CATALYTIC ACTIVITY OF IMMOBILIZED LIPASE ON GRAPHENE-CHITOSAN-SILICA NANO COMPOSITE

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Chapter 1
INTRODUCTION

The low cost and natural sorbents are increasing their applicability in research field. Chitin is a natural polymer, highly soluble material resembling cellulose in its solubility and low chemical reactivity[1]. Mesoporoussilicas are characterized by advanced surface stability in the acidic medium and highly developed surface, tunable pore size, acceptable kinetics, thermal stability, resistance to microbial attack, and low cost. Graphene has layered structure .Graphene-based nanocomposites with excellent mechanical and thermal properties. Graphene-based sheets have been tested as possible nanocarriers for delivering drugs [2] and also as functional biomaterials [3,4]. Chitosan fibre differs from other fibres inthat it possesses a positive ionic charge, gives it the ability to bond chemically with the negatively charged lipids, fats and bile acids [5-6].The Nano composites were synthesized to improve the properties. Properties of all the materials were utilized for the immobilization of enzyme.Immobilization is achieved by fixingenzymes to or within solid supports, as a result of whichheterogeneous immobilized enzyme systems are obtained and the systems stabilize the structure of enzymes,hence their activities. When compared to free enzymes in solution immobilized enzymes are more robust and more resistant to environmental changes. More importantly, the heterogeneity of the immobilized enzyme systems allows easy recovery of both enzyme and product, multiple reuseof enzymes, continuous operation of enzymatic processes, rapid termination of reactions and greater variety of bioreactor designs.Enzymes may be immobilized by variety of method [7]. Compared with the free enzyme, most

commonlythe immobilized enzyme has its activity lowered. In analytical applications immobilized enzymes are usedchiefly in biosensors[8-10]

OBJECTIVES OF THE PROJECT

- To extract enzyme from cheap source.
- To prepare graphene oxide from graphite by modified Hummer method
- To prepare mesoporous silica by hydrothermal method
- To Synthesis of chitosan-silica-graphene nanocomposite
- To immobilize Candida Rugosa Lipase in chitosan-silica-graphene nanocomposite
- To characterize the prepared graphene oxide via physico-chemical techniques like XRD,   FT-IR spectroscopy,SEMetc .
- To conduct Catalytic reactions of the immobilized enzyme in batch reactors and pack bed reactors.

Chapter 2

EXPERIMENTAL : MATERIALS AND METHODS

2.1 Chemicals and reagents used

TEOS (Tetra Ethyl Ortho silicate)	Sigma Aldrich Chemicals, Bangalore
Poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide), PluronicP123	Sigma Aldrich Chemicals, Bangalore
Trimethyl benzene	Sigma Aldrich Chemicals
Disodium hydrogen phosphate, sodium potassium tartarate	Merck
Commercial Candida rugosa lipase (Type VII)	Sigma Aldrich Chemicals, Bangalore
p-Nitro phenyl palmitate	Sigma Aldrich Chemicals, Bangalore
Graphite	Merck
$KmnO_4$	Merck
H_2SO_4	Merck
H_2O_2	Merck
Ethanol	Merck

2.2

(1) Lipase enzyme synthesized from oil spread on sand.

2.3 Synthesis of Graphene Oxide

GO wassynthesized via the simplified Hummers method[12] in which into a 100ml sulfuric acid 0.75g of graphite was added. Potassium permanganate (4.5g) was gradually added and the solution was left to oxidize for 24 hours whilst being continuously stirred. After 24 hours, the resulting solution was cooled to room temperature and poured onto ice (400ml) alone with 5 ml of 30% hydrogen peroxide. The solution was centrifuged and washed six times with water. Then it was washed with ethanol. Dispersed in 100ml ethanol and ultra sonicated

for 30 minutes and then concentrated into a highly viscous liquid. Finally the prepared GO was extracted with ether and dried under vacuum at room temperature.

