CATALYSIS BY ENZYMES IMMOBILIZED ON TUNED MESOPOROUS SILICA

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RENI GEORGE

Under the Supervision of

Dr. S. SUGUNAN

Department of Applied Chemistry

Cochin University of Science and Technology

Kochi - 682 022

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Author:

RENI GEORGE
Research Fellow, Department of Applied Chemistry
Cochin University of Science and Technology
Kochi -682 022
E mail: renivgeorge@gmail.com

Research Guide:

Dr. S. SUGUNAN
Emeritus Professor
Department of Applied Chemistry
Cochin University of Science and Technology
Kochi - 682 022
Email: ssg@cusat.ac.in

Department of Applied Chemistry
Cochin University of Science and Technology
Kochi - 682 022
India

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DEPARTMENT OF APPLIED CHEMISTRY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI - 682 022, INDIA

Dr. S. Sugunan
Emeritus Professor

20-05-2013

Certificate

Certified that the thesis work entitled "**Catalysis by enzymes immobilized on tuned mesoporous silica** " submitted by Ms. Reni George is an authentic record of research work carried out by her under my supervision at the Department of Applied Chemistry in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry of Cochin University of Science and Technology and has not been included in any other thesis previously for the award of any other degree.

Dr. S.Sugunan
(Supervising Guide)

Declaration

I hereby declare that the work presented in the thesis entitled **"Catalysis by enzymes immobilized on tuned mesoporous silica"** is my own unaided work under the supervision of **Dr. S. Sugunan**, Emeritus Professor in Department of Applied Chemistry, Cochin University of Science and Technology, Kochi-22, and not included in any other thesis submitted previously for the award of any other degree.

Kochi-22 **Reni George**
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Preface

Enzyme has its own unique advantages and disadvantages, and has been successfully utilized in various applications. Enzymes were immobilized into supports to preserve their stability and catalytic activity under extreme conditions.

Mesoporous silica nanoparticles provide a non-invasive and biocompatible delivery platform for a broad range of applications in therapeutics, pharmaceuticals and diagnosis. Additionally, mesoporous silica materials can be synthesized together with other nanomaterials to create new nanocomposites, opening up a wide variety of potential applications. The ready functionalization of silica materials makes them ideal candidates for bioapplications and catalysis. These properties of mesoporous silica like high surface areas, large pore volumes and ordered pore networks allow them for higher loading of drugs or biomolecules. Comparative studies have been made to evaluate the different procedures; much of the research to date has involved quick exploration of new methods and supports. Requirements for different enzymes may vary, and specific conditions may be needed for a particular application of an immobilized enzyme such as a highly rigid support.

In this endeavor, mesoporous silica materials having different pore size were synthesized and easily modified with active functional groups and were evaluated for the immobilization of enzymes. In this work, Aspergillus niger glucoamylase, Bovine liver catalase, Candida rugosa lipase were immobilized onto support by adsorption and covalent binding. The structural properties of pure and immobilized supports are analyzed by various characterization techniques and are used for different reactions of industrial applications.

Still there is no universal support and optimal technique that it is suitable for all enzymes and their applications. Search for a good support is a fascinating challenge in biotechnology.

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Contents

Enzymes have very relevant application in industries. Despite its drawbacks, enzymes are quite attractive catalysts for performing organic synthesis and have been considered to match the fundamental principles of environmentally benign manufacturing, sustainable development and green chemistry, which represent a bonus of increasing significance as environmental pollution becomes one of the most serious threats to mankind. Immobilization overcomes many disadvantages and finds vast opportunity in heterogeneous catalysis with the aim of reducing production costs by efficient recycling and control of the process with tremendous potential. These findings opened new possibilities for their applications in biotechnology. However, novel methods and materials are still needed to achieve a massive implementation of enzymes as catalysts for complex chemical processes. Three enzymes (catalase, glucoamylase and lipase) having different application in industry have been selected for the present study. Porous silica materials are ideal candidates for bioapplications and catalysis. Ordered mesoporous materials open a challenging pathway to tailor immobilized enzyme. This chapter aims to provide some background about enzymes and evaluation of different supports and methods of immobilization.

1.1 Introduction

In ancient culture man has been using enzymes, in different forms, as extracts obtained from vegetables, algues, and fungus or animal organs or as microbes. Enzymes have found various potential applications in the fields of chemical, food, medical industries. But, their applications are not completely exploited. Many important environmental problems can be avoided by the adaptation of enzyme technology.

1.2 Enzymes as biocatalyst

Enzymes are catalysts of biological origin having an extraordinary catalytic power and high degree of specificity. Most of the enzymes are proteins found in living organisms with complex structure and their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into subunits, its catalytical activity is lost. Thus the primary, secondary, tertiary and quaternary structures of enzyme protein are essential to their catalytic activity [1-3]. Enzymes offer a distinct advantage due to their specificity: chemo, regio and stereo selectivity, ability to function in aqueous solutions. They require only mild conditions and are free from the limitation of side product formation. Enzymes are ecofriendly and nontoxic in nature. Free enzymes are labile and not always sufficiently stable under operational condition and one time usage, as catalyst is costly. They are highly sensitive to reaction conditions and separation of enzyme from solutions very difficult. They can be poisoned by waste products. These disadvantages could be overcome by the use of immobilized enzymes.

1.3 Immobilization

Immobilization in a solid support reduced the boundaries of homogeneous, heterogeneous and biocatalysis. The term "*immobilized enzymes*" refers to

"Enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities and which can be used repeatedly and continuously" [4]. Heterogenization of a biocatalyst may serve three main functions including catalyst retention, catalyst concentration and catalyst stabilization. These advantages are balanced against disadvantages such as cost of immobilization and reduction of catalytic efficiency. Sumner immobilized enzyme and that was the milestone in enzyme catalysis. The advantages of immobilized enzyme include ease of reutilization, enhanced pH and thermal stability, a rapid separation of the biocatalyst from the reaction mixture. It can be reused and finds application of automated continuous process. It can be most suitable for practical, industrial, medical, food and analytical application. The use of immobilized and stable enzymes as biosensors has immense potential in the enzymatic analysis of clinical, industrial and environmental samples. Immobilization improves the stability of enzyme under the reaction conditions, enhances enzyme activity and makes the repeated use of the enzyme feasible. It permits the use of enzyme for diverse applications and thus lowers production costs. Immobilization provides a better environment for the enzyme to act and also offers better product recovery [7, 8].

Furthermore, immobilization is important to maintain constant environmental conditions in order to protect the enzymes against changes in pH, temperature, or ionic strength; this is generally reflected in enhanced stability. Moreover, the solid matrix may serve as a shield for harsh environmental conditions like pH variation, temperature alteration, and shaking condition.

1.4 Criteria for an ideal enzyme supports

Broad collections of new carriers for enzyme immobilization are coming up. So the selection of a proper support is essential in this field allowing the

researchers to specifically choose a support with different features depending on the enzyme and the given application like availablility at low cost, environmentally acceptable, structurally more stable, chemical functionality, length of spacer arm, porosity, high surface area, mechanical, physical, chemical and thermal stability, insolubility, binding capacity, chemically inert, resistant to microbial and chemical attack ,the hydrophile - lipophile balance of the microenvironment surrounding the enzyme to achieve higher product yield etc [9-13].

Supports are mainly classified into organic and inorganic supports. Organic supports include natural polymers, polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate, proteins like collagen, albumin, Carbon, Synthetic polymers like polystyrene, polyacrylate, polymethacrylates, polyacrylamide, polyamides, vinyl, and allyl-polymers. Organic polymeric carriers are the most widely studied materials because of the presence of rich functional groups, which provide essential interactions with the enzymes. However, the organic supports suffer a number of problems such as poor stability towards microbial attacks and organic solvents and disposal issues. The major problem with polymer supports is lower pH and thermal stabilities. Inorganic supports provide better thermal stabilities. Inorganic supports include natural minerals clay [14], zeolites, ceramic materials, silica, and processed materials. glass (nonporous and controlled pore), metals, and controlled pore metal oxides. They are found to be thermally and mechanically stable, non-toxic, and highly resistant against microbial attacks and organic solvents and hence there is immense scope for research in this area [15-19].

1.4.1 Mesoporous silica materials as support for enzyme immobilization

According to various reports inorganic porous materials like clay, zeolite etc were not suitable for complete entrapment because of inappropriate pore

size. So for complete entrapment of enzyme on large pore diameter material are required. In this present study mesoporous silica is selected as immobilization support. According to IUPAC, mesoporous materials have pore diameter in the range 2-50 nm. Ordered mesoporous silica materials open a challenging pathway to tailor enzymes due to their unique features including high surface area (300-1500 m^2/g), chemical, thermal, and mechanical stability, highly uniform pore size distribution and tunable pore size, high adsorption capacity, sufficient hydroxyl group for modification, low isoelectric point (~3.8) and an ordered open porous network for free diffusion of substrates and reaction products.

Among these available supports, mesoporous materials meet all the criteria for a perfect immobilization support. Mesoporous silica materials with tunable pore size have been a source of growing interest because of their industrial applications in the catalytic conversion of bulky molecules and also for adsorption and host-guest chemistry [20-22]. In 1990, Kuroda and co-workers first reported the preparation of mesoporous silica with uniform pore size distribution from the layered polysilicate kanemite (FSM-16) [23, 24]. A significant breakthrough in the mesoporous materials research has come when Mobil scientists disclosed the M41S family of materials, which have large uniform pore structures, high specific surface areas and specific pore volumes, including hexagonal-MCM-41(Mobil Composition of Matter) [25, 26], cubic-MCM-48 [27.], and lamellar-MCM-50 [28, 29] were synthesized using cationic surfactant. Very recently alkanes of different chain length have been used together with the surfactant to synthesize MCM-41 with different pore diameter [30]. Michigan State University researchers synthesized MSU-1 by using polyethylene oxide (PEO) as a structure directing agent. It has a disordered channel structure [31]. This material possesses large wall thickness and small particle size with considerable textural mesoporosity due to pores

formed between the relatively small particles. Santa Barbara group developed mesoporous materials called SBA-15 with thicker pore walls by using amphiphilic triblock –copolymer of poly (ethylene oxide) and poly(propylene oxide) (Pluronic 123) as structure directing agent in highly acid media [32, 33]. Both MCM-41 and SBA-15 exhibits 1D arrangement of hexagonal mesoporous with *p6mm* symmetry [34]. A cubic mesoporous silica structure (SBA-11) with *Pm*3h*m* diffraction symmetry has been synthesized in the presence of $C_{16}H_{33}(OCH_2CH_2)_{10}OH$($C_{16}EO_{10}$) surfactant species, while a 3D hexagonal (*P*63/*mmc*) mesoporous silica structure (SBA-12) results when $C_{18}EO_{10}$ is used [35]. Sugunan & Ajitha synthesized hydrothermally highly ordered mesoporous SBA-15 with different pore size. They studied the immobilization behaviour of α-amylase in these materials [36]. Fan *et al* was examined the bioimmobilization ability of rod and con SBA-15 using Lysozyme [37].

1.4.2 Mesocellular foam (MCF)

Siliceous mesocellular foam (MCF) is a facile and versatile support material for heterogenizeous catalysis. This material is attractive for its robust, well-defined pore structure with interconnected, ultra large pores that facilitate diffusion. Catalytic complexes and enzymes were successfully immobilized on MCF and easily recycled. By tuning the property of the linker groups and the microenvironment, these heterogenized catalysts were effectively applied towards useful reactions. Excellent activity and extremely low level of leaching (metal or enzyme) were attained with these robust supports. These heterogenized catalysts would facilitate the development of environmentally friendly and more cost-efficient industrial processes [38].

MCF materials posses well defined spherical pores ranging from 220 to 420 Å in diameter. Addition of swelling agents like trimethylbenzene produces

materials with large pore diameter but with having short range order [39-41]. MCM-41 and SBA-15 materials have monodimensional hexagonal mesopore structures where as MCF are composed of uniformly sized large spherical cells (upto 500Å) with high surface area interconnected by uniformly sized windows [~ 20 nm] to create a continuous 3D cage like structure [42-47]. Mesocellular form (MCF) materials can be prepared by using triblock-copolymers, organic additives and TEOS. It is well known that the poly-ethylene oxide (PEO) chains are hydrophilic while the poly-(propylene oxide) (PPO) chains tend to be hydrophobic, thus driving the formation of micelles with the PPO as core and the PEO chains as corona [48-50]. It is generally accepted that the organic additives (TMB and alkanes) can penetrate into the core of the surfactant micelles to swell the surfactant micelles, which will lead to mesoporous materials with large pore size [51, 52]. Fig.1.1 represents Schematic diagram of a micelle in presence of swelling agent.

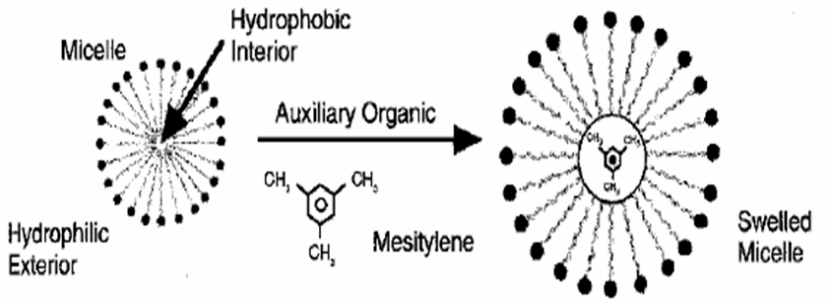


Fig.1.1 Schematic diagram of a micelle in presence of swelling agent.

A unique 3D cage like structure Fig.1.2 [42] of MCF was found to be critical factor which renders siliceous mesostructured foam a very promising material for immobilization of enzymes. Their surface can densely be covered with various anchoring groups due to high surface area and presence of pores with larger diameter (dimensions larger than enzyme molecule). The open structure as well as the uniform pore size that can enhance enzyme loading improve the stability of immobilized enzyme. Enzyme immobilization on

mesoporous silica based material has led to two important observations, protein loading strongly depends on pore size and surface structure and leaching of enzymes from carrier are significantly less. Among different types of developed mesoporous silica, mesocellular foams are found to be more efficient support for immobilization because of their cage like meso pore structure up to 40 nm diameter and mesopores are connected by windows upto 20 nm. It can host bulky enzyme molecules comfortably [13]. Functionalized MCF materials for enzyme immobilization were first demonstrated for organophosphorous hydrolase. Han *et al* successfully entrapped Lipase from Candida Antarctica in the cage like pores of MCF using a pressure- driven method [53]. Organo functionalized MCF materials were used to immobilize invertase and glucoamylase [54]. Pandya *et al* synthesized MCF materials having different pore diameter and functionalized with 3-APTES and glutaraldehyde to improve their enzyme binding capacity. They observed high specific activity for starch hydrolysis using α-amylase [55]. Zhang *et al* successfully immobilized glucose oxidase in MCF [56]. Sugunan & Reshmi synthesized large mesoporous cellular foam (LMCF) materials using the microemulsion templating route and β-glucosidase was immobilized via by glutaraldehyde (GA) crosslinking [57].

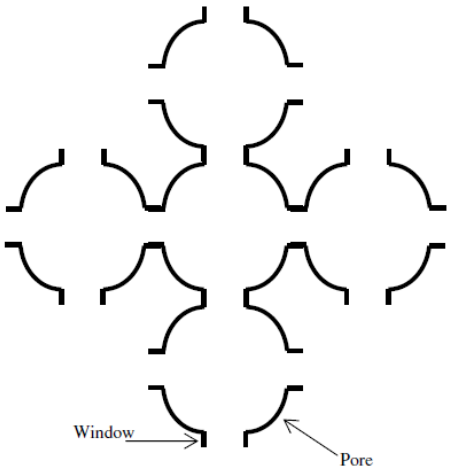


Fig.1.2 Schematic representation of cage like structure of MCF

1.4.3 Synthesis of mesostructured materials

Kresge and co-workers first introduced a sol-gel method to prepare mesoporous silicates and aluminosilicates in alkaline conditions [25]. Shortly after, Huo and co-workers [58, 59] reported the first synthesis of periodic porous silicates in acidic conditions. By adjusting the pH, it is possible to vary the charge density and geometry of the silicate species that interact with the surfactant head groups, and therefore, greatly influence the degree of polycondensation of silicate species and the morphology of the synthesized materials. The hydrolysis and condensation rate of the silica species are pH-dependent. If pH is lower than the isoelectric point (pH -2), the condensation is acid-catalyzed and becomes faster as the pH decreases. At pH > 2, the condensation rate increases with pH until pH=8 and then decreases. The particles of micelle templated mesoporous silica (MMS) made in acidic conditions tend generally to be bigger than MMS particles made in alkaline conditions. This is due to the slower nucleation rate in acidic conditions [60, 61]. In acidic conditions, silica species are less condensed linear oligomers, while in alkaline solution the silica species are more cross-linked clusters. The acid made MMS particles appear to be softer, stickier due to weaker surfactant-silicate interaction. It contains more surface silanol group resulting in richer morphologies. In alkaline conditions, one could often obtain small sub micrometer size particles.

Different surfactants (cationic, anionic and non-ionic) were also used to synthesise mesoporous materials. Pinnavaia and co-workers used nonionic surfactants in aqueous solutions to synthesize wormlike disordered mesoporous silica [62, 63]. With cationic surfactants synthesis carried out in HCl media below the aqueous isoelectric point of silica, the key interactions are among the cationic surfactant, chloride anion, and the cationic silica species (designated as S+X-I+, where S+ is the cationic surfactant, X- is the

halide anion, and I+ is a protonated Si-OH moiety, i.e. $[SiOH\ H]^+$, and the overall charge balance is provided by association with an additional halide anion). Various synthesis routes were proposed and it is based on the way in which the surfactant interacts with inorganic species.

1.4.4 Mechanism of formation of mesosilica

The neutral templating mechanism ($S^0H^+X^-I^+$) based on hydrogen bonding interactions has been proposed to synthesize mesoporous silica materials, in which randomly ordered rod like micelles interact with silica species to yield tubular silica deposited around the external surface of the micelle rods. The spontaneous ordering results in the formation of hexagonal structure. The assembly of the mesoporous silica organized by nonionic alkyl-ethylene oxide surfactants or poly (alkylene oxide) triblock copolymer species in acid media occurs through an $(S^0H^+)(X^-I^+)$ pathway. First, alkoxysilane species are hydrolyzed and transformed to a sol of silicate oligomers. The EO moieties of the surfactant in strong acid media associate with hydronium ions.

$$Si(OEt)_4 + rH_2O^+ \xrightarrow[pH<2]{hydrolysis} Si(OEt)_{4-n}(OH_2^+) + rEtOH$$

$$REO_m + yHX \xrightarrow{H_2O} REO_{m-y}[(EO).H_3O^+]y.....YX^-$$

Where R is alkyl or poly (propylene oxide) and X^- is (Cl^-, Br^-,I^-, NO_3^-, $H_ySO_4^{-2+y,}$ $H_yPO_4^{-3+y}$).

$$REO_{m-y}[(EO)H_2O^+]_y\ldots yX^-\ldots]^+ \equiv S^0H^+X^-]^+$$

These charge- associated EO units and the cationic silica species are assembled together by electrostatic, hydrogen bonding and Vander Waals interactions and are designated as $S^0H^+X^-I^+$. During the hydrolysis and

condensation of the silica species, intermediate mesophases, such as hexagonal, cubic, or lamellar mesostructures are observed. Further condensation of the silica species, organization of the surfactant and inorganic species result in the formation of the lowest energy silica surfactant mesophase structure allowed by the solidifying inorganic network [64]. Schematic pathway for the formation of mesoporous materials were reported by Corma *et al* [65].

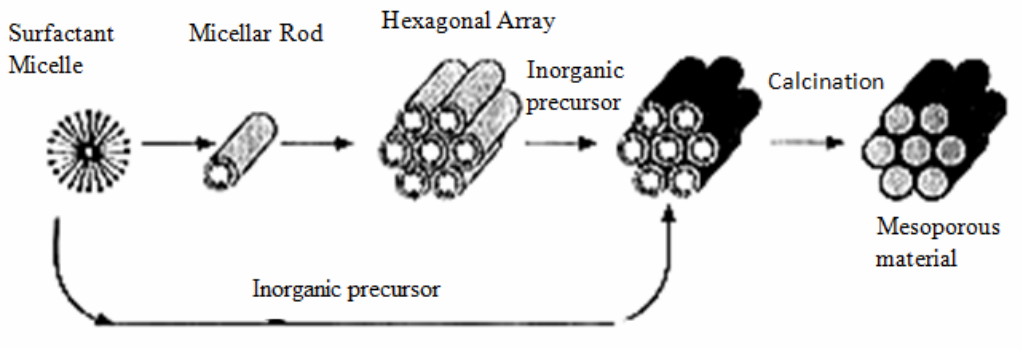


Fig.1.3 Mechanistic pathway for the formation of mesoporous materials

1.5 Enzyme immobilization

Enzyme immobilization procedure must exhibit high immobilization efficiency, conversion efficiency, long half life and operational stability to find industrial application. It exhibits slightly altered chemical and physical properties. Parameters like catalyst size, pH, ionic strength, temperature, substrate concentration etc must be carefully controlled to yield optimum conversion. Enzyme immobilization influences enzymatic activity, optimum pH, affinity to the substrate, stability etc. The extent of these changes depends on the enzyme, carrier support and the immobilization conditions [66, 67]. Immobilization inside porous materials will stabilize the enzyme against interaction with molecules, preventing aggregation, auto-proteolysis etc. These enzymes are not in contact with external hydrophobic interface. Hence air bubbles cannot inactivate the enzymes immobilized on porous solid [68-71]

Enzyme catalysis takes place in a small region of active site, which is formed by aminoacid residues. So in any adopted chemical modification studies there is a possibility that the active site of the enzyme is protected but some reagents react catalytically with the active site and thereby inactivate enzyme [72]. Gluaraldehyde is an excellent crosslinking agent which reacts with amino groups at neutral pH. It is widely employed in the field of immobilized enzymes [73-75]. It has dual nature, as crosslinking agent and as coupling agent. It reacts very rapidly with amino groups at around neutral pH. The cross-linking of proteins is either to a carrier or between protein molecules [76, 77].

1.6 Types of immobilization

Enzyme immobilization is done through carrier binding or attaching, crosslinking of enzyme using bifunctional reagents, entrapment or encapsulation of enzymes. Carrier binding involves the formation of interactions between enzyme and a support. This method can be further categorized as ionic binding, physical adsorption or covalent binding depending on the method. Covalent immobilization needs functional groups at the support surface with amino acid side chains that are available on the enzyme surface. Among the different modes of attachment, physical adsorption is often too weak to keep the enzyme to the support material [78]. The major drawback of this method is the easy desorption of enzyme by temperature fluctuations, changes in pH, substrate concentration and ionic strength in the activity measurements. When an immobilized enzyme is used to catalyze reactions in organic media, a strong enzyme-support interaction is not required, and due to the enzyme insolubility in the apolar medium physical adsorption may be a suitable method of immobilization [79, 80]. The ionic and covalent bonding is strong enough to overcome detachment of the enzyme. However, these two types of attachments are highly dependent on the structure of the enzyme and the support material. Although ionic binding is stronger than

physical adsorption leaching of enzyme is observed due to change in pH and ionic strength of the medium. Individual biocatalytic units are joined to one another with bi or multi functional reagents. High molecular insoluble aggregates are formed. Glutaraldehyde and di-isocynates are commonly employed as crosslinking agents. Crosslinking of a crystalline enzyme by using glutaraldehyde was achieved by Quiocho and Richards in 1964 .The main objective of this immobilization technique was to stabilize enzyme but it is not suitable for packed bed operations.

In matrix entrapment biocatalysts are embedded in water insoluble supports (natural, synthetic polymers or gel like structure etc) [81, 82]. Matrix entrapped biocatalyst can be spherical, cylindrical, fiber or sheet forms. It can be achieved by three methods micro-encapsulation, liposome technique and membrane reactors [83]. The different methods of immobilization are depicted in Fig.1.4.

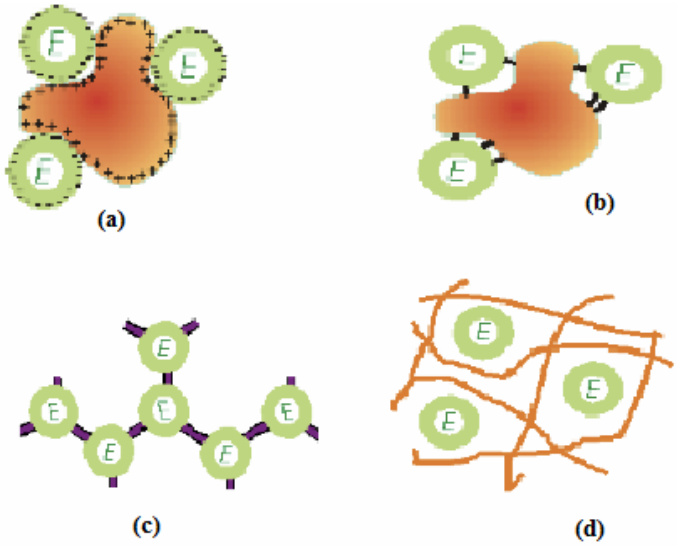


Fig.1.4 Pictorial representation of different methods of enzyme immobilization. (a) Ionic binding (b) Covalent binding (c) Crosslinking (d) Entrappment

1.7 Surface modification of supports

Pore surface of the silica materials functionalized (activated) with chemical species. It is useful for covalent coupling of protein and to modify their adsorption

properties. Due to lack of strong binding force between enzyme molecules and the support, adsorption method causes leaching of enzyme which results to poor enzyme loading and stability. The organo-functionalization of mesoporous materials can be done by the post-synthesis (grafting), and the co-condensation (direct incorporation/synthesis). Functionalization can be achieved by post grafting of as-synthesized ordered materials [84] or directly by co-condensation of a tetra-alkoxysilane and one (or more) organoalkoxysilanes in the presence of a surfactant template [85-89] leading to more uniform composition of the mesostructure [90, 91]. Post synthesis treatment with organic groups can maintain strong interaction and the immobilized molecule should be fabricated to the internal pore of the support. Post synthesis treatment is commonly used for surface modification by covalent linking of organo silane species with surface silanol groups (free and germinal silanol) under refux conditions [92]. Chemical modifications done via thiol, amine, nitrile or carboxyl groups protect the biocatalyst. The walls of silicon oxide contain large amounts of silanol groups. These groups are the anchoring point for functionalization**.** Amorphous silica gel has been grafted with aminopropyl and mercaptopropyl were studied by Walcarius *et al* [93]**.** After functionalization, the Si-OH bond is converted to Si-C and the nonhydrolyzable character of the Si-C bond prevents leaching of organic groups out of materials when used in solution. However, the grafting method has several shortcomings: (1) reduced pore size due to the attachment of a layer of functional moiety on the surface, leading to a less desirable product because the reduced pore size will cause a stronger diffusion resistance to protein molecules having a kinetic diameter of the similar size to the reduced pore size, (2) time consuming as it needs two steps to accomplish the modification process, (3) limited accessible surface silanol groups on the mesoporous silica materials, therefore only a low concentration of the organosilane can be attached; and (4) difficulties in controlling the loading and position of the organosilane. Some

unexpected advantages foreseen with suitable organically functionalized mesoporous materials were reported by Lei *et al* [94]. It has been found that the interactions of the enzyme - support depend strongly on the nature of functional groups attached to the surface. Both amino and gluaraldehyde functionalized supports are most popular for immobilizing enzymes (Fig 1.5.).

Generally co-condensation methods (one pot synthesis) were adopted and it enables homogeneous distribution of functional groups within short reaction time [95]. The co-condensation method was first reported by two research groups in 1996. This method allows modification of the surfaces of the mesoporous materials in a single step by copolymerization of organosilane with silica or organosilica precursors in the presence of a surfactant. A tight association exists between a biocatalyst and the carrier by means of shared pair of electrons and moreover the stress between the support and enzymes can be reduced by joining a spacer group. The spacer molecule provides a greater degree of mobility to the immobilized catalyst. It is stronger and stable than ionic bond and reduces leaching of enzyme into surrounding solution. This means immobilization increases the stability of the biomolecules and thereby increasing catalytical behavior. It has many advantages, after functionalization. Ordering and hydrothermal stability of mesoporous materials increases. A variety of functional groups have been incorporated into mesoporous materials such as aliphatic hydrocarbons, thiol, vinyl g, phenyl, amine, and perfluoro groups [96, 97]. Wang *et al* explains that functionalization process enhance the loading amount and activity of the lipase [98]. SBA-15 materials fuctionalized with different surface functional groups (–SH, –Ph, –Cl, $–NH_2$, and –COOH) to immobilize trypsin were explained by Yiu *et al* [99]. Sugunan *et al* successfully modified montmorillonite K-10 and SBA-15 using 3-APTES and gluaraldehyde [13, 14, 36]

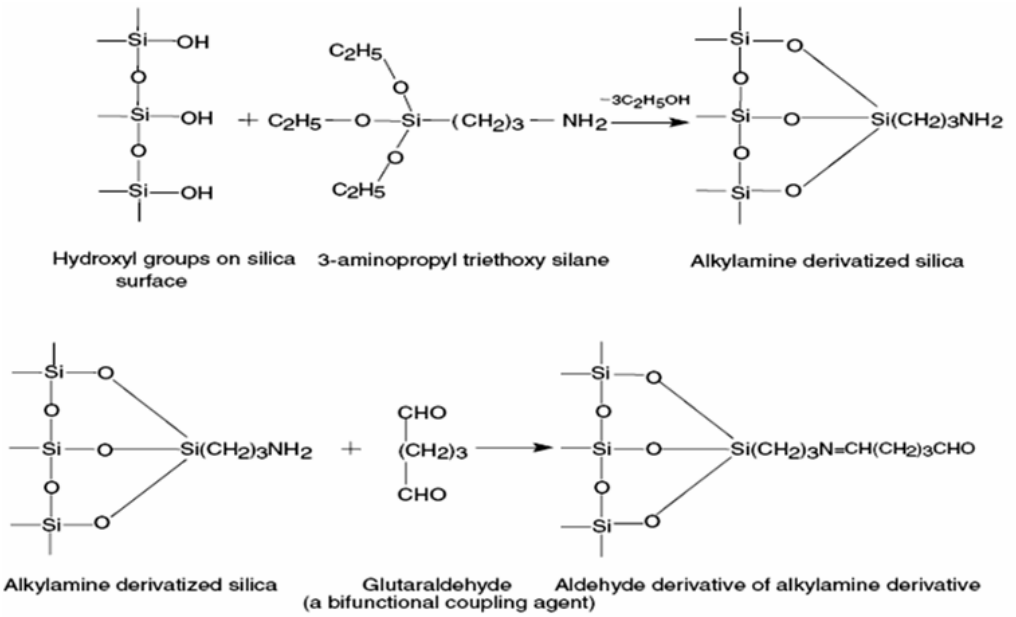


Fig.1.5 Two steps for surface modification of mesoporous silicas.

1.8 Significance of enzymes chosen

Enzymes have fascinating application in industrial, analytical, and biomedical fields. Biocatalysis is an active area of research and involves attempt to create enzyme catalyzed reactions with novel applications.

Pollution is the major concern of the environment. Phenol and phenolic compounds are ubiquitous pollutants which come to the natural water resources from the effluents of a variety of chemical industries such as refineries, phenol manufacturing, pharmaceuticals and industries of resin paint, dying, textile wood, petrochemical, pulp mill, etc [100]. So it must be eliminated from the environment by ecofriendly biocatalyst. Catalase is the enzyme which can decompose phenolic compounds to carbon dioxide and water in presence of hydrogen peroxide.

Amylases have great importance in fermentation industry and hydrolyse starch into sugar. It has considerable commercial significance and extensive

applications in food and beverage industry. Polylactic acid biodegradable polymer synthesized from sugar has diverse properties and various applications. Its biodegradability is adapted to short-term packaging, and its biocompatibility in contact with living tissues is exploited for biomedical applications (implants, sutures, drug encapsulation). Further, they are used in the manufacture of pharmacologically active digestive aids.

Lipases are the most pliable biocatalyst and bring about a wide range of bioconversion reactions, such as hydrolysis, esterification, alcoholysis, acidolysis and aminolysis. Lipases can act on a variety of substrates including natural oils, synthetic triglycerides and esters of fatty acids. They are resistant to solvents and are exploited in a broad spectrum of biotechnological applications. Lipase catalyzed transesterification, hydrolysis and esterification are the important class of reactions for food technology applications in fats and oil industry, dairy industry, pharmaceuticals and bakery industry. Lipases are very peculiar as they hydrolyse fats into fatty acids and glycerol at the water-lipid interface and can reverse the reaction in non-aqueous media. Novel biotechnological applications like biopolymer synthesis, biodiesel production, treatment of fat containing waste effluents, enantio pure synthesis of pharmaceuticals and nutraceutical agents have been established successfully. Esters of short and medium chain carboxylic acids and alcohol moieties synthesized by lipase play a relevant role in the food industry as flavour and aroma constituents. Esterification by lipases appears to be an attractive alternative to bulk chemical routes. In fact, ester synthesis using lipase can be performed at room temperature, ambiance pressure and at neutral pH in reaction vessels operated either batch wise or continuously [101]. Because of their applications these three enzymes are chosen for our studies.

1.8.1 Catalase (EC 1.11.1.6)

Catalase (CAT) (ferricatalase) are found in living organisms with molecular dimensions of (90 x 60 x 20) Å and isoelectric point at 4.8. It is a tetrameric enzyme with four phorphyrine with 57 kDa in each subunit. It functions in two way catalytically decomposing H_2O_2 to H_2O and O_2 and peroxidatively oxidizing alcohols, phenols etc [102-104]. Catalase has interesting therapeutic uses, it accelerate both healing and correct hereditary defiencies with hydrogen peroxide. Main function of this enzyme is to catalase the decomposition of 500 million mM H_2O_2 to H_2O and O_2 in 1mintues and finds more application in food industry and textile industry for removing H_2O_2 [105,106]. Catalase oxidizes alcohols and phenolic compounds in presence of H_2O_2.

1.8.2 Glucoamylase (EC 3.2.1.3)

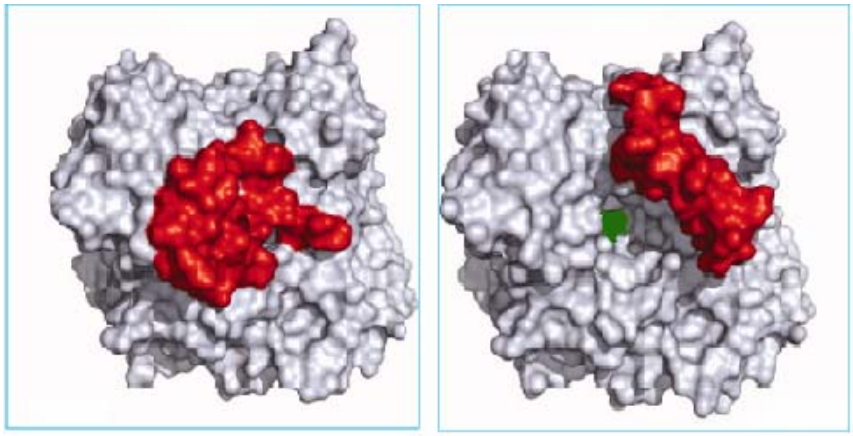
Glucoamylase (amyloglucosidase, exo-1,4 –α-D-glucan-glucanohydrolase, EC 3.2.1.3)(Glucan 1,4-α –glucosidase, exo-1- α –glucosidase ,γ-Amylase, lysosomal α –glucosidase) is an exoacting enzyme that yields β-D-glucose from the non reducing chain ends of amylase, amylopectin and glycogen by hydrolyzing α-1,4 linkages in a consecutive manner [107-109]. It hydrolyses α-1,6 and α-1,3 linkages at slow rate. It is an industrially important enzyme used in large scale for liquefaction and saccharification of starch in the food and beverages industry [110, 111]. The molecular weight of the enzyme was estimated to be 90 kDa by SDS-PAGE and gel permeation chromatography. The pI of the enzyme is 3.4.

1.8.3 Lipases (Hydrolases) (EC 3.1.1.3)

Lipase is a versatile and frequently used enzyme due to their widely diversified enzymatic properties and has a broad variety of industrial applications due to multiplicity of reaction they catalyze. Lipases have ability

to perform a wide range of organic reactions like esterification and transesterification reactions in non aqueous media, peptide synthesis, hydrolyzing carboxylic ester bonds and enatioselective hydrolytic reactions. Of all known enzymes, lipases have attracted the most scientific attention [112]. Versatility of lipase leads to multiple industrial applications in food and flavour making, pharmaceutical, synthesis of carbohydrate esters, amines and amides, bio detergent, cosmetics, perfumery, biomedical applications and biosensors[113, 114]. Its molecular dimensions are (40x36x35) A^{o}. The isoelectric point (P.I) of enzyme is 4.6. It is also called triacylglycerol hydrolases because they hydrolyze triacylglycerol at the lipid/water interface. Synthesis of biodiesel is now becoming economically attractive process. This biofuel offers several interesting and attractive properties like biodegradability and non-toxicity, compared to petroleum-based diesel. The most important advantage of biodiesel, as a renewable material, is in maintaining a balanced carbon dioxide cycle. Additionally, biodiesel combustion results in reduced emission of carbon monoxide, sulphur, aromatic hydrocarbons and soot particles [115, 116].

Two structural conformations are responsible for the substrate binding. Fig.1.6 represents two conformations of lipase. In close conformation the active site is shielded or covered by a polypeptide mobile lid. So it is called inactive conformation. By contact with hydrophobic solvent or interface, the lid opens. This is the open structure and active site is accessible for substrate binding [117]. The open lid form is more favoured thermodynamically than closed one [118]. The parameters like co solvent and modification in the pH of the microenvironment or in the dielectric constant of the active site could play a role in lid movement and in the enzyme activity [119-122]. This interfacial activation mechanism is responsible for enzyme activity.



(a) Closed conformation **(b) Open conformation**

Fig.1.6 Lipase from Candida rugosa in closed and open conformation. The lid depicted in red and active site highlighted in green which can be seen in open conformation.

1.9 Objectives of the present research work:

This present work deals with immobilization of industrially important enzymes on different mesoporous silica. The mesoporous materials were tuned by varying synthesis conditions and modified using organosilanes and glutaraldehyde. Mesoporous materials with various pore diameters for enzyme adsorption and their activities are compared in the present study. Three enzymes Aspergillus glucoamylase, Bovine liver catalase and Candida rugosa lipase were selected because of their various applications. Adsorption and covalent binding methods were adopted to immobilize enzymes. The kinetics and thermodynamic parameters of free and immobilized enzymes were determined. The main objectives of the present study are summarized here.

i) Synthesis of mesoporous silica with three different pore diameters via hydrothermal method

ii) To functionalize the silicas with 3-APTES and glutaraldehyde.

iii) Immobilization of Bovine liver catalase, Aspergillus glucoamylase and Candida rugosa lipase onto silica via two independent techniques namely simple adsorption and covalent binding.

iv) Physico-chemical characterization of the pure supports as well as the immobilized systems by various techniques like XRD, FT-IR, NMR, XPS, CHN, thermal analysis, surface area measurements, SEM, TEM and contact angle measurements.

v) To study the influence of pH and temperature on immobilization and activity of enzymes.

vi) To study the activity of immobilized glucoamylase for starch hydrolysis.

vii) To evaluate the biodegradation of phenol using free and immobilized bovine liver catalase.

viii) To synthesize esters (ethyl valerate green apple flavor and amyl isobutyrate apricot flavor) by immobilized lipase and optimization of reaction conditions.

ix) To compare the properties of the free and the immobilized lipases for the hydrolysis reaction of *p*-nitrophenyl palmitate in aqueous media.

x) To examine the scope of reusability and storage capacity of immobilized systems with respect to free enzyme.

xi) To estimate the kinetic parameters using Michaelis-Menton and Lineweaver-Burk plot and the substrate inhibition in esterification.

xii) To evaluate the kinetics data for enzyme adsorption by the Avrami model.

xiii) To study the adsorption of the enzyme onto different supports and the leaching properties of the immobilized enzymes.

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Chapter 2

MATERIALS AND METHODS

Contents

Novel methods are designed to develop synthesis of porous materials, architecting their structural morphologies and fabrication of enzyme. Adequate characterization of the catalyst can be used to evaluate and classify the materials. Various characterization techniques critically analyses quantitatively as well as qualitatively the structural behaviour of the catalyst. From fundamental point of view the ultimate goal of fundamental catalytic research is to characterize the surface of a catalyst at the microscopic level. Synthesis of porous material and their application found sustainable development in heterogeneous catalysis, biosensors, green chemistry etc. This chapter presents a detailed description of the techniques and its theoretical basis. It also describes synthesis pathway for mesoporous silica materials with different pore size distribution using surfactant and auxiliary chemical. Catalytic activity of enzymes varies when it is immobilized in porous materials having different pore diameter and pore size dependence were evaluated by adsorption and covalent binding. Depending upon the different spacer or binding, enhancement of adsorption as well as specific activity of immobilized enzymes are observed.

2.1 Introduction

Ordered mesoporous silica materials are porous inorganic supports having variable pore diameter as well as surface area which are the major

factors for a catalyst. It can be attained by varying the synthesis conditions and auxiliary chemical. Functionalization is used to increase the immobilization efficiency and to minimize the leaching effects of the supports. This chapter deals with the experimental procedure for the synthesis of mesoporous silica, functionalization and immobilization of enzymes onto the prepared samples. Techniques used for characterization and determination of catalytic activity are summarized.

