in many ways. Most widely used process is alkaline catalysis of transesterification using sodium hydroxide. However, this results in saponification and decreases the efficiency of the process.

Acid catalysis is also often used but it is associated with many drawbacks like corrosiveness, lower yield, and slow reaction and requires high temperature condition.

On the other hand use of lipase for the production of biodiesel or esters is an alternative to the above mentioned process.

Immobilization of enzyme on solid support results in increase of activity, tolerance to pH and thermal conditions.

Moreover utilization of immobilized *thermomyces lanuginosus* lipase on ZnO nanoparticles for esterification of oleic acid has many advantages over the traditional solid supports. Few are mentioned as below:

1. The ZnO nanoparticles can be conveniently made from easily available raw materials like Zinc acetate, Glycine and Gluteraldehyde by using a simple process. The materials used are environment friendly and does not cause any harm to the surroundings.
2. The immobilized enzyme has higher stability with respect to pH and thermal conditions.
3. This immobilized enzyme retains its synthetic activity during recycling. The results are as follows:

Sr. No.	Cycle	Yield
1	--	90%
2	1st Cycle	84%
3	2nd Cycle	82.5%
4	3rd Cycle	81%
5	4th Cycle	81%

4. ZnO is an optically active semiconducting material. It shows different optical behavior when pristine or capped with different ligand molecules. This can be advantageous to characterize the immobilization of enzyme.

This material, being non-toxic, can also be used as sensor for tracking biological processes taking place on its surface.

5. This process is suitable for industrial continuous process.

Sr No	Reference	Work reported	Remark	Comparison with the work reported in this study
1	**Title:** Improvements of lipase performance in high-viscosity system by immobilization onto a novel kind of poly(methylmethacrylate -co-divinylbenzene) encapsulated porous magnetic microsphere carrier. **Authors:** Xiao Meng, Gang Xu, Qin-Li Zhou, Jian-Ping Wu, Li-Rong Yang. **Ref.** Journal of Molecular Catalysis B: Enzymatic 89,**2013** 86 –92.	Mucorjavanicus lipase was supported on porous magnetic polymeric microsphere as carrier. To make the polymeric beads magnetic Fe_3O_4 was selected as core and poly(MMA-co-DVB) was layered on the surface of core as a shell. The enzyme molecules were embedded **inside** the polymer matrix. It was claimed that this system can work in the highly viscous medium or solvent free synthesis of ester can be carried out.	The possible drawbacks of the synthesized assembly may be the diffusion parameters. It may be very difficult to diffuse the reactants molecules inside the polymer matrix and undergo chemical reaction in a highly viscous medium. The same is the case with the product formed inside the polymer matrix. Secondly it was claimed that the catalyst can easily be recovered in magnetic field.	In our work we have reported the immobilization of enzyme on the surface of the NPs. Because of the interfacial like environment the enzyme has to change its conformation favorable for esterification reaction. As shown pictorially in the graphical TOC oleic acid molecules can easily be diffused towards the enzyme present on the surface (compared to the polymer matrix) may result into high yield of

				the product. Application of magnetic field for bulk production is not feasible. On the other hand, the fixed bed catalysis containing immobilized enzyme can be carried out using the enzyme reported in this study. We have proposed the flow-sheet diagram for the process in the revised ms.
2	**Title:** Covalent immobilization of organic solvent tolerant lipase on aluminum oxide pellets and its potential application in esterification reaction. **Authors:** Davender Kumar, Sushil Nagar, Indu Bhushan, Lalit Kumar, Rajinder Parshad,Vijay Kumar Gupta. **Ref.** Journal of Molecular Catalysis B:	This study was carried out to covalently immobilize the partially purified lipase from Bacillus sp. DVL2 on glutaraldehyde-activated **aluminum oxide** pellets and subsequently use the immobilized enzyme for esterification of oleic acid and ethanol. In this study, the potential of free and the immobilized lipases were compared in esterification of oleic acid with ethanol to form ethyl oleate in hexane. The immobilized lipase showed maximum **63%** conversion of oleic acid into ethyloleate in **16h** whereas free lipase showed maximum 60%	Alumina may cause environmental problem during active recovery and also affect the quality of the final product.	Compared to alumina ZnO NPs are environmentally friendly and non-toxic. Being in nano phase less quantity provides high surface area due to high surface to volume ratio. This reduces the mass transfer barrier of the substrate and enhances the

	Enzymatic, 87,**2013,** 51–61.	conversion of oleic acid into ethyloleate in 24h**.**		activity of an enzyme. The maximum esterification yield (**90%**) is obtained in presence of TLL enzyme (20 mg/mL) with oleic acid (C18) and methanol in the molar ratio of 2:1 in petroleum ether as solvent at 40 ^{o}C in **14h**.
3	**Title:** Thermally Responsive Reversed Micelles for Immobilization of Enzymes. **Authors:** Hong Chen, Li-Hong Liu, Li-Shan Wang, Chi-Bun Ching, Hong-Wei Yu, and Yi-Yan Yang. **Ref.** Adv. Funct. Mater. 18, **2008,**95–102.	These authors discuss the thermally responsive micellar system for enzyme immobilization. They synthesized amphiphilic copolymers poly(N-isopropylacrylamide-co-acrylic acid) and utilized to fabricate reversed micelles and immobilize lipase enzymes. Reversed micelles have also been widely studied for enzyme immobilization as they enable enzymatic reactions in organic solvents. The reversed micelles possess a core-shell structure. The hydrophilic core is used for the immobilization of enzymes, providing a favorable aqueous environment for achieving high enzyme activity. The hydrophobic shell makes the micelles soluble in organic solvents, and prevents the direct contacts of the enclosed enzymes with unfavorable	Synthesis of organic copolymers (here, poly(N-isopropylacrylamide-co-acrylic acid) is a tedious task and requires critical maintenance of reaction parameters.	Synthesis of semiconductit ng inorganic nanoparticles is easy and cost effective. In present study we have also proposed the concept of hydrophilic-hydrophobic balance directly affect the performance of an enzyme.

		organic solvents and thus enhances the stability of enzymes.		
4	**Title:** Ultrasound technology and molecular sieves improve the thermodynamically controlled esterification of butyric acid mediated by immobilized lipase from *Rhizomucormiehei* **Authors:** Lucas P. Fallavena, Fa´bioH. F. Antunes, Joana S. Alves, Natalia Paludo, Marco A. Z. Ayub, Roberto Fernandez-Lafuente and Rafael C. Rodrigues. **Ref.** RSCAdv., 4, **2014,**8675-8681.	In this work, the effects of ultrasound stirring and the addition of molecular sieves on esterification reactions between butyric acid and several alcohols catalyzed by immobilized lipase from *Rhizomucormiehei* (Lipozyme RM-IM) were studied.	Ultrasound technology shows good results to improve the performance of an enzyme. However, at present not cost effective.	Immobilized enzyme reported in this study shows better activities in term of esterification product in presence of molecular sieves.

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