2.4 Synthesis of Mesoporous Silica

The mesoporous silica materials are prepared in aqueous hydrochloric acid using triblock co-polymer surfactant Pluronic P123 (poly(ethylene oxide)-block-poly (propylene oxide)-block-poly (ethylene oxide), EO_{20}-PO_{70}-EO_{20}, M_{av}=5800) with TMB (Trimethyl benzene) as organic swelling agent [13-14]. P123 (2.0g, 0.3 mmol) is dissolved in 2M HCl containing TMB at 40-60^{0}C.The resultant solution was rapidly mixed with silica precursor under vigorous stirring to form a reactive gel having composition 1g P123:3g TMB:2g TEOS: 8g HCl :2mol.pH of the resulting mixture was maintained below 2(pH<2). The synthesis was carried out under conventional hydrothermal conditions by treating the precursor gel at 100^{0}C for 24h in Teflon lined autoclave .The solid samples were separated by filtration, washed thoroughly with deionized water ,1% ammonium nitrate solution and then 5% aqueous ethanol dried at ambient temperature and calcined at 500^{0}C for 12h

2.5 Synthesis of the Chitin – Graphene-Silica nanocomposite

The 1 wt% chitosan solution was prepared by dissolving chitosan in 2% (v/v) aqueous acetic acid solution using a magnetic stirrer at 200rpm for 1 h and filtered with a filter paper followed by, a desired amount of GO and silica was added into the chitosan solution. The solution was then stirred at 600rpm for 1 h, followed by sonication for 10 min to remove the bubbles. After that, the GO/chitosan/Silica suspension was poured into a plastic dish and placed in fume hoodat room temperature to allow water to evaporate [15].

2.6 Characterization

Prepared systemswere characterized using powder XRD.,FT-IR spectroscopy,BET Surface area and SEM.

2.7Experimental procedure

The photometric assay substrate (pNPP) was prepared as described by Winkler and Stuckmann with slight modifications[16-19]. Solution A contained p-nitrophenylpalmitate (pNPP) dissolved in 10 ml of 2-propanol to concentrations of 20mM, with a sonicator for 10

min at room temperature. Solution B for the pNPP assay consisted of 50 mMTrisHCI buffer (pH 8) containing 0.4% Triton X-100 and 0.1% Gum Arabic. It was observed that the solutions were stable for about 2 weeks when refrigerated. The reaction mixture consisting of 1 part solution A and 9 parts solution B was prepared fresh before the assay.

In the standard conditions the reaction mixture was composed of 1mL of 20mM *p*-NPP in an Erlenmeyer flask. The reaction was started by the addition of 10 mg free lipase preparation (or 20 mg immobilized lipase preparation). The mixture was incubated at 37°C. After 5 min of reaction, agitation was stopped, the lipase powder was allowed to settle for 30s, and the clear supernatant was withdrawn. 0.5mL of supernatant was immediately mixed with 1.0mL of 10mM NaOH, directly in 1.0mL cuvette of the spectrophotometer. It displayed a yellow colour in aqueous alkaline phase because of the alkaline pH and hydrolysis activity was measured spectrophotometrically at 410 nm. The same was repeated in the next 25 min at regular time intervals. A standard calibration curve constructed with p-NP of known concentration and absorbance. The unknown concentration was determined from the absorbance of standard solutions of *p*-NP in the reaction medium. One unit of lipase activity was defined as the amount of enzyme that liberated 1μmol of p-nitro phenol from p-NP per min.