2.2 Chemicals and Reagents Used

Chemicals	Company
TEOS (Tetra Ethyl Ortho silicate)	Sigma Aldrich Chemicals, Bangalore
3-APTES (Aminopropyl triethoxy silane) Glutaraldehyde	Sigma Aldrich Chemicals, Bangalore
Poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide), PluronicP123	Sigma Aldrich Chemicals, Bangalore
Trimethyl benzene	Sigma Aldrich Chemicals
n-Decane	S.d. Fine Chemicals Ltd., Mumbai.
Disodium hydrogen phosphate, sodium potassium tartarate	Merck
Aspergillus niger glucoamylase	Sigma Aldrich Chemicals, Bangalore
Bovine liver catalase	Sigma Aldrich Chemicals, Bangalore
Commercial Candida rugosa lipase (Type VII)	Sigma Aldrich Chemicals, Bangalore
Phenol, *p*-Nitrophenol	Merck
Starch	Merck
Pentanol, Ethanol,	S.d. Fine Chemicals Ltd., Mumbai.
Pentanoic acid	S.d. Fine Chemicals Ltd., Mumbai.
Isobutyiric acid	Sigma Aldrich Chemicals, Bangalore
Folin reagent	Sigma Aldrich Chemicals, Bangalore
KI, Iodine	S.d. Fine Chemicals Ltd., Mumbai.
p-Nitro phenyl palmitate, *p*-Nitro phenyl acetate	Sigma Aldrich Chemicals, Bangalore
Gum arabic and Triton X-100	Sigma Aldrich Chemicals, Bangalore
Acetone ,Dimethylsulfoxide,Dioxane, Heptane, Hexane ,iso octane ,propan-1-ol	S.d. Fine Chemicals Ltd., Mumbai.
Hydrochloric acid	Merck

2.3 Synthesis of mesoporous silica materials

Mesoporous silica materials with various pore diameters have been synthesized with and without auxiliary chemicals. The mesoporous silica materials were synthesized as per method described by Zhao *et al* [1, 2].

2.3.1 Synthesis of SBA-15 (MS-9)

Siliceous SBA-15 was synthesized 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), P123 (2.0 g, 0.3 mmol). P123 was dissolved in 2M HCl at 40-60°C.The resultant solution was rapidly mixed with silica precursor under vigorous stirring to form a reactive gel having composition 1 g P123 :2 g TEOS: 8 g HCl. 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 120°C for 48h in Teflon lined autoclave. The solid samples were separated by filtration, washed thoroughly with deionized water, 1% ammonium nitrate solution and 5% aqueous ethanol, dried at ambient temperature and calcined at 500°C for 12h.

2.3.2 Synthesis of MS -13

The mesoporous silica materials was 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 n-decane as the organic swelling agent [3]. P123 (2 g, 0.3 mmol) was dissolved in 2M HCl containg n-Decane at 40-60°C.The resultant solution was rapidly mixed with silica precursor under vigorous stirring to form a reactive gel having composition 1 g P123: 1 g n-Decane: 2 g TEOS: 8 g HCl. 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 130°C for 48h in Teflon lined autoclave .The solid samples were separated by filtration, washed thoroughly with deionized water, 1% ammonium nitrate and finally with 5% aqueous ethanol. It was dried at ambient temperature and calcined at 500°C for 12h.

2.3.3 Synthesis of MCF-25

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 [4, 5]. P123 (2.0g, 0.3 mmol) was dissolved in 2M HCl containing TMB at 40-60°C.The resultant solution was rapidly mixed with silica precursor under vigorous stirring to form a reactive gel having composition 1 g P123:3 g TMB:2 g TEOS: 8 g HCl :2 mol. 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°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. It was dried at ambient temperature and calcined at 500°C for 12h.

2.3.4 Functionalization of supports

The synthesized mesoporous silica was amino functionalized by condensing 0.5g solid with 1-5mmol 3-APTES solution in 50mL acetone at 40°C for 8 h with constant stirring. Products were cooled, separated by filtration, washed with soxhlet extraction and dried at 60°C for 6 h [6]. Following this amino functionalized silica sample was treated with 1-5 mmol solution of glutaraldehyde in distilled water for 8 h at 40°C. Glutaraldehyde is a bifunctional monomer with a molecular weight of 100 Da. It is used to pre-

stabilize the enzyme [7, 8]. The products were cooled, filtered and washed with distilled water till excess glutaraldehyde was removed which was tested by Tollens reagent and then dried at 60°C for 6 h.

2.3.5 Enzyme immobilization

Adsorptions as well as covalent binding methods were used to immobilize enzyme on to the support. 1 g supports was mixed with 20mL of 100 mM buffer [pH 3-5.5 phthalate and 6-8 phosphate] solution and 20 mg enzyme and shaken in a Remi CIS.24BL refrigerated incubator shaker at 30°C for 1 h and shaking speed was maintained at 200 rpm. The immobilized enzyme was centrifuged in Remi PR-24 Compufuge, refrigerated centrifuge at 14500 rpm and 4°C for 30 min, after that it was separated by filtration and washed with the same buffer solution until no protein is detected. The same procedure was repeated with functionalized materials.

2.3.6 Protein assay

The protein concentration was estimated using spectroscopic method of Lowery [9] with Folin – Ciocclataues's phenol reagent and absorbance was measuread at 640 nm using bovine serum albumin (BSA) as the standard by Chemito Spectroscan UV-2600 Double beam UV-Visible spectrophotometer.

2.4 Catalytic activity measurements

Batch reactor was used to evaluate the activity of immobilized enzymes. In this work, we have employed batch reactors to determine activity.

2.4.1 Batch reactor

1 mL of free enzyme solution (50 mg immobilized enzyme) was mixed with required volume of buffered substrate solution of required concentration

incubated and in shaken in a Remi CIS.24BL refrigerated incubator shaker at room temperature for fixed time and shaking speed was maintained at 200 rpm. After reaction time, an aliquot (1 mL) of the product was removed from the reaction mixture and analyzed quantitatively.

2.5 Biochemical characterization

2.5.1 Effect of pH

The influence pH during immobilization process and activity of free enzyme and immobilized enzyme was studied at different pH range 3-8 (100 mM buffer pH 3-5.5 phthalate and 6-8 phosphate) by keeping temperature and other conditions constant. The reactions were carried out in a Remi CIS.24BL refrigerated incubator shaker and shaking speed was maintained at 200 rpm

2.5.2 Effect of temperature

The reaction was carried out at various temperatures (10- 60°C) to study the effect of temperature on immobilization and activity. All other optimal conditions were kept constant.

2.5.3 Effect of buffer concentration

To evaluate the effect of buffer concentration, activities of free and immobilized enzyme were determined by using 0.01, 0.025, 0.05, 0.1 M buffer solution at predetermined optimal pH.

2.5.4 Effect of incubation time

Different incubation periods (10-240 minutes) were employed at different temperatures to study the effect on activity. All other optimal conditions were kept constant.

2.5.5 Effect of substrate concentration

The effect of amount of substrate on activity was studied by changing the substrate concentration. The study was carried out by keeping the optimum levels. The optimum amount was achieved and fixed for further experiments.

2.5.6 Effect of solvents

The effect of solvent on esterification activity of immobilized lipase was studied by using different solvents having different polarity. Solvents like hexane, heptanes, 1, 4-dioxan, dimethyl sulphoxide etc were used to determine the activity study. Enzymes show stability in organic solvents than water for esterification reaction.

2.6 Determination of activity of enzymes

Product analysis was carried out by adopting different methods like UV-visible spectrophotometer, gas chromatography, titrimetry etc. Different calculation methods are adopted to describe activity of enzymatic reactions, activity yield, specific activity, enzyme units respectively.

2.6.1 Activity of Aspergillus niger glucoamylase

Starch hydrolysis activity was measured colourimetrically by spectrophotometer, colour was developed using iodine - potassium iodide reagent and the absorbance was read at 605nm. The results were compared with absorbance of standard starch solution and the amount of starch converted was calculated.

2.6.2 Activity of Bovine liver catalase

In the case of phenol decomposition the product analysis was performed on a Chemito Gas Chromatograph (8610) equipped with flame ionisation

detector. The 2 m×3.2 mm stainless steel column packed with 10% OV-17 on chromosorb W was used for the analysis by internal standard method with ethanol.

2.6.3 Activity of Candida rugosa lipase

Lipase catalyzed esterification reaction, both gas chromatographic analysis and titrimetry are adopted. The process of esterification was monitored by determination of the residual acid content by titration against sodium hydroxide using phenolphthalein as an indicator and mixture of methanol and acetone (1:3) as a quenching agent. The ester formed was calculated as being equivalent to acid consumed. This was tested by determination of ester concentration on Chemito gas chromatograph equipped with stainless steel column packed with 10% SE-30 on chromosorb W and a flame ionization detector. Nitrogen was used as carrier gas.. Quantification of data was done by calibration with standard samples.

Ester hydrolysis activity measured colourimetrically at 410 nm by spectrophotometer. *p*-nitro phenyl esters can produce *p*-nitro phenol which can produce a yellow colour in NaOH.

2.6.4 Immobilization yield, Specific activity, Enzymatic unit, Relative activity

Different parameters were adopted to explain the percentage of immobilization and activity. Immobilization yield (IY) was calculated by measuring difference between concentrations of protein in the supernatant liquid before after immobilization

$$IY(\%) = \frac{C_i - C_f}{C_i} \times 100$$

Where C_i is the initial protein concentration and C_f is the concentration of protein present after immobilization.

The amount of protein adsorbed on the support P_g (mg g^{-1}) and immobilization efficiency (%) were determined from the following equations.

$$P_g = \frac{(C_0 - C_f)V}{w} \quad [10]$$

Immobilization effiency is calculated by two equations.

$$\text{Immobilization Efficiency (\%)} = \frac{[(E_0V_0 - E_fV_f)]}{E_0V_0} \times 100$$

Where E_0 =intial enzyme activity and E_i = final enzyme activity

$$\text{Immobilization Efficiency (IE)} = \frac{\text{Activity Yield}}{\text{Immobilization Yield}}$$

$$IE = \frac{AY}{IY}$$

In the case of lipase catalyzed esterification reactions, both percentage conversion and esterification activity were used to determine the product formation.

$$\text{Esterification Activity} = \frac{V \times m \times 100}{E \times t}$$

Where V is the difference in volume in mL of NaOH present in the blank and samples after time T (incubation time in minutes), M is the molarity of NaOH and E is the amount of enzyme in mg. Specific activity can also be used for esterification reaction using the equations.

$$\text{Specific Activity} = \frac{Vi - Vo \times m}{t \times w}$$

V_i and V_0 are the volumes of NaOH solution consumed in the lipase solution and blank experiments (mL), respectively; m is the concentration of NaOH solution (mol/L); *t* is the reaction time (min), *w* is the weight of protein (g) [11].

One enzyme unit (IU) is defined as the amount of enzyme required to produce 1μmol of product in a reaction per minute per mg of enzyme.

Relative activity is used to compare the activity,

$$\text{Relative Activity\%} = \frac{\%\ \text{of Activity at no. of day}}{\text{Maximum \% Activity}}$$

$$EF(\eta) = Vmax_{(\text{immobilized enzyme})} / Vmax_{(\text{free enzyme})}.$$

If the value of 'EF'≤1, no diffusion is assumed due to immobilization process. If 'EF' is >1 then the immobilization may have an effect on the substrate and product diffusion. The 'EF' value is usually <1 in the case of immobilized system that provides a barrier to transport of substrate and product [12].

2.7 Determination of kinetic parameters

The kinetic parameters (Michales constant K_m, V_{max}) of free enzymes and immobilized enzymes were determined by measuring the rates of reaction at various substrate concentrations at optimal conditions. The k_m and V_{max} were computed by adopting Michaelis-Menten plot, Lineweaver-Burk plot and Ping-Pong mechanism using Graph Pad prism and sigma plot nonlinear regression curves.

2.8 Determination of thermodynamic parameters

The thermodynamic parameters for thermal stability were calculated by rearranging the Erying's absolute rate equation derived from transition state theory.

$K_{cat} = (k_b/h)\ e^{(\Delta H^*/RT)}\ e^{(\Delta S^*/R)}$,

where h = Planck's constant,

k_b = Boltzmann constant,

R= gas constant (8.314 $JK^{-1}\ mol^{-1}$), N = Avogadro number, T = temperature,

Enthalpy of activation $\Delta H^* = Ea - RT$,

Free energy of substrate binding $\Delta G^*_{E\text{-}S} = RT \ln K_a$, where $K_a = 1/K_m$

Entropy of activation $\Delta S^* = (\Delta H^* - \Delta G^*)/T$[13]

2.8.1 Activation energy (Ea)

Kinetic parameter k changes with temperature according to Arrhenius equation.

$K = k_0 \exp(-E_a/RT)$

Activation energy was determined by assaying free & immobilized enzymes for constituting reaction at various temperatures ranging from 10 - 60°C. This data were plotted to get Arrhenius plot. Arrhenius plot was applied to determine the activation energy.

2.9 Reusability Storage stability and Leaching studies

Storage, reusability and leaching studies are important for immobilization. These parameters are used to determine significant advantageous of immobilized enzymes.

2.9.1 Reusability

Reusability of the immobilized enzymes was tested in a batch reactor. After each reaction, the mixture was centrifuged catalyst was separated, washed with deionized water and mixed with fresh substrate solution. The reaction was continued upto 15 cycles.

2.9.2 Storage stability

The storage stability of free and immobilized enzymes were determined after storage in 0.1M buffer at optimal pH at 4°C for a period of 3 months and the residual activities were analyzed in a regular interval. The residual activities were compared with initial activity.

2.9.3 Leaching studies

Leaching studies were investigated in the batch reactor. The immobilized enzyme was treated with optimal immobilization buffer solution and shaken for 30 minutes. It was centrifuged and the centrifugate was estimated for protein. This leaching process was repeated for 10 cycles. All results were expressed as percentage of enzyme retained.

2.10 Enzyme adsorption isotherms

To investigate the effect of temperature on the adsorption of enzymes on meso silica support, the mixtures were incubated with shaking at constant temperatures. Adsorption to the support silica was followed by removing aliquots at various times, clarifying the suspension by filtration and measuring the protein content of the filtrate. The protein content was measured by the Lowry method with BSA standard at 610nm.

The amount of protein adsorbed on the mesoporous silica P_g (mg g^{-1})

$$P_g = \frac{(C_0 - C_f)V}{w}$$

The degree of adsorption (α) from the KEKAM reaction model was calculated as

$$\alpha = P_g / P_{gm}$$

Where P_{gm} (mg g^{-1}) is the maximum amount of protein adsorbed on the mesoporous silica. The kinetic curve $\alpha = f(t)$ were analyzed by Kolmogorov - Erofeev - Kazeeva - Avrami - Mapel (KEKAM) equation for the calculation of the kinetic characteristics.

$$\alpha = 1 - \epsilon\exp(- kt^n)$$

Where α is the degree of adsorption, k is the overall rate constant of adsorption and n is the specific parameter which can be related to the adsorption mechanism. The double logarithmic form of the KEKAM equation was used.

$$\ln [-\ln (1-\alpha)] = \ln k + n \ln t$$

This form of the KEKAM equation can be successfully applied to test the experimental data to prove the protein adsorption by linear plot {ln [-ln (1-α)]} against ln t [14].

2.11 Catalyst Notations

Notation	Catalyst
MS-9	Hydrothermally synthesized silica
MS-9A	3-APTES functionalized MS-9
MS-9G	Glutaraldehyde functionalized MS-9
MS-9E	Enzyme adsorbed on MS-9
MS-9GE	Covalently bound enzyme
MS-13	Silica prepared via hydrothermal decane as auxiliary
MS-13A	3-APTES functionalized MS-13
MS-13G	Glutaraldehyde functionalized MS-13
MS-13E	Enzyme adsorbed on MS-13
MS-13GE	Covalently bound enzyme
MCF-25	Silica prepared via hydrothermal TMB as auxiliary
MCF-25G	Glutaraldehyde functionalized MCF-25
MCF-25E	Enzyme adsorbed on MCF
MCF-25GE	Covalently bound enzyme

2.12 Catalyst Characterization

All the prepared supports were characterized to study solid surfaces and the properties of catalyst by different physico-chemical techniques viz Low Angle X-ray Diffraction (XRD), BET Surface area and pore size measurements (BET), Solid state MAS NMR studies, FT-IR spectroscopy, Transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), Scanning Electron Microscopy (SEM), Thermo gravimetric analysis (TGA), Contact angle measurement and CHN Analysis These techniques are briefly described below.

2.12.1 Powder XRD

X-ray diffraction is the commonly used powerful tool to determine phase formation, lattice parameters, geometry, etc. X-Ray diffraction is one of the oldest and most frequently applied techniques in catalyst characterization. This technique is widely used for qualitative and quantitative analysis of solid phase catalysts [15]. It is used to identify bulk phases, to monitor the transformations and to estimate particle size. The XRD method [16-21] involves the interaction between the monochromatic X-rays (like Cu K α or Mo Kα) with family of planes (identified by a system of miller indices hkl) in poly crystalline materials. A fixed wavelength is chosen for the incident radiation and the Braggs peaks are identified as a function of scattering angle 2θ. The inter planar distances (d spacing) are calculated from the Braggs equation

$$n\lambda = 2d\sin\theta$$

Where, λ is the wavelength of the X-rays, d is the distance between two atomic planes in the crystalline phase, n is the order of the diffraction, and θ angle of incidence. Small angle scattering of both X-rays (SAXS) and neutrons (SANS) are

used. Conventional mesoporous silica materials like MCM-41 and SBA-15 are amorphous in nature and reflexes are observed at low angles $0.5 < 2\theta < 1^{o}$ in X-ray diffractograms and are due to the long range order induced by regular ordered arrangements of the pores. However mesoporous materials possess large d-spacing and wall thickness. Due to this reason the reflexes will appear at low angles. Unit cell parameter (a_0) of a hexagonal lattice can be calculated using the equation $a_0 = 2d_{100}/\sqrt{3}$.This unit cell parameter is also used to calculate the frame wall thickness (FWT) of the channels of mesoporous materials. Wall thickness = unit cell parameter (a_0) – pore diameter calculated from surface area measurements.

Low Angle X-ray diffraction studies (SAXS) of the silica and the immobilized supports were taken on a Panalytical Xpert PRO MPD model with Ni filtered Cu K_α radiation (λ-1.5406A^{o} 40 kV) within the 2θ range 0.1-10^{o} at a speed of 1°/minute at room temperature.

2.12.2 Nitrogen adsorption-desorption studies

Measurements of gas adsorption isotherms are widely used for determining the surface area and pore size distribution of solids. Surface area determination is an important factor in predicting the catalyst performance. The Brunauer, Emmett and Teller (BET) method [22] is widely used to determine total surface area. It is based on the extension of the Langmuir theory to multilayer physical adsorption. In the BET theory, it is assumed that solid surface possess uniform localized sites and the adsorption at one site does not affect the adsorption at neighboring sites. The adsorption is multilayer and the heat of adsorption of second and subsequent layer are identical and is equal to the liquefaction of the adsorbate.

The BET equation can be represented as

$$\frac{P}{V(P_0-P)} = \frac{1}{CV_m} + \frac{(C-1)}{CV_mP_0}$$

Where,

P = Adsorption equilibrium pressure

P_0 = Saturated vapour pressure of the adsorbate

V = Volume occupied by molecules adsorbed at equilibrium pressure

V_m = Volume of the adsorbate required for monolayer coverage.

C = Constant related to the heat of adsorption.

A plot of $P/[V (P_0-P)]$ against P/P_0 is a straight line with slope $(C-1)/V_mC$ and intercept $1/V_m C$. From the slope and intercept, V_m can be calculated and the specific surface area of the sample can be calculated using the relation,

$$\text{Surface area } (m^2g^{-1}) = \frac{V_m \times N_0 \times a_m}{22414 \times \text{weight of the catalyst}}$$

Where,

N is Avogadro number

a_m = Molecular cross sectional area of the adsorbate (for N_2 molecule 0.162 nm^2)

In BET method, adsorption of N_2 is carried out at liquid nitrogen temperature. Previously activated samples were degassed at 300°C under nitrogen for 3h and then brought to -196°C using liquid nitrogen for N_2 gas at various pressures. The pore volume is measured by the uptake of N_2 at a relative pressure of 0.9. Pore size distributions for micro pores as well as meso- and macropores were calculated from N_2 -desorption isotherms by

differential HK (Horvath-Kawazoe) and BJH (Barrett-Joyner-Hallender) methods respectively [23].

Adsorption isotherms

The amount of N_2 adsorbed over a range of partial pressures at a single temperature is known as adsorption isotherm. Capilary condensation is normally characterized by a step in the adsorption isotherm. Materials with uniform PSD, the capillary condensation is sharp. [24] These isotherms can have different shapes depending upon the type of adsorbent, adsorbate and interaction between the gas and the surface. According to IUPAC adsorption isotherms are classified into six and are described in Fig.2.1 Adsorption at high p/p_0 on porous materials is characterized by hysteresis.

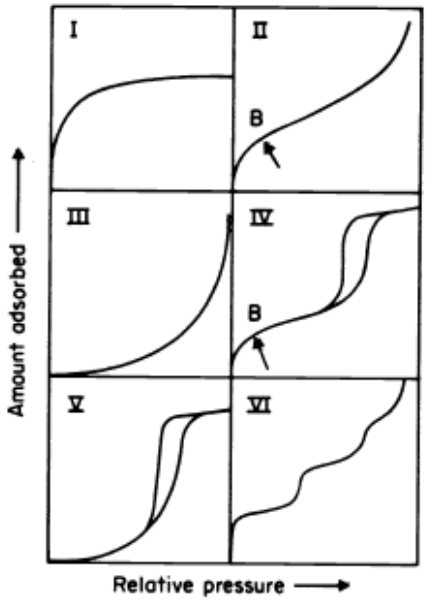


Fig. 2.1 Type of physisorption isotherms

Type I shows adsorption isotherms characteristic of microporous adsorbents. Adsorption isotherms on macroporous adsorbent with strong and weak affinities are characteristic of Type II and Type III. Type IV and

Type V isotherms characteristic of mesoporous adsorbent with strong and weak affinities. Finally, type VI isotherm represents stepwise multilayer adsorption on a uniform non-porous surface.

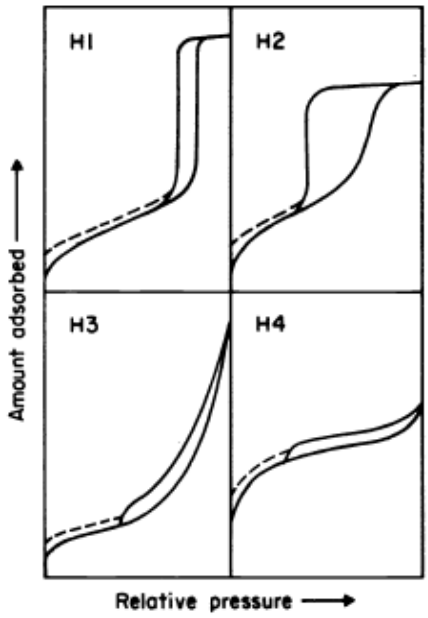


Fig 2.2 IUPAC classification of hysteresis loops

Standard Hysteresis loops according to IUPAC classification is based on change of geometry during adsorption and desorption process and the Kelvin equation was used for theoretical justification [25-27]. The hysteresis loop is seen in materials consisting of slit –like pores, cylindrical like pores and spherical pores i.e. ink-bottle pores [28**]** H1 is typical for type IV isotherms, H2 is characteristic for ink-bottle pores and are distorted and their PSD are not well defined and H3 and H4 are commonly associated with thin slit pores which are shown in Figure 2.2 [29-31].

Pore Size Distribution (PSD)

The pore width is equal to diameter in the case of cylindrical shaped pores and the distance between opposite walls in the case of slit shaped

pores. The PSD is a plot of $\Delta V_p/\Delta D_p$ vs D_p where V_p is the pore volume accumulated upto pore width D_p.

The average pore size at which 50% of the volume is larger and 50% smaller can be determined from the pore size distribution. Early calculations of pore size distribution were based on adsorption desorption isotherms. Pore size distributions were calculated by BJH method. In this approach the filled pores are taken as a starting point. The emptying of the filled pores with decreasing relative pressure is incrementally evaluated to obtain a pore diameter distribution. For each incremenent the pore diameter of pores emptied is calculated according to the Kelvin equation.

$$\frac{P}{po} = \exp\left(-\frac{2\gamma\gamma^1}{rK * RT}\right)$$

Where, P/P_0 is the relative vapour pressure over a curved surface, γ is the surface tension, υ is the molar volume of the liquid and r_k is the radius of curvature. The Kelvin equation shows that the smaller the pore radius, the lower the vapour pressure, P.

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°C followed by degassing of 16h at 200°C. Functionalized materials were degassed at 60°C for 16 h. Immobilized enzymes were degassed at room temperature overnight.

2.12.3 Solid State NMR spectroscopy

Solid state NMR Spectroscopy is a kind of nuclear magnetic resonance (NMR) spectroscopy. It is an important tool to investigate structural properties of different kinds of solid material and is characterized by the presence of

anisotropic (directionally dependent) interactions. It is a straight forward application of NMR techniques for the determination of structure of porous materials, polymers, crystalline materials and finds several applications in catalysis, analytical chemistry and biology. The principal use of NMR spectroscopy in heterogeneous catalysis is to characterize the chemical and structural environment of atoms in the catalyst s or in species adsorbed at the catalyst surface.

Solid-state NMR studies of heterogeneous catalysts are carried out on powder samples. Special techniques have to be applied to remove or reduce the line broadening effects. The most important techniques for line narrowing are

(i) Dipolar decoupling
(ii) Magic angle spinning(MAS)
(iii) Double oriented rotation (DOR)
(iv) Cross –Polarization (CP)

Heteronuclear dipolar interactions between two nuclei can be removed by irradiation of a strong Rf field at resonance frequency of the other nucleus. High –power dipolar decoupling is widely applied to remove line broadening effect due to heteronuclear dipolar interactions with proton. eg. ^{13}C-$_1H^1$ interactions in organic molecules rigidly bound at the surface of catalysts, or ^{29}Si-$_1H$ interactions of surface Si-OH groups in silica and related materials [32, 33].

MAS (Magic angle spinning)

The most popular technique for line narrowing in powders is fast mechanical sample rotation about an axis inclined at the magic angle $\Theta = 54\ 44^{o}$ to the direction of B_0. This is called magic angle spinning (MAS) which removes

line broadening from dipolar interactions, chemical shift anisotropy and quadrupole interactions.

DOR (Double oriented rotation)

Second order quadrupole line broadening can't be removed by MAS but effectively eliminated by spinning the sample simultaneously around two axes. In this experiment a large outer rotor rotating at the magic angle contains a smaller inner rotor with the sample, the spinning axis of which inclined at an angle of 30.6 relative to that of the out rotor dynamic angle spinning (DAS) involves successive rotation of sample around two different axes by switching the direction of the spinning axis in subsequent time periods.

CP (Cross Polarization)

It does not affect the line width but is applied to improve sensitivity. Signal to noise ratio of spectra of nuclei with low natural abundance (for ^{13} C, ^{29} Si) and to monitor the spatial proximity of the nuclei. This technique employs indirect excitation of the observed nucleus Y (less abundant nucleus) through magnetization transfer from an abundant spin system X (usually $_1H^1$) to spin system Y

According to Hartmann-Hahn condition $\gamma_x B1_x = \gamma_y B1_y$, Where γ_x and γ_y are gyro magnetic ratios and $B1_x$ and $B1_y$ the radiofrequency field strength of the nuclei X and Y respectively. The maximum gain in sensitivity that can be achieved in CP experiments depends on the ratio γ_x/γ_y. It is very useful technique for sensitivity enhancement in NMR spectra of nuclei with low abundance. CP between nuclei located at the surface of a catalyst (for surface hydroxyl groups or adsorbed molecules) and other nuclei present in the bulk and surface. It can be applied to surface selective studies and used to distinguish between mobile and immobile components of surface system.

Most of the structural information that can be derived from a high resolution NMR spectrum of solid catalyst relies on the following parameters.

(i) The number of signals in the spectrum.

(ii) The relative signal intensities *I*

(iii) The isotropic chemical shift δ_{iso}

(iv) The quadrupole coupling constant QCC

(v) The assymetry parameter of electric field gradient.

Solid State ^{13}C NMR

The ^{13}C CPMAS NMR spectra of as-synthesized materials give us information about the incorporation of intact organic structure directing agents inside the channels of these materials. ^{13}C CP/MAS NMR is a valuable tool to interpret the organic moiety present in the mesosilica material after functionalization with 3-APTES, glutaraldehyde and enzyme immobilization.

Solid-State ^{29}Si NMR

Si environment of the samples were analyzed by ^{29}Si MAS solid state NMR. Solid State MAS NMR experiments were carried out over a Bruker DSX-300 spectrometer at a resonance frequency of 78.1MHz. For all measurements a standard 4 mm double-bearing Bruker MAS probe was used. The sample spinning frequency was 8 kHz with a single pulse excitation corresponding to $\pi/2$ flip angle. The pulse lengths for the experiments were 10 μs whereas pulse delay was 2 s .The number of scans collected was 1024. XWIN NMR software was employed to acquire and retrieve data.

2.12.4 FT-IR Spectroscopy

Infrared absorption spectroscopy is a rapid, economical and non-destructive physical method universally applicable for structural analysis [17]. The technique is so versatile that it can be used both as a source of the physical parameters of crystal lattice determination and as a means of eliciting purely empirical qualitative relationships between specimens. It is an intrinsically simple technique that deserves to be more widely used in clay mineralogy and soil science.

The absorption of infrared radiation by solid materials depends critically on atomic mass, length, strength and force constants of inter - atomic bonds in the structures of these materials. It is also controlled by the constraints of the overall symmetry of the unit cell and the local site symmetry of each atom within the unit cell. The total number of potentially active internal vibrations is given by 3n-6, where n is the number of atoms in the unit cell. Not all of these vibrations are active in the infrared; only those that undergo a change in dipole moment during the vibrations are IR active.

In non-dispersive FTIR spectrometers, the detector continuously monitors the entire wave number range of radiation emitted by the IR source, providing an inherently more sensitive system. Fourier transform instruments use interferometers and they require a dedicated computer to transform their output an interferogram into an absorption spectrum. Fourier transform infrared spectrometers may be either single beam, in which the sample spectrum must be rationed against a background or double beam, in which rationing against background is carried out continuously. The infrared spectra of the prepared samples were recorded by DR-IR spectrometer, Thermo FT-IR Nicolet 380 in the range 400-1400cm^{1}.

2.12.5 Transmission Electron Microscopy

TEM uses electrons to determine the structure with high resolution. The light source is replaced by cathode filament that acts as a source of electrons. The electron source which is on the upper side of TEM must be placed in a vacuum and its position is reversed with respect to the position of the light source. Electrons are accelerated toward a given sample by a potential difference of 100,000V. A series of cylindrical magnets and metal apertures are used to focus the electron beam into a monochromatic beam. This beam collimated by condenser lenses collides with sample and interacts with it depending on the density of the material. These interactions are affected by the specimen. A beam of electrons is transmitted through specimen the image formed is magnified, directed to appear on a fluorescent screen or layer of photographic film and is detected by a sensor (CCD camera –charge couple device) [34]. TEM analysis was carried out in ultrahigh resolution analytical electron microscope JEOL 3010.This gives lattice resolution of 0.14nm and a point to point resolution of 0.12 nm. Bulk materials have to be thinned to make them electron transparent. A sonicated solution of the sample in acetone which evaporates on the TEM grid to form a dry film was prepared. The typical column vacuum in machine is $< 1x10^{-5}$Pa.

2.12.6 Scanning Electron Microscope (SEM) analysis

Scanning Electron Microscopy (SEM) was used for high magnification imaging. It is based on the strong interaction of electrons with matter and appreciable scattering by quite small atomic clusters. Electrons can be conveniently deflected and focused by electric or magnetic fields so that magnified real-space images can be formed in addition to simple diffraction patterns. This property of electron beam is used in SEM analysis. In SEM the electron optics act before the specimen is reached to convert the beam into a

fine probe, which can be as small as 100 Å in diameter [35]. It is employed by bombarding the specimen with a scanning beam of electrons and collecting the slow moving secondary electrons that the specimen generates. These electrons are collected, amplified, and displayed .The electron beam and cathode ray tube scan synchronously so that an image of the surface of specimen is formed [36]. Images are taken at a very slow rate of scan in order to capture greater resolution. The technique is of high interest in catalysis because of its high resolution. It is used to find out the topology and composition of the surface. In addition to the morphological information given by secondary electrons, the SEM used to sense X-ray fluorescence (XRF) signals from the analyzed sample which are used to determine the elemental composition of the sample. However, serious drawback is that the results need not be really representative of the whole sample. This can be overcome by making many analyses at different locations of the sample particles and many catalyst particles. The scanning electron microscopy of the samples were carried out on a JOEL JSM 840 A (Oxford make) model 16211 SEM analyzer with a resolution of 1.3 eV. The samples were adhered to the sample holder with adhesive tape and then coated at vacuum by cathode sputtering with a layer of gold to minimize charge effects.

2.12.7 Thermogravimetric analysis

Thermogravimetric analysis (TGA) is a simple analytical technique that measures the weight loss or gain of the material as a function of temperature or time during controlled heating. As the materials are heated weight loss due to water desorption, structural decomposition of materials or from chemical reactions are observed. Some materials can gain weight by reacting with the surrounding atmosphere. A plot of mass or mass percent as a function of time is called thermogram, or a thermal decomposition curve (weight loss or gain

converted to percent weight loss on the y-axis plotted versus the sample temperature in degree Celsius on the x-axis [37]. The horizontal region of the thermogram indicated the stability of the sample while the curved portion indicates weight loss. DTG is the first derivative plot of the TG curve from which a better understanding of the weight loss can be obtained from the dip in the curve. The main advantage is the results are specific for particular sample. It is also used to determine the thermal stability of mesoporous materials.

TG analysis of the samples was conducted on a Perkin Elmer Pyris Diamond 6 thermo gravimetric /differential thermal analyzer by heating the sample at the rate $10^{o}Cmin^{-1}$ from 40°C to 800°C in Nitrogen/ inert atmosphere.

2.12.8 Organic elemental analysis (CHN analysis)

Dynamic elemental analysis is concerned with the quantitative and qualitative determination of one or more chemical elements in an organic sample. In the CHN analyzer, organic compounds are oxidized at high temperatures to yield carbon dioxide, water and oxides of nitrogen (NO_x)

$$\text{CHN compound} \xrightarrow[1000^0 C]{O_2} CO_2 + H_2O + NO_x$$

Oxides of Nitrogen are then converted to nitrogen gas in the presence of metallic copper.

$$NO_x\ NO_x \xrightarrow[600^0 C]{Cu} N_2$$

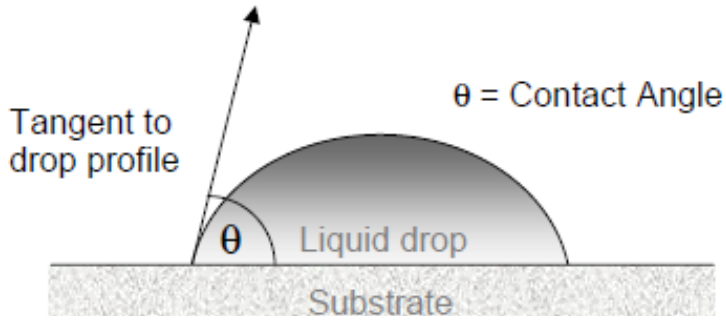
CO_2, H_2O, and N_2 are separated quantitatively and measured individually. Commercial CHN analyzers are classified into two categories. In

one category CO_2, H_2O and N_2 are separated by gas chromatography where as in the other three products are separated by selective adsorption.

Organic elementary analysis (C, H, and N) was performed on a Elementar Vario EL III apparatus. The organic material in a sample of 2 mg of functionalized silica material was combusted at 1000°C in a flow of He with O_2. The amount of evolved CO_2, H_2O, and N_2 were used to calculate the content of C, H, and N and the products were separated by selective adsorption [38].

2.12.9 Contact Angle measurements

The contact angle measurements are used to demonstrate the relationship between the properties and chemistry of a surface and also the surface free energy of the monolayers by measuring contact angles as a function of surface tension of a series of liquids.

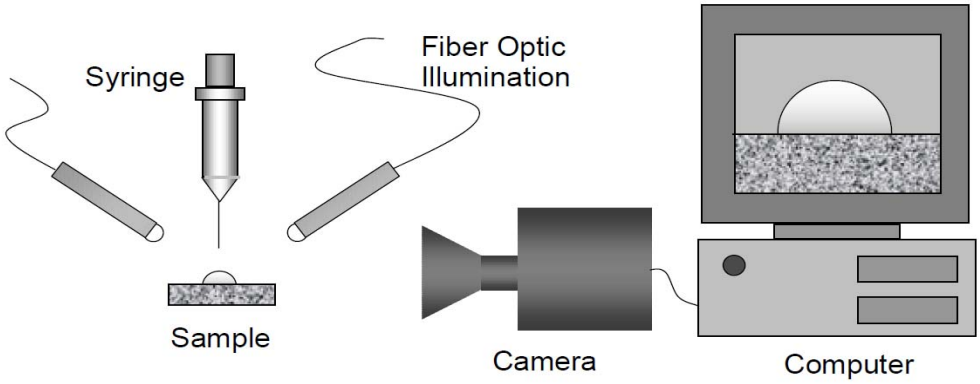


$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \Theta$$

Θ is the apparent contact angle of water with the surface, γ_{lv} the interfacial surface tension of liquid and vapour interface, γ_{sl} the interfacial surface tension of solid liquid interface, γ_{sv} is the apparent surface free energy of the solid. From the equation it is clear that the contact angle is function of surface tension of solid and liquid. The liquid with surface tension greater than the surface tension of solid $(\gamma_{lv} > \gamma_{sv})$ make definite contact angle with solid

surface and if the surface tension of liquid is less than the surface tension of solid then total wetting occurs which indicates $\Theta = 0^0$,γ = surface tension (the energy required to create a unit area of an interface [39].

If the energy required to create the solid-liquid (sl) interface is greater than that required for creation of a solid -vapour (sv) interface, then the critical angle will be greater than 90^o i.e. the liquid will be bead up on the surface to minimize the solid –liquid interfacial area.



A sessile drop of water was manually deposited onto the surface of sample. The drop radius usually was larger than 3 mm. A sequence of images of the drop was then recorded and analyzed by the Rame-hart contact angle meter at ambient humidity and temperature using distilled water as the medium [40].

2.12.10 X-ray Photoelectron Spectroscopy (XPS)

It is most widely used method for surface chemical analysis. It provides quantitatively analyze the chemical composition in the surface region and to identify different chemical states of an element. The core electrons are excited by X-ray through photoelectric effect and the kinetic energy of the emitted

electrons are measured in terms of binding energy. Depending on the chemical environment of atoms, the binding energy of core electrons varies; it helps to get the information about the chemical nature of elements present in the sample. It gives quantitative information of the sample. It is also used to find out the oxidation state. If the element with higher binding energy shows its higher oxidation state, but the lower binding energy is the evidence for an increased electron density around the element.XPS spectra were recorded in an electron spectrometer equipped with Thermo VG Clamp -2 Analyzer and a Mg Kα X-ray source (1253.6 ev, 30 mA x 8 kv). A thin sample wafer of 12 mm in diameter was used for measurements.

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Chapter 3

PHYSICO – CHEMICAL CHARACTERIZATION

Contents

Designing a catalyst is a fascinating challenge and correlating logical extension to reveal the structural properties. Numerous popular characterization techniques are employed to evaluate and identify the synthesized, functionalized and immobilized materials. Immense strides have been taken in characterizing the surface and bulk structure of catalysts. It is essential to evaluate the structure and properties of the synthesized materials qualitatively and quantitatively. The main aim of the physcio-chemical characterization is the ready identification of hidden information of the 2D surface structure, nature of bonding and porosity. Immobilization has been used to impart novel characteristics to an enzyme, thereby intentionally modifying its catalytic behaviour which can be verified by characterization techniques.

3.1 Catalyst characterization

Several characterization approaches are adopted to investigate the synthesized catalyst. The performance of the catalyst can vary widely depending on the nature of the sample which can be evaluated by the characterization

studies. Combinations of different techniques give an idea about physical, electronic, chemical and surface properties of the catalyst. This chapter summarizes the results of the physicochemical characterization of different mesoporous silica materials and their modified forms. Various techniques like Low Angle X-ray Diffraction Analysis (XRD), thermal analysis (TG/DTG), N_2-adsorption-desorption, Fourier Transform infrared spectroscopy (FTIR), CHN elemental analysis, CPMAS Nuclear magnetic resonance spectroscopy (NMR), X-ray photoelectron spectroscopy (XPS), contact angle measurements, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) of bare, functionalized and enzyme immobilized samples are discussed in this chapter.

3.2 Low Angle X-ray Diffraction analysis

Mesophase formation was monitored by different characterization techniques and the important one is low angle XRD. Since mesoporous materials do not possess three dimensional crystallinity at atomic level, reflections are not observed at higher angles but, reflections are observed at very low angles [1]. Ordered mesoporous material shows three well resolved diffraction peaks indexed as (100), (110), (200) planes which are characteristic for meso phase and are associated with a hexagonal p6mm symmetry. A very strong peak due to (100) and the decrease in intensity of (110) and (200) plane are observed after post synthesis modifications. Hexagonal unit cell parameter 'a' was calculated by the equation, $a = 2d_{100}/\sqrt{3}$. Both functionalization and immobilization can cause change (decrease or increase) in 2θ value.