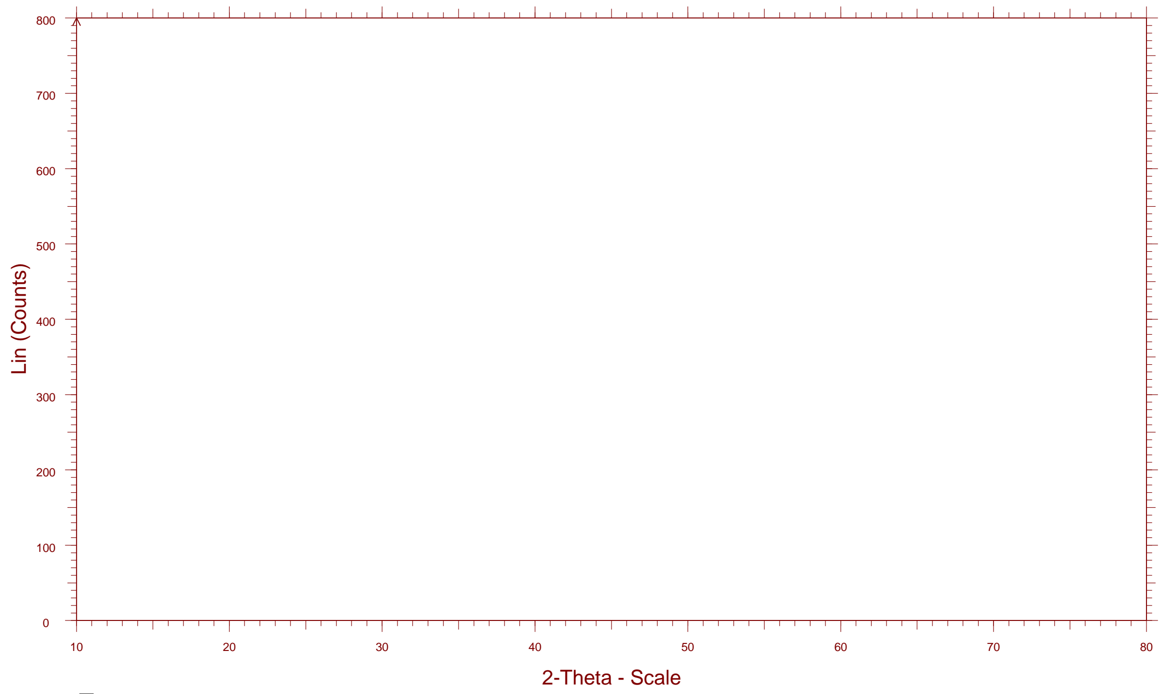
Chapter 3

RESULTS & DISCUSSIONS

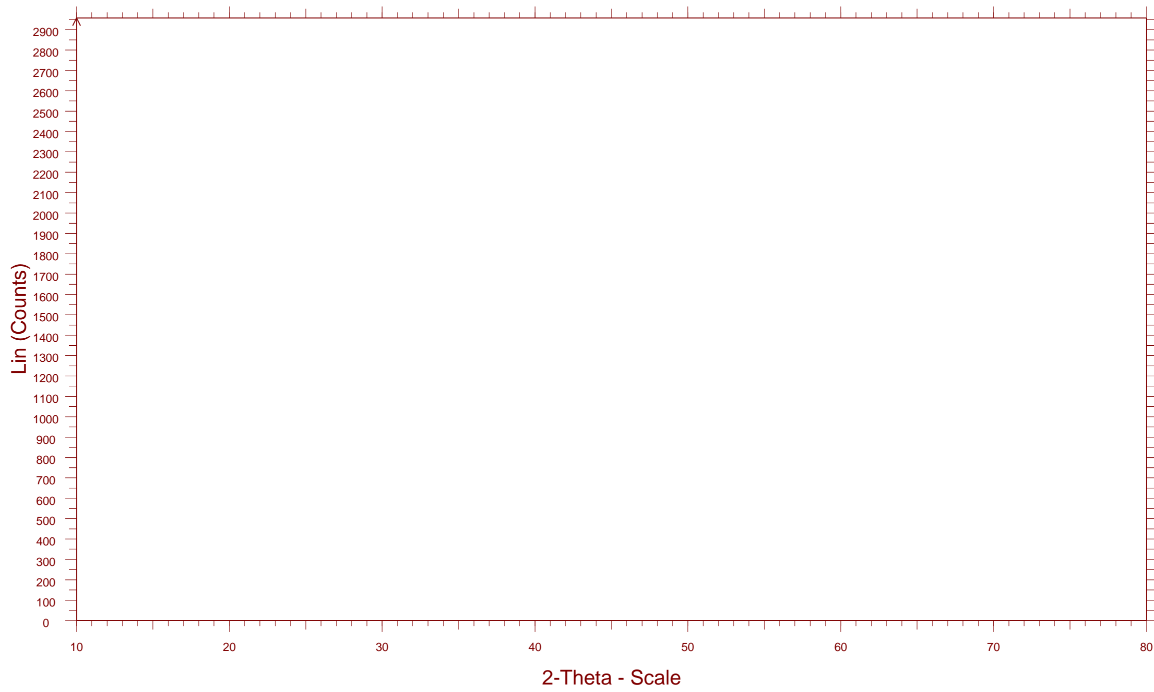
This chapter summarizes the results of the physicochemical characterization of different prepared materials and their modified forms. Various techniques like X-ray Diffraction Analysis (XRD), N2-adsorption-desorption, Fourier Transform infrared spectroscopy (FTIR,Ttransmission electron microscopy (TEM) and scanning electron microscopy (SEM) of bare, functionalized and enzyme immobilized samples are discussed in this chapter.

3.1 XRD

The XRD patterns of graphene oxide are shown in figure 1and 2. Small angle XRD pattern of graphene oxide displays two well resolved peaks, a very intense peak at $2\theta = 10$, $2\theta = 25$ and one distinct peak at $2\theta = 45^0$ which confirmed that the sample is graphene oxide. The XRD signals were indexed as (001) , (002) and (100).

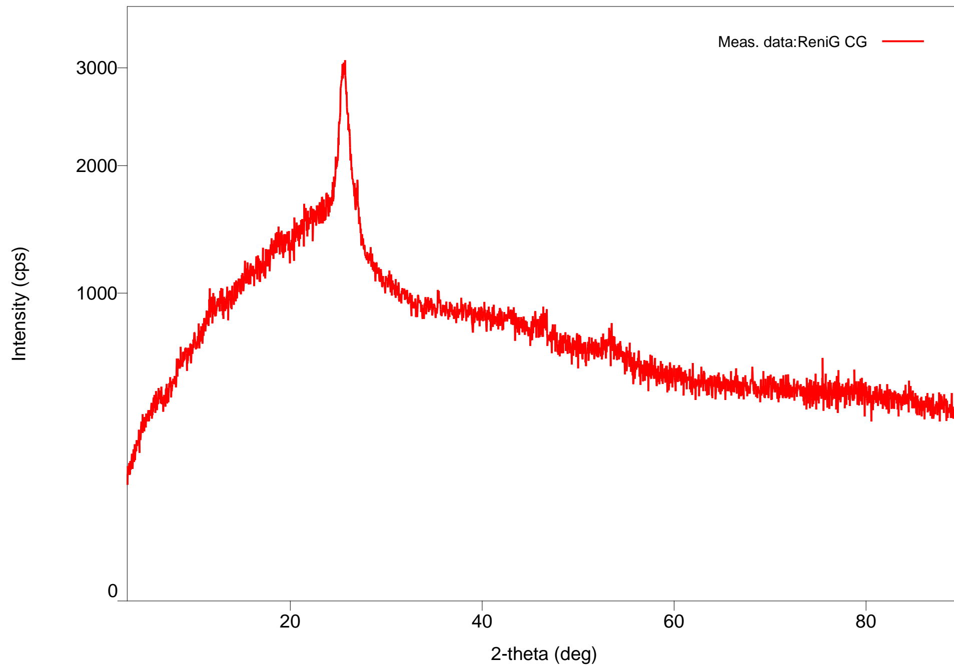


G1
Lin (Counts)
800
700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
80
2-Theta - Scale
File: SAIFXR170426D-01(G1).raw - Step: 0.020 ° - Step time: 59.7 s - WL1: 1.5406 - kA2 Ratio: 0.5 - Generator kV: 40 kV - Generator mA: 35 mA - Type: 2Th/Th locked
Operations: Smooth 0.250 | Background 0.813,1.000 | Import