XRD patterns of synthesized, functionalized and enzyme immobilized MS-9 are shown in Fig.3.1. The peaks obtained are the reflection of mesostructure .After functionalization, the peaks shifted to the right is an indication for pore

shrinkage but ordered structure is preserved. A very intense peak at 0.88° and another two peaks at 1.50° and 1.74° represent (100), (110) and (200) planes respectively. When 3-APTES and glutaraldehyde was loaded to MS-9, the 2θ value is shifted towards lower value (0.87°) and slight increase in d-spacing and unit cell parameter are observed. This indicates that functionalization slightly disturbs the ordered structure and it is due to the large contrast in density between the silica walls and the empty pores relative to that between the silica walls and the pores filled with enzyme molecules [2-5].

Table 3.1 XRD results of pure, functionalized and immobilized samples of MS-9

Sample	2θ (°)	d spacing	a (Å)	Wall thickness(Å)
MS-9	**0.88**	**100.12**	**115.61**	**26.61**
MS-9A	**0.87**	**100.77**	**116.36**	**44.36**
MS-9G	**0.87**	**100.06**	**116.69**	**48.69**
MS-9GE	**0.86**	**100.69**	**117.42**	**54.42**

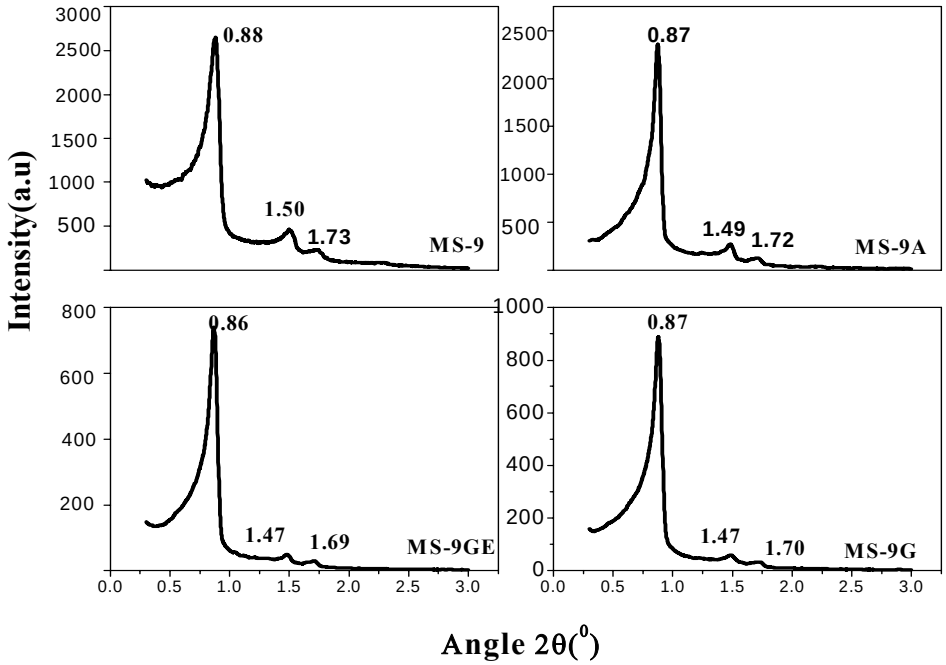


Fig. 3.1 X-ray diffraction patterns of MS-9 family

The Small angle X-ray diffractograms of MS-13, MS-13A, MS-13G and enzyme immobilized samples are presented in Fig 3.2. MS-13 shows an intense peak at 2θ = 0.84° and corresponding d-spacing value is 105.09 Å. After functionalization and immobilization, 2θ value shifted towards lower angle and one diffraction signal vanished in the case of enzyme loaded MS-13A and MS-13G indicating that immobilization perturbs the ordered structure of mesoporous silica. The (100) values and the unit cell parameters calculated were shown in Table3.2. The d-spacing shifted to higher values and unit cell parameter also changed after post treatment and enzyme immobilization. A similar trend was observed for wall thickness. The result describes that the d-spacing value increases up to 30Å when MS-13 were modified with functional group and enzyme. This indicates that functionalization and immobilization take place in the pore and it can cause the pore blockage.

Table 3.2 XRD results of pure, functionalized and immobilized samples of MS-13

Catalyst	2θ	d(100)Å	a(100)Å	Wall thickness
MS-13	0.84	105.09	121.35	0.35
MS-13A	0.83	106.40	122.86	14.86
MS-13G	0.65	135.86	156.88	69.88
MS-13E	0.68	129.91	150.01	38.01
MS-13AE	0.67	131.84	152.25	73.25
MS-13GE	0.61	144.82	167.22	90.22

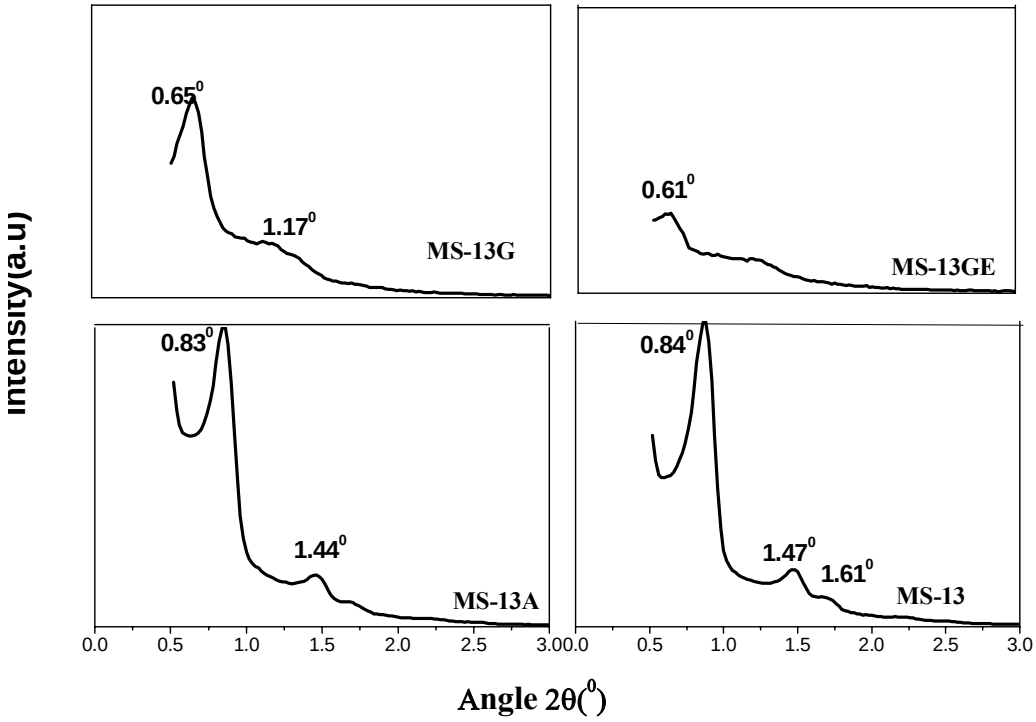


Fig. 3.2 X-ray diffraction patterns of MS-13 family

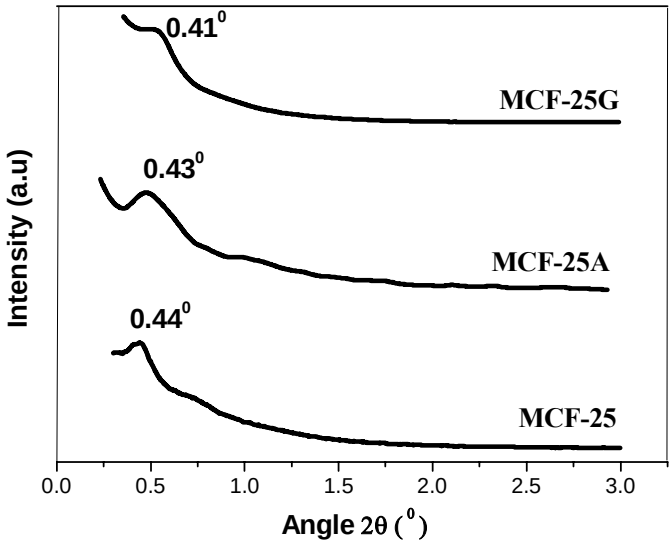


Fig. 3.3 X-ray diffraction patterns of MCF-25 family

Table 3.3 XRD results of the pure, functionalized samples of MCF-25

Sample	2θ (°)	d spacing Å
MCF-25	0.44	199.98
MCF-25A	0.43	202.27
MCF-25G	0.41	211.45

Synthesized MCF material shows only one XRD peak in the 2θ range ~ 1-2°, it is quite different from SBA-15 and other mesoporous materials. MCF - 25 silica shows one peak at 2θ= 0.44° corresponding to a d spacing of 199.98 Å. This peak represents the (100) plane and indicates the presence of mesopores. It also shows that cells in MCF materials are spherical and have uniform size. After functionalization with 3-APTES and glutaraldehyde this peak shifts to lower 2θ values of 0.43°, 0.41° for MCF-25A and MCF-25G respectively. But they show increase in d spacing after functionalization and immobilization probably due to the disordering of the pores and the functionalization takes place within the pores. The d-spacing increases from 199.98-211.45Å. These materials exhibit broader peaks and its intensity is lower than SBA-15. Result demonstrates that MS-9 and its functionalized materials retain ordered structure than MS-13 and MCF-25 materials.

3.3 N_2-adsorption desorption Studies

According to literature mesoporous material show type IV isotherm and exhibits an H1– type hysteresis loop at relatively high pressure which are the characteristics of hexagonal cylindrical open channels in these materials. But the H1 hysteresis loop indicates the presence of open channels [6, 7]. Pandhya *et al* reported that solid materials with uniform pore sizes and shapes give H1 hysteresis and those with non-uniform pore sizes and shapes give H2 hysteresis [8]. Narrow pore size distribution (PSD) is a characteristic feature of

well ordered porous materials described by Katiyar *et al* [9]. The pore size distribution curves are based on the BJH (Barrett-Joyner-Hallender) method.

Fig 3.4 shows the nitrogen adsorption desorption isotherms of the calcined mesoporous material MS-9 and the changes are observed when functionalized with 3-APTES, glutaraldehyde and immobilized with enzymes. Details of porosity and surface area of calcined, functionalized and immobilized samples are summarized in Table 3.4. All these samples show type IV isotherms typical for mesoporous material but a clear hysteresis loop of H1 type is observed and capillary condensation occurs at high relative pressure, indicating large cylindrical mesopores (hexagonal cylindrical open meso channels). The pore distribution curves (BJH plots curves are not shown here) show a clear shift of pore diameter from 89 to 68 Å, are when functionalized with 3-APTES and glutaraldehyde. The pore volume is found to be 1.3707, 0.8649 0.7858, 0.5655 cm^3/g for MS-9, MS-9A, MS-9G and MS-9G respectively. The reduction in volume of nitrogen adsorbed observed in amino, glutaraldehyde and enzyme loaded samples is due to external adsorption of the molecule. There is no steep uptake of nitrogen at low relative pressures that indicates the low micro porosity in MS-9 and the functionalized materials. This is well correlated with the Harkin-Jura t-plot. This micro pores may be formed due to the removal of template by calcination. The hydrophobic ethylene oxide tails partially penetrated to silica walls during preparation. The micro pore volume estimated from t-plot for MS-9 is 0.0656 cm^3/g. After glutaraldehyde functionalization it decreases to 0.00932 cm^3/g. It indicates that microporsity decreases upon surface modification.

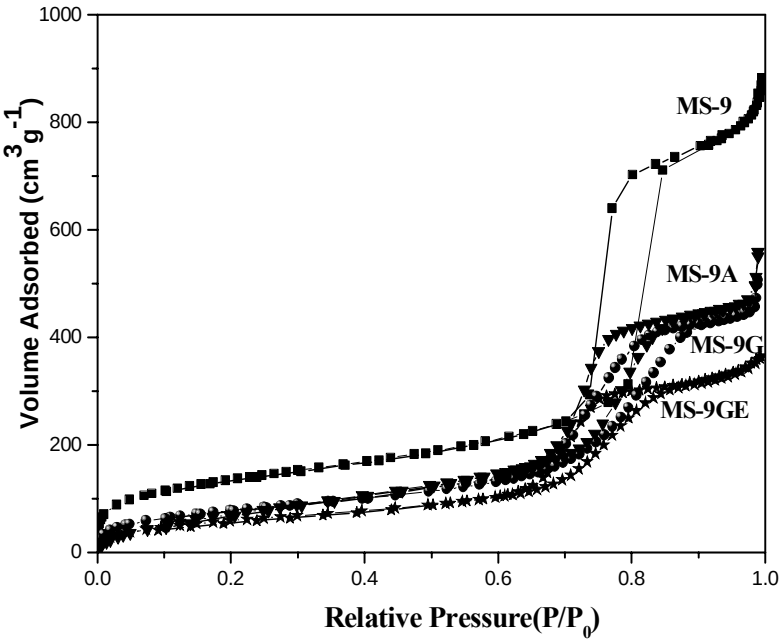


Fig. 3.4 Nitrogen adsorption isotherms of MS-9 family

In adsorption isotherm, pressure range P/P_0 is less than 0.30. It indicates the monolayer formation of nitrogen gas adsorbed on mesopore walls and which is used for calculation of BET surface area. Inflection observed around P/P_0 = 0.50 shows capillary condensation within mesopores. A sharp inflection in N_2 adsorption at P/P_0 = 0.75, represents the narrow pore size distribution in MS-9 which is characteristics of highly ordered mesoporous materials. There is no steep uptake of nitrogen at low relative pressures that indicates the low micro porosity in MS-9 and the functionalized materials. There is a large decrease in sharpness of inflection after surface modification with 3-APTES and glutaraldehyde. Upon enzyme immobilization the inflection step almost diminished which indicates the pore blockage.

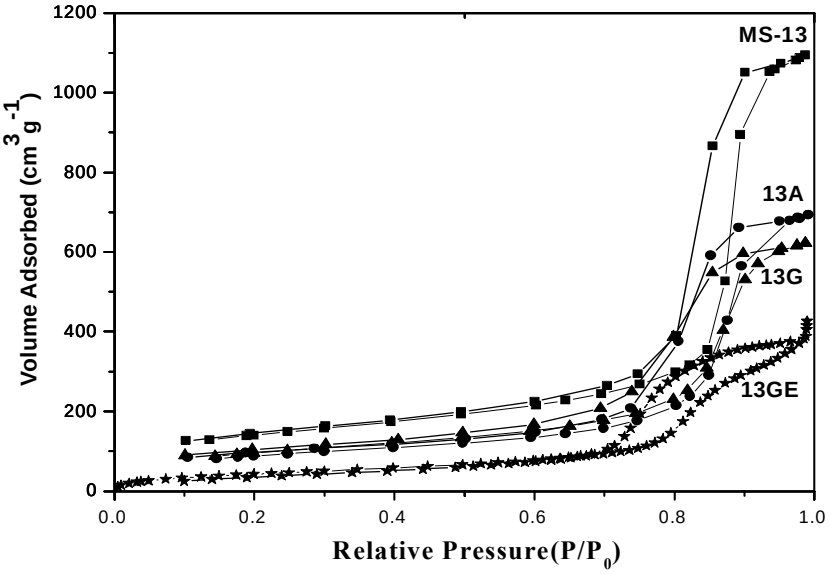


Fig. 3.5 Nitrogen adsorption isotherms of MS-13 family

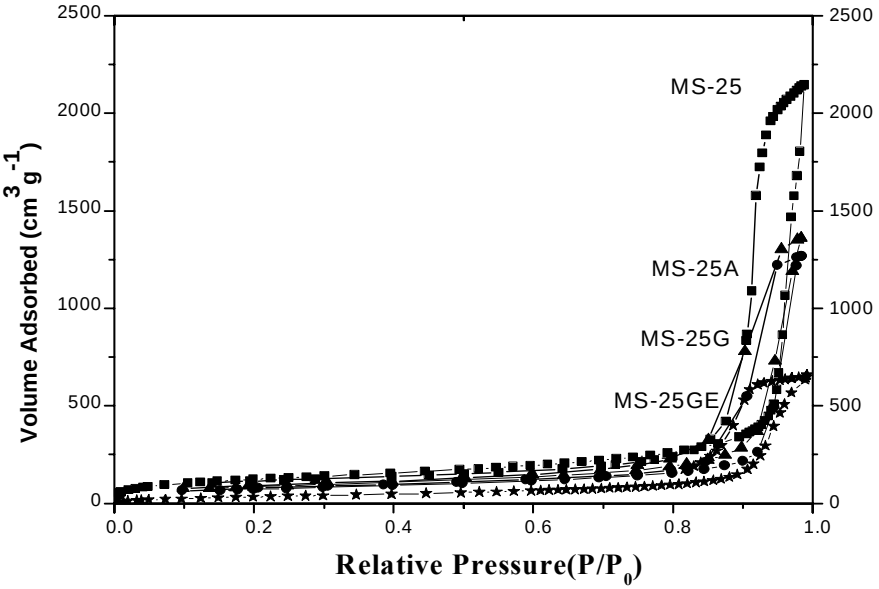


Fig. 3.6 Nitrogen adsorption isotherms of MCF-25 family

Table 3.4 Textural parameters of pure, functionalized and immobilized samples

SAMPLE	Pore Diameter (Å)	Surface Area (m^2/g)	Pore Volume V_p (cm^3/g)	Micropore volume V_{mi} (cm^3/g)	Mesopore volume V_{ms} (cm^3/g)	Macropore volume V_{ma} (cm^3/g)
MS-9	89	474	1.3707	0.0660	1.1835	0.1212
MS-9E	82	342	1.2325	-	1.1663	0.0662
MS-9A	72	288	0.8649	0.0195	0.6446	0.2008
MS-9AE	69	258	0.7502	-	0.7205	0.0297
MS-9G	68	259	0.7858	0.0093	0.6521	0.1244
MS-9GE	60	256	0.5655	-	0.5101	0.0554
MS-13	121	479	1.6899	0.0033	1.5814	0.1052
MS-13E	112	278	1.3220	-	1.1056	0.2164
MS-13A	108	334	1.0779	0.0031	1.0252	0.0496
MS-13AE	79	203	0.8906	-	0.7390	0.1516
MS-13G	87	303	0.9594	0.0030	0.8798	0.0766
MS-13GE	77	165	0.6599	-	0.5703	0.0896
MCF-25	241	396	2.9888	0.0262	2.8501	0.1125
MCF-25E	135	358	1.6921	-	1.2489	0.4432
MCF-25A	211	300	2.1027	0.0248	1.1104	0.9675
MCF-25AE	168	167	1.2910	-	1.2450	0.0460
MCF-25G	189	255	1.9606	0.0219	1.2597	0.6790
MCF-25GE	184	135	1.0173	-	0.9155	0.1018

Textural parameters of pure, functionalized and immobilized samples are summarized in Table 3.4. MCF materials show significant increase in pore diameter. It is observed by varying synthesis parameters (synthesis time, synthesis temperature) or post synthetic treatment. It mainly depends on the template used and addition of auxiliary organics [10-12]. The transition from SBA-15 to mesocelluar foam occurs at TMP-template mass ratio greater than 0.2. This gradual transition to the MCF phase is characterized by a sharp increase in the pore size. The increased hysteresis in Nitrogen sorption isotherm indicates a change in pore morphology [13] and the sharp rise at

relatively high pressures (P/P_0 near 1) indicates the presence of large mesoporous material. Normally MCF materials possess sharp rise at relative pressure $P/P_0 > 0.9$. In MS-9 N_2 adsorption occurs at $P/P_0 = 0.75$, MS-13 N_2 adsorption at $P/P_0 = 0.85$ but in MCF-25 N_2 adsorption occurs at $P/P_0 = 0.95$. MCF-25 has large mesoporocity than other prepared silica material as it is evident from the data given in Table 3.4. The mesopore volume of MS-9, MS-13 and MCF-25 are 1.1835 cm^3/g, 1.5814 cm^3/g, 2.8501 cm^3/g respectively.

Type C hysteresis is the characteristic of materials with spheroidal pores with circular cavity radius with various dimensions for entranced pore. Open and closed ink-bottles with a heterogeneous distribution of neck radii would also cause such a hysteresis loop. The hysteresis loop is significantly large as shown in Fig 3.6. According to BdB (Broekhoff – de Boer) model the pores are treated as spherical cells with small openings or windows. MCF-25 is MCF type material, having large pore diameter (241 Å) and high pore volume (2.9860cm^3/g).

Maximum volume of nitrogen adsorbed in MS-9, MS-13 and MCF-25 are 2250cm^3g^{-1}, 1100 cm^3g^{-1},900 cm^3g^{-1}.So MCF-25 has higher pore volume than MS-9 and MS-13.

3.4 CHN analysis

Table 3.5 Elemental (CHN) analysis data of the samples

Sample	C%	H%	N%	C%/m^2x10^{-2}	N%/m^2x10^{-3}
MS-9 A	9.17	1.74	4.37	1.93	9.22
MS-9G	11.40	1.39	3.61	2.41	7.62
MS-13 A	6.53	1.52	2.18	1.37	4.55
MS-13G	12.92	1.70	1.41	2.70	2.94
MCF-25 A	7.21	1.98	2.16	1.82	5.43
MCF-25G	7.31	1.71	1.61	1.84	4.07

CHN analysis was carried out to estimate the amount of materials incorporated as functional groups. Calcined silica has no C, H and N. However, on APTS loading, % of C, % of H and % of N is increased. On further binding of glutaraldehyde to amino functionalized silica, % C and % N are expected to increase whereas overall % N is expected to decrease. All the synthesized samples show similar trend and CHN data are given in Table 3.5. Data have been normalized with respect to surface area of the samples. These data show that loading of APTES and glutaraldehyde increase in the order MCF-25 > MS-13 > MS-9. During binding of glutaraldehyde molecules with APTES loaded silica, free NH_2 groups of 3-APTES interact with –CHO of glutaraldehyde to give R–CH=N group . The % N decreases on binding with glutaraldehyde as glutaraldehyde molecule does not have any nitrogen atom in it. These data confirm loading of APTES as well as glutaraldehyde in mesoporous silica. The data on % C/m^2 and % N/m^2 calculated (Table 3.5) show that amount of APTES and glutaraldehyde follow the order MCF-25 > MS- 13 > MS-9 confirming that loading is highest in MCF-25**.** This data along with FT-IR, TG, NMR and XPS result confirm the functionalization of silica material.

3.5 Contact Angle measurements

Hydrophilic or hydrophobic properties of surface (wettability) are characterized by contact angle measurements (sessile drop method), made between a water droplet and a surface. A surface is hydrophilic if the value of the contact angle is less than 90°. Surface is hydrophobic if the value of the contact angle is greater than 90°. Surfaces with the contact angle between 120° and 180^0 are called super hydrophobic [14]. The hydrophobicity can also be increased by increasing surface roughness and creation of air pockets. Bo He *et al* demonstrated that surface roughness amplifies hydrophobicity [15]. It is

found that contact angle increases with the addition of silylating agent [16]. The surface modification of silica from hydrophilicity to hydrophobicity was carried out using 3-APTES and glutaraldehyde. In the case of MS-9A contact angle is less than 90, it is hydrophiliic in nature. But after glutaraldehyde functionalization it is nearer to superhydrophobicity. As the pore diameter increases hydrophobic character also increases in the case of MS-9, MS-13 and MS-25. But after functionalization pore diameter decreases whereas contact angle increases which is due to surface roughness and air pockets formed during post synthesis. It is concluded that functionalization leads silica material to hydrophobic nature [17]. The contact angle of pure supports was less than 5° indicating that the surfaces are hydrophilic in nature. This is due to the hydroxyl groups of the different supports that make it superhydrophilic. Fig 3.6 shows the contact angle photograph of functionalized samples. Table 3.6 summarizes the contact angle values of the functionalized materials.

Table 3.6 Contact angles of the pure and functionalized samples

Sample	Contact Angle (°)
MS-9A	44.0
MS-9G	109.5
MS-13 A	60.3
MS-13G	115.1
MCF-25A	102.6
MCF-25G	119.5

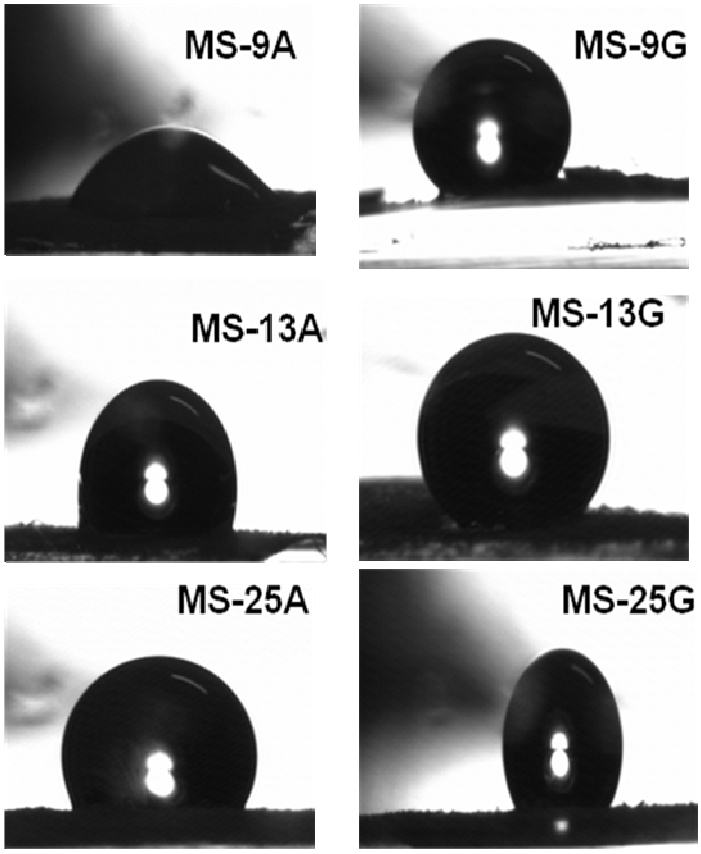


Fig. 3.7 Contact angle photographs of the pure and the functionalized samples

3.6 Thermogravimetric Analysis

In thermogravimetry, the change in the mass of a sample is studied while the sample is subjected to a controlled temperature programme. Enzymes cause denaturation when it is subjected to heat. It gives important information about the thermal stability of the samples and is used to optimize the calcination temperature of hydrothermally synthesized materials.

A small weight loss around 100°C is due to the loss of adsorbed water. Another weight loss around 170-500°C is due to the decomposition of P123

and other organic materials. But no weight loss is observed after 450°C for uncalcined MS-9 and MS-13, so 500°C is taken as the calcination temperature.

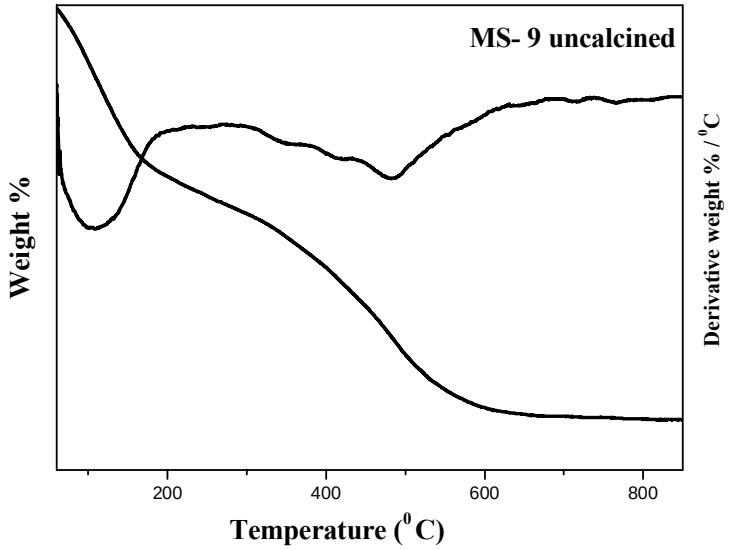


Fig. 3.8 TG/DTG spectra of uncalcined MS-9

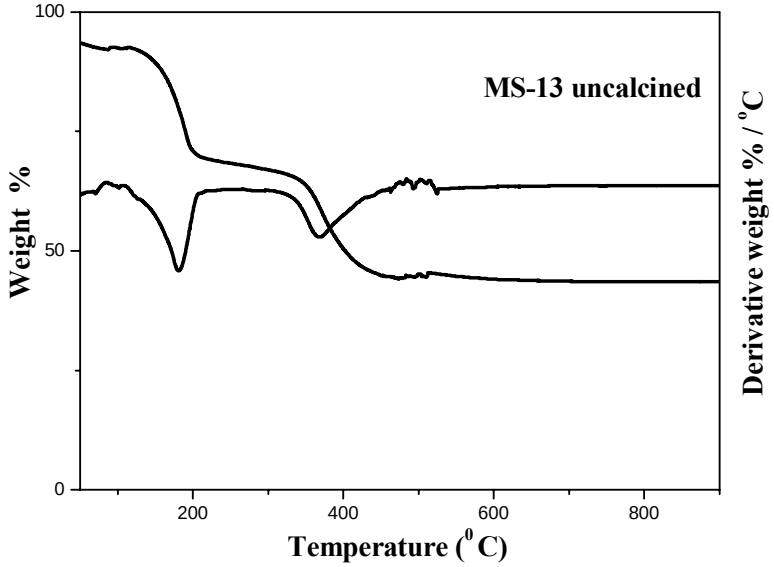


Fig. 3.9 TG/DTG spectra of uncalcined MS-13

A small weight loss (about 7.67%) below 100°C is due to adsorbed water molecule. Another weight loss (29.3%) around 170^0C is due to organic materials which were used as swelling agent for the synthesis of silica. Another major weight loss around 350°C is mainly due to the thermal removal of surfactant P123. So thermogravimetry was used to optimize the calcination temperature. No weight loss was observed after 550°C, so it was taken as the calcination temperature for MS- 13.

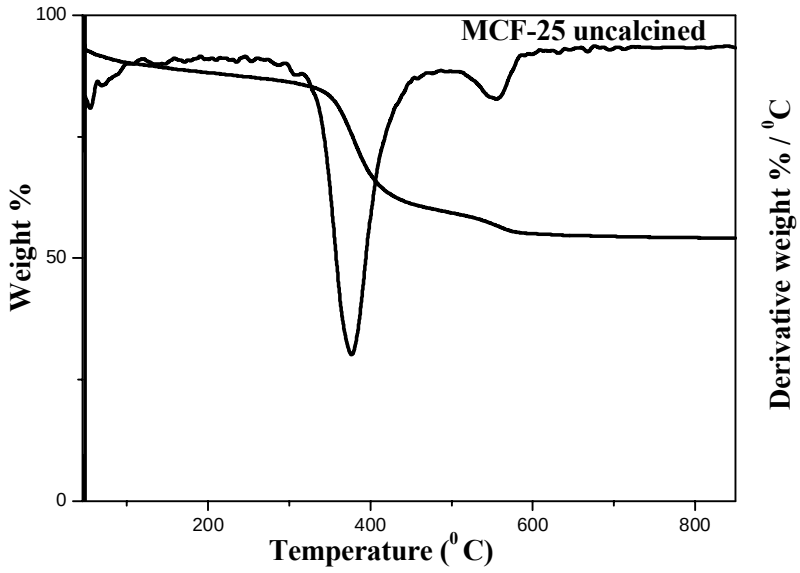


Fig. 3.10 TG/DTG spectra of uncalcined MCF-25

A weight loss (nearly 1%) below 100°C is due to physisorbed water. 18% weight loss at about 375°C and 1% weight loss at 556°C are observed. In the case of MCF-25 a small weight loss is observed at 555°C and therefore 600°C taken as calcination temperature.

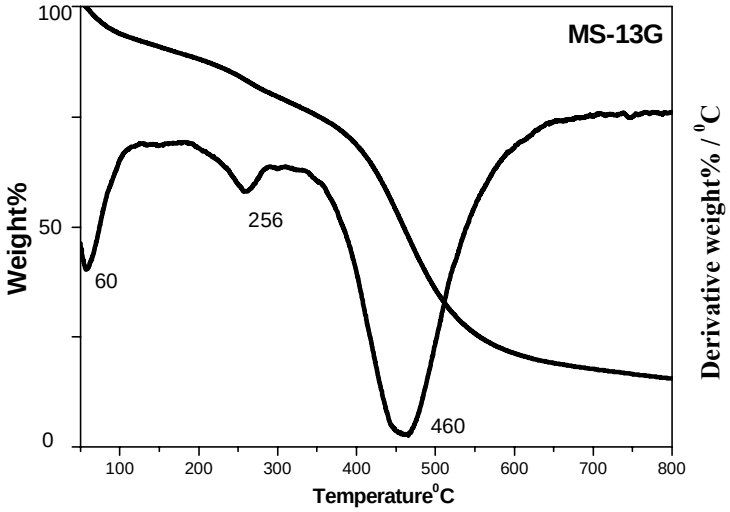


Fig. 3.11 TG/DTG spectra of functionalized MS-13

Weight loss at 256°C and 460°C from functionalized samples are due to the decomposition of silane and glutaraldehyde moieties from external surface (Fig.3.11).

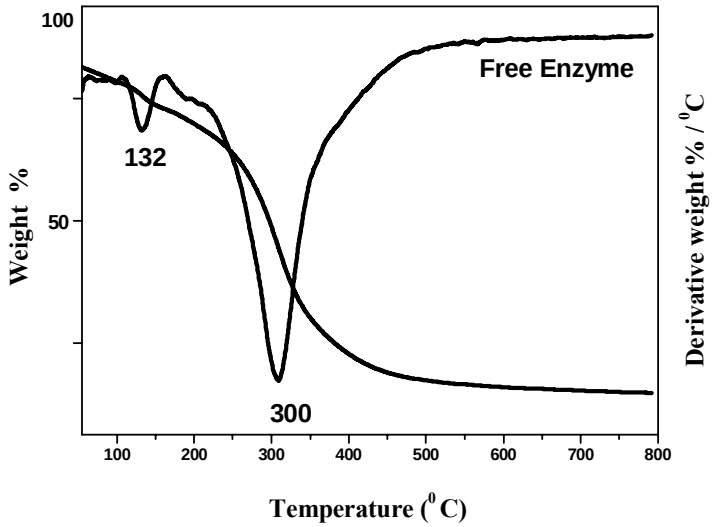


Fig. 3.12 TG/DTG spectra of free enzyme

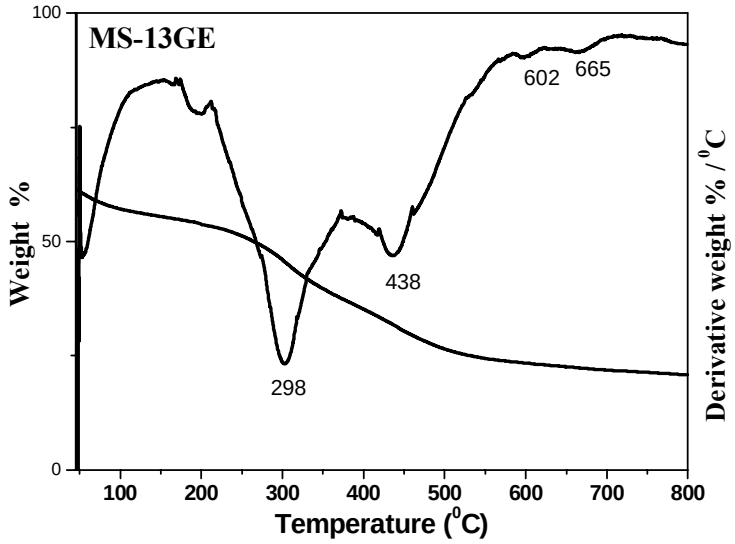


Fig. 3.13 TG/DTG spectra of covalently bound enzyme

At 150°C weight loss due the dehydration of interstitial water in free enzyme is observed. A weight loss at 300°C indicates the complete decomposition of the organic structure of free enzyme (Fig.3.12). A weight loss at 298°C and 438°C are due to the decomposition of enzyme, silane & glutaraldehyde spacer groups as can be seen from Fig.3.13. Weight loss around 600°C is due to decomposition of protein.

3.7 Fourier Transform Infrared spectroscopy

FT-IR is widely applied in surface science to identify the active species of the catalyst and to identify the functional group present in the prepared catalyst. It is a sensitive technique to probe the secondary structure of proteins. The samples of pure silica and immobilized derivatives were submitted to the FT-IR analysis. The spectra were obtained in the wavelength range from 400 to 4000 cm^{-1} for the evaluation of the immobilization procedures.

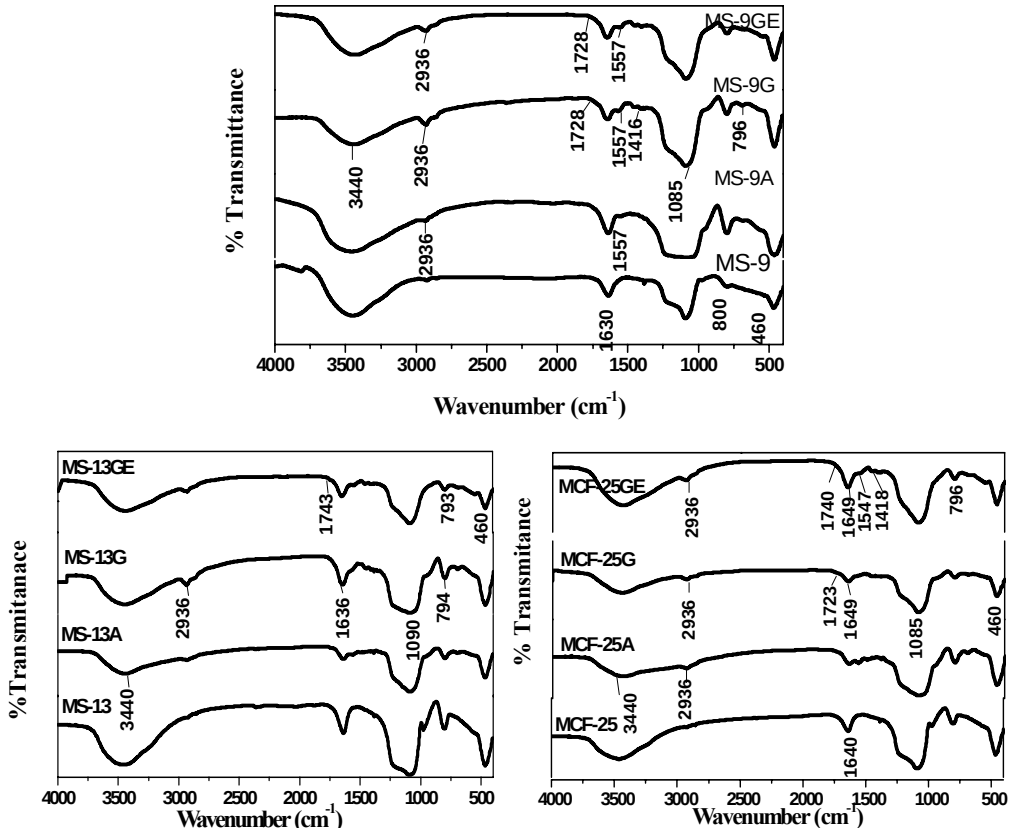


Fig. 3.14 FT-IR spectra of (A) MS-9 (B) MS-13 (C) MCF-25 families

Fig. 3.14(A)–(C) shows the IR spectra of MS-9, MS-13 and MCF-25 as such and after surface modification. The peaks at 800 cm^{-1} and 460 cm^{-1} indicate symmetric Si–O stretching and symmetric Si–O bending modes. IR absorptions of siloxane (Si-O-Si asymmetric stretching vibrations) occur in the range 1000-1100 cm^{-1}. It also exhibits a broad band at 3440 cm^{-1} (O–H silica) and weak absorption peak at 970 cm^{-1} which is due to stretching vibration of silanol group. The band around 1630 cm^{-1} is due to O-H bending vibration of adsorbed water molecules. There are no other functional groups detected for MS-9, MS-13 and MCF-25.

It is observed that after functionalization and immobilization a band at 2936 cm^{-1} appears confirming the presence of –C-H group which is not observed in the FT-IR spectra of bare silica material. A band at 2936 cm^{-1} indicates of asymmetric stretching vibration of aliphatic C-H group. It confirms the contribution from the organosilane, glutaraldehyde, and the enzyme molecules in mesosilica materials [18, 19]. At 1416 cm^{-1}, a weak peak is observed, which is due to the C-H bending vibrations. Another peak at 796 cm^{-1} is due to the Si-C bond which demonstrates the incorporation of alkyl groups into silica.