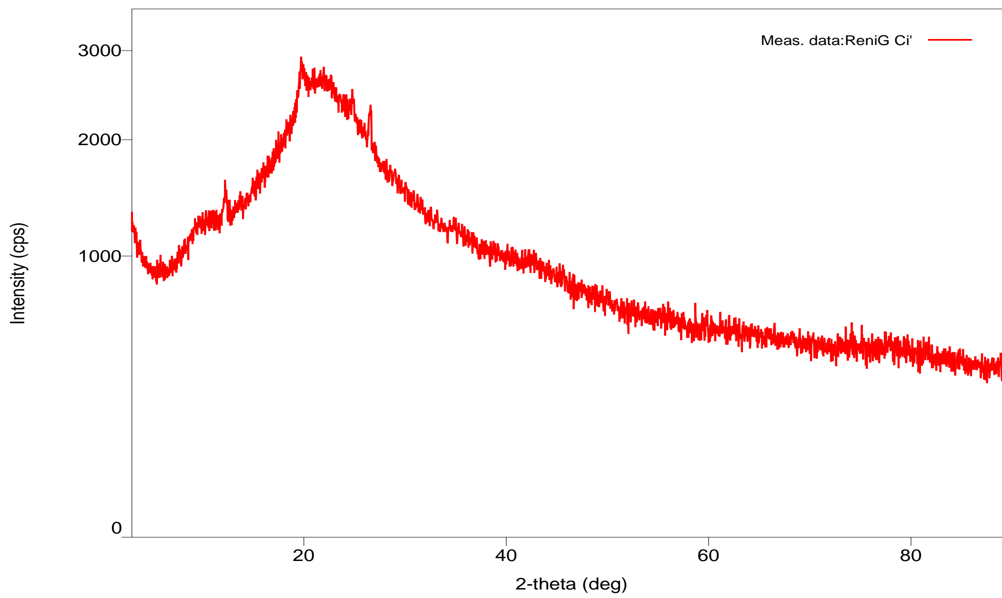


Near 10 a peak obtained is due (001) plane and a peak at 25 obtained is due to (002)plane and 45 due to (100) plane.

Catalyst	2θ
GO	10
	25
	45



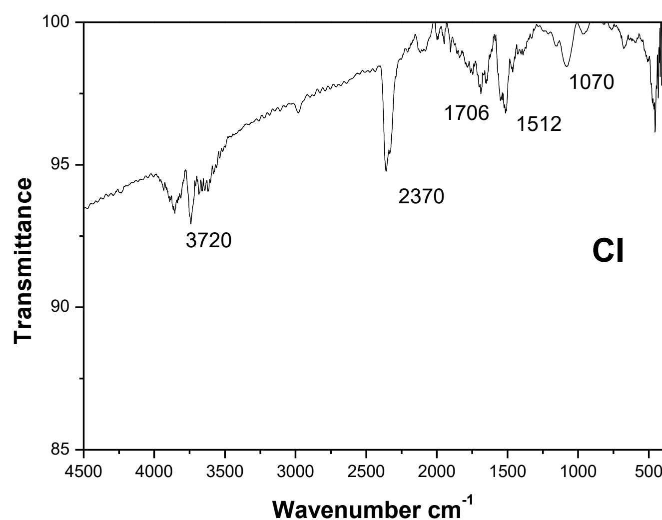
The above shows the XRD pattern of the Chitin –Graphene-Silica Nanocomposite .It shows a sharp peak around at an angle 2θ= 25.66^0 and its d-spacingaround 3.49 A^0.

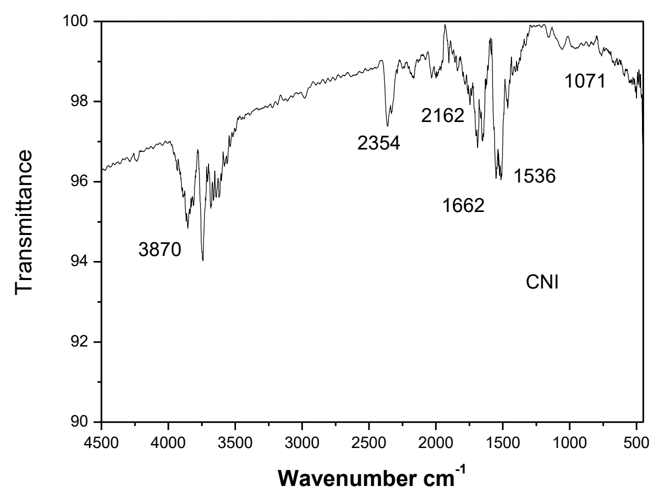


The above XRD pattern shows Lipase immobilized Chitin –Graphene-Silica Nanocomposite. Here we got the exactly similar pattern of the bare support but there is a small deviation from the original pattern.

3.2 FT-IR SPECTRA

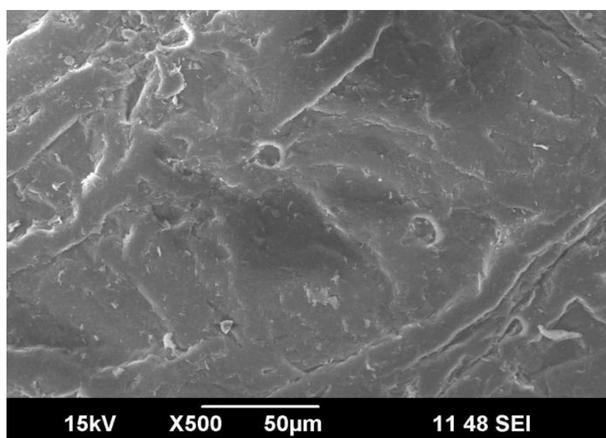
The FT-IR Spectra of the silica, graphene, chitin ,grapheme-silica-chitin nano composite and the immobilized nano composite were done. The IR spectra of synthesized samples were done. The FT-IR spectra of the synthesized nanocomposite Chitosan-Graphene-Silica and immobilized systems were depicted below. It was used to very the amide linkage between GO ,chitosan and silica . The peak at 1070 cm-1 corresponds to a stretching vibration from C-O bonds .In the spectrum dominant peaks exist at 1070 cm-1 and 1512 cm-1. These peaks correspond to an absorbance of glucosidic bond, stretching vibration from C=O of-NHCO- and the N-H bending of NH_2, respectively. The peak located at 1706 cm^{-1} is attributed to C=O incarboxylic acid and carbonyl moieties. In the spectrum of chitosan, dominant peaks exist at 1070 $cm^{-1}$and around 1593 cm^{-1}[19-24].