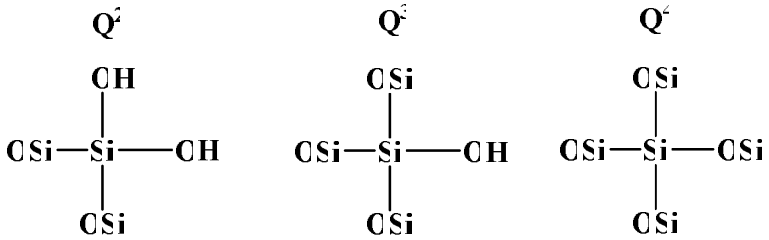
A band around 1560 cm^{-1} is associated with the $–NH_2$ bending vibration indicates the presence of aminopropyl functional group in all the silane modified samples [20, 21]. At 689 cm^{-1}, a weak peak is observed, which is due to the bending N-H vibration. The presence of aldehydic group is confirmed by the –C=O stretching at 1728 cm^{-1}. The C-N stretching vibration is normally observed in the wavenumber range 1000-1200 cm^{-1}. However, this peak is not resolved due to the overlay with the IR absorptions of Si-O-Si in the range 1000-1130 cm^{-1}. On glutaraldehyde loading, free $–NH_2$ group of 3-APTES binds to one –CHO group of glutaraldehyde molecule. Bands between 1690–1630 cm-^{1} and 1718–1730 cm^{-1} have been shown for R–CH=N and -C=O of –CHO respectively [22]. Therefore, band at 1655 cm^{-1} is due to R–CH=N confirming that $–NH_2$ group of 3-APTES loaded silica is covalently bonded to –CHO group of glutaraldehyde. No band corresponding to N–H bending vibration band observed confirming the absence of any free $–NH_2$ group of 3-APTES. It is observed that in presence of glutaraldehyde the intensity of C–H band (2936 cm^{-1}) increases.

A similar trend in spectral frequencies is observed for MS-9, MS-13 and MCF-25, as seen in Fig. 3.14(A)–(C) confirming that 3-APTES and

glutaraldehyde are covalently bonded to silica surface. The strong binding of the enzyme on the surface of mesoporous silica is also identified from the FT-IR spectra. These results further confirm that enzyme has been successfully immobilized into silicas.

3.8 CPMAS Nuclear Magnetic Resonance spectroscopy

^{29}Si-MAS-NMR is used in the evaluation of the degree of cross-linking of the network by examining the contribution of the various silicon species present in the matrix. The NMR data were analyzed according to the chemical shifts known as the Q^n and T^n Si sites [23, 24]. Q^n and T^n representation system is explained in detail in Fig. 3.15. The local silicon environment is described as Q^n (n = 0, 1, 2, 3, 4), -Si (O-Si)$_n$, which represents the extent of cross linking. The Q^4 structural units represented interlinked SiO_4 tetrahedra in the interior of the mesopore walls, while Q^3 and Q^2 structural units are present on the wall surface associated with silanol group. The successful incorporation of APTES and glutaraldehyde in functionalized mesoporous silica materials is confirmed by NMR spectroscopy. There were abundant silanol groups on the internal surface of the parent mesoporous silica which served as sites for the incorporation of organic functional groups.



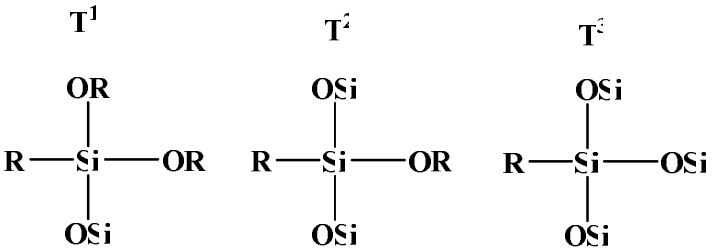


Fig. 3.15 The structural units in silica corresponding to Q^n and T^n Si sites

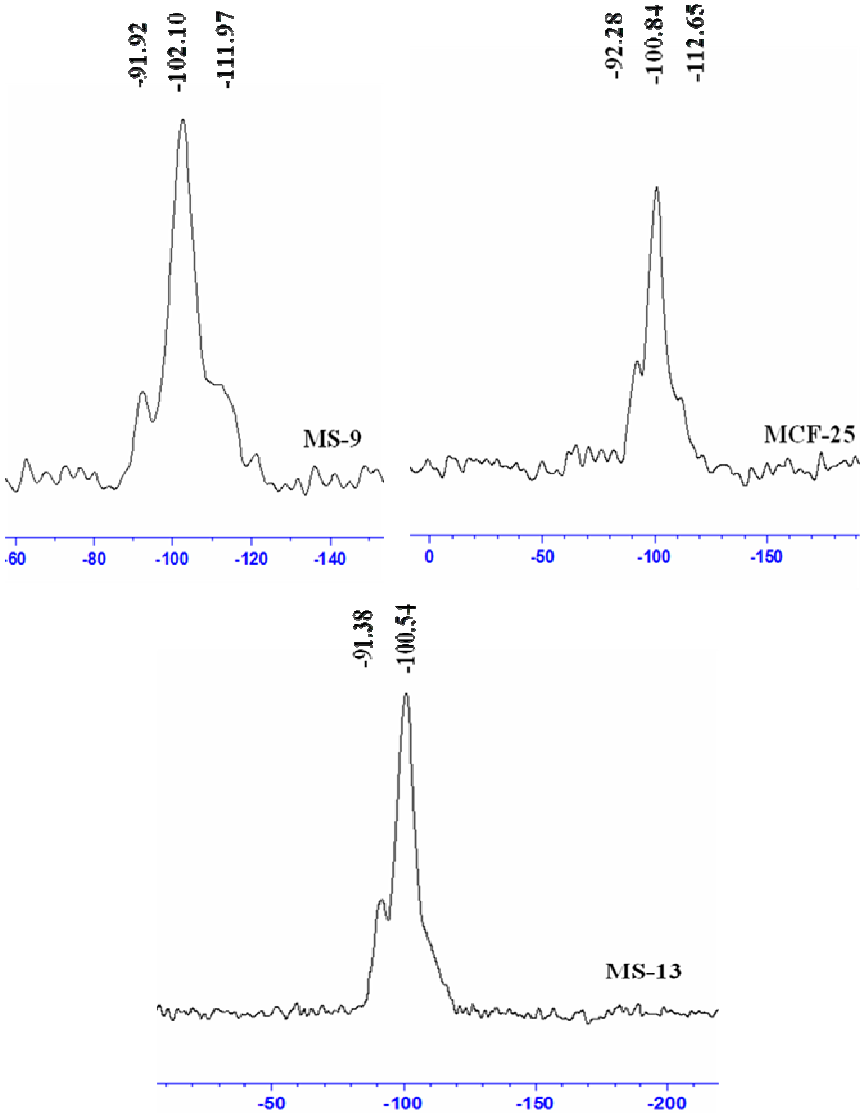


Fig. 3.16 ^{29}Si NMR spectra of MS-9, MCF-25and MS-13

Fig 3.16 depicts the ^{29}Si CP-MAS NMR spectra of MS-9, MS-13 and MCF-25 samples. Three resonance peaks of Q^4 (δ-110 ppm), Q^3 (δ -102 ppm), and Q^2 (δ -92 ppm) can be seen in the case of MS-9 and MCF-25. MS-13 exhibits one intense peak at -100.54 ppm for a Q^3 environment together with a shoulder at -91.38 ppm responsible for Q^2 species. It was observed that Q^4 peak is absent which indicates a lower degree of condensation of silicate species. The three samples exhibit intense Q^3 peak which is characteristic of mesophase, prepared under acidic conditions. Intense Q^3 peak denotes rich and isolated silanol group which may be free or hydrogen-bonded in MS-13 sample used for functionalization whereas the Q^2 sites have the geminal silanol sites. Generally, the free and geminal silanol sites are the active sites participating in the condensation reactions with the silylating agents, whereas the hydrogen-bonded silanol groups do not actively participate due to the efficient hydrophilic networks formed among them [25, 26].

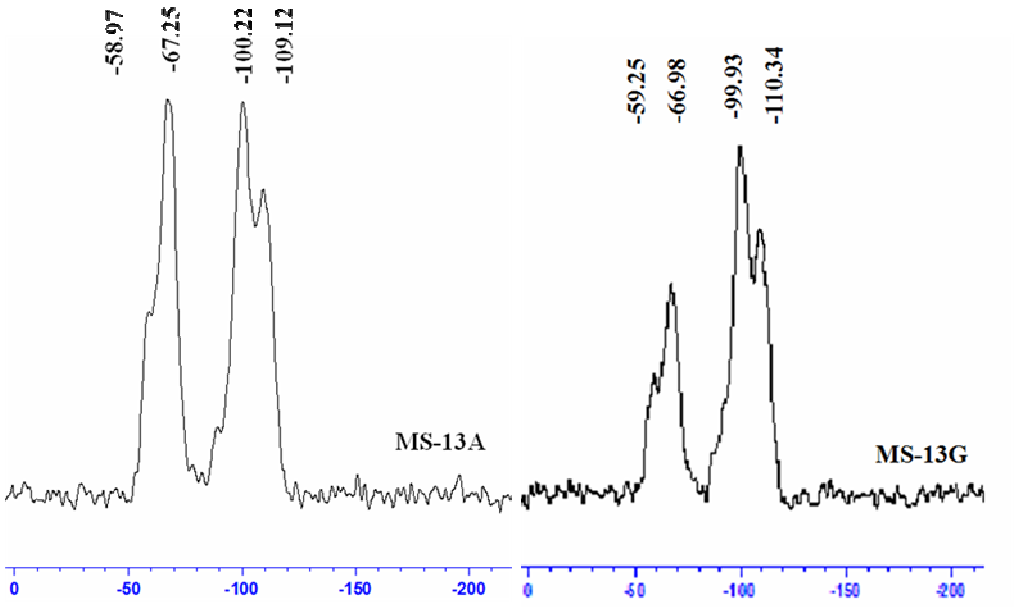


Fig. 3.17 ^{29}Si NMR spectra of MS-13 A and MS-13G

^{29}Si MAS spectrum of the MS-13A and MS-13G are shown in Fig 3.17. In the case of MS-13A two additional peaks T^2 at -59.97ppm and T^3 at -67.25ppm [27] are observed which confirms the silane organic moiety. The shift in resonance of T^2 from -59.97 to -59.25 ppm and peak due to T^3 from -67.25 to 66.98 was observed after covalent binding with glutaraldehyde. It was also observed that after glutaraldehyde binding there is not much change in peak position. In the case functionalized samples Q^4 peak is observed (Fig.3.17) which indicates that organic functionalization during post synthesis leading to an increase in the cross polymerization of silica species

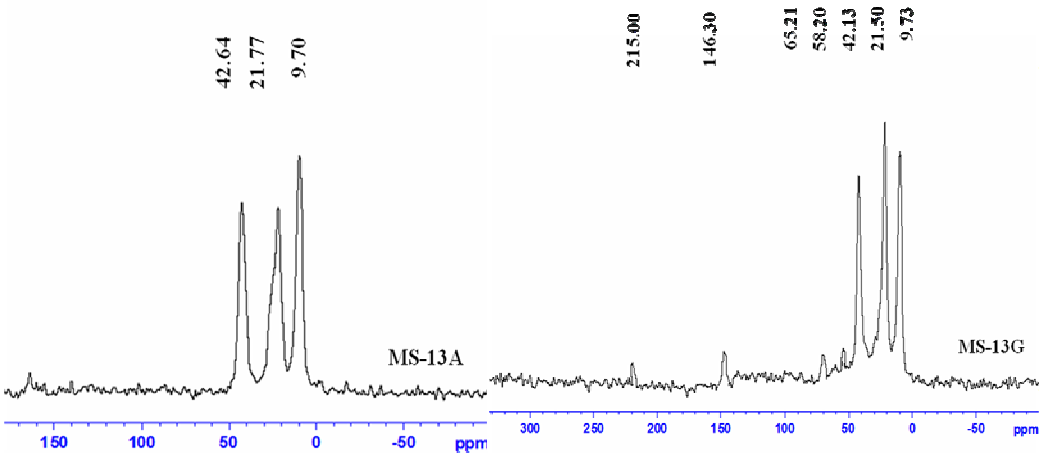


Fig. 3.18 ^{13}C NMR spectra of MS-13A and MS-13G

The ^{13}C NMR spectra of 3-APTS anchored mesoporous materials clearly display three sharp, distinct resonance peaks at 9.7, 21.8, and 42.6 ppm corresponding to the C atoms of the aminopropyl groups on the Si-CH_2-CH_2-CH_2-NH_2 group (sequence from right to left). This result indicates that aminopropyl groups were successfully incorporated on MS-13 silica. After glutaraldehyde binding peaks due to different C atom were observed at 9.7, 21.5, 42.13, 58.2, 65.2, 146 and 215 ppm. The peak at 146 ppm is due to the

imine group formed between $–NH_2$ (3-APTES) and glutaraldehyde. The presence of free aldehyde group is confirmed by the peak at 215 ppm [28].

3.9 X-ray Photoelectron Spectroscopy

XPS analysis is used to determine the incorporation of functional groups on the surface of the material. It is also used to determine the atomic configuration, chemical structure and elemental composition. X-ray photoelectron spectroscopy has been used interpret the different bonding states of silicon atom.

Organo functionalized silica sample shows relatively strong C-1s and Si-2p peak. These peaks indicate the presence of organosilanes. Incorporation of APTES results a N-1s peak in the spectrum which is attributed to NH_2 group.

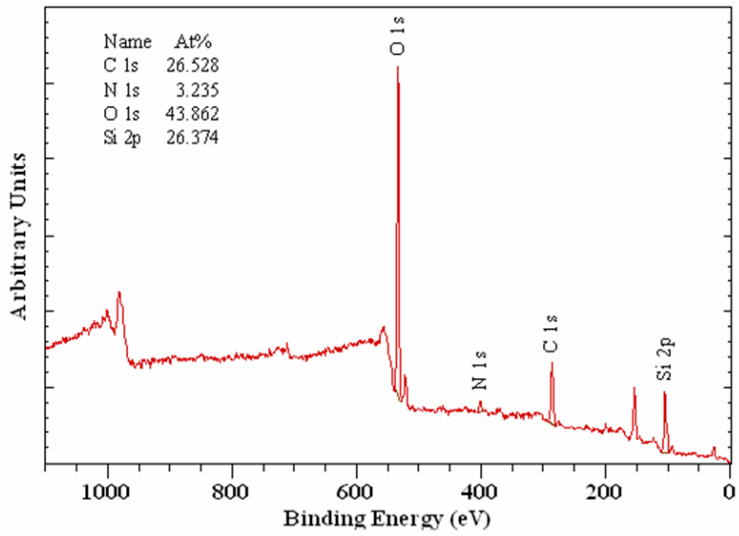


Fig. 3.19 XPS survey scan of MS-9A

Table 3.7 Structural Assignment of the Various Peaks

Groups	Binding Energy (eV)
C-Si	438
C-N +C-OH	510
C-O-Si	183
$C-NH_2$	452
$-NH_3^+$	427

Fig 3.19 represents the survey scan of MS-9A. Structural assignment of the various peaks observed in the high resolution X-ray Photoelectron Spectra (Fig.3.20) of 3-APTES-functionalized MS-9 mesoporous Silica are summarized in Table 3.7.

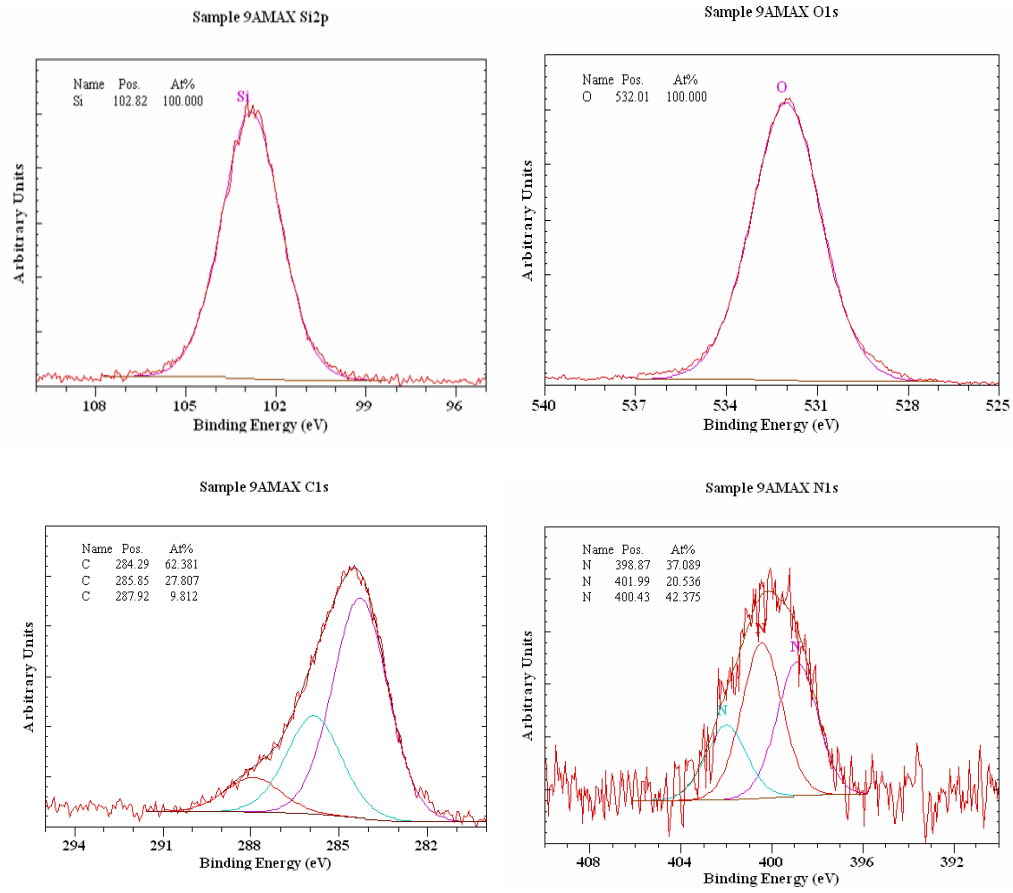


Fig. 3.20 High resolution XPS Spectra of Si, O, N, C in amino functionalized MS-9

The binding energy value of 399 eV corresponds to N 1s of C-NH_2 and other at 401.9 eV corresponds to NH_3^+ [29, 30]. The C 1s binding energy value of 284.29 eV shows the presence C-Si bond which confirms the presence of organosilane molecule in the sample. The presence of C-H and C-N bonds are confirmed by the C 1s binding energy values of 285.85 eV and 287.92 eV. Oxygen

1s binding energy of the Si-O bond is observed at 532.01 eV. This result along with IR and NMR confirms the functionalization of 3-APTES.

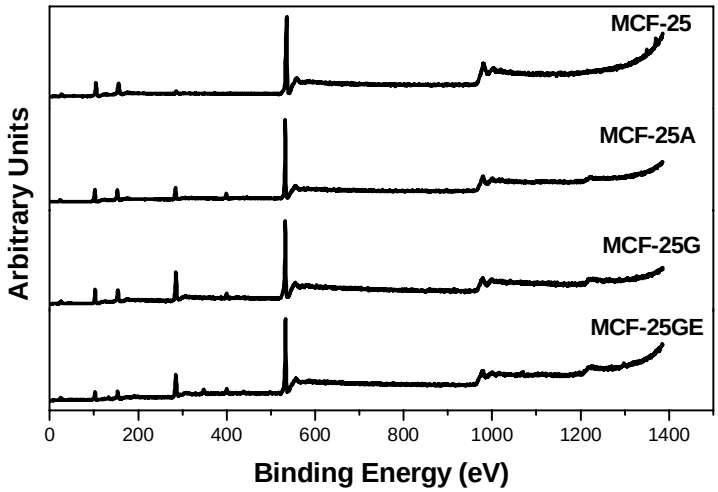


Fig. 3.21 XPS survey scan of MS-25 family

All MCF sample show the Si 2p peak mostly at 100 eV and Si 2s peak at 150eV. A binding energy value found at 103.8 eV indicates the presence of Si–O–Si bonds [31]. Fig 3.21 shows the XPS survey spectra of nonfunctionalized, organo-functionalized and enzyme immobilized MCF-25 materials that were used to determine the chemical structure. In general, the spectra of organo-functionalized silica samples show relatively strong C1s and Si 2s peaks which indicate the presence of organosilanes. MCF-25A, MCF-25G, MCF-25GE shows a peak near 400 eV confirms the presence of N1s in the sample. The incorporation of APTES in the silica matrix shows the presence of N1s peak in the MCF-25A spectrum attributable to incorporated NH_2 groups. Functionalization with glutaraldehyde groups into the matrix results enhanced intensity of C 1s peak while the enzyme immobilization further decreases the intensity as observed in the Fig 3.21.

3.10 Scanning Electron Microscopy (SEM)

Textural properties of meso compounds are quite different from one another. There are different kinds of meso silica and their morphology can be varied by controlling synthesis conditions (silica source, presence of co-surfactant, auxiliary chemical used etc). The morphology of the silica materials can also be controlled by condensation rate of silica species (pH and silica source), stirring rate, micelle shape and concentration of inorganic species.

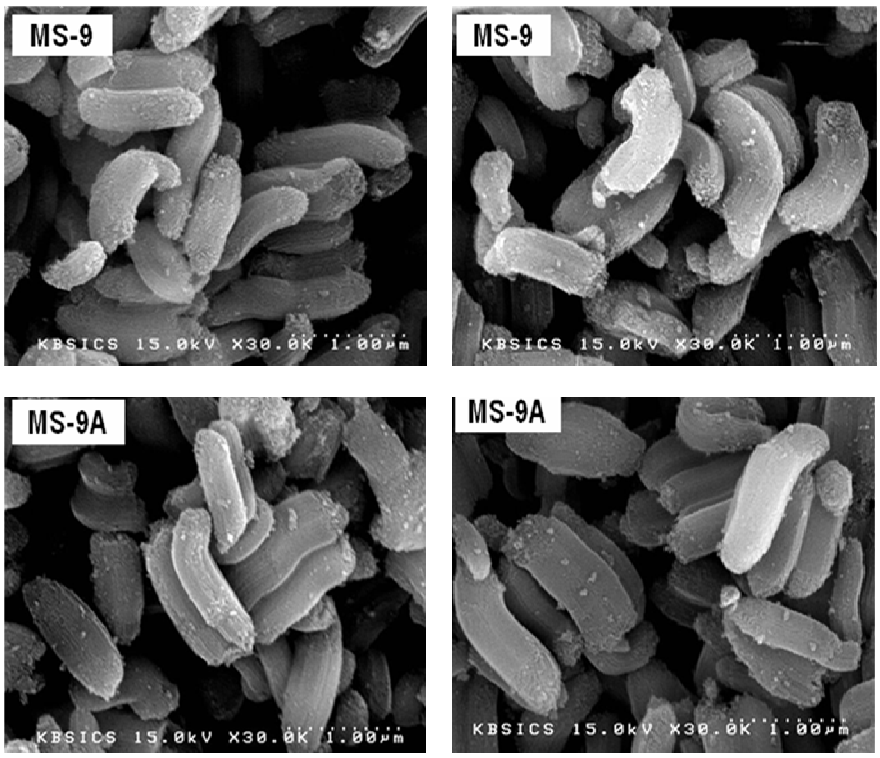


Fig. 3.22 Scanning electron micrographs of MS-9 and MS-9A at two different magnifications

The images (Fig.3.22) show that the calcined mesoporous silica MS-9 have uniform, ordered and rectangular rod like morphology. When synthesis time was increased the rodes grow into fibers. This type of morphology was reported by several researchers [5, 32].

It is observed that after functionalization with 3-APTES similar morphology was maintained. It indicates that overall structure was maintained even after functionalization and immobilization.

The SEM image (Fig.3.23) of the calcined MS-13 displays hexagonal spherical morphology that is different from MS- 9. It is also shorter than that for MS-9

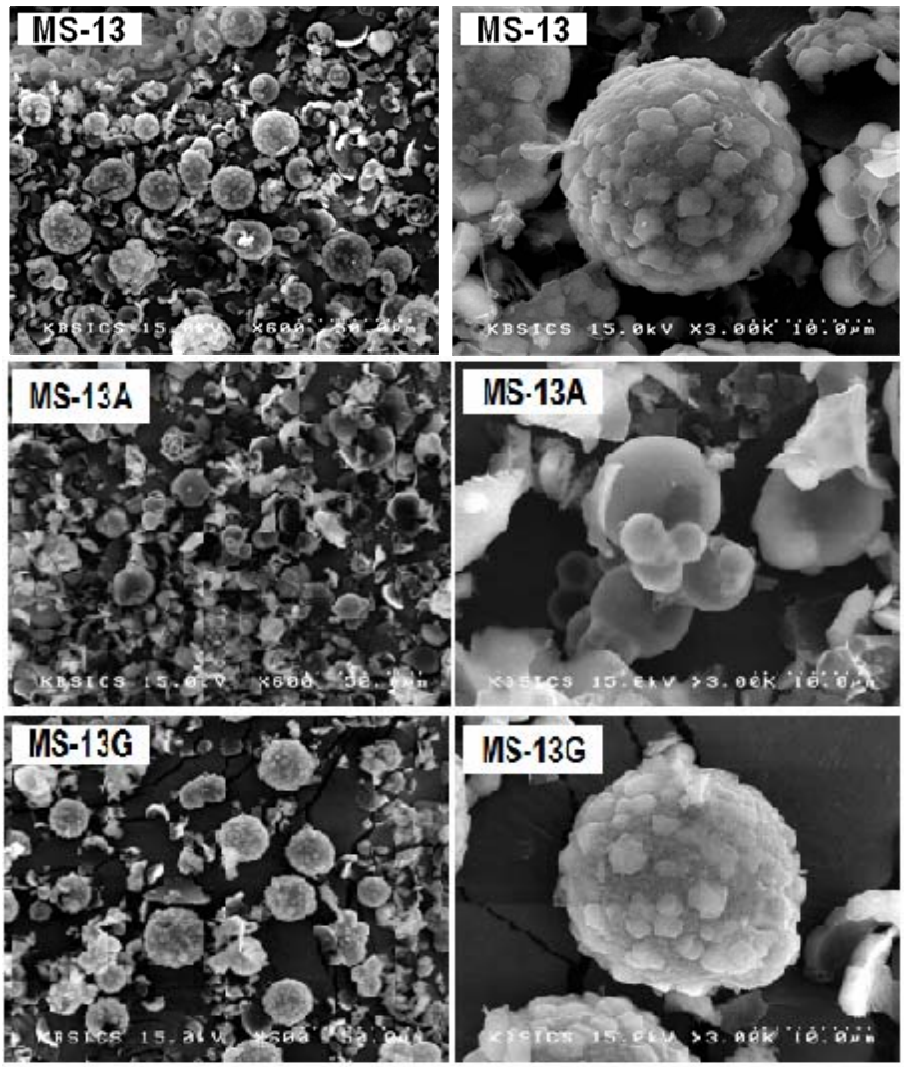


Fig. 3.23 Scanning electron micrographs of MS-13, MS-13A and MS-13G at two different magnifications

MCF has irregular particle morphology with a very broad particle size distribution. Spherical agglomerated blocks are observed with MCF material.

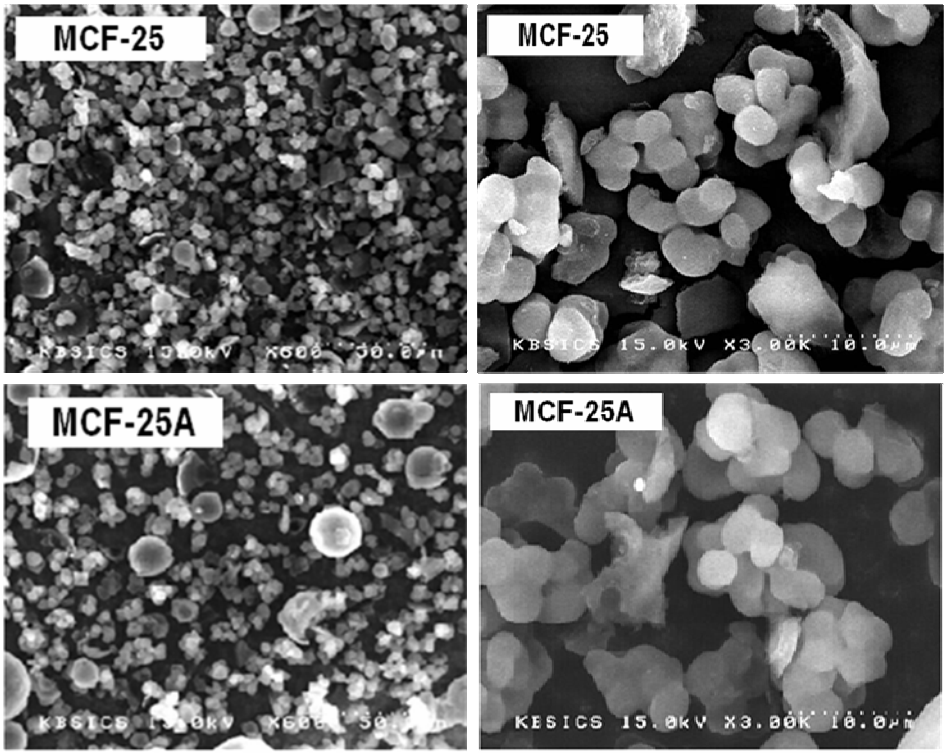


Fig.3.24 Scanning electron micrographs of MCF-25 and MCF-25A at two different magnifications

3.11 Transmission Electron Microscopy (TEM)

High resolution electron microscopy can in unique fashion, identify the known phases and reveals the structure of previously unknown phases etc. Both MS-9 and MS-13 show relatively ordered parallel cylindrical pore channels. MS- 9 has well ordered 2D hexagonal mesostructure, as expected. However MS-13 also shows an ordered structure.

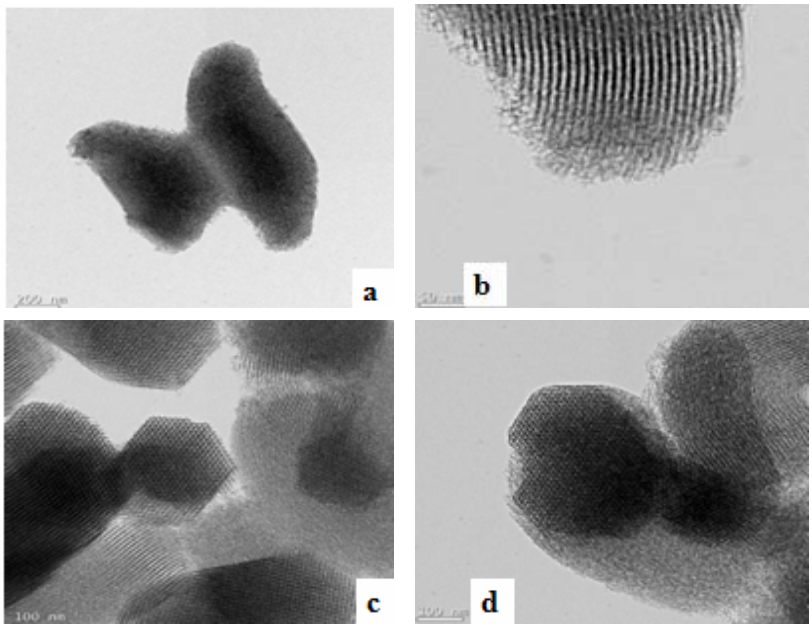


Fig. 3.25 TEM images of MS-9 material at different magnifications.

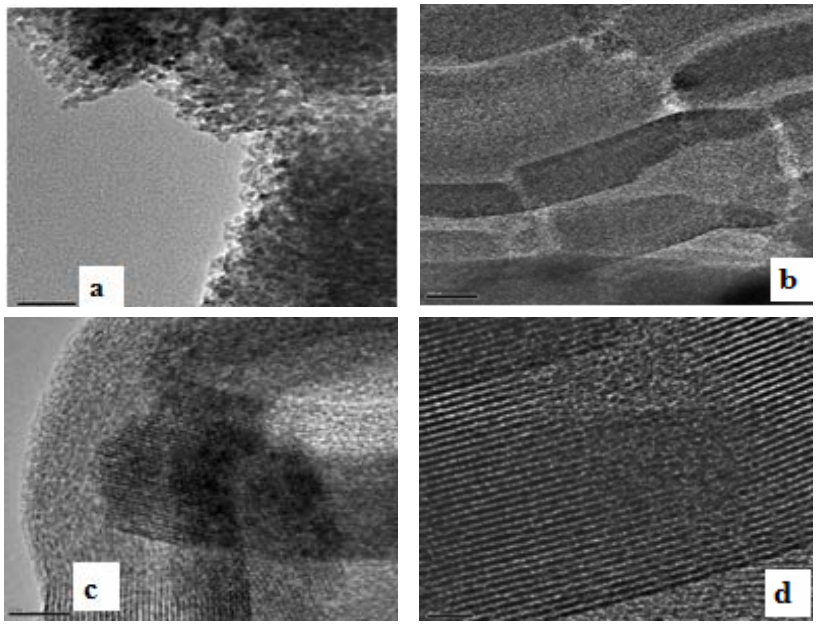


Fig. 3.26 TEM images of MS-13 material at different magnifications

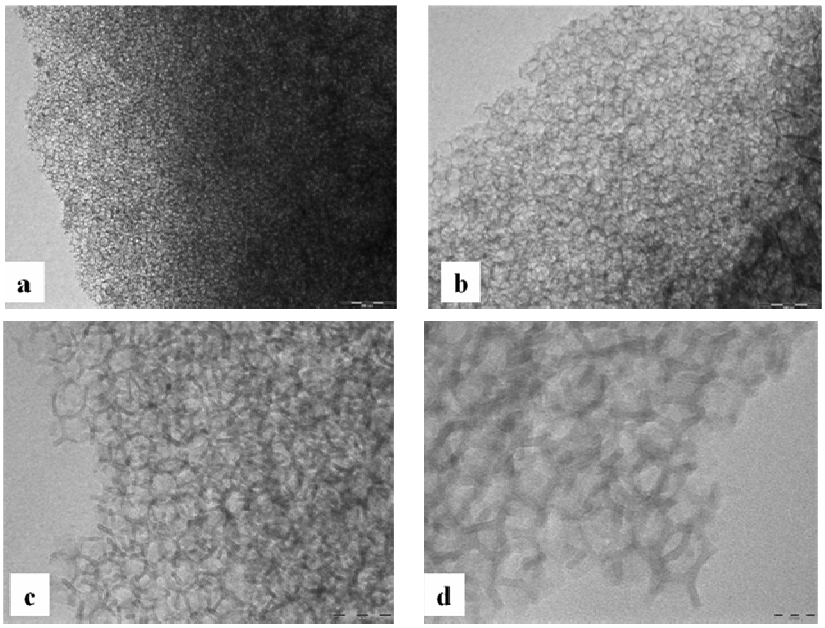


Fig. 3.27 TEM images of MCF-25 material at different magnifications.

Fig 3.27 shows TEM images of MCF- 25 sample. It has worm hole type pore structures [33, 34]. It can be easily seen that MCF-25 synthesized through hydrothermal method using TMB as swelling agent shows the typical three dimensional and ultra large pore structures of the pure MCF. These large cells are interconnected by narrow windows and it possesses ink bottle structure and uniformly sized cellular pores as evident from TEM image. These TEM images show that MCF-25 has a three dimensional mesocellular arrangement which is in good agreement with the reported MCF materials [35- 37]. It is observed that swelling agents are responsible for change in pore structure.

3.12 Conclusions

Mesoporous materials having different pore diameter were synthesized by hydrothermal route. The synthesized materials were functionalized with different organic molecules to improve their enzyme immobilization. Various

characterization techniques were employed to analyze the synthesized materials. The important conclusions obtained from the characterization studies are given below.

- Ordered mesoporous silica having different pore size were synthesized via hydrothermal method and functionalized by co-condensation of 3-APTES and glutaraldehyde.
- Decrease in the 2θ value and increase in the d spacing after functionalization and immobilization were probably due to the disordering of the pores and functionalization taking place within the pores.
- The BJH pore diameter of the mesoporous silica materials were increased by the addition of different organic auxiliary chemicals (e.g. decane and trimethyl benzene) during the hydrothermal synthesis process. The result indicated that the pore size of MCF-25 molecular sieve was 241 Å when trimethyl benzene was used as auxiliary chemical.
- All synthesized mesoporous silica materials showed a type IV isotherm with H1 hysteresis loop, which is typical of mesoporous materials.
- MS-9, MS-13 and MCF-25 samples show relatively sharp increase at around $P/P_0 = 0.85$, which confirms that the material possess a uniform pore structure.
- The thermal stability of the samples were evident from the thermograms.
- The C, H, N elemental analysis and IR spectroscopy demonstrated the presence of functionalization and immobilization.

- The hydrophobicity and hydrophilicity of the functionalized samples were determined from contact angle measurements. MCF-25G showed highest contact angle value confirming the enhanced hydrophobicity of the material.
- Transmission electron micrograph confirmed the textural properties of mesopororous silica and modified samples. The two dimensional mesocellular arrangements are clearly observed in the TEM images.
- SEM images showed that there is no change in morphology after functionalization and immobilization.
- Low angle XRD, N_2 adsorption studies and TEM analysis confirmed the mesoporosity of the prepared supports.
- Solid state NMR and XPS studies confirmed the incorporation of the organic groups onto the supports.

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Chapter 4

IMMOBILIZED GLUCOAMYLASE FOR STARCH HYDROLYSIS

Contents

Glucoamylase is widely used in food industry for the production of glucose from starch and play an important role in the improvement of starch based food products. A wide variety of immobilization carriers were used to improve enzyme stability. A critical parameter for successful immobilization was the binding capacity of enzyme to the mesomeric channels. It depends on the pore diameter, pore volume of the support, size of the enzyme molecule and bonding. Silica material was tuned in such a way as to incorporate the glucoamylase and effect of increased pore diameter was compared for the starch hydrolysis.

4.1 Introduction

Aspergillus Niger or Rizhopus Sp. Glucoamylase (EC 3.2.1.3*)* (GA) has wide industrial application for large scale saccharification of malto-oligosaccharides into β-D-glucose units. It involves two steps, liquefaction followed by saccharification. Saccharification is time consuming (72h) and it requires high operation temperature, hence high temperatures are employed for immobilized enzyme. During saccharification, glucoamylase gets inactivated partially. It has large capacity to hydrolyse α-D-1, 4 linkages, α-D-1, 6 and rarely α-D-1, 3 glucosidic linkages. But in the third case rate is low [1] and it

uses single active site with approximately 500 fold higher activity for the α-1, 4 linkages [2]. Aspergillus Niger glucoamylase occurs in two natural forms. The large one GAI consists of two globular parts with separate functional residues. 1-471 amino acid residues form the catalytic globular domain (CD) and 509-616 amino acid residues form the globular starch binding domain (SBD). The smaller, the natural proteolytic fragments GAII comprises 1-512 only and thus neither binds to nor hydrolyze raw starch. Due to the high costs required for the isolation and purification, the catalytic processes were performed with reutilization of biocatalyst by immobilization on to water insoluble solid supports [3]. The enzymes have been immobilized in various supports by different coupling methods adsorption, covalent binding and encapsulation respectively.

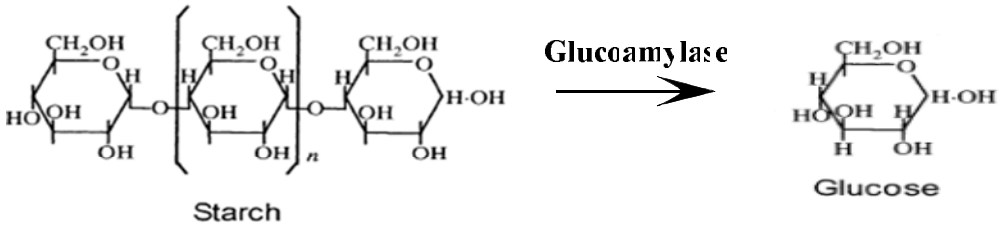

Fig.4.1 Scheme for starch hydrolysis

Starch hydrolysis by glucoamylase led to glucose [4]. (Fig.4.1). The use of gelatin as a support matrix for immobilization of glucoamylase by entrapment followed by cross linking with glutaraldehyde were used as a commercial process [5] and it was further modified by chelating method with titanium (IV) reported by Kennedy *et al* [6]. It is also immobilized by adsorption to gelatinized corn starch and subsequent fiber entrapment, prevent the leakage of glucoamylase. Glucoamylase effectively immobilized on different supports like porous glass, chitin, polymers [7-9], carboxyl – functionalized magnetic nano particles [10], bacterial cellulose bead [11],

glyoxal agrose [12] and on carbon support sibunit, bulk catalytic filamentous carbon (bulk CFC), activated carbon (AC) [13] and calcined chicken bone particles [14]. Support with appropriate pore diameter is very essential to incorporate the enzyme. There should be a perfect match in size between the enzyme and pore structure of the support because enzyme has its own specific size [15]. The molecular size of glucoamylase is 80 Å, that the prepared silica materials were tuned in such a way to accommodate the enzyme molecules inside the channels. Wilson *et al* were the first group to carry out immobilization of glucoamylase by covalent binding [16]. The fibers were treated with glutaraldehyde and isocynate treatment stabilized the beads by cross linking the enzyme molecule making them hard to leak out [17, 18]. Glutaraldehyde activation increases the immobilization yield. Several inorganic carriers like ceramics, glass, and alumina [19] have been used by treating with glutaraldehyde to improve stability. Ipsita *et al* showed that mixture of immobilized glucoamylase and pullulanase on to calcium alginate beads in a sequential fashion is more efficient than immobilized glucoamylase alone [20]. The bacterial cellulose beads coupled with glutaraldehyde by epoxy method showed better results at low temperature even at 20°C [21]. Glucoamylase was covalently immobilized through the spacer-arm of the MPMMA (Magnetic poly -methylmethacrylate) microspheres by using either carbodiimide (CDI) or cyanogen bromide (CNBr) as a coupling agent. The activity yield of the immobilized glucoamylase was 57% for CDI coupling and 73% for CNBr coupling [22]. Several reports described new supports (magnetic chitosan carriers, Fe_2O_3 clay nanocomposites, carbon nanotubes functionalized with dendrimer) for glucoamylaseimmobilization [23-26]. GA was immobilized on montmorilonite via adsorption and covalent binding and its activity of enzyme for starch hydrolysis was tested in fixed bed reactor and batch reactors [27].