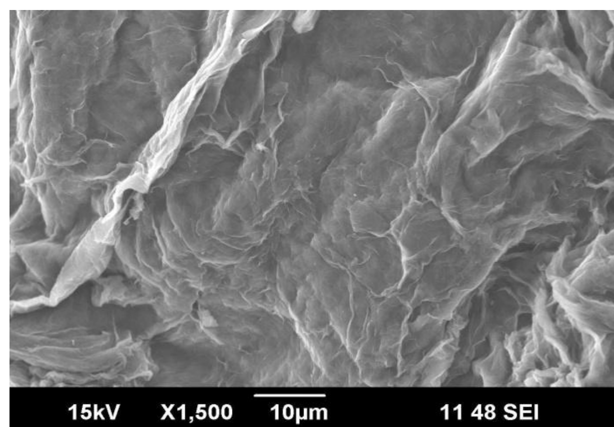


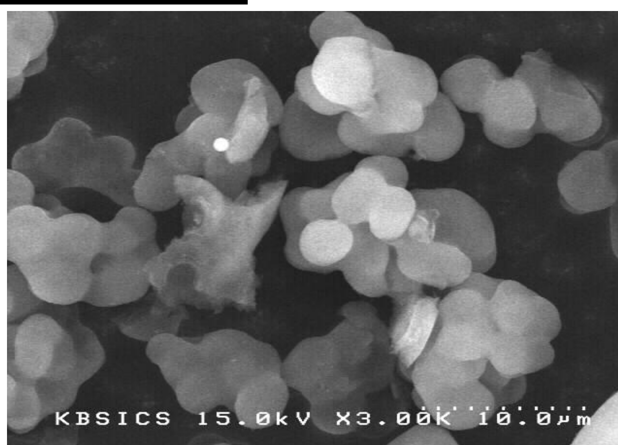


3.3 SEM image

Surface morphology of the prepared systems were individually analysed and the images of Chitosan, Graphene oxide and silica were visualized below.







3.4 BET-surface Area and pore diameter analysis

Nitrogen adsorption – desorption measurements were done in a micromeritics Tri-Star 3000 surface area and porosity analyzer. Prior to the measurements the samples were degassed for 30 minutes at 90^0C followed by 2 h at 200^0C.Immobilized enzymes were degassed at room temperature overnight BET surface area and pore diameter gives an idea about the pore diameter which is essential for immobilization. The pore diameter of the mesoporous silica was $89A^0$, Graphene Oxide, and the Nano composite were also analyzed.

3.5 Immobilization Studies

The enzyme Candida Rugosa Lipase were immobilized in the Graphene-Silica-Chitosan support in different pH

3.6 Effect of pH

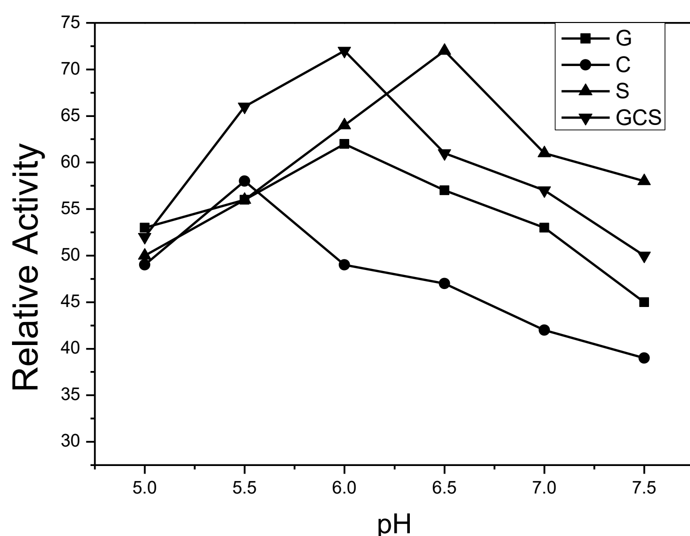
pH is unique of each enzyme and pH change will affect the activity and stability of enzyme. The changes depended on the enzyme, the type of support and method of immobilization. It controls charging of enzyme as well as support. The pH is varied from 4 to 8.The enzymes were dissolved in corresponding pH and supports were added and shaken for 1hour at 30^0C.The supports were filtered and washed pH solutions. Hung et al reported that immobilized C. rugosa lipase was stable in the pH range of 5-8 with optimum pH 9**.**Immobilization protects enzyme from change in pH. Isoelectric point (pI) of CRL is 5 to 6.5; the protein is kept at its most stable conformation at these pH values. Although the active and the stable confirmation and the stable conformation of a protein may not be the same, in this case, the neutral protein surface may enhance the hydrophobic interaction between the lipase and the modified silica surface. Maximum amount of enzyme adsorption occursat a pH near the pI of the protein–substrate complex and not at the pI of the protein.