Porosity plays important role in immobilization. Macroporous materials like porous glass [28] and glycidyl methacrylate [29] were used for enzyme immobilization. Arica *et al* reported that small microsphere materials with pore diameter 50-100 μm have high enzyme loading than the 100-200 μm microspheres [30]. Lomako *et al* studied the dependence of catalytic activity and influence of pore diameter of silica carriers, macroporous silica gels, silica chromes and porous glass [1, 31]. Lee *et al* explained immobilization of glucoamylase on very small particles as a means of increasing glucose yield [32]. They also suggested that as the particles became smaller, the effect of slow pore diffusion of the substrate and intermediates would be expected to decrease and this would lead to results closer to those obtained with soluble glucoamylase.

Drastic changes in conformation of polymers were observed by Dudra *et al* when enzymes were immobilized in thermosetting polymers [33]. It was reported that when GA was immobilized on polyaniline material high storage and reusability were obtained [34-35]. Haupt *et al* demonstrated that the immobilization of GA on spherical brushes fully preserve the activity [36].

The literature reveals that immobilization leads to change in pH, temperature, activity and structural stability and it enhances storage, operational stability and reusability. These changes depend upon the enzyme, supports and immobilization conditions [37-38].

4.2 Experimental procedure

Activity of enzyme was measured in batch reactor. The reaction was carried out with 1mL of free enzyme solution (0.05 g immobilized enzyme) and buffered 3% (w/v) starch solution. After the reaction time, the reaction mixture is centrifuged and 1ml of filtrate was analyzed colorimetrically.

Colour was developed using iodine solution and absorbance was recorded at 600 nm. The results obtained were compared with absorbance of standard starch solution.

4.3 Biochemical characterization of free and immobilized biocatalyst

The influence of different parameters like pH, temperature, buffer concentration and effect of substrate concentration on activity was studied to get optimum results.

4.3.1 Effect of pH on immobilization

Enzymes are amphoteric molecules including acidic and basic groups; therefore pH may affect activity and structural stability of the enzyme since the charges on these groups may change with pH. Enzymes are very sensitive to changes in pH and optimal pH is very important factor for activity studies. The immobilization process may also affect these groups and it can cause change in the optimum pH of an enzyme. Generally, the enzyme activity was decreased after immobilization. It was attributed to the modification in the three-dimensional structure that may lead to the distortion of amino acid residues involved in catalysis. Random immobilization hindered the analytical approach to the active site of the enzyme. The immobilization pH is different for different supports and that is due to the charge effects of the support. If the support is negatively charged, the use of a more basic pH is required to maintain the enzyme conformation after immobilization [39, 40].

The influence of pH on immobilization was studied using 0.1M buffer solution at different pH range 3-8(3-6 phthalate buffer & 6.5-8 phosphate buffer) by keeping temperature at 30°C and other conditions constant. Immobilization was carried out in different buffer solutions in order to

determine a suitable buffer for immobilization of glucoamylase on different mesoporous silica materials. Figure 4.2 shows the influence of immobilization pH on different carriers. Free enzyme showed optimum pH of 5.5 whereas in the case of MS-9 and MCF-25G bound enzyme, pH shifts towards 6 but enzyme bound MS-13G and MCF-25 the optimum pH remains the same as that of free enzyme (Figure. 4.2). The optimum pH for covalently bound MS-9G and enzyme adsorbed MS-13 was 6.5. The morphology of mesoporous silica materials also play an important role in immobilization. The conformation of the enzyme is another factor which is more favourable at higher pH range. The enzyme activity is very low at very low pH values due to enzyme deactivation.

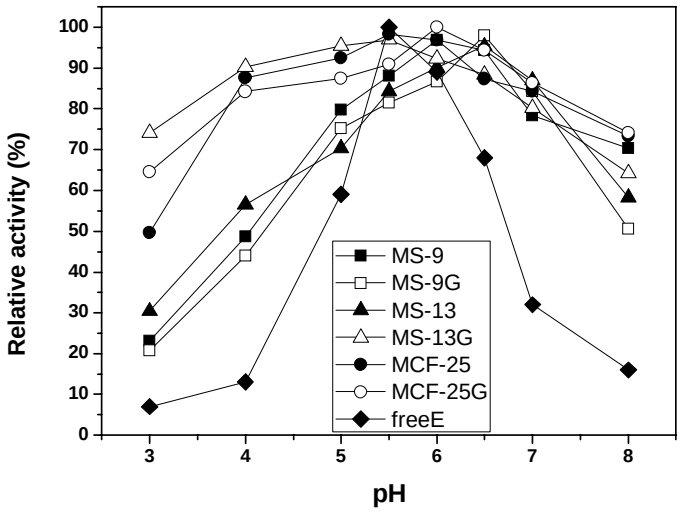

Fig. 4.2 Effect of immobilization pH

4.3.2 Effect of pH on the activity

The influence of pH on activity was studied at different pH range 3-8 (3-6 phthalate buffer & 6.5-8 phosphate buffer, 0.1M concentration) by keeping temperature at 30°C and other conditions constant. Deviation from the

optimum pH value alters the state of protonation of the groups involved and withdraws them from catalytic process. The variation in activity with pH of glucoamylase immobilized on different support is shown in the Figure 4.3. After immobilization the pH profile is widened that indicates the stability of immobilized enzyme [40]. This indicated that the immobilization procedure improved the stability of glucoamylase appreciably particularly in the alkaline region. From the Figure 4.3 it is clear that the optimum pH of MS-9, MCF-25 and MCF-25G shifted to alkaline region and for MS-9G, MS-13 and MS-13G the optimum pH was shifted to the acidic region relative to free glucoamylase.

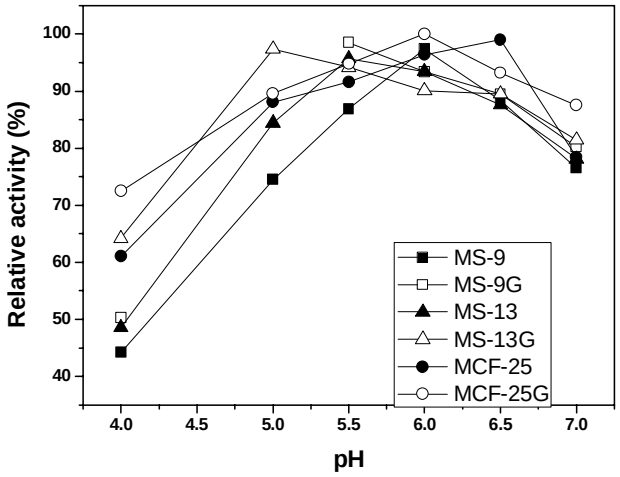

Fig.4.3 Effect of pH on activity

4.3.3 Effect of buffer concentration

The buffer concentration of the solution is an important parameter which effects enzyme activity. It is very important to control the concentration of buffer solution as well as with the pH. To determine the effect of buffer concentration, optimum pH is taken for this study and buffer concentration is varied in the range 0.01 to 0.1M.

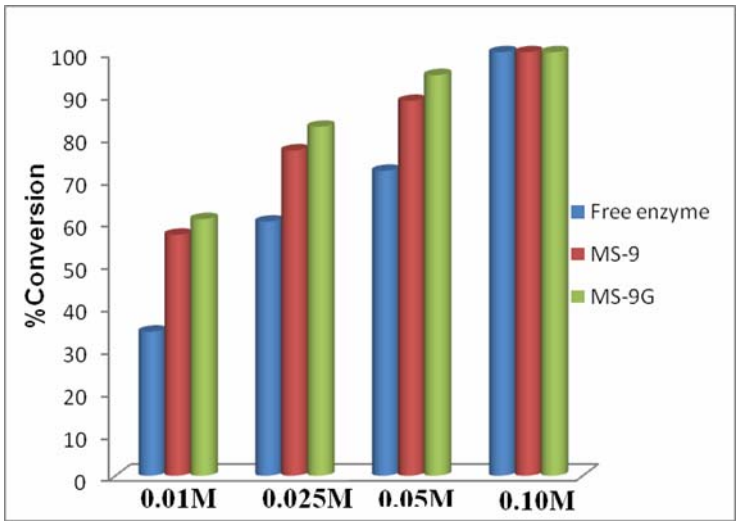

Fig.4.4 Effect of buffer concentration

The result (Fig.4.4) shows that when the buffer concentration is increased, the activities of free and immobilized systems are also increased. At higher buffer concentration (0.1M) both free and immobilized glucoamylase retained 100% of its maximum activity. The catalysis depends on the movement of charged molecules relative to each other. Thus both the binding of charged substrates to enzymes and the movement of charged groups within the catalytically 'active' site will be influenced by the ionic composition of the medium. If the charges of reacting species and ionic strength of the solution are opposite then there is a decrease in the reaction rate with increasing ionic strength whereas if the charges are identical, an increase in the reaction rate will occur.

4.3.4 Effect of temperature on the activity

Enzyme requires relatively low temperature for reaction. Determination of optimum temperature is very important since immobilization can bring about a change in the optimum values. Optimum temperature measurements of immobilized enzymes are conducted in optimum pH (phosphate buffer, 0.1M, pH = 6) for 1 h at different temperature range 10-70°C, keeping all other

conditions constant and shaking speed is maintained as 200 rpm. After 40°C, free glucoamylase shows decreased activity because of denaturation of enzyme while immobilization significantly retained about 85% activity even after 60°C (Fig. 4.5). The free glucoamylase shows a sharp drop in activity with time indicating a high rate of inactivation. Immobilization brings about marked changes in the thermal stability of biocatalyst. Immobilization increases the optimum temperature because their mobility is restricted thereby resisting denaturation of protein. Covalently bound GA demonstrates a higher optimum temperature compared to adsorbed GA. Covalently bound GA was able to withstand higher temperature without denaturation [41- 44].

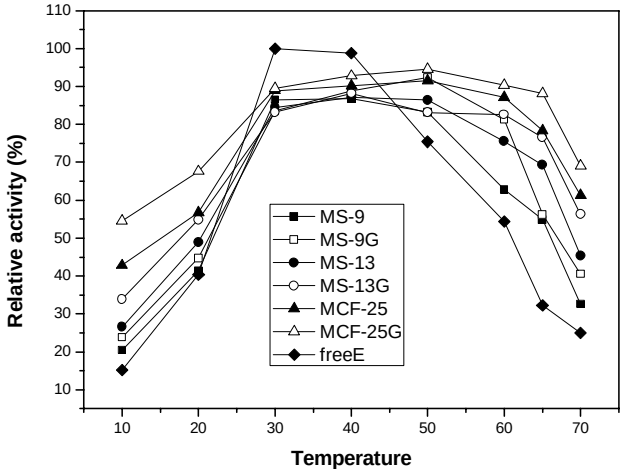

Fig. 4.5 Effect of reaction temperature

After immobilization enzyme needed higher temperature for optimum activity, it conveys the effective protection to enzyme by immobilization process against thermal denaturation. Above 50°C, the free glucoamylase activity showed a serious decrease, probably due to the predominance of thermal denaturation of the enzyme, while the activity of the immobilized glucoamylase raised even up to 70°C. This shows that the immobilized glucoamylase became more stable against heat than free enzyme.

4.3.5 Thermal stability

The thermal stability of an immobilized enzyme is one of the most important criteria for their industrial applications because the immobilized enzyme is more resistant against heat and denaturing agents compared to the free form. An enzyme with more thermal stability has more resistance to denaturation at high temperatures. Thermal stability studies of free and modified enzymes are evaluated at various time intervals at 60°C in batch experiments. During preincubation activity studies, the enzymes were exposed to corresponding temperature without substrate. After reaction, aliquots were removed at various intervals and activities were evaluated. In this study all the reactions were performed in duplicate to reduce error. It is observed that thermal stability was significantly increased after immobilization on various supports [45, 46].

After 180 minutes preincubation, free enzyme loses almost all activity while covalently bound enzyme hold over 80% of its initial activity. This result indicates the enhanced thermal stability of glucoamylase after immobilization. Immobilized enzymes enhance its sensitivity to temperature inactivation, so covalently bound GA on MCF-25G and MS-13G shows 90% of the initial activity at 60°C. All the immobilized enzymes show more than 60% activity because immobilization protects enzyme from denaturation. The findings would play key role in developing a bioreactor for the continuous applications. The decrease in the activity and stability after successive cycles may due to enzyme denaturation and/or physical loss of enzyme from the carriers [47].

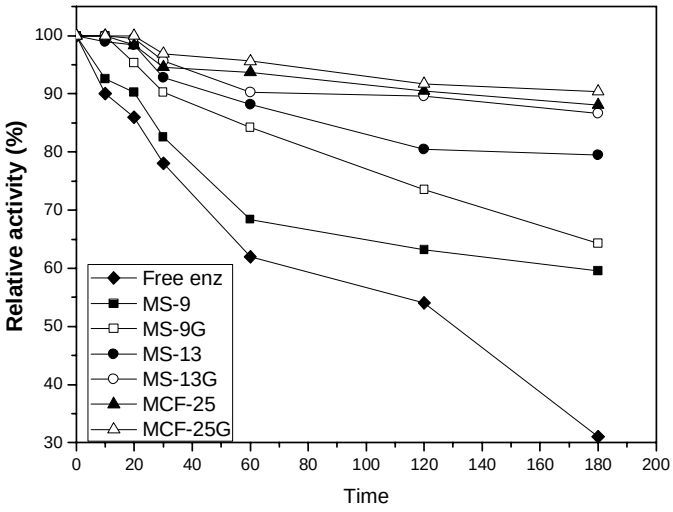

Fig. 4.6 Thermal stability of free and immobilized enzymes at 60°C

4.4 Effect of chemicals

To identify the role of activators and inhibitors, various chemicals are selected randomly. Non specific substances which are necessary for the activity of an enzyme or which activate a precursor of an enzyme are often called activators. In most of the enzymatic reactions, metallic ions perform this role. In metal- activated enzymatic reactions, enzymes may have to be activated by the addition of metal ions. It has been shown that ternary complexes are formed between an enzyme, metal ion and substrate. Enzyme inhibitors are substances which alter the catalytic action of the enzyme and consequently slow down, or in some cases, stop catalysis. The decrease in catalytic activity means that the inhibitor has affected the active site of the enzyme directly, by damaging or physically clogging up the active site. Indirectly it may change the three dimensional shape of the entire protein. The binding of an inhibitor can stop a substrate from entering the enzyme's active site and/or hinder the enzyme from catalyzing its reaction. Inhibitor binding is either reversible or irreversible. Irreversible inhibitors usually

react with the enzyme and change it chemically (e.g. via covalent bond formation). These inhibitors modify key amino acid residues needed for enzymatic activity. In contrast, reversible inhibitors bind non-covalently and different types of inhibition are produced depending on whether these inhibitors bind to the enzyme, the enzyme-substrate complex, or both. Activators and inhibitors are termed effectors. Enzymes obtained from different sources show different responses to a particular activator or inhibitor.

To determine the effect of different chemicals both free and immobilized (GA adsorbed on MS-9 and covalently bound GA onto MS-9G) enzymes were incubated with 1mM solutions of electrolytes at 30°C for 1h. After preincubation, the enzyme activity was determined at optimal conditions. Appropriate blank was also performed. The data obtained are presented in Table 4.1.

Table 4.1 Effect of selected chemicals on the activity of enzyme
Control without the addition of any substance
Relative activity (%) is the activity obtained after incubating the enzyme with various chemicals at 30^0C for 30min compared to control

Chemicals	mM	Relative Activity %		
		Free Enzyme	MS-9	MS-9G
Control	-	100	100	100
$MnCl_2$	1	235	246	240
$CuSO_4$	1	240	275	284
$CaCl_2$	1	160	184	192
$MgCl_2$	1	175	184	176
$CoCl_2$	1	106	104	111
EDTA	1	65	68	71
$ZnSO_4$	1	10	8	6
$HgCl_2$	1	0	0	0

Enzymes are stable to some metal ions Mn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+} ions due to the protective factors present in the enzyme. The enzyme is also active in the absence of metal ions that has been reported to be an advantage in terms of industrial usage [48, 49]. The presence of 1 mM Mn^{2+} and Cu^{2+} appeared to increase glucoamylase activity by 235% and 240%, respectively. Nguyen *et al* also found the increased activity of glucoamylase with Mn^{2+}ions [50]. The Mn^{2+} and Cu^{+2} were the best activators at 1mM and the glucoamylase activity increased 2.35 and 2.40 times. Low concentration of metal ions increases the catalytic activity of an enzyme because in low concentrations metal ions act as cofactors of many enzymes but at high concentrations metal ions reduce the activity which may be due to the partial denaturation of the enzyme in the presence of excessive free ions [51, 52] .The soluble enzyme was inhibited in the presence of Zn^{2+}, Hg^{2+} and EDTA. The enzyme was completely inactivated in the presence of 1mM Hg^{2+} ions. The inhibitory effect could be exerted by the destruction of disulphide bonds on the enzyme. The involvement of tryptophan and cysteine in catalytic activity and in maintaining the conformation of the enzyme is the suggested explanation.

4.5 Reusability

Free enzymes are soluble in reaction mixture so it cannot be separated during the course of the chemical reaction. They are not permanently changed or destroyed which means that they can be reused. Enzymes immobilized on solid support facilitate easy separation of the catalyst from the reaction mixture and it can operate continuously. The retention of the immobilized enzyme activity was examined by measuring the relative activity of the immobilized enzyme preparation at optimum temperature and pH at intervals of 30 min. 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 measurements were conducted under standard assay conditions. Reusability of the catalyst balances the cost of the enzyme. Results demonstrated (Table 4.2) that immobilized enzymes could be reused and it sustains above 75% activity after 10 cycles. After 10 cycles MS-13G and MCF-25G retained 90% activity. Covalently bound enzyme defences against natural inactivation on account of time dependent denaturation of the enzyme protein. Cu ion chelated with FeSBA-15-PGMA imidazole shows similar results in reusability studies. It was reported by Zaho *et al* [53]. It has been reported that salt and base forms of poly (o-toluidine) retained 80-85% of initial activity after 8 cycles [35].

Table 4.2 Reusability of free and immobilized glucoamylase

Catalyst	Relative activity % after 10 reuses
MS-9	76
MS-9G	80
MS-13	88
MS-13G	90
MCF-25	89
MCF-25G	94

4.6 Storage stability

Enzyme has a short life span so it cannot be stored for a long time. After immobilization the life of enzyme can be improved and it can be kept for long time. The stability for long term storage for immobilized enzyme was measured by calculating their activities after being stored at 4°C for 30 days. After 8 days of storage free GA lost activity completely while the covalently bound enzyme retains more than 75% activity. Covalently bound enzyme on MCF-25G and enzyme adsorbed on MCF-25 show more than 90% activity

after 30days. From these results we can conclude that covalently bound GA shows better stability than adsorbed GA. The decrease in activity after repeated usage may be due to the inactivation of the enzyme and the leakage of protein from the support upon use.

Table 4.3 Storage stability of free and immobilized glucoamylase at 4°C

Catalyst	Relative activity %		
	after 5 days	after 10 days	after 30 days
Free Enzyme	62	0	0
MS-9	84	76	61
MS-9G	89	87	77
MS-13	96	92	87
MS-13G	89	88	88
MCF-25	98	93	91
MCF-25G	98	96	93

After immobilization half life of biocatalyst increases. Free Glucoamylase has half life less than 6 days if it is wetted with buffer and stored at 4°C [54]. Bhatti *et al* found that free GA showed half life of 24.75 min and GA modified with copper showed 2 fold increase in half life time i.e. 57.76min [55] where as if it is immobilized on to porous glass it increases to 8 days [56]. Gaoiang *et al* described that when GA was covalently bound to porous glass its half life drastically increases to 104 days [7].

4.7 Evaluation of kinetic parameters

Kinetic studies were carried out by batch reactors. Kinetic constants of free Glucoamylase and immobilized glucoamylase for starch hydrolysis were determined at starch concentration from 0.2 to 15 μM in optimum pH conditions. It can be simply described by the pseudo-first-order kinetic equation.

The kinetic constants K_m (Michaelis constant) and V_{max} (maximum rate) were calculated from Michaelis-Menten and double reciprocal Lineweaver-Burk plots using graph pad software. Fig.4.7 shows Michaelis menton Lineweaver-Burk plots of GA and GA immobilized on various supports**.** The kinetic parameters obtained from these plots are compared in the Table 4.4. Comparison of the K_m value for a given free and immobilized enzyme could provide information about interaction between enzyme and its support [57]. The kinetic parameters for the immobilized enzyme may be different from those of the soluble enzyme because of diffusion restrictions and interactions with the carrier or deactivation due to immobilization. The K_m value of free glucoamylase is calculated and it is found to be 3.32 μM and K_m value of the immobilized glucoamylase was higher than free enzyme. Increased K_m value indicates lesser affinity for the substrate towards the active site. Decrease in V_{max} indicates structural changes of enzyme and difficulty in diffusion of substrate to reach the active site of the enzyme thereby resulting in loss of activity. Immobilization brought decrease in V_{max} and increase in K_m [58, 59].

Table 4.4 Kinetic parameters of free and immobilized glucoamylase for starch hydrolysis

Sample	K_m (μM)	V_{max} (μMmin^{-1})	R^2	η Effectiveness factor	Catalytic Efficiency (min^{-1})
Free GA	3.32	0.2239	0.9877		0.0674
MS-9	7.44	0.1964	0.9755	0.8675	0.0264
MS-9G	7.10	0.1853	0.9838	0.9876	0.0268
MCF-13	6.60	0.1770	0.9984	0.7905	0.0267
MS-13G	6.02	0.1787	0.9947	0.7981	0.0296
MCF-25	5.21	0.1217	0.9781	0.5435	0.0233
MCF-25G	4.96	0.1076	0.9916	0.4806	0.0217

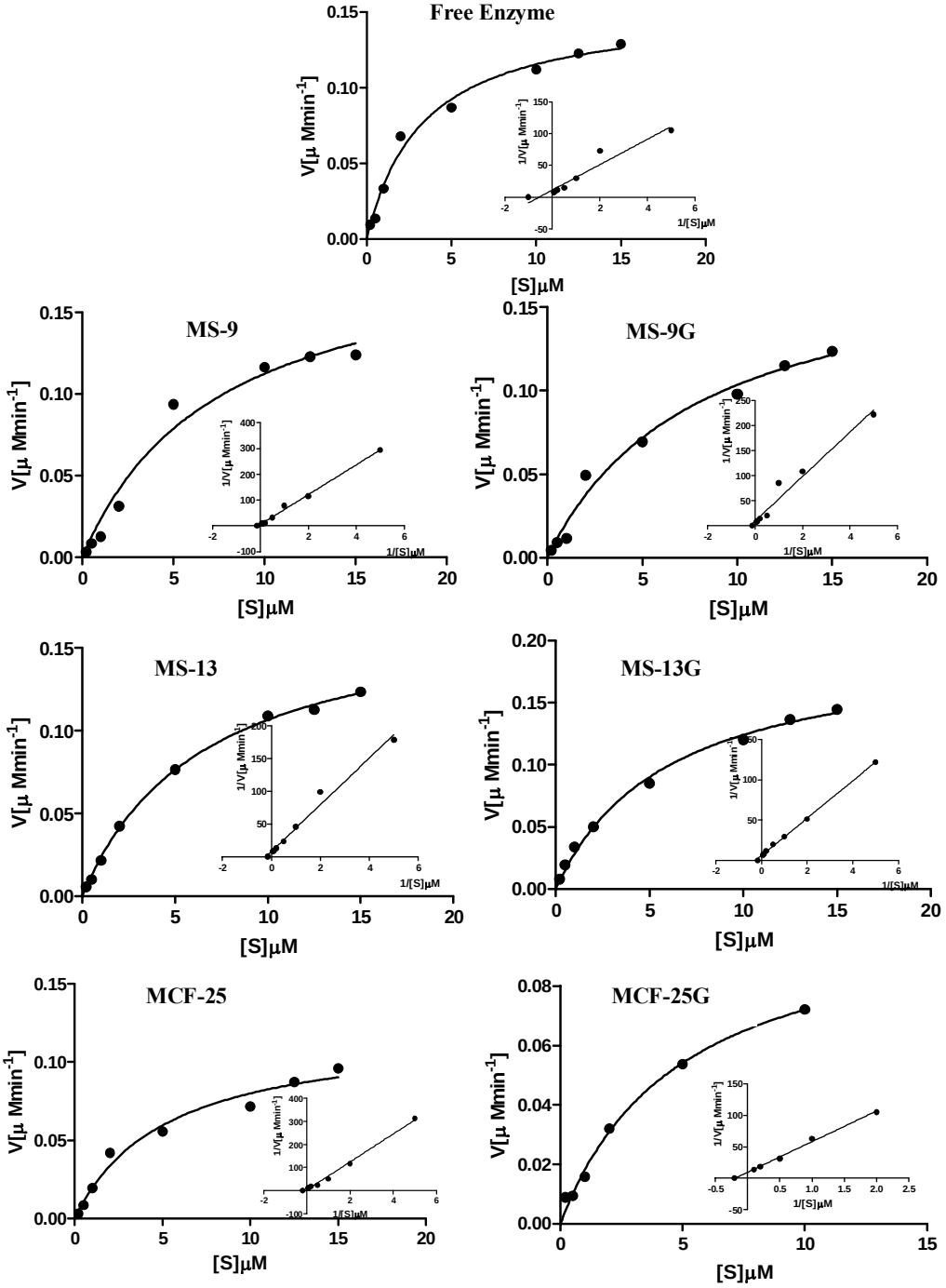

Fig.4.7 Michaelis-Menten and Lineweaver-Burk plots to estimate K_m and V_{max} for free and immobilized glucoamylase

The K_m value for immobilized GA on MS-9 and MS-9G were approximately 2 fold higher than that of free enzyme. Here it was observed that K_m value of all immobilized samples was greater than that for free GA and that is due to the change in the affinity of the enzyme to its substrate. It is probably caused by structural changes in the enzyme introduced by immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme [43, 44]. V_{max} value was lesser in the case of MCF-25G because the hydrophilic starch molecules had lesser affinity towards highly hydrophobic MCF-25G. This lower Vmax value was due to steric hinderance imposed by the support on the macromolecular substrate or due to chemical modification. Evidently, linear fitting is reasonably good (correlation coefficient, R^2 ~0.9 for all systems).

Effectiveness factor (η) was calculated from the rate of the reaction and it provides information about the role of diffusion in the reaction. η =1 under conditions of complete diffusion, i.e. in the case of homogeneous reaction with the free enzyme. Efficiency of immobilization is defined as the relative activity of free enzyme retained after immobilization and is calculated as η x100. The efficiency is 48% and 54% respectively for covalently bound GA on MCF-25G and adsorbed GA on MCF-25. The decrease in η value after immobilization demonstrates that there is a significant role played by mass transfer restriction due to diffusional limitations. But in the case of MS-9G and MS-9 the η values are higher and these values suggest that diffusional limitations are minimized and the native conformation of GA is maintained after immobilization.

The free enzyme K_m value was lower for a soluble starch substrate, the decrease of K_m with the increase of the molecular weight of substrate was remarked by Kusunoki *et al* and Imai *et al* [60, 61]. A slight decrease of K_m

registered with the decrease of substrate molecular weight was observed by Vallat and Monsan in experiment with immobilized glucoamylase and with substrates of different molar mass (soluble starch and hydrolyzed manioc starch) [62].

Cabral *et al* compared the performance of three types of immobilized reactors, PBR, FBR and continuous feed stirred tank reactor (CFSTR) and assessed the long term operational stabilities of enzyme on alkyl amine derivatives of Ti (IV) – activated porous silica. From the comparative study it is concluded the FBR is the most suitable [63]. Glucoamylase immobilized on chitin and glass beads showed K_m values 7.8g L^{-1}, 1.2g L^{-1} respectively [64, 65].

4.8 Thermodynamic parameters

The thermodynamic parameters for starch hydrolysis were calculated by measuring activity at different temperatures. Arrhenius plot was analyzed by taking ln k against 1/T in the temperature range 10-70°C. Activation energy was found to be 20.43, 22.11 and 23.99 $kJmol^{-1}$ for free and GA immobilized on MCF-25G and MS-13G respectively. The covalently bound GA increased the conformational flexibility, there by resulting in lower activation energy for the molecule to reorganize and attain the proper conformation for binding to the substrate. Similar results were obtained to Kennady *et al* [66]. The activation energy obtained was 19.3 $kJmol^{-1}$ and which is in agreement with our results.

Table 4.5 Thermodynamic parameters of free and immobilized glucoamylase for starch hydrolysis

Samples	Ea [kJmol^{-1}]	ΔH [kJmol^{-1}]	ΔG substrate binding [kJmol^{-1}]	ΔS [kJmol^{-1}K^{-1}]
Free GA	20.43	17.95	-2.94	0.0700
MS-9	47.29	44.81	-4.97	0.1671
MS-9G	39.80	37.32	-4.86	0.1415
MS-13	38.26	35.78	-4.68	0.1358
MS-13G	23.99	21.51	-4.45	0.0871
MCF-25	34.01	31.53	-4.09	0.1195
MCF-25G	22.11	19.63	-3.97	0.0781

The thermodynamic parameters calculated are summarized in Table 4.5. The enthalpy of activation (ΔH), Gibbs free energy (ΔG substrate binding) and entropy of activation (ΔS) for soluble starch hydrolysis by glucoamylase were calculated as 17.95 kJmol^{-1}, -2.94 kJmol^{-1} and 0.070 kJmol^{-1}K^{-1} respectively. The lower enthalpy value of glucoamylase showed that the formation of transition state or activated complex between enzyme-substrate was very efficient. Moreover, lower ΔG value suggested that the conversion of its transition complex into products was more spontaneous. The feasibility and extent of a chemical reaction is best determined by measuring change in Gibbs free energy (ΔG) for substrate hydrolysis, i.e. the conversion of enzyme substrate complex into products. If the free energy change is lower the more feasible is the reaction, i.e. the conversion of the reactant to product will be spontaneous. The entropy was slightly lower, which explained that the transition complex is more ordered [67].

4.9 Conclusions

The results of glucoamylase immobilized on different mesoporous silica materials are summarized as follows:-

- Aspergillus niger glucoamylase was immobilized on various mesoporous silica materials and demonstrated more than 60% activity.
- pH and temperature stabilities were enhanced by immobilization.
- The immobilized enzymes showed broader pH and temperature range than free enzyme.
- Low concentration of Mn^{2+} and Cu^{+2} ions activated the catalytic activity of immobilized systems.
- Decrease in V_{max} and increase in K_m were observed after immobilization.
- Lower values of ΔG proved the affinity of enzyme to catalyze starch hydrolysis.
- Immobilized glucoamylase showed better storage stability even after 30 days storage at 4°C.
- Reusability was enhanced after immobilization for all systems and MCF-25G retained more than 90% activity even after 10 reuses.

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Chapter 5

BIODEGRADATION OF PHENOL USING IMMOBILIZED BOVINE LIVER CATALASE

Contents

Catalase is one of the most potent biocatalyst which can oxidize potentially harmful toxins. Catalase is an abundant natural enzyme which is used in food industry, analytical applications (bio sensor) and textile industry to remove pollutants because of its high activity. Since catalase has many industrial applications, immobilization of catalase in solid support makes it heterogeneous catalyst which is advantageous for commercialization of the catalyst. The most recent and interesting advancement in size, morphology control and surface functionalization of mesoporous silica have enhanced the biocompatibility of these materials with high surface area and pore volume. The unique properties of mesoporous silica materials have attracted substantial interest for use as enzyme-immobilization matrices. Current research demonstrates that enzymes immobilized via adsorption and covalent binding on mesoporous silica retain their biocatalytic activity and are more stable than enzymes in solution. In this work, the influence of pH, temperature, buffer concentration and thermodynamic parameters of activation are evaluated.

5.1 Introduction

Catalase(EC 1.11.1.6) (ferricatalase, oxidoreductase)(CAT) is a cellular enzyme produced during oxidative metabolism of aerobic living systems which degrades toxic hydrogen peroxide produced during oxidative metabolism in cells[1-3]. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the

enzyme to react with the substrate. The optimum pH for reactivity of catalase is approximately 7, and it has a fairly broad maximum (the rate of reaction does not change appreciably at pH between 6.8 and 7.5). In 1937 James B. Sumner and Alexander Dounce crystallized catalase from cattle liver

Catalase has interesting therapeutic uses, it accelerate both healing and correct hereditary defiencies in combination with hydrogen peroxide. Catalase has the highest turnover number among enzymes: One catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. It decomposes 500 million mM H_2O_2 to H_2O and O_2 in 1 minute and finds more application in food industry and textile industry mainly for removing H_2O_2 [3-7]. Catalase is a redox enzyme, oxidizes alcohols, aromatic phenolic compounds and formic acid in presence of H_2O_2. It functions in two ways catalytically decomposing H_2O_2 to H_2O and O_2 and peroxidatively oxidizing alcohols, phenols, formic acid etc [8, 9]. Phenol (Ph) is an aromatic compound commonly found as a pollutant in industrial effluents, including pulp paper bleach, textiles, pesticides, tannery, oil, and petrochemical products [10, 11]. Phenolic compounds are toxic in very low concentrations [12, 13] and causes central nervous system disorders, muscle weakness, corneal whitening, blindness, hepatic damage and anorexia [14, 15]. So degradation of phenolic compounds play important task in analytical fields. Biocatalysts are ecofriendly and remove the pollutants without causing any harmful effects [16-18]

Interestingly, immobilization of catalase is broadening its application to various fields. Immobilization of CAT on three supports alumina, chitosan and cellulose were performed by Eberhardt *et al*. They performed pre-treatment with $FeCl_2$ in the case of alumina and with glutaraldehyde for other

biopolymeric supports. Glutaraldehyde treatment of biopolymeric supports improves the chemical and mechanical resistance [4, 19, 20]. Senay *et al* explained thermal stability of catalase on chitosan films, cibacron blue F3GA attached chitosan beads and also explained the resistance against thermal and pH denaturation [21-24]. A simple, easy and cheap method to fabricate enzyme on egg membrane surface based biosensor for detection of hydrogen peroxide was demonstrated by Choi *et al* [9]. Several supports for immobilization such as egg shell [25, 26] silica sol gel films [27], single walled carbon nano tubes [28], silk fibroin[29], agarose hydrogel film[30], modified glassy carbon electrode [31], chitosan film[21], controlled pore glass [32], non porous glass [33], asymetric cellulosemembrane [34], nylon membrane [35], collagen membrane[36], alumina [37], dextran [38, 39], bioskin which is a natural polymer containing aminosugars [40], ultrafine silica particles [41], calcium hydroxyapatite [42] by adsorption. CAT was immobilized on polyacrylamide gels [43] by entrapment and it was cross linked chitosan beads [23] have been reported. Aptekin *et al* [44] investigated the immobilization of catalase onto controlled pore glass modified with 3-APTES and glutaraldehyde. In this work pore size tuned mesoporous silica were used to immobilize CAT because of their ordered hexagonal structure and higher thermal stability.

5.2 Catalase immobilization methods

Catalase immobilization was carried out in mesoporous silica materials through physical adsorption and covalent binding. The activated support (50 mg) was mixed with 20 mL of catalase (600 units) in buffer solution (pH 4-8) and shaken in a shaking incubator at 30°C for 1 hour and shaking speed is maintained at 200 rpm. The immobilized enzyme was centrifuged in a cooling centrifuge at 14500 rpm and 4°C for 30 minutes after that it was separated by filtration and washed with buffer solution. The immobilization was carried out at different pH range 3-8. The amount of

enzyme protein was estimated using the spectroscopic method of Lowery using Folin – Cioclataues's phenol reagent and absorption is measured at 600nm.

5.3 Activity measurements

Activity of enzyme was measured in batch reactor. 10mL 1% phenolic solution, 4 ml 30 % H_2O_2 and 0.3 mg/mL catalase were shaken in a shaking incubator at 30°C for 1 hour. Shaking speed is maintained at 200rpm. After the reaction time the reaction mixture was analyzed chromatographically. The amount of phenol is quantified through internal standard method using ethanol. A fixed quantity of reaction mixture and 1 mL ethanol was made upto 10 mL with deionized water. 1 µmL of the reaction mixture was run on the Gas Chromatograph; (GC-8610 Chemito) integrator with FID detector, packed column [OV-17 10 wt%].

5.4 Biochemical characterization of free and immobilized catalase

Optimization of reaction conditions was very essential to obtain maximum activity of free and immobilized enzyme. A small change in reaction and immobilization condition may affect the activity. So parameters like pH, temperature and buffer concentration were carefully evaluated during this study.

5.4.1 Effect of immobilization pH and temperature

To investigate the optimum pH catalase solution with concentration 0.25 mg/mL was prepared in 0.05M buffer (pH 3-5.5) and phosphate buffer (pH6-8)]. Immobilization was carried out using 20 mL catalase solution for 50mg of support at 30°C for 1 hr. The catalytic activity was measured using 10mL 11mM phenolic solution (in pH 7 phosphate buffer) and 4 mL, 9.7M H_2O_2. The reaction was carried out for 1hr and shaking speed was

maintained at 200 rpm and the reaction was stopped by adding 1mL of 1M HCl. The % conversion was measured by gas chromatography using standard curve.

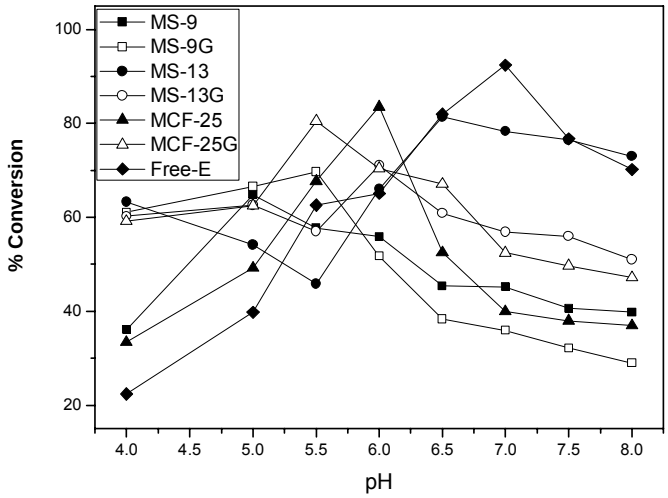

Fig. .5.1 Effect of immobilization pH

Optimum immobilization pH was investigated by changing the pH of the medium between 4 and 8. The amount of bound protein is increased by increasing pH and at low pH (pH-4) the bound protein was less. After pH 5 it slightly increases because pH 5.4 was the isoelectric point of catalase. The amount of bound protein is less below pH 4 .That is due to the instability of imine bond (broken to amine and aldehyde) in acidic medium, particularly pH ≤ 4. As seen from Fig.5.1, the activity of immobilized catalase increased up to pH 6.5 and then decreased by increasing pH. The effect of pH on immobilization is different for both types of immobilization. In the case of free catalase, maximum activity is shown at an immobilization pH of 7 while immobilized catalase shows optimal activity below pH 7.

At higher temperature enzyme inactivation takes place. So room temperature was opted as the immobilization temperature.

5.4.2 Effect of pH on catalytic activity

To study the effect of pH, activity of free and immobilized catalase were measured by using 11.2 mM phenolic solution in 0.05 M phthalate buffer (pH 4-6) and 0.05 M phosphate buffer (6-8)

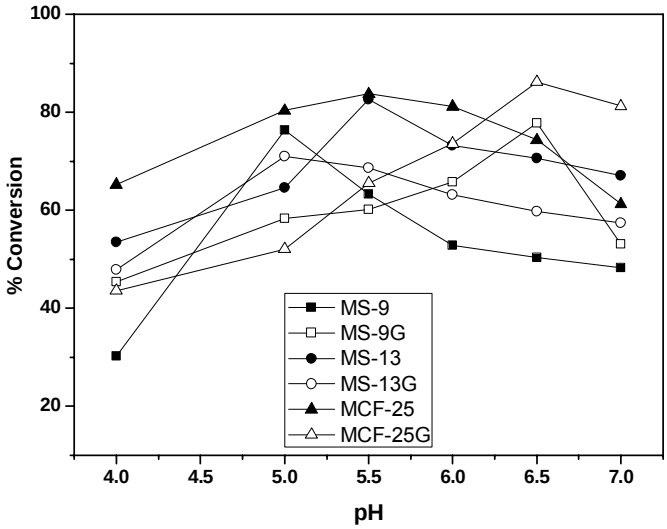

Fig. 5.2 Effect of pH on catalytic activity

The influence of pH on enzyme activity is shown in Fig.5.2 and it is observed that immobilized enzyme was less sensitive to pH changes. The immobilized enzyme had an optimum pH value which was lower than that of free enzyme. Free and immobilized catalase showed maximum activity at pH 7 and 6 respectively. Free catalase was more sensitive to pH changes as compared to catalase immobilized on different mesosilica, especially at acidic pH. One unit shift in pH of immobilized enzyme towards acidic side could be due to the basic properties of the support. Optimum pH value of 5 was reported for MS-9 & MS-13G and pH of 5.5 was reported for MS-9G & MCF-25G. For the supports MS-13 and MCF-25 the optimum pH obtained for activity was 5.5. This decrease in pH may be related to the existence of

positive charges on the surface of the particle, probably associated with residual amino groups. Depending upon the ionic strength of the solution, these charges contribute to the establishment of a pH gradient near the particle and thus the immobilized enzyme is submitted to a pH which is different from the pH of the bulk solution. Arica *et al* described that the shift in pH is due to the secondary interactions (ionic, polar interactions and hydrogen bonding) between the enzyme and support [24].

5.4.3 Effect of buffer concentration

Buffer concentration plays a major role in homogenous systems. The concentration of buffer had a marked effect on soluble enzyme compared to the immobilized enzyme. Immobilized enzymes exhibits slightly altered chemical and physical properties. The parameters such as temperature, pH, ionic strength, substrate concentration etc must be carefully controlled in order to yield maximum conversion. So the concentration of buffer solution is an important parameter in the stability of the immobilization process and enzyme activity.

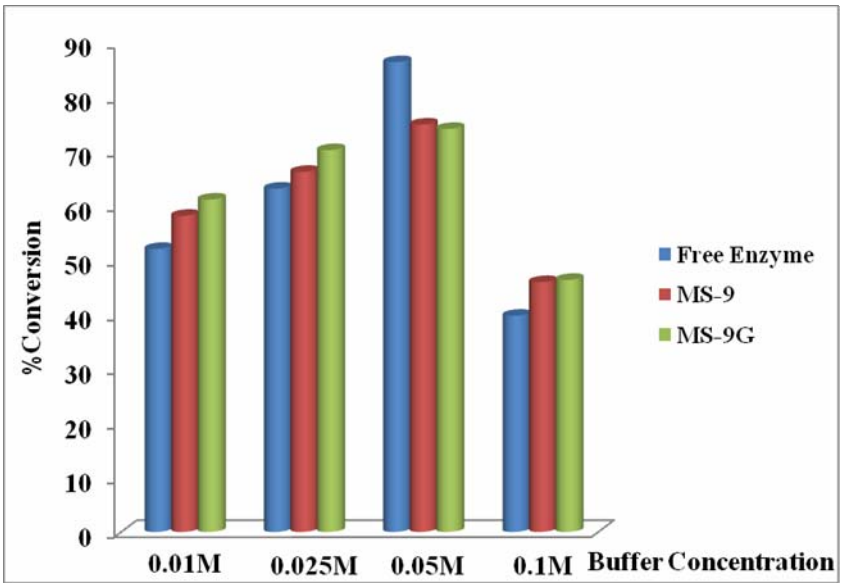

Fig. 5.3 Effect of buffer concentration

The effect of buffer concentration on the activity of free and immobilized catalase were determined at optimal conditions. Buffer concentration is varied from 0.01M to 0.1M and results are presented in Fig. 5.3. For all preparations the optimal buffer concentration was determined as 0.05M. At 0.1M buffer concentration free enzyme retained 39.8% of its maximum activity. Catalase immobilized by adsorption retained 46.5% of its maximum activity and catalase immobilized via glutaraldehyde through covalent binding retained 46% of its maximum activity. This result shows that when the buffer concentration is increased, the activities of both types of immobilized catalase were decreased. Akgol and Dinçkaya reported that increasing the buffer concentration to 0.1M caused a significant decrease in response of a catalase electrode [45].

5.4.4 Effect of temperature on the activity

Reactions were conducted at the temperature range 20-60°C and activities were measured at each temperature. Temperature plays an important factor for enzyme activity. After reaching an optimum temperature, further increasing temperature enzyme was deactivated due to denaturation. Immobilized catalase is also more stable against heat-induced denaturation, resulting in longer maintenance of activity. After immobilization enzyme needed higher temperature for optimum activity, it conveys the effective protection to enzyme by immobilization process against thermal denaturation.

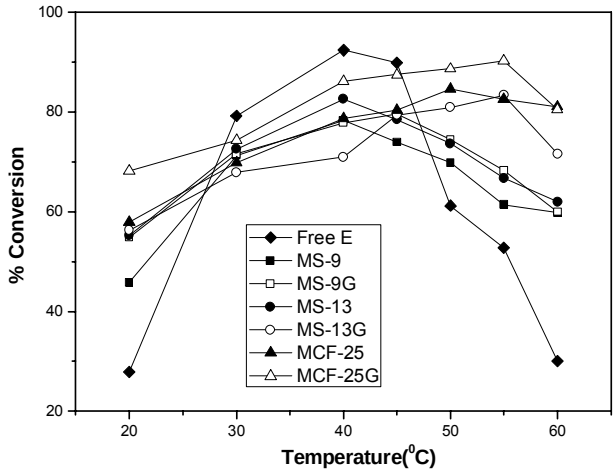

Fig.5.4 Effect of reaction temperature

The temperature profiles of free and immobilized catalase are shown in Fig. 5.4. Optimum temperature is found at 40°C for free catalase. Fig. 5.4 shows that the loss of the activity of immobilized catalase was lower than that of the free catalase for high temperatures. After 45°C free CAT activity is suddenly decreases to 56%. Between 50-60°C all the immobilized catalase shows higher activity than free CAT. Covalently bound catalase on to MCF-25G and shows more than 80% conversion from 40-60°C. Catalase immobilized on MCF-25 and MS-13 shows above 75% conversion and they shows negligible change in their activity from 40-60°C. So these systems are considered as better support to immobilize catalase for phenol degradation. The support has a protecting effect at the high temperatures at which enzyme deactivation takes place. It indicates that conformational flexibility of the enzyme affected by immobilization. Immobilization of catalase in meso porous silica caused an increase in the enzyme rigidity which is commonly reflected by increase in stability towards denaturation by raising the temperature [44, 46]. It was found that catalase immobilized on crab shell-based chitosan flakes is able to attain a relative activity of over 70% only at 45–60°C.

5.4.5 Thermal stability of free and immobilized catalase

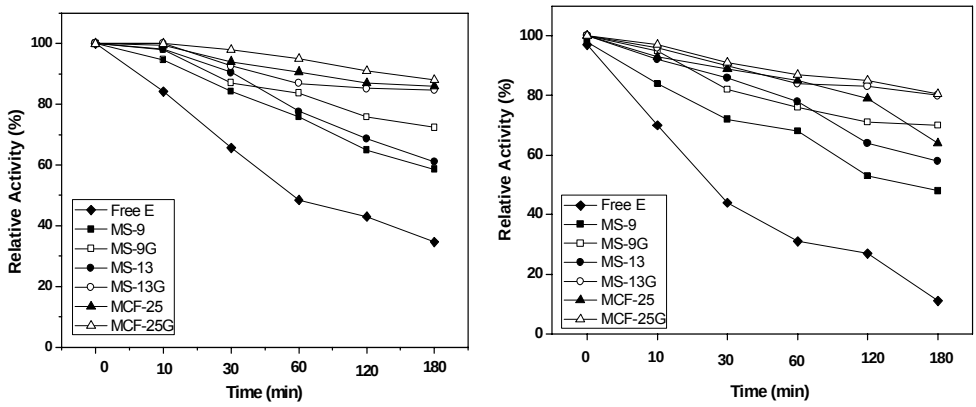

Fig.5.5 Thermal stability of free and immobilized catalase at 50°C and 60°C

Fig.5.5 shows the activities of free and immobilized enzyme at 50°C and 60^0C as function of preincubation time. The immobilized catalase retained above 80 % of its initial activity at 50°C, while free enzyme lost 50 % its initial activity during a 60 minutes incubation period. At 60°C the immobilized enzyme retained 50% and free enzymes retained 30% of their initial activities. The immobilized enzymes retained more than 60 % of their initial activity even after 180 minutes of incubation at 50°C, which indicates that the immobilized form was inactivated at a much slower rate than the free enzyme. These results suggest that the thermal stability of immobilized catalase increased considerably as a result of immobilization onto mesoporous silica. The activity of the immobilized enzyme, especially in a covalently bound system, is more resistant against heat and denaturing agents than that of the soluble and adsorbed form. The denaturation resistance of the covalently immobilized catalase is due to multipoint attachments onto silica through aldehyde. A higher thermal stability of immobilized enzyme was observed than free enzyme because of the reduction of conformational flexibility in the immobilized enzyme [47]. There was no appreciable improvement in the

thermal stability of catalase after immobilization by gel entrapment. The conformational flexibility of enzyme was not affected by gel entrapment [48]. Cu-Chitosan-CAT showed high stability at 25–35°C. Free catalase and Chitosan - CAT showed high stability at 25 and 35°C as reported by Senay *et al* [49]. Itoh *et al* explained that catalase encapsulated to nanochannels of an inorganic composite membrane showed higher stability than free enzyme [50]. Free and immobilized catalase on florisil retained 93 and 83% of their initial activity when preincubated at 20°C and 25°C for 24h [51].

5.4.6 Storge stability

The life time of a biocatalyst can be improved by immobilization and can be attributed to improved resistance to temperature and denaturing agents [52]. It can be stored in optimum pH at low temperatures but the activity is gradually decreased. The storage stability of free and immobilized enzymes at 4^0C is determined by measuring the enzyme activities at a regular interval and the results are summarized in Table 5.1. The free enzyme lost its activity completely within 10 days. It was observed that except catalase immobilized on MS-9 retained more than 70% of its initial activity. The retained activities were found to be 74, 76 and 78% for MS-9G, MS-13G and MCF-25G respectively upon 30days storage. From these results it can be concluded that immobilization makes the enzyme into a more stable position in comparison to free enzyme and stability of enzymes are substantially improved by covalent binding. This decrease in activity was explained as a time-dependent natural loss in enzyme activity and this was prevented to a significant degree upon immobilization.

Table 5.1 Storage stability of free and immobilized catalase.

Support	Relative Activity (%)			
	After 5 days	After 10days	After 20days	After 30 days
Free BLC	46	3	0	0
MS-9	79	64	53	50
MS-9G	98	89	85	74
MS-13	88	77	73	69
MS-13G	98	90	85	76
MCF-25	97	87	81	72
MCF-25G	100	94	80	78

Silva *et al*. [53] reported that the covalent immobilization of catalase on the teflon membrane showed an excellent lifetime of more than 4 months, the membrane being stored at room temperature. Arıyca *et al* immobilized catalase on the poly (2-hydroxyethyl metacrylate)-Cibacron Blue F3GA (poly HEMA-CB) and poly(2-hydroxyethyl metacrylate)-Cibacron Blue F3GA-Fe(III) (poly HEMA-CB-Fe(III))-derivatized membranes by adsorption. They found that the free enzyme lost all activity within 20 days. Immobilized preparations of poly HEMA-CB-CAT and poly HEMA-CB-Fe(III)-CAT lost 40% and 25% of their activities during the same period [24]. Solas *et al* reported that free catalase lost its activity within 3 days and immobilized catalase on bioskin by ionic adsorption retained at about 70% of its activity level for 16 days at room temperature [40]. Native catalase isolated from *Bacillus Sp* was formulated with different additives (glycerol and glutaraldehyde). It showed the best performance for long-term storage at 30°C and neutral pH [54]. Ozyilmaz *et al* demonstrated the presence moisture content of the medium for the stability of immobilized catalase. Free CAT lost its complete activity with in 15 days but immobilized CAT on florisil lost 81.5% activity within 8 days at 25°C but retained 14.5 and 56.5% of their initial activity at the end of 60 days at 4°C [51].

5.4.7 Reusability

Immobilization makes heterogenization of biocatalyst and facilitates easy separation of enzyme from the reaction mixture, reusability and it can be applicable to continuous process. Operational stability is another important factor for consideration because it can be operated continuously without any break. So evaluation of reusability and operational stability is very essential for immobilized biocatalyst. Literature reveals that the immobilized enzyme activity decreased when recycling number was increased (the activity decays exponentially with time). Fig.5.6 shows the reusability of immobilized catalase on different mesoporous silica. Covalently bound catalase on MCF-25G and MS-13G retained more than 90% of its initial activity even after 15 runs. But catalase adsorbed on MS-9 shows gradual decrease in activity on repeated use and could retain only 60% activity after 15 runs. This is because of leaching out of enzyme (desorption) during repeated use. The better reusability of immobilized enzyme could be explained by improved resistance to denaturation and conformational changes caused by the surrounding conditions such as buffer solution, reaction mixture etc [55].

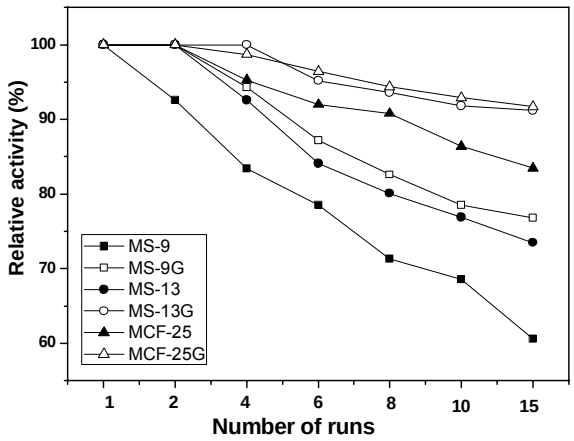

Fig.5.6 Reusability studies of immobilized catalase

The catalase immobilized in FSM still retained its original activity after being applied 50 times, whereas the native catalase lost its activity after 20

times recycling [56]. CAT immobilized on Polyacrylonirile carbon nanotube retains above 60% of its initial activity after 10 uses [57].

5.5 Thermodynamic parameters of activation

To determine and compare thermodynamic constants of the free and immobilized catalase similar experiments were conducted at optimal conditions by varying the temperature of the reaction. Thermodynamic parameters of activation are determined by Arrhenius plot. Data are given in Table 5.2.

Table 5.2 Thermodynamic parameters of free and immobilized catalase

Samples	Ea [kJ mol^{-1}]	ΔH[kJ mol^{-1}]
Free CAT	13.30	10.82
MS-9	29.60	27.12
MS-9G	19.45	16.98
MS-13	16.53	14.05
MS-13G	17.17	14.69
MCF-25	18.62	16.14
MCF-25G	15.60	13.13

Activation energy is found to be 13.3kJmol^{-1}, 15.6 kJmol^{-1} and 16.53 kJmol^{-1} for free and CAT immobilized on MCF-25G and MS-13 respectively. Activation energies of covalently bound catalase on MS-9G and MCF-25G are higher than adsorbed catalase. The lower activation energy is due to the increased conformational flexibility. The lower enthalpy value of catalase showed the high efficiency of the transition state or activated complex between enzyme–substrate formed during the reaction.

Whitaker *et al* reported the activation energy for spontaneous decomposition of peroxide to be 18 kcalmol^{-1} [58, 59]. The activation energy of free catalase, immobilized catalase via glutaraldehyde and immobilized catalase via glutaraldehyde + spacer were 14.4 ± 3.5 kJ mol^{-1}, 14.8 ± 2.4 kJ

mol^{-1} and 15.3 ± 2.4 kJ mol^{-1} respectively [60]. Elena Horozova suggested that ΔH and ΔS depend on the matrix used for immobilizing the enzyme [61].

5.6 Conclusions

The important results of immobilization of catalase are summarized as follows:-

- Different methods for the immobilization of catalase by physical adsorption and chemical modification on to different mesoporous silica were evaluated.
- The concentration of buffer solution was found to be one of the most important factors in the activity of both free and immobilized catalase and activity significantly enhanced in buffer solutions with an ionic strength of 0.05M.
- The optimum pH and temperature varied after immobilization.
- The highest phenol degradation rate was obtained in the case of covalently bound catalase on MCF-25G
- The thermal stability improved significantly by the immobilization process.
- The immobilized catalase could be reused efficiently in batch reactor and covalently bound catalase on MS-13G and MCF-25G retained about 85% of the initial catalytic activity, even after 15 cycles.
- All the immobilized systems retained more than 50% activity even after 30 days storage at 4°C indicating that the immobilization improved storage stability.
- Thermodynamic parameters such as activation energy and enthalpy of enzyme substrate complex showed an increase after immobilization.

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Chapter 6

SYNTHESIS OF ESTERS WITH IMMOBILIZED CANDIDA RUGOSA LIPASE

Contents

Traditionally esters are extracted from plant materials and direct biosynthesis by fermentation. These methods for flavor and fragrance production exhibit high cost for commercial use and low yields. Esters are synthesized by a chemical reaction of alcohol with an organic acid in presence of strong acids such as sulphuric acid, p-toluene sulphonic acid and phosphoric acid as homogeneous catalysts. Chemical routes are fraught with many drawbacks such as high temperature or pressure requirement, use of hazardous chemicals, longer reaction times, low conversions and pollution. Although several heterogeneous catalysts have been developed in recent years to improve the yield and selectivity of the reaction they also share the drawbacks such as high temperature and harsh conditions. Different strategies such as modification of reaction conditions, development of reusable catalyst and use of biocatalyst have been adopted to address these problems and make them green and environmentally benign process. Organic esters are found in numerous potential applications in the perfume, cosmetic, food, beverage and pharmaceuticals industries. They are also used as plasticizers, solvents, lubricants in machinery industries, biological additives and hydraulic fluids.

6.1 Introduction

Lipases (E.C. 3.1.1.3) from different sources are used in various biochemical reactions including triacylglycerol hydrolysis, esterification between fatty acids and alcohols. The rising interest in lipase mainly lies on its

wide industrial applications, including detergent formulation, oils/fats degradation, pharmaceutical synthesis, and cosmetics production [1-3]. Candida rugosa lipase (CRL) is a stable mesophilic lipase that has high activity and broad specificity in reaction medium [4]. These enzymes are very important because of many reasons: they do not require cofactors, they remain active in organic solvents, they can be used with a broad range of substrates, and they often exhibit high enantio and regio-selectivity [5]. Biocatalysis in organic solvents has several potential advantages over biocatalysis in aqueous media: (i) increased solubility of hydrophobic compounds (ii) ability to carry out new reactions, that are kinetically or thermodynamically restricted in water (iii) suppression of undesirable side reactions (iv) control or modification of enzyme selectivity (substrate, enantio, regio, prochiral); (v) the possibility of recovery of some products by the use of low boiling point organic solvents; and (vi) an increased enzyme thermostability [6]. Lipase catalysis mostly occurs *via* an interfacial activation process. The presence of a water–lipid interface is required for efficient catalysis [7]. The basic reactions, kinetics, and mechanisms of lipase-catalyzed reactions (hydrolysis, esterification, acidolysis, alcoholysis, and ester exchange) have been discussed [8-11]. In the presence of a suitable lipase as the biocatalyst, the following reactions are possible (the reactions are also reversible under appropriate conditions). In spite of these advantages, enzymes do not always meet desired levels of activity, productivity and most importantly stability in organic solvents [12]. Hydrophobic solvents typically lead to higher enzymatic activity and stability than their hydrophilic counterparts, which tend to strip some of the water required for enzymatic function, thereby lowering the catalytic activity [13]. The availability of lipases that were stable in polar solvents would favour new applications in biotechnological processes with polar substrates. Ajaikumar *et al* suggested that hydrophobic properties of the catalysts play important role in

the esterification reaction [14]. Surfactant coated enzymes have been regarded as very competitive and promising biocatalyst in organic media [15]. Lipase stabilization and activation is partly related to the peculiar structural features of lipases, i.e. the presence of a mobile domain acting as a "lid" for the enzyme active site, which is able to modulate the entrance of substrates into the catalytic pocket. In the presence of a hydrophilic environment the lid is closed and the active site almost unaccessible. The contact with hydrophobic interfaces or the presence of drops of hydrophobic substances induces a conformational movement of such mobile domain and the consequent exposure of wide hydrophobic patches that surround the active site. The catalytic site becomes accessible to substrates and the enzyme turns to its activated form [16, 17]. Most lipases display a large increase in activity when adsorbed on hydrophobic supports. This characteristic has been shown to be associated with conformational changes in the enzyme upon adsorption, creating an open, substrate-accessible active site. Thus, lipases recognize hydrophobic surfaces similar to those of their natural substrates and they undergo interfacial activation during immobilization. Special emphasis is paid to the selective adsorption of lipases on tailor-made strongly hydrophobic support surfaces [18].

The esterification reaction is a liquid-phase process, where the limiting conversion of the reactants is determined by equilibrium. Typically, esterification reactions are very slow; it requires several days to attain the equilibrium in the absence of catalyst. Therefore, the reaction is enhanced by an added catalyst. Acidic catalyst creates many disadvantages to commercialize the esterification process. Hence there is a need to develop a green catalyst. Among various enzymes lipases are used for the synthesis of natural flavor esters. The long chain ester synthesis gave very low conversions and it can be increased by varying

conditions. There are some papers reporting on short chain ester synthesis catalyzed by lipases immobilized on variety of supports, such as silica matrices [19-21] or celite [22, 23] and very few catalyzed by lipase–hydrogel conjugates [23, 24]. Candida rugosa lipase was immobilized in micro emulsion based organogels (MBG) and used for production of a variety of flavor esters [25-27]. Lipase from Candida rugosa was coated with surfactants and immobilized on silica to catalyze esterification reaction to produce ethyl butyrate. Among various surfactants, non-ionic surfactant performed better than cationic surfactant whereas inhibitory effect was observed with an anionic surfactant. Hence surfactant coated lipase immobilized on silica can be exploited for higher ester production [28].

Different esters of isoamyl alcohol were synthesized with various aliphatic acids ranging from acetic acid to octanoic acid by three different enzymes, Novozym 435, Lipolase 100T and HPL (Hog pancreas lipase) in supercritical carbon dioxide and in solvent free conditions. In supercritical carbon dioxide, results showed that the conversion increased with increasing chain length of the acid when the reaction was catalyzed by HPL. The conversion decreased with increasing chain length of the acid for the reactions catalyzed by Lipolase 100T. The conversion is nearly independent of the chain length of acid for the reaction catalyzed by Novozym 435 [29].

This chapter is divided into two parts. In first part we are dealing with synthesis of ethyl valerate green apple flavor and in other part we explain synthesis and kinetics of amyl isobutyrate. The experimental procedure and analytical methods used for both syntheses are the same.

6.2 Experimental procedure

The reaction was carried out in 250 mL glass stoppered flasks. The reaction mixture consists of 10 mL of solvent and equimolar concentration of

valeric acid and ethanol (except for substrate effect studies). To initiate the reaction, required amount of enzyme was added to the reaction mixture and kept on orbital shaker at 35°C (except for temperature effect studies) and 250 rpm. Samples were withdrawn periodically and analyzed by gas chromatography (GC) and titration methods.

Analytical procedure

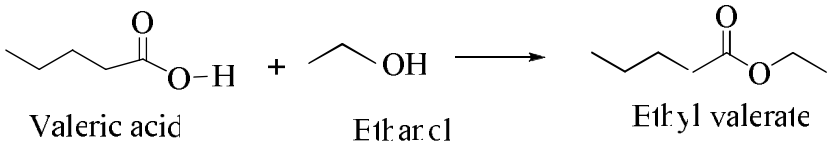
Esterification reactions were analyzed by both gas chromatographic method and titrimetry. In titrimetry, esterification was monitored by determination of the residual acid content by titration against sodium hydroxide using phenolphthalein as an indicator and mixture of methanol and diethyl ether (1:3) as a quenching agent. The ester formed was calculated as being equivalent to acid consumed. The reaction mixture was withdrawn periodically and immediately analyzed by Gas Chromatography (Chemito) equipped with flame ionization detector and a 10% SE-30 on chromosorb W 2 m × 3.2 mm stainless steel packed column. Quantification of data was done by calibration with standard samples. Esterification activity determined by GC analysis and titrimetry were in good agreement.

The unit of enzyme activity is defined as μmol of acid consumed (in an esterification reaction with alcohol) per min per mg of the enzyme.

Part I

Synthesis of Ethyl valerate

Ethyl valerate, also known as green apple flavor is an important ester used as a constituent in many fruity flavors such as plum, apricot, peach, strawberry and pear aromas [30]. It is well known for its wide applications in the areas of food, pharmaceuticals and cosmetics industries [31, 32]. The aminated polypropylene (APP) membrane was used for covalent immobilization of Candida rugosa lipase via glutaraldehyde coupling. The retained esterification activity of the immobilized lipase for ethyl valerate was 76%. [33].



6.3 Biochemical characterization of free and immobilized lipase

6.3.1 Effect of immobilization pH

The effect of pH on the activity of CRL for catalyzed esterification of ethyl valerate was studied by varying pH from 4 to 8 at 40°C (Fig. 6.1). For this reactions, results clearly indicate that the optimal pH for free CRL is 7 where as the optimal pH is different for different immobilized systems. Optimal pH for MS-9 is 5.5. In the case of glutaraldehyde bound enzyme pH shifts to 6.5. All the immobilized samples retain more than 50% activity in the range of pH 5-7. These results indicate that immobilized CRL possesses excellent adaptability over a wider pH range compared to free CRL. This may be due to the hydrophobicity of the synthesized samples on surface, which

causes the partitioning of protons between the bulk phase and enzyme microenvironment. Consequently, a shift in the optimal pH value occurs [34]. Additionally, hydrogen bonds formed between the support surface and CRL protect the active sites from being directly exposed to the medium and thus limit the sensitivity of immobilized lipase to a higher pH [35]. These results clearly prove that the functionalization on meso silica improve the activity of immobilized CRL.

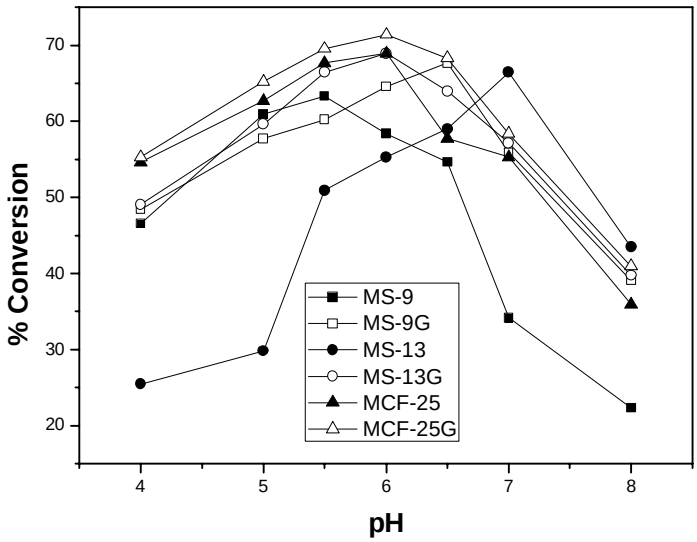


Fig. 6.1 Effect of immobilization pH

Lipase immobilization occurs in pH around the isoelectric point ensures the relatively high activity. Enzyme conformation plays a vital role for enzymatic activity and it is around the isoelectric point of lipase. Correlations between the pH, movement of the lid and the catalytic behaviour have been reported [36-39]. The influence of some organic compounds in stability, activity and enantioselectivity of the enzyme was also reported [40-45].

6.3.2 Effect of solvent

Proper selection of organic solvent is primarily the most important criterion in any enzymatic reaction and to predict which solvent would be better for performing any enzymatic reaction. It has been well reported that solvent has the ability to alter the enzyme activity by changing its conformational structure [46]. Thermal stability and enzyme activity were increased in presence of organic solvent [12, 47-49]. Hydrophobic solvents lead to higher enzymatic activity and stability because they will strip essential water required for the enzymatic function, and lower the catalytic activity [6, 13]. Lipases are used as catalyst in organic solvents for many reactions including esterification and transesterifications. Lipase catalyzed esterification requires a nonpolar environment with a low water content to shift the thermodynamic equilibrium in favour of esterification over hydrolysis. A biphasic reaction medium (immobilized enzyme/substrate) provides lipases with an inter phase to enhance the catalytic performance of enzyme. Recently lipases have been used for organic synthesis because of their stability in non aqueous media and mild reaction conditions [50].

Different solvents such as heptane, hexane, isooctane, DMSO and 1,4-dioxane are used with conversions of 75, 72, 73, 19 and 22 %, respectively. It is found that (Table 6.1) heptane is the best solvent. Therefore, all further studies were carried out in heptane. In presence of suitable solvent the lid is open, the catalytic triad at the active center becomes accessible for a substrate. Because of this reason lipase shows higher activity. Karra- Chaabouni *et al* produced ethyl valerate and hexyl acetate in hexane, heptane and solvent-free medium and found remarkably higher ester yield in both hexane and heptane medium than those obtained in the absence of organic solvents [51].

Table 6.1 Effect of various solvents on the activity of free and immobilized lipases

Samples	% Conversion				
	Hexane	**Heptane**	**Isooctane**	**DMSO**	**1,4 Dioxane**
Free CRL	72	75	73	19	22
MS-9	61	63	62	30	36
MS-9G	65	68	67	52	58
MS-13	63	67	63	42	43
MS-13G	68	69	69	50	52
MCF-25	61	69	67	51	59
MCF-25G	67	70	68	52	57

When the reaction medium was changed from water to an organic solvent the overall efficiency of the enzyme changed dramatically. In aqueous medium, it acts as hydrolyzing enzyme whereas in organic medium it favours the esterification reaction. Enzymes are more stable in solvents having log *p* greater than 4. Water plays a major role of 'molecular lubricant' in enzymes resulting in its conformational flexibility and leads to enhanced activity in non-aqueous media. A minimum quantity of water is essential for lipase catalyzed esterification. These solvents do not distort the essential water coat, thereby leaving the bio-catalyst in an active state [52-54]. It is clear that activity of enzyme is strongly affected by the solvent. A control experiment in the absence of enzyme did not show any conversion.

In the case of immobilized enzymes, the support may prevent the water monolayer from being stripped and maintain the three dimensional structure of its active protein conformation for catalysis [55]. Therefore, enzymes may be stable in organic solvents than they are in water and this is the reason why they are used as reaction medium in enzymatic reaction. The adsorbed as well as covalently bound systems showed higher activity than the free enzyme in each solvent.

6.3.3 Effect of temperature

The effect of temperature was studied at 30,35,40,45, 50, 60 and70°C and the final conversions after 48h were as 61, 65, 71, 72, 74, 75 and 19%, respectively. With an increase in temperature from 30 to 60°C, the rate of the reaction and conversion increased from 61 to 76%. Further increase in the temperature 70°C, resulted in a decrease in conversion due to thermal degradation of the enzyme (Table. 6.2). Temperature affects the enzyme stability, the thermodynamic equilibrium and kinetics of the reaction [56]. These factors may interact leading to the observed trend. Therefore, all further experiments with free and immobilized lipases were carried out at 40°C.

Table 6.2 Effect of temperature on the activity of free lipase

Temperature(°C)	% Conversion	Esterification activity
30	61	0.1041
35	65	0.1123
40	71	0.1235
45	72	0.1256
50	74	0.1286
60	76	0.1317
70	19	0.0337

Literature results suggest that the immobilization matrix might be able to protect the enzyme against denaturation at higher temperatures as was observed on the immobilized lipase in kaolin [57]. Lee *et al* also found that the optimum temperature for the activity of immobilized enzyme in uncoated calcium alginate beads, chitosan coated calcium alginate beads and poly-L-lysine coated calcium alginate beads were higher than those of free aminoacylase [58].

6.3.4 Effect of amount of biocatalyst

The synthesis of ethyl valerate was studied by taking different amount of lipase (25–125 mg) in 10 mL of reaction mixture containing 100 mM and 50 mM each of pentanoic acid and ethanol in *n*-heptane at 40°C in 48h under shaking (250 rpm).

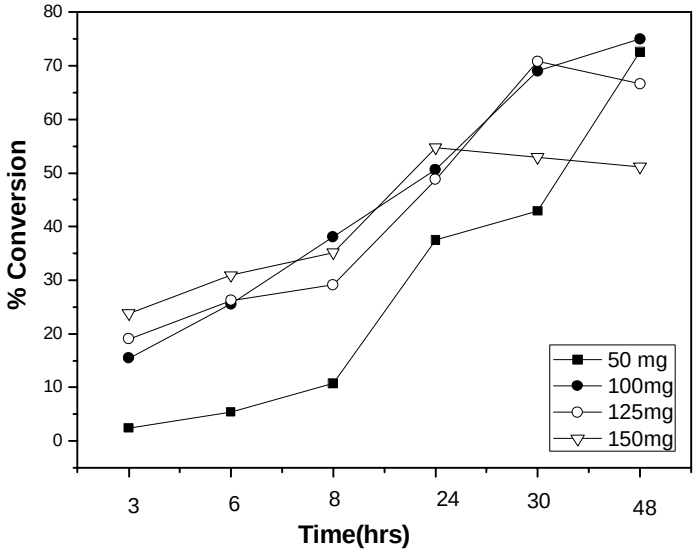


Fig.6.2 Effect of amount of biocatalyst

Enzyme concentration is known to influence the esterification behaviour. Different amounts of lipase were used and the progress of the ester synthesis was studied. Maximum esterification (72% conversion) was achieved when 100 mg of lipase was used. The results (Fig 6.2) showed that a lipase above 100 mg did not significantly increase the reaction rate, i.e. further increase in the amount of lipase failed to enhance the rate of esterification. Hence, 100 mg lipase was used in the subsequent experiments. An increase in the quantity of biocatalyst concentration resulted in a decrease in the apparent enzyme activity in the production of ethyl valerate. Decrease in activity is due to lack of substrate to access the active site of enzyme at high concentration.

6.3.5 Effect of initial addition of water

Water plays multiple roles on lipase-catalyzed esterification reactions. Water participates directly or indirectly in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes and in esterification/hydrolysis reactions [59, 60]. Water content affects the equilibrium conversion of the reactions as well as the distribution of products in the media as a result of water acting as a substrate [61-63]. Most biocatalysts are inactive in a fully dehydrated medium, and a minimal quantity of water is necessary to maintain their activity. Proper amount of water for lipase activity depends on the nature of the support, polarity of the substrate, substrate concentration and solvent [52]. As Svensson *et al* reported, for these reactions the activating effect of water dominates at water contents below the optimum, while at higher water contents the net esterification rate decreases. It may be because of water acting as a substrate in hydrolysis of the acyl-enzyme intermediate [64, 65].

The effect of initial water content on the esterification reaction was investigated. The reaction was carried out at various amounts of added water ranging from 0.05 to 0.3 mL .In the absence of any added water the biocatalytic activity seems to be inhibited as it does not exceed 8% after 6h. The esterification reaction is triggered with the addition of water and the conversion limit fluctuates according to the initial water level. The high conversion yield was obtained by the addition of 0.15 mL of water. These results (Fig. 6.3) suggest that as the initial addition of water increased, the hydrolysis was accelerated and hence reduced the conversion percentage gradually. Above 0.15 mL of added water, it is likely that water will start to accumulate on the external surface and cover the enzyme molecule which becomes unavailable for biocatalysis. If the substrate molecules dissolved in heptane there will be a physical separation between the reactants and the lipase

active sites were reported by Ghamgui *et al* for the enzymatic synthesis of butyl oleate [66]. Mukherjee *et al* suggested that low water content favours ester synthesis over hydrolysis [67].

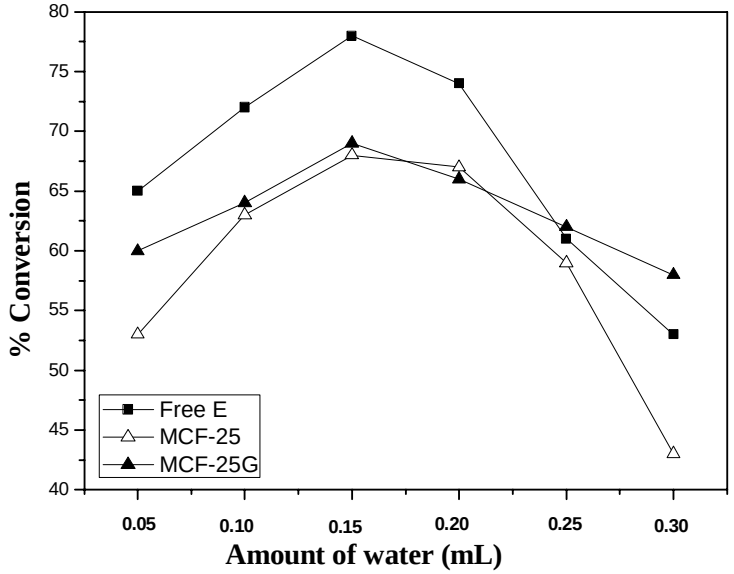


Fig. 6.3 Effect of amount of initial water

6.3.6 Effect of molecular sieves

The main drawback of esterification reactions using alcohol is that water is formed as a by-product, requiring azeotropic distillation to remove water during the reaction which otherwise would deactivate the catalyst [68]. It is well known that in order to obtain higher yield of esters, the reactions must be forced to completion by removing the water which was formed during the course of reaction and/or by operating with an excess of one of the two reactants (acid or alcohol). 4Å molecular sieves were added into the reaction system to adsorb the byproduct water. In order to verify the effect of water removal, reactions were carried out with 4Å molecular sieves which were added to the system by several batches. The addition of molecular sieves at the

beginning of the reaction did not improve the final conversion. When molecular sieves was added at the beginning % conversion decreases indicating that some amount of water is essential for esterification and if it exceeds the limit reverse reaction (ester hydrolysis) can occur.

The molecular sieves (4Å) were added at the concentration from 25 to 150 mg per reaction volume as mentioned above and synthesis of ethyl valerate in the reaction mixture was determined. The esterification reaction results in formation of water as a by-product of the reaction, and its removal using molecular sieves might enhance the synthesis of ester by pushing the reaction equilibrium in the forward direction [69, 70]. Before each reaction, molecular sieves were dried at 300°C for 3h. They were added to the reaction medium after 6h, 12h and 24h of reaction. However, when the effect of molecular sieve was studied by adding molecular sieve at concentrations of 25 to 150 mg per reaction mixture, a gradual increase in the synthesis of ethyl valerate was noticed up to 100 mg (80%) and any further increase in the amount of molecular sieves resulted in a decrease in the amount of ethyl valerate (Table 6.3). Esterification markedly increased in the presence of molecular sieves is observed in the present study.

Table 6.3 Effect of molecular sieves on the activity of free lipase

Amount of molecular sieves(mg)	% Conversion	Esterification activity
Control	75	0.1369
25	76	0.1380
50	77	0.1402
100	80	0.1458
125	70	0.1270
150	56	0.1016

Several researchers explained that excessive stripping of the essential water decreases enzyme activity [71]. Lumor *et al* determined increased esterification activity by reacting caprylic acid with glycerol in the presence of molecular sieves (30%, w/w) and adapted the use of molecular sieves to improve esterification reaction rate [72].

6.3.7 Effect of mole ratio

To study effect of ethanol concentration for production of ethyl valerate using lipase, the concentration of valeric acid was fixed at 0.05 mol while concentration of ethanol was varied from 0.02 to 0.09 mol. The total liquid volume was kept at 15 mL with heptane. The reactions were carried out at 40°C and shaking speed was maintained as 250 rpm. It was observed that after 0.05 mol alcohol concentration % conversion decreases. Fig. 6.4 a shows that ethanol inhibits the lipase catalyzed reactions when its concentration was higher than 0.05 mol. These results reveal the hypothesis of inhibitory effect of ethanol on lipase from Candida rugosa since the decrease of initial rate with increase of alcohol concentration above 0.05 mol was observed. Several researchers reported that high alcohol concentrations may slow down the esterification reaction rates [73-75].

To study the effect of valeric acid concentration, acid concentration was varied from 0.04 to 0.08 mol while concentration of ethanol was fixed at 0.05 mol. It can be noted that (Fig. 6.4b) a contrary effect of ethanol is observed. Valeric acid enhances the product conversion as the concentration of valeric acid increases. But after 0.01mol concentration % conversion slightly decreases and remains unchanged when the concentration of acid is increased. The maximum % conversion was achieved at 0.05 mol alcohol and 0.1 mol valeric acid. So this mol ratio was optimized for further studies.

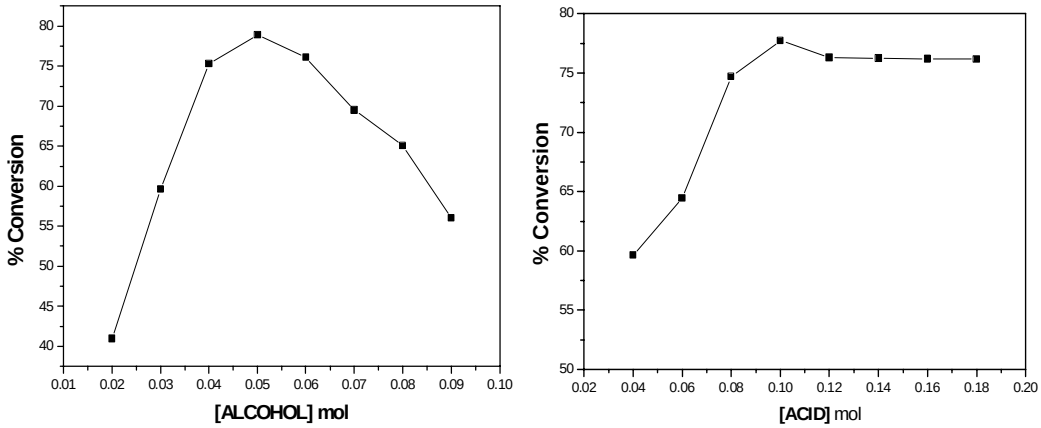


Fig. 6.4 (a) and (b) Effect of concentration of alcohol and acid

As previously reported, in the lipase-catalyzed esterification reaction the first step consists of the preferential binding of the acid molecule to the enzyme molecule [76]. At high alcohol molar ratio, a large increase in alcohol concentration may promote the binding of alcohol molecules to the lipase, during the first reaction step, competing with the acid. As a result, a decrease in the amount of bound acid occurs. Thus, this situation would lead to a decrease in the reaction rates, since the reaction will be limited by the amount of acid in the vicinity of the enzyme [74].