Lipase has been immobilized in PMOs (Periodic mesoporousorganosilicas) with large cage-like pore and an extended study was carried out to investigate the influence of the nature of the support on the immobilization efficiency of lipase as well as the resulting catalytic activity in hydrolysis **.**

3.6.1 Effect of Immobilization pH

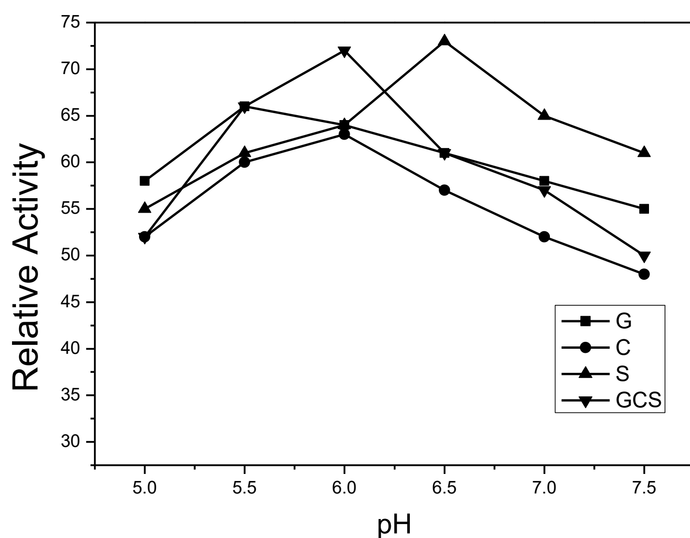
The optimum immobilization pH for the adsorbed systems Graphene, Chitin, Silica and GCS a were found to be 6.0, 5.5, 6.5 and 6 respectively. There was decrease in the immobilization capacity at alkaline side of the pI of the lipase. Lipase immobilization in pH range of 5.0 – 6.5 provided relatively high activity because enzyme conformation and it is essential for enzymatic activity changed with pH.The results obtained were summarized in Figure

.



3.6.2 Effect of Reaction pH

The reaction pH also varied from 5 to 7 and the activities were studied to get the optimum reaction pH. The results obtained were summarized in Figure.



3.7 Effect of temperature

The effect of reaction temperature on the activity of free and immobilized lipase was also investigated at various temperatures ranging from 30 to 60°C. The optimum temperature obtained for hydrolysis reaction for the free lipase was 37^0C and immobilized lipase shifted into the higher region relative to the temperature of free enzyme.

3.8 Reusability

The most important advantage of immobilization is repeated use of enzymes and its applications in a batch or continuous reactor. Reusability studies were performed at optimum conditions. After each run, the immobilized enzyme was filtered off and was washed several times with buffer. They were reintroduced into a fresh reaction medium and activity measurement was conducted under standard assay conditions. Here the enzyme immobilized on Chitin-Graphene-Silica and its activity were checked for 15 days .The enzyme retains its activity for 20 days and gradually decreases after 30 days.

Support	**Residual Activity (%)**			
	After 5 days	**After 10days**	**After 20days**	**After 30 days**
Free LIPASE	**80.2**	**72.5**	**60.7**	**10.2**
CNI	**92.1**	**90.3**	**83.2**	**57.7**

Chapter 4
CONCLUSIONS

Graphene oxide was synthesized by modified Hummer method and mesoporous silica also successfully synthesized. Chitosan-silica-grapheneNano composite were also prepared. The enzyme Lipase were successfully immobilized into the support.The synthesized Chitosan-silica-grapheneNano composite and enzyme immobilized support were characterized by powder XRD,BET surface area,FT-IR,SEM and TEM. Enzymatic activities of lipase immobilized on Chitosan-silica-grapheneNano composite materials are evaluated using pNPP. The experimental results showed that lipase after immobilization had good thermal stability. The immobilized enzyme could be reused, keeping around 80% of its original activity after 20 reaction cycles without any treatment.

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