6.3.8 Thermal stability

Thermal stability of enzymes is important for bioprocesses operated at high temperature in industrial application to improve productivity and to lower production costs. In order to survey the thermal stability of immobilized lipase, the relative activities of free and immobilized CRL for catalytic esterification were assessed by incubating the lipase at 50°C for 120 minutes and then they were assayed at 40°C for 48hr reaction time. (Fig. 6.5). After 60 min of incubation, immobilized CRL maintained more than 80% of their initial activities, while free CRL lost 50% of its activity after 60 min. This indicates

that the immobilization procedure enhances the thermal stability of the lipase. Similarly, Nara *et al* [77] reported that racemase adsorbed on folded-sheet mesoporous silica showed higher thermal stability than free racemase. The excellent thermal stability of immobilized lipase could be attributed to the architecture of the support which may provide protection to the enzyme molecules from direct exposure to high temperature.

Results demonstrated that immobilized lipase were stable at 50°C while free lipase became less stable at temperatures higher than 40°C. This result is consistent with the reports by Chiou and Wu [78] and Hung *et al*., [79]. Rahim *et al* reported that free lipase was stable only up to 30°C, while the lipase immobilized by binary method using chitosan remained fully active at 40°C [79, 80]. However immobilized enzyme was found to be deactivated on heating to temperatures higher than 50°C. Matsumoto and Ohashi reported that the thermal stability of lipase was enhanced when immobilized in calcium alginate gels, inorganic microcapsules of calcium silicate and macroporous acrylic beads [73].They also observed a higher thermal stability than free lipase at 50°C but the activity was completely lost at 80°C within 15 min [81]. This indicates that the immobilization procedure enhances the thermal stability of the lipase. The excellent thermal stability of immobilized lipase MCF-25G could be attributed to the architecture of the support which may provide protection to the enzyme molecules from direct exposure to high temperature.

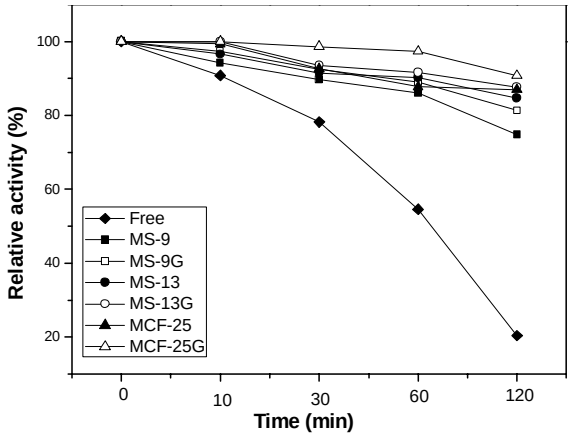


Fig. 6.5 Thermal stability of the free and immobilized lipase at 50°C

6.3.9 Reusability

Enzymes are relatively expensive and therefore discarding them after a single use is not economical. The re-usage of enzymes is the main problem that restricts their use in industrial applications. One method to circumvent this issue is to immobilize the enzyme on a suitable supporting medium [82, 83]. Not only does this facilitate easier recovery and reuse of the enzyme, but also in many cases, immobilized enzymes show a higher stability than free species [84]. The immobilization method can solve this problem to a great extent**.** Heterogeneous catalysts which are widely used in industry have good thermal stability and can be easily separated from the reaction mixture they can often be readily regenerated and reused. Reuse of immobilized lipase for continuous reactions allows the reduction of production cost and improves economic efficiency [4]. The reusability of immobilized CRL was studied using the same substrate concentration, optimum temperature and reaction time as mentioned above. The initial activity of immobilized CRL was compared with the activity of the used CRL obtained after repeated use for 10 cycles.

The decrease in enzyme activities of the samples might be ascribed to the leakage of immobilized lipase as a result of weak interaction between support and

enzyme during catalytic reactions. Results showed (Fig. 6. 6) that the two catalysts MS-9 and MS-9G have low stability than other supports. Supports having high pore size showing better stability must be needed to achieve higher interaction between enzyme and catalytic support and allow any commercial application.

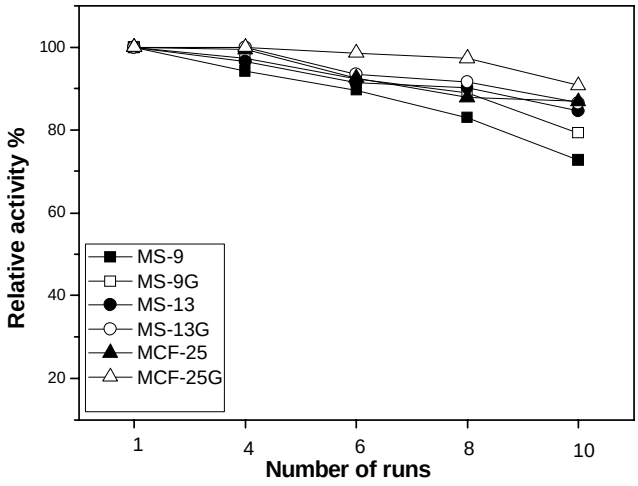


Fig. 6.6 Reusability characteristics of free and immobilized lipase

6.3.10 Storage stability

Enzymes are not stable during storage in solutions and their activities decrease gradually by the time. The storage stability of immobilized enzymes without appreciable loss of enzyme activity is important for the economic viability of a biosynthetic process [85-87]. Free and immobilized lipase was stored in buffer (0.1M) at 4°C and activity measurements were carried out for a period of 60 days. The free enzyme lost its initial activity within 40 days. Table 6.4 represents the results for storage stabilities for 60 days at 4°C of free and immobilized lipase. The immobilized lipase showed higher storage stability compared to free lipase. Immobilized enzymes lost 30% of its initial activity after 60 days. The immobilized lipase retains more than 70% of its initial activity at 60days storage. The immobilized lipase however, retained its high catalytic activity of 71% and above,

while the free lipase retained only about 58.4% of catalytic activity after 10 days and just about 26% after 30 days of storage at 4°C. The enhancement in stability provided by the present immobilization method is more than three-fold. Covalently immobilized enzymes show higher activity than adsorbed one because it holds the enzyme in a stable configuration in comparison to the free counterpart [33]. After 10 days of storage at 4°C, the immobilized lipase seemed to retain its activity by more than 90% of its initial activity, indicating that the enzyme is easily protected after immobilization. It was concluded that the immobilized lipase possess high resistance to denaturation and enhanced enzyme stability.

Table 6.4 Storage stability of free and immobilized lipase

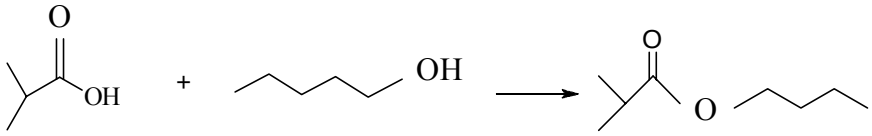
Support	Relative Activity (%)			
	After 5 days	After 10days	After 30days	After 60 days
Free CRL	82.7	58.4	26.0	0
MS-9	95.9	92.0	87.8	71.3
MS-9G	96.5	94.3	91.2	78.4
MS-13	99.7	99.4	94.5	87.6
MS-13G	100	98.6	96.9	90.4
MCF-25	100	100	99.2	98.7
MCF-25G	100	100	100	99.0

Supports of different chemical structure, hydrophilicity/hydrophobicity as well as immobilization methods gave different storage stability. For example, the lipase from Candida rugosa immobilized in poly(N,N-dimethylacrylamide-co-acrylamide) and poly(N-isopropylacrylamide-co-acrylamide)/-carrageenan hydrogel systems retained only 54% and 42.5% of their initial activity after storage in 0.05M phosphate buffer for 60 days at 4°C [88]. In another example, the lipase from Mucor miehei adsorbed on styrene–divinylbenzene copolymer retained more than 71% of its initial activity after storage in phosphate buffer (50 mM, pH 6.0) at 4°C for a period of 8 weeks [33].

Part II

Synthesis of Amyl isobutyrate

Amyl isobutyrate is apricot flavor ester. The Candida rugosa lipase catalyzed esterification of butyric acid with amyl alcohol in isooctane and in solvent-free system were studied by Bezbradica *et al* Nearly complete conversion (>95%) of substrates was achieved using low enzyme amount of 0.5% (w/v) at 45°C [89]. Candida rugosa lipase was immobilized into NiPAAm/IA hydrogel (poly (N-isopropylacrylamide) and its activity for n-amyl isobutyrate synthesis in organic medium (91.2% for 48h) was evaluated. The results showed fairly good stability and other characteristics led that immobilization method to many industrial applications [87]. A wide range of sources of lipase in an organic solvent using the reverse micelle immobilization method was studied by using two surfactants AOT and CTAB in n-hexane studied by Hong *et al* [90]



Isobutyric acid Amyl alcohol Amyl isobutyrate

We have investigated various parameters to optimize the reaction and the optimized conditions are summarized in Table 6.5.

Table 6.5 Optimized parameters for the synthesis of amyl isobutyrate

Amyl alcohol	0.05 mol
Isobutyric acid	0.12 mol
Reaction time	48h
Temperature	45°C
Reaction medium	Isooctane

6.4 Kinetics and mechanism of Candida rugosa lipase for esterification reaction

The thorough knowledge of kinetics is of great importance not only to elucidate mechanism of this reaction, but reliable information about the rate of product formation and changes in experimental systems are necessary for the design of suitable reactors and later industrial scale-up. A range of substrate concentration was examined and the initial rates were attempted to study the kinetics of esterification. Michaelis constant K_m is used to determine the substrate affinity between enzyme and substrate at the initial stages of reaction or low conversion phase. If two substrates are present one of the substrate can cause inhibition which is explained by Ping –Pong Bi-Bi mechanism. In Ping-Pong mechanism only one substrate is bound to enzyme to form the acyl-enzyme complex and one product formed is released. The other substrate binds to the enzyme to form a second product. Many researches accepted Ping-Pong mechanism for two substrate two product (Bi-Bi) reactions for lipase catalyzed esterification and transesterification reaction in organic media [91-93].

6.4.1 Kinetic studies of Candida rugosa lipase for amyl isobutyrate synthesis

The kinetics of the free lipase was studied at 45°C in a batch reactor with isobutyric acid and amyl alcohol (1-pentanol) as substrates .In this study, molar quantity of isobutyric acid and amyl alcohol was varied from 0.01 and 0.3 mol. The 100 mg of enzyme is used for the study and isooctane was taken as solvent. The results are illustrated in Fig. 6.7 and Fig.6.8. Reactions were carried out up to 10% conversion and the initial rates (r_0) were determined. The concentration of one of the two substrates was maintained constant while the full series of concentration for the second one was investigated.

The effect of concentration of both the substrates on the rate of reaction was investigated. It was found that when the concentration of amyl alcohol (B) was increased, the rate of reaction increased and reached a maximum at a critical concentration. A subsequent increase in amyl alcohol concentration decreased the initial rate. Therefore, it may be concluded that amyl alcohol at higher concentration reacts with the enzyme to form a dead end inhibitory complex. There was no evidence of inhibition by isobutyric acid (A) at any concentrations tested. A mechanism in which the product is released between the addition of two reactants is called Ping-Pong Bi-Bi and when one of these reactants forms a complex with the enzyme that cannot participate in the reaction is called Ping-Pong Bi-Bi with dead end inhibition

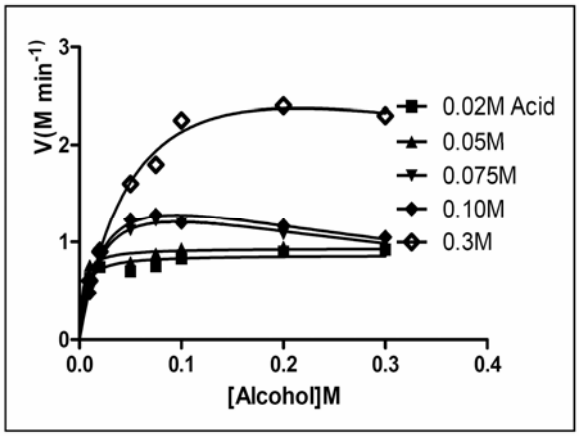


Fig 6.7 Initial conversion rates as a function of the mol number of Amyl alcohol for free lipase

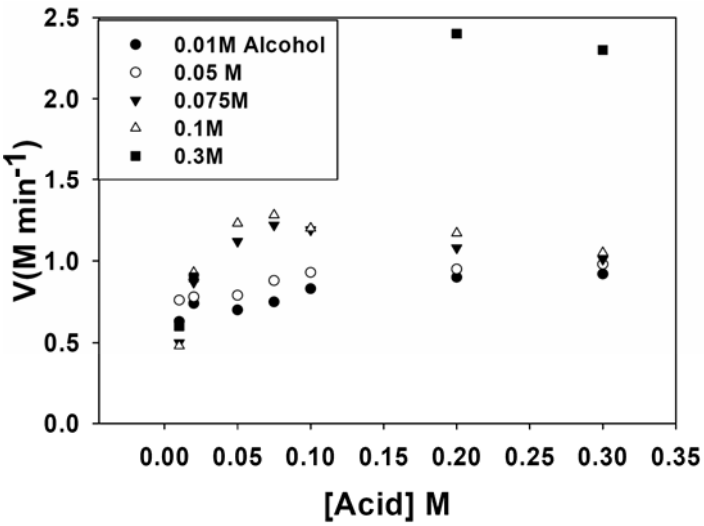


Fig 6.8 Initial conversion rates as a function of the mol number of Butyric acid for free lipase

Mechanism of the reaction

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^1 + P$$

$$E^1 + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E^1B$$

$$E^1B \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + Q$$

$$E + B \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + B$$

where A is acid, B- alchohol , E free enzyme, EA enzyme-acid complex, E^1-enzyme-acyl complex, E^1B- binary complex of acyl enzyme and alchohol, P-water and Q is ester. According to the Ping-Pong Bi-Bi kinetic mechanism, shown in Scheme 1, the acyl donor binds first to the free enzyme forming a

noncovalent enzyme-anhydride complex which is transformed by a unimolecular isomerization reaction to an enzyme–acyl intermediate with the concomitant release of the first product, acetic acid. In a second step, the second substrate, alcohol, binds to the binary enzyme–acyl complex and forms a tertiary complex enzyme–acyl–alcohol. This complex is also isomerized by a unimolecular reaction to an enzyme–ester complex, which results in the release of the second product, ester while the enzyme recovers its initial conformation.

The shape of the graphs in the Fig 6.10 was found to qualitatively correspond to a Bi-Bi Ping-Pong model with inhibition by amyl alcohol, according to the various kinetics models detailed by Segel [94]. This mechanism with competition between the substrates and inhibition by one of them is characterized by the existence of a maximum in the conversion rate (of amyl alcohol and isobutyric acid) as a function of the mole number of amyl alcohol (Fig. 6.9). The fitting of these experimental points with a theoretical model was achieved with the data-processing software Sigma plot 8.0 from SSPS. The equation of the function corresponding to the Bi-Bi Ping-Pong model with inhibition by one of the substrates [94] is the following:

$$\frac{r}{r_{\max}} = \frac{[A][B]}{K_{i(A)}K_{m(B)}K_{m(A)}[B] + K_{m(B)}[A] + [A][b]} \quad \text{----------(1)}$$

Lineweaver–Burk equation is obtained as follows initial rate data and concentration is used.

$$\frac{1}{r_0} = \frac{r_{\max}K_{i(A)}K_{m(B)}K_{m(A)}[B_0] + K_{m(B)}[A_0] + [A_0][B_0]}{[A_0][B_0]} \quad \text{----------(2)}$$

where $[A_0]$ is the initial concentration of isobutyric acid $[B_0]$ the initial concentration of amyl alcohol (mmol), $K_m(A)$ is the Michaelis constant of isobutyric acid, $K_m(B)$ the Michaelis constant of amyl alcohol, K_i the inhibition

constant of amyl alcohol, r_0 the initial rate of the reaction, r_{max} is the maximum rate of the reaction[95]. Most of the kinetic studies on the lipase-catalyzed synthesis of esters the direct esterification of alcohols with acids, and described a Ping-Pong Bi-Bi kinetic mechanism with inhibition by both or one of the reactants [92, 96].

Chulalaksananukul *et al*. carried out ester synthesis using lipase from Mucor miehei and proposed Ping-Pong Bi-Bi model with dead-end inhibition by alcohol [97]. The same model was proposed by Hazarika *et al* for synthesis of ethyl oleate with porcine pancreatic lipase [98] and by Yadav and Lathi for synthesis of butyl isobutyrate with the commercial immobilized lipase from Candida Antarctica [52]. On the other hand, in the case of the synthesis of citronellol laurate the random order Bi-Bi model with inhibition by lauric acid was reported [101]. Arcos *et al*. proposed the model that does not include any kind of inhibition [99]. Ping-Pong Bi-Bi kinetics with inhibition by both substrates was observed in studies with lipase from Burkholderia cepacia [100] and with the lipase from Rhizomucor miehei [91]. Garcia *et al* developed kinetic model for entire course of the reaction based on ordered Bi-Bi mechanism of esterification with the inhibition by both substrates and both products [102].

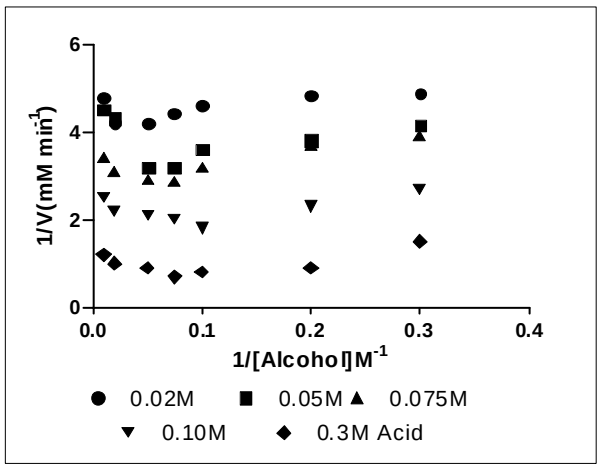


Fig 6.9 Lineweaver-Burk plots 1/ v versus 1/[Alcohol] obtained with free lipase at five fixed concentration of Acid

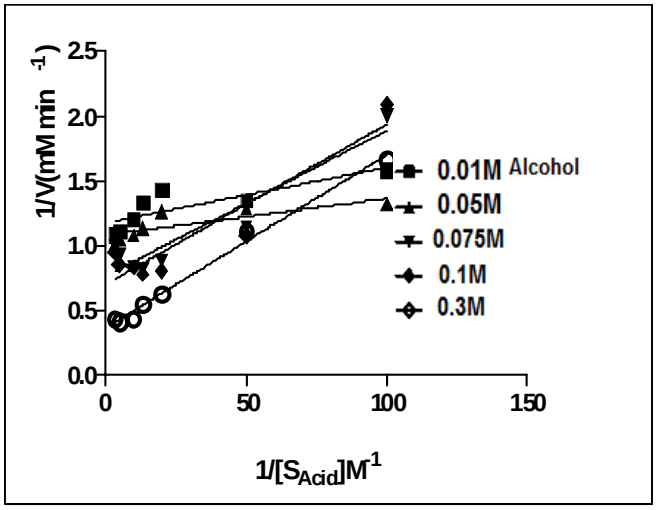


Fig. 6.10 Lineweaver-Burk plots 1/ v versus 1/[Acid] obtained with free lipase at five fixed concentrations of Alcohol.

The reaction mechanism has been elucidated by the plot of l/ V versus l/S (isobutyric acid). Data are presented in Fig. 6.10 which shows that at low fixed amyl alcohol concentrations (0.01M and 0.05M) the plots appear parallel. As the amyl alcohol is increased (0.075, 0.1 and 0.3M), the slope increases and the 1/ V axis intercept decreases as usual to a limit of l/V_{max}. These results agree with an assumed Ping-Pong Bi-Bi mechanism with dead-end inhibition by one substrate as described in Fig.6.10 [94]. In this typical reaction sequence, the lipase may react with amyl alcohol to yield a dead-end enzyme- amyl alcohol complex or with isobutyric acid to yield the lipase-isobutyric acid complex. Then the lipase- isobutyric acid complex transforms to a carboxylic-lipase intermediate and water is released. This is followed by interaction of carboxylic-lipase with amyl alcohol to form another binary complex which then yields the amyl isobutyrate and free lipase.

Once the Ping-Pong mechanism was confirmed, the kinetic parameters of the Eq. (2) were calculated by a multiple regression fitting of the experimental data. The results are shown in Table 6. 6. From the Equ (2) V_{max}, the maximum

rate of reaction, $K_{m\ alcohol}$ and $K_{m\ acid}$ the Michaelis constant of amyl alcohol and isobutyric acid respectively, K_i the inhibitor constant of amyl alcohol were determined. Based on the calculated kinetic parameters, affinity of the enzyme towards amyl alcohol seems to be greater than of iosbutyric acid, since $K_{m\ alchohol}$ was lower than $K_{m\ acid}$. In other words, the relatively large value of $K_{m\ acid}$ shows that the acid inhibition is much less than the alcohol inhibition. These results agree to a Ping-Pong Bi-Bi mechanism, which assumes that the acyl donor is the first reactant that binds to the lipase. Zaidi *et al* considered the influence of alcohol chain-length on kinetic constants in the esterification of oleic acid. Similar K_m constants were obtained for the acid and alcohols when short chain alcohols (methanol and ethanol) were used. However, for longer chain alcohols (more than four carbon atoms) a much higher Michaelis-Menton constant was observed [92].

The kinetics constants were determined by curve fitting with the theoretical kinetics function. The results are provided in Table 6.6. It appears that the kinetics constants $K_{m\ alcohol}$ and $K_{m\ acid}$ were increased after immobilization except for the inhibition constant by n-butanol ($K_{i\ alcoholol}$) and V_{max} which decreased. $K_{m\ alcohol}$ was also higher when compared to free enzyme but lower than $K_{m\ acid}$. The apparent Michealis-Menten constant for CRL lipase increased after immobilization. It was also observed that the V_{max} values of immobilized lipase by adsorption are higher than that of covalent binding and the K_m value of the covalently bound lipase (MCF-25G) were higher than that for adsorbed lipase (MCF-25) Data confirm the effect due to immobilization.

Table 6.6 Kinetic parameters of free and immobilized lipase

	V_{max} (M min^{-1})	$K_{m\ alcohol}$ (M)	$K_{m\ acid}$ (M)	$K_{i\ alcohol}$ (M)
Free Lipase	17.62	0.4177	1.0273	1.4829
MCF-25	12.42	1.3017	1.6829	1.2180
MCF-25G	11.32	1.5271	1.9272	1.0121

The increase in K_m value after immobilization could be due to the changes in structure of the enzyme upon immobilization and the difficulty in diffusion of the substrate to reach the active site of the enzyme. An increase in K_m indicates that the immobilized enzyme has an apparent lower affinity for its substrate than that of the native enzyme. It may be caused by the steric hindrance of the active site by the support, or the loss of enzyme flexibility necessary for substrate binding. So the V_{max} values for MCF-25G and MCF-25 were lower than that of the free enzyme indicating a lowering of activity of enzymes on account of immobilization. This decrease may be due to steric hindrance imposed by the support on the macromolecular substrate or due to chemical modification [103]. Additionally, the accumulation of reaction products in the enzyme microenvironment as a consequence of slow diffusion and interactions with hydrogen ions could have triggered competitive inhibition decreasing $V_{max.}$ The decrease in V_{max} value as a result of immobilization is considered to be associated with the external and internal diffusional resistances.

Chulalaksananukul *et al*. proved that mechanism of esterification is the inhibition by alcohol in Ping-Pong systems [97]. The study of transesterification previously reported by Zaks and Klibanov [104] did not show alcohol inhibition but also agreed with Ping-Pong mechanism. So, alcohol substrate molecule can act as a dead-end inhibitor in the esterification reaction (by preventing enzyme-acid complex formation) but not in transesterification (enzyme-ester complex is first formed).

6.5 Conclusions

The present study deals the synthesis of ethyl valerate (condensation of ethyl alcohol and valeric acid) and amyl isobutyrate (condensation of amyl

alcohol and isobutyric acid) using free and immobilized CRL in different mesoporous silica supports. The conclusions drawn from the present study are outlined as follows :-

- The immobilized lipase showed high activities at acidic to neutral pH (5–7) in which pH 7 was the optimum for free lipase while a steep decline was observed in basic pH.
- All the covalently bound systems gave higher conversion than the adsorbed systems due to the increased hydrophobicity of the supports. MCF -25G and MS-13G are the most efficient systems and can appear to be worthy of further application in industrial biocatalysis.
- Polar aprotic solvents such as DMSO showed less activity for both ethyl valerate and amyl isobutyrate esterification
- The ratio of acid: alcohol for maximum ester production and complete substrate utilization correspond to 0.05:0.1 mol for ethyl valerate and 0.05:0.12 mol for amyl isobutyrate while higher alcohol concentrations displayed substrate inhibition.
- The addition of 0.15 mL water is essential for enzymatic esterification as it maintains the proper catalytic conformation of the lipase. Increase in water concentration up to 0.3 mL resulted in decrease in the rate of reaction and conversion was found to be less than 50% which could be attributed to shifting the equilibrium towards the reverse reaction (ester hydrolysis).
- Addition of molecular sieves after 6h of the reaction resulted a marked increase in ester synthesis.

- All the immobilized systems achieved good operational stability and could be reused for 10 cycles with excellent activity retention.
- All the immobilized lipase showed enhancement in thermal stability at the incubation temperature 50°C MCF-25G and MS-13G showed more than 95% relative activity.
- Investigation on storage stability revealed that all the immobilized lipases could retain more than 71% activity even after 60 days storage at 4°C. MCF-25G showed 99% activity after 60 days. It is better than already reported values.
- A model based on Ping-Pong Bi-Bi with inhibition by amyl alcohol was found to fit the initial rate data in the kinetics of lipase-catalyzed esterification.
- $K_{m\ alcohol}$ was found to be lower than $K_{m\ acid}$ which showed greater affinity for the alcohol in both the native and immobilized lipase. The V_{max} as well as the $K_{i\ alcohol}$ were lowered after immobilization.
- The operational stability of the immobilized system in esterification reaction proved to be fairly good with 10 consecutive uses with a relative activity of 80% implies that the developed immobilized system could provide a promising solution for the flavor ester synthesis at the industrial scale.

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Chapter 7

HYDROLYSIS OF ESTER BY LIPASE IMMOBILIZED ON MESOSILICA

Contents

Lipases from Candida Rugosa have many excellent characteristics and they are widely used for preparative purposes for organic synthesis in many industrial applications and scientific research projects. One aim of the research dealing with enzymes in aqueous media is to determine the optimal conditions for a specific application. Very few studies have been devoted to direct comparison of lipase activity in aqueous and organic media. The features of support enable the immobilization of lipase via strong interactions enhancing the stability of immobilized enzyme molecules. Biocompatible mesoporous silica nanoparticle supports have been used for biocatalysis in various applications owing to their long-term durability and efficiency. In this study, the hydrolysis rate of p-nitrophenyl palmitate in a batch reactor with Candida rugosa lipase was investigated. The conditions such as p-PNPP concentration, temperature and pH were evaluated to achieve the optimum reaction conditions for both free and immobilized lipase. Esterasic activity is used to test a rapid assessment of lipase activity.

7.1 Introduction

Lipases (EC 3.1.1.3) (triacylglycerol acylhydrolases, glycerol ester hydrolases) are serine hydrolases which under physiological conditions catalyze the hydrolysis of ester bonds in the molecules of triacylglycerols to glycerol and free fatty acids. They also catalyze a variety of synthesis reactions under reduced aqueous conditions (*e.g.*, esterification, transesterification,

alcoholysis, acidolysis, aminolysis, acylation and resolution of racemic mixtures) [1-3].Thus, lipases have become important for biotechnological and industrial applications [4, 5]. In systems containing an aqueous phase, lipase activity is usually high compared to organic media. Lipases are able to hydrolyze esters of long chain fatty acids and it is faster than esterification.

A typical lipase catalyzed reaction in aqueous media is ester hydrolysis. This enzymatic conversion can be used for the synthesis of triglycerides as shown for the preparation of platelet-activating factor [6, 7]. Another application of the hydrolytic specificity of lipases is the partial hydrolysis of triglycerides to di- and monoglycerides in the food industry, where di- and monoglycerides serve as biocompatible emulsifiers and food additives. These and other applications of lipases in industry and research have been discussed in the review by Rajendran *et al* [8]. Enzymatic activity was assayed at different concentrations of proline (0–1 M) using para-nitrophenyl palmitate (*p*-NPP) as substrate and also used to determine unfolding and refolding kinetics [9]. Enzymatic activities of lipases from Chromobacterium viscosum, Pseudomonas fluorescens, Bacillus sp, Candida cylindracea, Aspergillus carneus and Penicillium sp. were compared with the spectrophotometric p-nitrophenylbutyrate (*p*-NB) assay at different concentrations of the detergents Tween 20, Triton X-100, polyvinyl alcohol, and linear alkylbenzene sulfonate (LAS) [10]. Sultana *et al* studied the activity assays of purified proteins (PE and PPE multigene families) using p-nitrophenyl esters of aliphatic carboxylic acids with varying chain length (C2–C16) to study the substrate specificity effectively inhibited by PMSF [11-12].

The catalytic conversion, the stability and the productivity of the lipase immobilized on silicate-1catalyst have been evaluated in the hydrolysis of methyl myristate to myristic acid [13]. The natural substrates of lipases are

practically insoluble in water so the reaction is catalyzed at the water lipid interface. The catalytic mechanism of most lipases involves a step called "interfacial activation". In homogenous aqueous medium, these enzymes are closed (inactive conformation) when the active site cleft is isolated from the polar solvent by a lid formed by a helical segment of the polypeptide chain [14]. Navarro *et al* reported that lipase activation involves the equilibrium states (closed, intermediate and open) which depend on the composition of the microenvironment. The state of lipase can change in response to detergent, medium and other molecules [15]. The kinetics for the tributyrin hydrolysis using lipase (Pseudomonas fluorscenes CCRC-17015) was investigated in the liquid–liquid and liquid–solid–liquid reaction systems in a batch reactor. The turnover numbers calculated for free and immobilized lipase were 29 and 5.7 s^{-1}, respectively [16]. Esterase activity of lipase from Humicola lanuginosa increased up to 20-fold after adsorption on octyl-agarose while the lipase from Pseudomonas fluorescens showed a much higher enantioselectivity towards the hydrolysis of ethyl-α hydroxy phenyl butyrate after adsorption on the octylagarose, octadecyl-Sepabeads support [17].

Hydrolysis of oil and fat is an important industrial operation. The products fatty acids and glycerol are basic raw materials for a wide range of applications. Olive oil hydrolysis is also used to determine lipase activity explained by different researchers [18-19]. Lipase catalyses oil hydrolysis at the oil–water interface. Their activity drastically increases upon binding to the interface. Sarda and Desnuelle reported a convincing evidence for interfacial activation of the lipase. A Surfactant, Gum Arabic (GA) was added to the reaction medium to enhance the formation of oil-water emulsions [20, 21]. The kinetics of the enzymatic hydrolysis of palm oil using lipase in a batch reactor has been investigated [22, 23]. The conjugated system of relatively

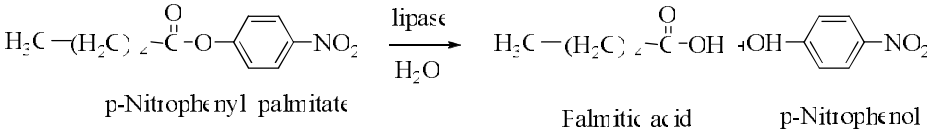
cheap lipase from Lipolase 100T (a bulk enzyme intended for the detergent industry) gave satisfactory results for olive oil hydrolysis at substrate concentrations up to 60% w/v at room temperature [24].

7.2 Experimental procedure

The photometric assay substrate (*p*-NPP) was prepared as described by Winkler and Stuckmann [25] with slight modifications. It was used to measure lipase activity. Solution A contained p-nitrophenyl palmitate (*p*-NPP) dissolved in 10 ml of 2-propanol to concentrations of 20 mM, with a sonicator for 10 min at room temperature. Solution B for the p-NPP assay consisted of 0.05 M Tris HCI buffer (pH 8) containing 0.4% Triton X-100 and 0.1% Gum Arabic [18, 19]. 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 were prepared freshly before the assay.

The enzyme activity of the free and immobilized lipase preparations in aqueous medium was determined according to the process described by Kordel *et al* [26-29]. In the standard condition, the reaction mixture was composed of 1 mL of 20 mM *p*-NPP in an Erlenmeyer flask. The reaction was started by the addition of 10 mg free lipase (or 20 mg immobilized lipase). 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 liquid was withdrawn. 0.5 mL of supernatant liquid was immediately mixed with 1 mL of 10 mM NaOH, directly in a 1 mL spectrophotometer cuvette. It displayed a yellow colour in aqueous alkaline phase. The 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.



The difference between the lipase activities measured by the *p*-NPP and *p*-NPA assays is attributed to the fact that the different substrates form emulsions of different qualities is also affected by the nature of the detergents, the differences in the amounts of substrates and detergents used and the substrate-detergent relationships.

7.3 Measurement of lipase activity

7.3.1 Effect of substrate

The investigation of specificity for different substrates revealed that esters of short-chain fatty acids are very poor substrates, which is typical for real lipases. Winkler *et al* explained dependence of activity towards chain length. The dependence of the enzyme activity on appropriate surface tension and viscosity, which vary with fatty acid chain length and the number of double bonds [28, 29]. Verger and co-workers demonstrated that enzyme activity changed with surface tension, yielding an optimum curve. They interpreted that short chain esters are bad substrates because they show a lower tendency to form micelles and the surface tension is sub optimal [30]. Very long-chain saturated esters are densely packed because of strong hydrophobic interactions between the hydrocarbon chains resulting in an increasing surface viscosity with increasing chain length. Consequently the lipase, which is supposed to penetrate partially into the lipid layer, can no longer come in close contact to

the substrate molecules. By the monomolecular film technique surface tension of lipid films and temperature effects can be controlled. Literature investigation showed that the explanation also holds for the substrate preference. The Inhibition studies revealed that lipase could act via an active serine residue [25]. For human pancreas lipase, two different sites for the hydrolysis of triglycerides and *p*-NPA were proposed but serine involvement at the *p*-NPA specific site is unclear [28].

Here we are selected *p*-nitrophenyl acetate (*p*-NPA) and *p*-NPP to investigate the enzymatic activity of lipase towards substrate 20 mM substrate solution was prepared in 2-propanol. They were tested using 10 mg lipase at 37°C. Activities were measured spectrophotometrically.

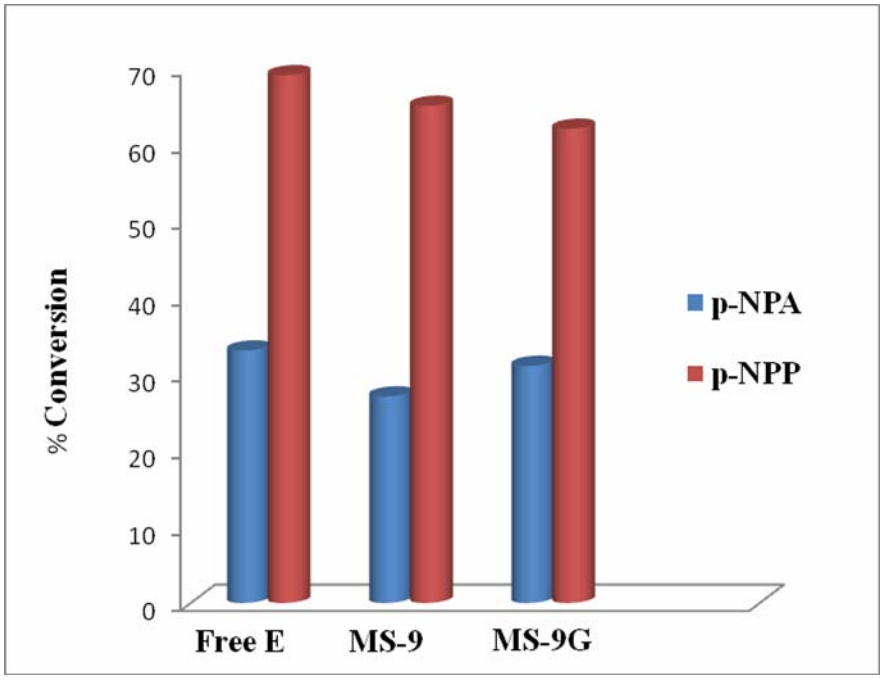


Fig.7.1 Effect of substrate

Results (Fig 7.1) demonstrate that *p*-NPA shows 33% conversion where as *p*-NPP shows 69% conversion i.e. a two fold increase in activity towards the substrate *p*-NPP. On comparing the results it is clear that water insoluble substrate

p-NPP was easily hydrolyzed when presented to the enzyme in the interface of a micelle.

7.3.2 Effect of immobilization pH

pH is characteristic of each enzyme and pH change will affect the activity and stability of enzyme. The extent of these changes depends on the enzyme the type of support and method of immobilization. It controls charging of enzyme as well as the support. Enzymes are considered as a heteropolymer of different amino acid residues. Amino, carboxyl, disulphide and hydroxyl groups are responsible for linking to a support. The protonation and deprotonation of the charged functional group depend on the pH of the solution and change in hydrogen ion concentration which can diminish the electrostatic attraction

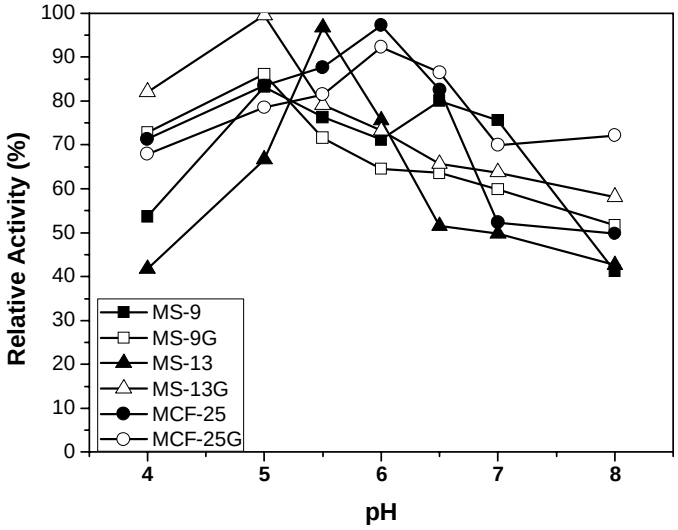


Fig.7.2 Effect of Immobilization pH

The optimum pH for the adsorbed systems MS-9, MS-13 and MCF-25 were found to be 6.5, 5.5 and 6 respectively. The pH at which maximum immobilization took place in the case of covalently bound systems was 5 in

the case of MS-9 and MS-13G while it was 6 in the case of MCF-25. There was decrease in the immobilization capacity at alkaline side of the pI of the lipase. Lipase immobilization in the pH range of 5.0 – 6.5 provided relatively high activity because enzyme conformation which is essential for enzymatic activity changed with pH. The ionization state of the active site of the lipase molecule is affected by the pH of the buffer used in the immobilization process and activity is very sensitive to the pH of the solution during the binding step [31]. The immobilized C. rugosa lipase was stable in the pH range of 5-8 with optimum pH 9 as reported by Hung *et al* [32]. Immobilization protects enzyme from pH change. Isoelectric point (pI) of CRL is 5 to 6.5; in which the protein is kept at its most stable conformation at these pH values. Although the active and the stable confirmation 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 occurs at 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 mesoporous organosilicas) 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 [33].

7.3.3 Effect of pH on the activity

The reactivity of free lipase decreased with decreasing pH value. The activity of enzyme cannot remain constant and must be reduced because the enzyme was denatured when the pH value was lower. The effect of pH on the enzyme activity was measured at pH ranges of 4 to 9. Buffer systems used were 0.05 M acetate (pH 4-5), 0.05 M phosphate (pH 6-7) and 0.05M Tris HCl (pH 8-9).

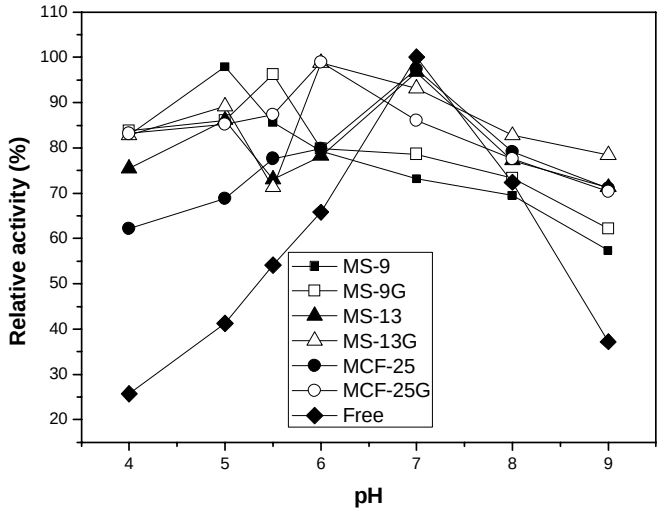


Fig.7.3 Effect of pH on the activity

Fig. 7.3 shows a plot of relative activity and pH value for free lipase and immobilized lipase. Free lipase is inactivated at lower and higher pH while immobilized systems retain high activity in both acidic and alkaline pH. The pH profile of the immobilized enzyme was much broader with respect to free enzyme. The change in immobilization pH depends on the method of immobilization as well as the structure and charge of the matrix. It should be noted that the pH shift towards acidic region when compared to the optimal pH of free enzyme. Generally, binding of enzyme to polycationic supports would result in an acidic shift in the pH optimum [34, 35]**.** If the support is polyanionic the optimum pH value is shifted towards basic. This means that the hydrogen and hydroxyl ions are distributed differently between the area close to the surface and the remaining solution with negative charges clustering close to the immobilized enzyme. A basic shift in pH optimum of Candida rugosa lipase immobilized on PVC, sepharose, chitin, agarose [36] and celite [37] have been reported in the literature. But Candida rugosa lipase immobilized on celite by acetone precipitation and adsorption, the optimal

reaction pH was shifted from 7 to 6.5. Yang and Chen reported no change in the pH optima of lipase entrapped in ENTP-4000 prepolymer, while a shift in pH optima of lipase immobilized on PVC, Sepharose, chitin, and agarose have been reported for olive oil hydrolysis. Fungal lipases were only active in acidic conditions [36, 38, 39].

7.3.4 Effect of temperature on the activity

The effect of reaction temperature on the activity of free and immobilized lipase was also investigated at various temperatures ranging from 20 to 70°C. It was observed that the optimum temperature for hydrolysis reaction for the free lipase was 37°C and immobilized lipase shifted slightly in the higher region relative to the temperature of free enzyme.

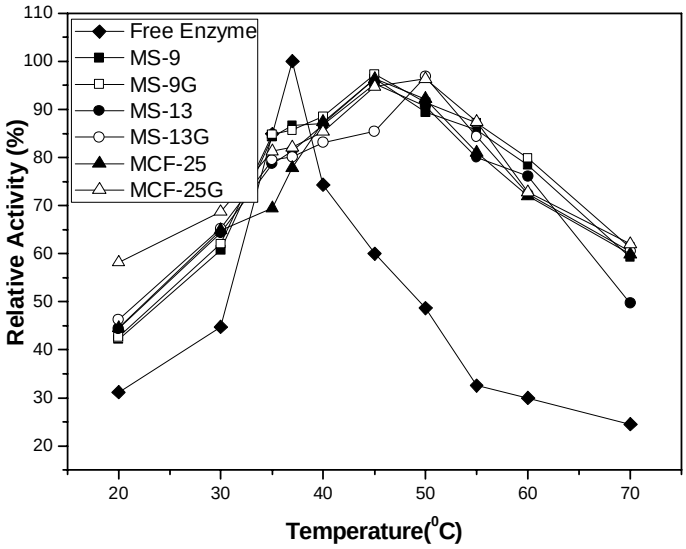


Fig.7.4 Effect of temperature

Fig. 7.4 shows a plot of relative activity and temperature value for free lipase and immobilized lipase. The optimum temperature value of hydrolysis of *p*-NPP using free lipase was around 37°C, but the temperature profile for

the immobilized lipase was widened considerably. The optimum temperature value of immobilized lipase was greater than that of free lipase. Immobilized lipases exhibit optimal temperatures in the range of 40 to 55°C, lipase immobilized on MS-9, MS-13G and MS-25G had an optimal temperature at 50°C, while the optimum temperature for immobilized lipase on MS-9G, MS-13 and MCF-25 were 45^0C. Immobilized lipase is much more heat resistant than free enzyme and it protects unfolding of enzyme at higher temperatures.

7.3.5 Effect of surfactants on the enzyme activity and stability

Surfactants have been described to have a complex effect on lipases. They are applied in lipase catalyzed reactions because they increase the water lipid interfacial area (improving the stability of emulsion) increases substrate solubility, stabilize the open conformation of lipases and prevent it from aggregation leading to improve their catalytic activity. But they may also have a negative effect on the lipase stability and even act as competitive inhibitors [40]. Moreover, detergents have been proposed as a simple way to modulate the enzyme selectivity [41]. This means that enzyme molecules with different rigidity, may present a different behaviour when incubated in the presence of detergents. Open 3D structure of lipase was obtained in the presence of some detergents and stabilizes the open conformation of the lid [42, 43].

To understand whether the main effect produced by the detergents was inhibition or inactivation, the free and immobilized enzymes were incubated in the presence of each of the three detergents at different concentration (w/v %) for 1h at 30°C. At the end of the incubation period, lipase activity was determined using *p*-NPP substrate under standard assay conditions. Control experiments were performed without surfactant.

Table 7.1 Effect of selected surfactants on the activity of enzyme
Control without the addition of any substance
Relative activity (%) is the activity obtained after incubating the enzyme with various surfactants at 30°C for 30min compared to control

Detergents	(w/v)%	Relative Activity (%)		
		Free Enzyme	MS-9	MS-9G
CTAB	1	9	39	18
	0.5	18	50	47
	0.1	32	61	20
Triton x-100	1	123	315	339
	0.5	109	242	287
	0.1	105	236	275
SDS	1	65	206	212
	0.5	88	238	243
	0.1	109	275	254

Table 7.1 shows the effect of different detergents in the activity of hydrolysis of ester. At a concentration of 1% (w/v) Triton X-100 produced an increase in the activity of lipase. It was also observed that as the amount of Triton X-100 increases enzyme activity also increases but Lima *et al*. reported that increasing concentrations of Triton X-100 decreased lipase activity. CTAB and high concentration SDS produced a decrease in the activity of lipase. These results suggest that the ionic detergents are responsible for the inactivation of the enzymes. Instead of more complex effect there is a positive conformational change were observed in the case of Triton X-100, while incubating in CTAB there must be a negative effect. This suggests that Triton X-100 could result in the "opening" of the lipase. But it has some other negative effects, which are less important in immobilized systems [53].

Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$) is a nonionic surfactant which has a hydrophilic polyethylene oxide chain (on average it has 9.5 ethylene oxide

units) and an aromatic hydrocarbon lipophilic or hydrophobic group. Free lipase increased the activity 5% at 0.1% Triton X-100 concentration, while with 0.5% the activity increased to 9% and with 1% the behaviour was quite different. Addition of 1% Triton X-100 enhanced enzyme activity more than 200% for immobilized systems.

CTAB caused a decrease in the activity even at the lowest concentration (0.01%). At 1% CTAB concentration the relative activity was around 8% for free enzyme 0.1% CTAB produced a slight increment in enzyme activity, although at higher concentrations it produced a decrease in enzyme activity. Similar reports were observed for lipase immobilized on cyanogen bromide [44].

SDS at 0.1(w/v %) slightly stimulated the free enzyme activity (9%) but significant activities were observed with immobilized systems. Incubation of the enzyme in the presence of these detergents (CTAB) suggested that they could inactivate immobilized lipase. Immobilized system was more resistant to this inactivation due to the higher stability of this immobilization method. Most nonionic detergents, such as Triton X-100 and Tween 20 slightly increased the lipase activity while SDS completely inhibited [45].

7.3.6 Effect of various chemicals on the activity and stability

Cofactors are not required for lipase activity but divalent cations such as calcium generally stimulate the activity. It has been postulated that this is based on the formulation of calcium salts of long-chain fatty acids [46]. The lipase activity is inhibited drastically by Co^{2+}, Ni^{2+}, Hg^{2+} and Sn^{2+}; and is slightly inhibited by Zn^{2+}, Mg^{2+} and EDTA [47]. In H. lanuginosa S-38, sulphahydryl-reducing agents like dithiothreitol did not alter the enzyme activity but did render it more susceptible to heat inactivation. Inactivation is accelerated by the addition of urea. Reducing compounds (cysteine, 2-mercaptoethanol), chelating agents

(EDTA, o-phenanthroline) and thiol group inhibitors (*p*-chloro mercuric benzoate, monoiodoacetate) did not show a detectable effect on lipase Lipase is not a metallo-enzyme and it does not require either free -SH group or an intact S–S bridge for its activity.

To evaluate activity and the stability of free and immobilized enzymes, they were incubated with 5 mL of various chemical solutions at 30°C for 30 min at 1 mM concentrations. The relative lipase activity after incubation was determined under standard assay conditions of *p*-NPP as substrate and the results expressed as percentage of the activity obtained without the chemical agent.

Table 7.2 Effect of selected chemicals on the activity of enzyme Control without the addition of any substance
Relative activity (%) is the activity obtained after incubating the enzyme with various chemicals at 30°C for 30min compared to control

Chemicals	mM	Relative Activity (%)		
		Free Enzyme	MS-9	MS-9G
Control		100	100	100
$MnCl_2$	1	128	129	197
$MgCl_2$	1	112	162	185
EDTA	1	123	133	226
KCl	1	174	236	208
NaN_3	1	109	112	176
$CaCl_2$	1	40	94	96
$CuSO_4$	1	23	26	26
$HgCl_2$	1	0	0	0

The effect will vary with metal ions and their concentration. Among the metal ions used chlorides of K^+, Mn^{+2}, Mg^{+2} ion increases 174%, 128%, 112% respectively while Ca^{+2} and Cu^{+2} caused decrease of enzyme activity by about 39% and 23% (in the case of free enzyme).The enzyme activity were strongly inhibited by Hg^{+2} (thiol group inhibitor) in the case of both free and immobilized enzymes. This may be due to the proximity of the SH group to the catalytic and

interfacial binding site but spacially remote from the catalytic site. This may have induced the marked loss of activity [30, 48]. The catalytic triad of lipases has been recognized to consist of Ser, His, and Glu or Asp [49]. Thus the bulky Hg^{2+} group might cause steric interference to the approach of the substrate to the active site. NaN_3 at levels of incorporation of 1mM activated free CR Lipase and covalently bound lipase on to MS-9G by 8% and 76% respectively. EDTA (1 mM) activated the free enzyme, adsorbed lipase and covalently bound lipase by 22%, 33% and 126% respectively.

Treatment with Cu^{2+} at both 1 and 10mM significantly inhibited the activity of grey mullet lipase with only 19% residual activity after 30 min incubation at 10 mM. This was similar to the findings of Choo *et al* [50] and Aryee *et al* [51], but different from what Sztajer *et al* [52] and Lima *et al* [53] reported for Penicillium simplicissimum lipase (4.7%) and Penicillium aurantiogriseum lipase (31%) by 1mM Cu^{2+}.

7.3.7 Effect of incubating medium on the activity and stability of lipase

Enzymes have been described to function in non aqueous media although, in at least the majority of cases a finite level of water associated with the protein must be present to retain conformational integrity and there by activity. Many researchers investigated a wide variety of common solvents to see which would support the enzyme for catalysis. In fact it seems very much like an on-off type situation. The enzyme was either fully or near- fully active or it was not active at all. The lipases were active in all water immiscible solvents. Conversely, the solvents in which the enzyme was not active were all water-miscible.

Water certainly plays a very key role in maintaining conformational integrity of proteins. Hydrophobicity is the main criteria to enhance the activity but that is more favourable in presence of small amount of water.

Consequently, it is proved that the water miscible solvents with decreasing hydrophobicity and increasing polarity extract essential water from the protein. A structural change in the protein accompanies water loss, resulting in a non-active conformation. The stability in organic solvents is an important characteristic of lipases. It is very interesting to determine whether the enzyme can be used to catalyze synthetic reactions and also to predict which solvent would be better for performing the reaction.

Lipases are diverse in their sensitivity to solvents, but there is a general agreement that polar water-miscible solvents ($-2.5 < \log P < 0$) are more destabilizing than water immiscible solvents ($2 < \log P < 4$) [54, 55]. Hydrophilic solvents exhibit a higher affinity to water and hence it is more likely that they will strip essential water from enzyme molecules, than hydrophobic solvents. The log P value describes the hydrophobicity of organic solvents, where as P is the partition coefficient of the solvent between octanol and water in a two-phase system [56, 57]. Both free and immobilized enzymes were incubated in different solvents at 30^0C for 1hr and the residual activities were measured.

Table 7.3 Effect of selected solvents on the activity of enzyme Control without the addition of any substance
Relative activity (%) is the activity obtained after incubating the enzyme with various chemicals at 30°C for 30min compared to control

Solvent	log P	Residual Activity (%)		
		Free	MS-9	MS-9G
Methanol	-0.76	75	93	101
Ethanol	-0.24	39	47	73
Acetone	-0.23	30	64	93
Toluene	2.5	143	127	129
Hexane	3.5	151	165	190
Heptane	4.0	120	143	152
Isooctane	4.5	98	87	84

After 1h of incubation, the enzyme was stable in toluene, hexane and heptane with a residual activity of 142%, 151% and 120% respectively as shown in Table 7.3.). The solvents may have kept the enzyme in open conformation during incubation with the water-immiscible organic solvents thus the lid covering the active site remained open even during the aqueous incubation period [58, 59]. It is observed that after log P= 4.5 relative activity decreases for free and immobilized lipases. Ethanol and acetone show inhibitory effect and inactivation of enzyme may be due to the stripping off essential water from enzyme surface. The organic solvents with log P <2 and log P > 3.5 are unsuitable for enzymatic reaction. Mainly water miscible organic solvents are toxic to the enzyme and causes great deactivation effect.

The low stability of the crude P. aphidis lipase in water-miscible organic solvents was the consequence of enzyme denaturation due to stripping of water from the enzyme surface as described above. The high relative activity of P. aphidis lipase (82.2%) in acetone after 1 h of incubation corresponds to the results obtained by other authors. Only a few reports show a high stability of lipases in acetone after a prolonged period. For example, the lipase produced by Pseudomonas sp. had relative activity values from 100 to 110% after 15 h at room temperature in acetone. Lipase from Mucor javanicus exhibited high stability and an increased activity after 2 h of incubation at 25 °C in acetone [60-62]. Lipase catalyzed reactions were carried out in media containing water immiscible organic solvents with a small amount of water to prevent denaturation. It is still debated whether solvent polarity (log *P*) is the key factor in enzyme deactivation, since the nature of the solvent and its structure have been shown to greatly influence solvent effect in non-aqueous milieu catalysis [63].

7.3.8 pH stability

The pH stability profiles were studied using phosphate buffers at 50 mM concentrations: The lipases that have been studied show profound stability around pH 6.0–7.5. For pH stability, the enzyme was incubated in 1 mL of various buffers (pH 6 and pH 7) at 37°C for different time intervals. After incubation the relative lipase activity was assayed. The activity was expressed as relative activity percentage.

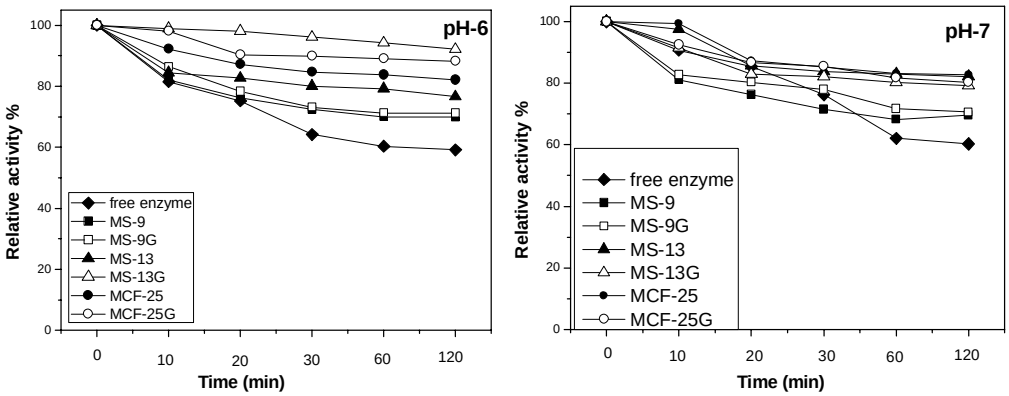


Fig.7.5 Stability of the free and immobilized systems at pH 6 and 7

The pH stability results (Fig. 7.5) again showed that the enzyme was stable at both pH 6 and 7. Even at pH as high as 10.0 and as low as 4.0, the immobilized enzyme still retained more than 50% of its initial activity for 30 min. prolonged incubation in buffer cause inactivation of free lipase. Immobilized systems show higher stability at pH 6. Covalently bound enzymes exhibit higher stability which limits the transition of enzyme conformation against the change of pH.

7.3.9 Thermal stability

The thermal stability of immobilized lipases is one of the most important criteria for their application. According to Klibanov *et al* [64] two types of enzyme instability should be distinguished. One of them is a heat-induced,

cooperative unfolding usually almost instantaneous and reversible. The other is a time-dependent gradual irreversible loss of enzymatic activity on exposure to high temperatures. The first kind was evaluated at the optimum temperature assays and the second one was evaluated in the experiments of thermal stability. As is well known, the immobilized enzymes are more resistant to heat and denaturing agents than that of the soluble form [16, 65]. For thermal stability studies the enzyme was incubated at 50 and 60°C for different time intervals. After each period of incubation the enzyme was immediately cooled and the relative activity was determined.

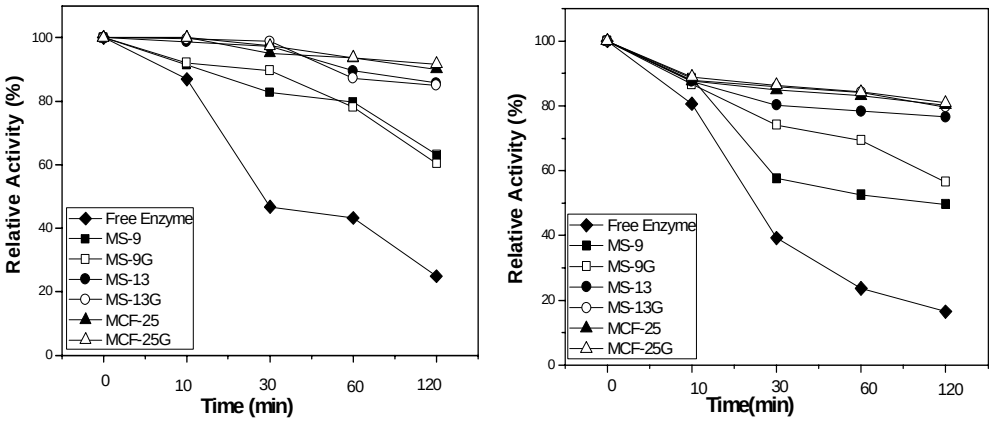


Fig.7.6 Thermal stabilities of free and immobilized lipase at 50°C and 60°C

The variation of reaction activity of lipase with different temperatures from 50°C and 60°C is shown in Fig. 7.6. The relative activities were determined for free lipase and immobilized lipase. The maximum activity of free lipase and immobilized lipase were around 35% and above 60% at 50°C .This finding demonstrates that the thermal stability of immobilized lipase was greater than that of free lipase. The thermal stability of free and immobilized lipase for hydrolysis reaction is higher at 50°C than 60°C. Temperature stability studies showed that covalently -bound lipase on MCF-25G and MS-13G were more stable at 50°C, retaining more than 90% of its initial activity after 120 min. Under

the same conditions the activity of free lipase was reduced to 16%. In fact, when incubated at 50°C for 24h, the covalently bound lipase still retained about 55% of its activity (data were not shown). The chemical interaction between the amino group and aldehyde group provides additional stability to the covalently immobilized lipases. It is observed that the greater thermal stability could be attained after immobilization. As can be observed, all the immobilized systems were more thermally stable than the free enzyme and the highest thermal stability was achieved by immobilizing the lipase in MCF-25G.

7.3.10 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. Results were demonstrated in Fig.7.7.

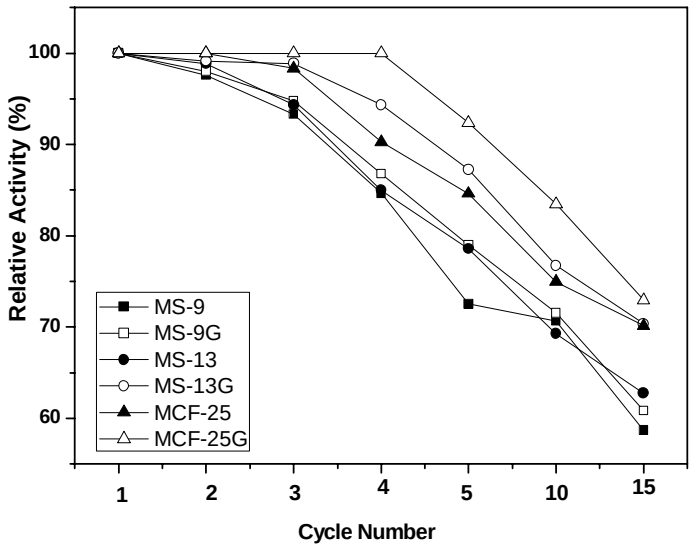


Fig.7.7 Reusability of immobilized lipase

Immobilized lipase was active and retained 55% of activity after 15 uses. After 15 continuous cycles, the covalently bound lipase on MCF-25G and MS-13G retained 70% initial activity while lipase adsorbed on MS-9 lost 40% of its initial activity. The observed decrease in activity may be due to desorption of the enzyme. Immobilized enzyme lost its activity in repeated use is a common phenomenon [66]. Nakane *et al* explained that the loss in activity was attributed to inactivation of enzyme due to continuous use. [67]. Results demonstrate that even if the enzyme activity progressively decreases, the total productivity of the same enzyme, reused for 12 cycles of reaction, is higher than the total productivity achieved using the free enzyme.

Candida rugosa lipase adsorbed on biodegradable poly (3-hydroxybutyrate-co hydroxyvalerate) showed 94 % residual activity after 4 h at 50°C and showed good reusability till 12 cycles [68]. But Lee *et al* explained that lipase from candida rugosa on PANCMA retained 62% activity in aqueous media [31]. Lipase 'immobilized' on Eudragit retained 44% hydrolysis after four reuses as observed by Charusheela *et al* [24]. Nylon-immobilized lipase treated with HCl and activated with glutaraldehyde showed a faster activity loss i.e. it retained 4% of initial activity after the third use. For M. miehei lipase immobilized on nylon-PVA shows a slightly better performance of 16% activity was achieved after the first use [69].

7.3.11 Storage stability

One of the most important parameters to be considered in enzyme immobilization is storage stability. The stabilities of the free and the immobilized lipase were determined. They were stored in optimum buffer (50 mM) at 4°C for 30 days. Under the same storage conditions, the activities of the immobilized lipase decreased slower than that of the free lipase (Fig. 7.8).

The free enzyme lost all its activity within 30 days. The immobilized lipase preserved about 80% of its initial activity during a storage period of 30 days. The covalent immobilization definitely holds the enzyme in a stable position in comparison to the free counterpart i.e. it prevents enzyme from structural denaturation.

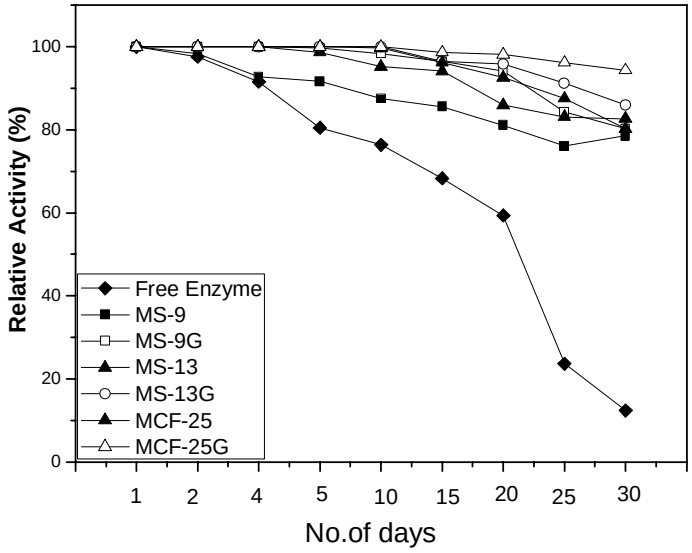


Fig.7.8 Effect of storage stability on the activity of free and immobilized lipase

As seen in Fig. 7.8, 90% loss of activity was observed for free enzyme after storage for 30 days at 4°C. This might be due to protein–protein interaction (autolysis of the lipase). This enhanced stability is probably a result of the prevention of autolysis by immobilization. The activity of lipase covalently immobilized on MCF-25G did not change even after 30 days and they could maintain more than 90% activity. The results mentioned above indicated that the immobilized enzyme had much better storage stability than free enzyme. In conclusion, the enhanced stability of the immobilized lipase offers an attractive platform for biocatalysis. The purified lipase from Pseudomonas fluorescens Lp1 was immobilized onto functionalized magnetite using glutaraldehyde as the

coupling agent exhibited excellent reusability for 4 cycles and storage stability upto 15 days retaining about 75% of its initial activity [70].

7.4 Kinetic parameters of free and immobilized lipase in aqueous medium

The kinetic behaviour of enzymes when they are bound to a solid matrix (the conformation of fixed enzyme) can be different from that of the free enzyme in solution. The local environment provided by the matrix for the enzyme can be significantly different from the reaction medium. The changes in diffusion parameters can slow down the overall process. The changes in the catalytic behaviour of enzyme due to immobilization are due to the changes in conformation of enzyme molecule and the heterogeneous nature of the local enzymatic environment in which the concentration of substrate, products and other cofactors are quite different from those in solution.

An experiment was conducted to determine the rate of enzymatic hydrolysis of *p*-PNPP in aqueous medium using lipase at different substrate concentration (0.2 -1.5 mM). The kinetic parameters were estimated by fitting the data to the Lineweaver-Burk model. The parameters of the Michaelis-Menten kinetic equation were determined from the double-reciprocal plot of the lipase activities. The quality of the fit for Lineweaver-Burk plots in Fig 7.9 is quite good with average correlation coefficients of 0.90. The kinetic parameters K_m and V_{max} are obtained from slope and intercept of these straight lines are listed in Table 7.4. It can be observed that immobilization shows a pronounced effect on the K_m and V_{max}. The immobilization can cause increases in K_m and reduction in V_{max}. The increase in K_m means that the affinity of enzyme to its substrate decreases when enzyme is immobilized and whereas the decrease in V_{max} indicates that an activity loss occurred during immobilization.

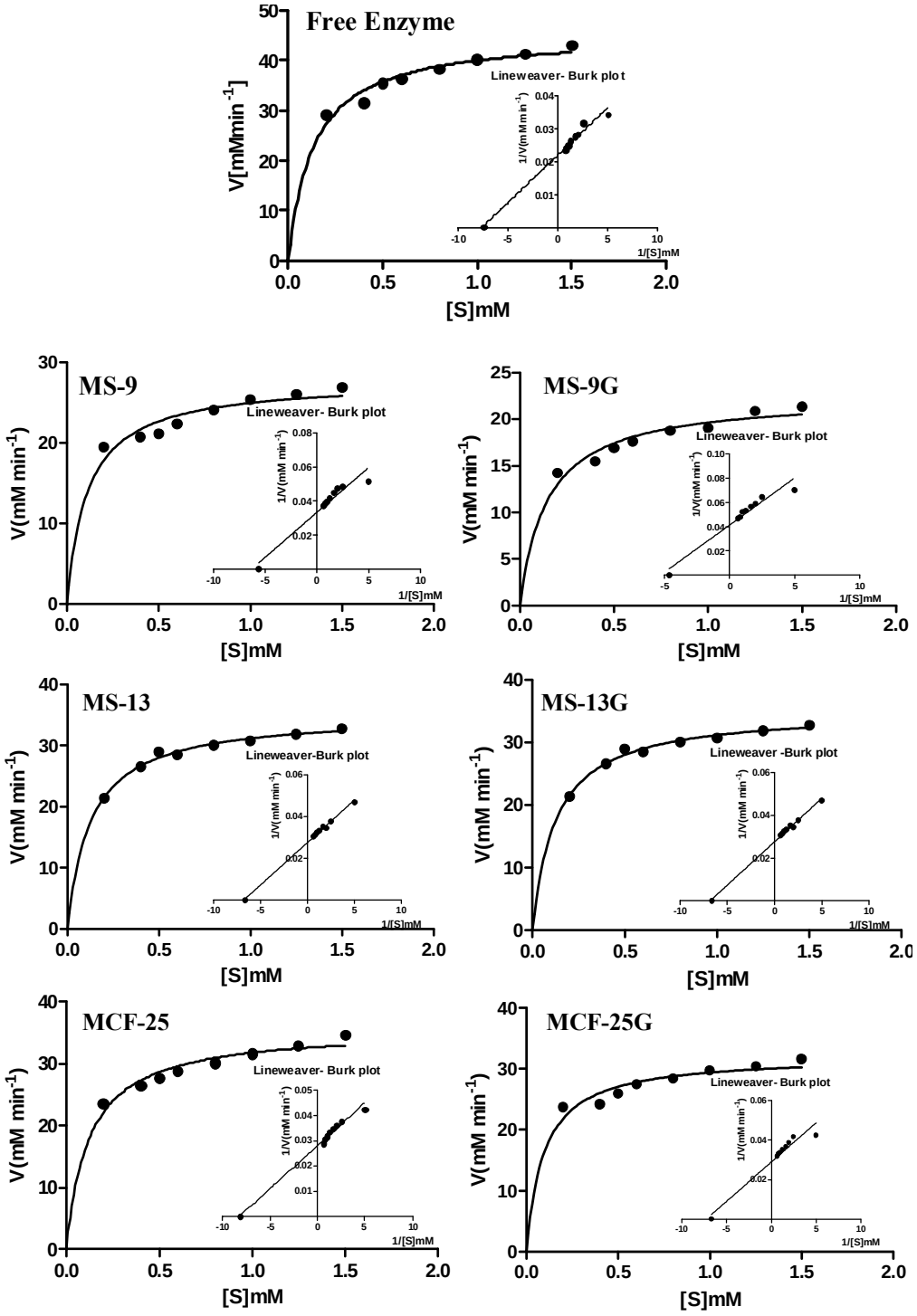


Fig.7.9 Michaelis-Menten and Lineweaver-Burk plots to estimate K_m and V_{max} for free and immobilized lipase forester hydrolysis in aqueous medium

Table 7.4 Kinetic parameters of free and immobilized lipases

Sample	V_{max} (mM min^{-1})	K_m (mM)	R^2	η (Effectiveness factor)	Catalytic efficiency (min^{-1})
Free Enzyme	45.36	0.13	0.92	-	339.01
MS-9	30.06	0.16	0.90	0.66	190.13
MS-9G	24.26	0.19	0.98	0.53	128.29
MS-13	36.29	0.14	0.98	0.80	255.56
MS-13G	29.01	0.15	0.98	0.64	196.94
MCF-25	35.67	0.12	0.97	0.84	310.69
MCF-25G	34.89	0.15	0.95	0.77	232.44

Rogalski *et al*. reported that the changes in the kinetics of immobilized enzymes are controlled mainly by four factors, (i) change in enzyme conformation (ii) steric effects (iii) microenvironmental (iv) bulk and diffusional effects. The higher K_m values for the solid-phase enzymes may be a result of a number of effects. The migration of substrate from the solution to the microenvironment of an immobilized enzyme can be a major factor for increase in K_m values [71, 72]. The K_m for an enzyme depends on the particular substrate and on conditions of assay such as temperature & pH. K_m represents the strength of binding or affinity of the substrate for the enzyme. Tightly bound substrates have a low Km and loosely bound substrates have a high K_m. Also the K_m is that concentration of substrate at which half the active sites of the enzyme are filled.

The K_m and V_{max} values of immobilized and free enzyme were compared by using the classical Michaelis-Menten enzyme kinetics. The K_m of the lipase immobilized by adsorption on MCF-25 is almost identical to that of the free enzyme. On the other hand, the V_{max} of the immobilized lipase is lower than that of the free enzyme suggesting that the activity of the lipase

decreased in the course of immobilization. From Table 7.4 it was observed that K_m values of covalently bound lipase was higher than adsorbed lipase. Similarly the V_{max} values of the immobilized lipases by adsorption are higher than that of covalent binding.

The internal diffusional effects can be quantitatively expressed by the effectiveness factor η. The effectiveness factor is defined as the ratio of the actual reaction rate inside the particle to the rate in the absence of diffusional limitations [73]. Immobilized systems showed effectiveness factor less than 1 in aqueous medium and it is due to diffusion of substrate and product. The decrease in activity is attributed either to conformational changes in the three dimensional structure (active site) of the enzyme molecule to the shielding effect of the matrix which makes the active centre less accessible to substrate and cofactors [74].

7.5 Conclusions

Lipases are amongst the most important biocatalysts that carry out novel reactions in both aqueous and nonaqueous media. Enzymatic activity of lipase immobilized on different mesoporous silica materials are evaluated using *p*-NPP. The main conclusions from the present study are given below.

- The lipase displayed different degrees of hydrolytic activity against different esters depending on the number of carbon atoms of the hydrocarbon. The CR lipase shows higher activity towards *p*-NPP than *p*-NPA.
- The experimental results showed that lipase after immobilization had good thermal stability and greater pH stability.

- Hydrolytic activity was enhanced by Mg^{2+}, K^{+} and Mn^{2+}, but was inhibited by Cu^{2+} and Ca^{2+}.
- Sodium azide did not show any inhibitory effect on the activity of Candid rugosa lipases.
- The free and immobilized lipases were strongly inhibited by $HgCl_2$.
- Ionic detergents showed a more impact in hydrolytic activities
- The enzyme showed a good stability in organic solvents with high log *P* values ($2 < \log P < 4$), the best result was obtained in n-hexane (151% relative activity).
- Lineweaver-Burk method was used to estimate the kinetic parameters of the Michaelis-Menten model by fitting the experimental data.
- The immobilized enzyme exhibited mass transfer limitation as reflected by a higher apparent K_m value.
- The immobilized enzyme could be reused, keeping around 60% of its original activity after 15 reaction cycles without any treatment.

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Chapter 8

KINETICS OF ADSORPTION STUDIES OF ENZYMES ONTO MESOPOROUS SILICA:–EVALUATION OF AVRAMI MODEL

Contents

Adsorption was one of the special interesting area arose in ancient age because it was traditionally used in purification of water. Carbon materials widely used and nowadays it is replaced by activated carbon having large surface area. Adsorption of biomolecules find a potential application in biochemistry for various purposes. The current experience of adsorption gathered in the areas of medical implantation, controlled drug delivery, biosensing and immobilization of biomaterials is allowing the design aimed to new application for future decades. This present study deals with adsorption of enzymes lipase onto different pure and functionalized mesoporous silica materials having different pore diameter. The result showed that MCF-25 has highest adsorption capacity. It was also observed that MS-25G has highest stability towards enzyme.

8.1 Introduction

The adsorption of a protein molecule on solid support is a worthy scientific problem due to their applications. Mainly it is adapted in biorelated technologies such as medical implant, biosensing and drug delivery. It is used to understand protein surface interaction. The structure of support should influence adsorption. The first step to an efficient adsorption process is the search for an adsorbent with good capacity and long lifetime and availability at

economical cost. There are two types of adsorption physical adsorption and chemisorptions. In physical adsorption the attractive forces between adsorbed molecules and the solid surface are Vander Waals forces and being weak in nature results in reversible adsorption. If the attraction forces are due to chemical bonding, the adsorption process is called chemisorption. Due to the higher strength of the bonding in chemisorption, it is difficult to remove chemisorbed species from the solid surface [1]. Adsorption process is widely used for dye removal, waste water treatments and enzyme immobilization. The amount of adsorbate can be calculated from the adsorption isotherms. The adsorption isotherms are constant-temperature equilibrium relation between the quantity of adsorbate per unit of adsorbent (q_e) and its equilibrium solution concentration (C_e).

Different supports are used for this process. The use of naturally occurring easily biodegradable materials having low cost are preferred as supports. Among them most important is chitosan and clay [2]. A porous material having high surface area should be good adsorbent and the time taken for attaining adsorption equilibrium is less. Hata *et al* first reported the effect of pore size and influence of solvent on the loading [3]. Vallet-Regi and co-workers confirmed the role of pore size as an important factor determining the adsorption and release of biomolecules [4]. There are other pieces of evidence where pore size has effectively altered the loading and release of protein. Hartono *et al* describes that, for enzyme immobilization inside mesoporous silica pore size is a crucial factor [5]. Due to the small pore sizes of the MCM-type materials, the molecular size that could be incorporated and loaded inside the pores was limited to 2–3 nm. Therefore, larger organic molecules, proteins, enzymes and DNA could not be used. Rate of adsorption influences several experimental variables such as solution pH, temperature, ionic strength,

protein size and bulk and surface concentration [6]. Small pores tend to limit the diffusion of enzyme through the pore, while large pores increase the possibility for enzyme immobilization. The pore diameter of mesoporous materials is large enough to allow penetration of the protein molecule into the large internal surface area/pores of these materials. A more of protein molecule is adsorbed in modified silicate materials than MCM-41 as shown by Deere *et al* [7]. Therefore mesostructure has a significant effect on bio-adsorption. Variety of mesoporous materials have been synthesized with varying sizes, shape and morphology. They provide easy and direct access to host large molecules mesoporous silica material therefore hold great promise for use as support to immobilize enzymes [8-10]. For e.g. SBA-15 type nano rods [11], SBA-15 nano spheres [12], IBN type mesoporous silica nano particles with a 3D cubic (Im3m) structure [9]. Cytochrome C is a small (12 384 Da) redox protein, with an approximate spherical diameter of 40 Å immobilized on MPS as explained by Diaz and Balkus [13, 7]. The various adsorption protocols used with lipases have been extensively reviewed by Malcata *et al* [14]. A great number of synthetic or natural carriers with different shapes/ sizes, porous/non-porous structures, different aquaphilicities and binding capacity have been used for lipase immobilization. Among the supports that have been studied are alumina, silica, celite, ceramics, metal oxides, porous glass, sepharose, sephadex, cellulose, zeolites, polyethylene, polypropylene, polystyrene, nylons polyacrylates, and others.

The adsorption of lipase onto carrier materials depends on factors such as pH, ionic strength, isoelectric point of the lipase, surface and protein properties, as well as the history dependence of lipase-adsorption kinetics. Most supports usually bind from 2 to 50 mg protein per gram of support. While some supports are claimed to bind as high as 170 mg protein per gram

of support, such high binding capacity may result in steric interference problems and loss of enzyme activity [15]. In general, the maximum adsorption is observed at pH close to the isoelectric point of the lipase. In addition, porous particulate supports are superior to nonporous supports for immobilization of lipases due to their greater surface area. However, porous supports must have an internal morphology that allows not only the lipase binding but also an easy accessibility to substrate molecules in order to minimize diffusional limitation. The pore sizes best suited for lipase adsorption are at least 100 Å in diameter as identified by Mojovic *et al*. [16]. Katiyar *et al* observed that the adsorption capacity and rate of adsorption is dependent on the solution pH, protein, pore size, pore volume and morphology [17]. Sugunan *et al* also studied influence of pore diameter on adsorption of enzymes. The highest equilibrium capacity of 220 mg amylase/g supports was achieved for mesoporous silica material having 84 Å pore diameter [18].

Surface modified silica shows higher protein adsorption capacities compared to the unfunctionalized one. Pore surface may be functionalized with chemical species to modify their adsorption properties. This makes them suitable to host different species and to protect them for long time, under appropriate conditions. In aqueous solution, the amino groups of amino functionalized silica are much easier to cationize and they adsorb enzyme anions strongly by electrostatic attraction. The ionic strength was found to strongly influence the amount of protein adsorbed, with little absorption occurring at high ionic strengths [7].

Many kinetic models were developed in order to find intrinsic kinetic adsorption parameters. The expressions which are originally given by Lagergren are special cases for the general Langmuir rate equation [2, 19]. A simple kinetic analysis of adsorption of protein shows the pseudo-first-order .

The kinetics of protein binding on silica was proven by KEKAM model. A group of authors developed a general kinetic equation known as a Kolmogorov-Erofeev-Kazeeva-Avrami-Mampel (abbreviated KEKAM) equation. This model implies that the reaction is located on the surface active sites of the solid support.

$$\alpha = 1 - \epsilon xp(- kt^n)$$

The double logarithmic form of the KEKAM equation is

$$\ln [-\ln (1-\alpha)] = \ln k + n \ln t$$

Based on the proposed model, the equation was used to estimate specific kinetic parameters (k rate constant for enzyme binding and n specific kinetic parameter). It might suggest the mechanism of enzyme binding to the support. The value of n, which is related to the adsorption mechanisms changes.

8.2 Lipase immobilization

Lipase solutions (1 mg/mL) were prepared by adding appropriate amounts of lipase powder to phosphate buffer solution (0.1M, pH 7.0) at room temperature. 100 mg of the support was added to the stock solution .The supports with the lipase solution is shaken gently in Remi orbital shaker with a stirring speed 200 rpm at 30°C for 3h. Finally, the mixture was centrifuged and filtered and rinsed with the buffer till no soluble protein was detectable in the washings. Protein concentration in the solutions was determined by developing colour with Folin Phenol Ciocaltaue's reagent.

8.3 Adsorption procedure

In this chapter, we investigated the kinetics of adsorption of enzyme at optimum pH. The effects of the size of enzyme structure, temperature and

contact time on adsorption capacity were evaluated. Changes in adsorption rate were also evaluated as a function of contact time and temperature using specific Avrami adsorption equation. The adsorption experiments were performed in controlled pH using batch procedure and protein sample concentration was determined in triplicate runs by spectroscopic method.

8.3.1 Adsorption isotherms

Adsorption (binding) capacities of all the samples were evaluated by typical adsorption experiment. Lipase was dissolved in phosphate buffer at pH 7 at 30°C to make a stock solution (1.0 mg mL). 100 mg of mesoporous silica materials was added to the stock solution (20 mL). Kinetic experiments to determine the amount of lipase adsorbed as a function of contact time were conducted by contacting protein solution with mesoporous silica with stirring at pH 7.0 at 30°C temperature in a vessel covered to prevent evaporation. Samples were withdrawn periodically for immediate analysis. Adsorbed amount was determined from the difference in concentration of the enzyme before and after adsorption by UV-Visible spectroscopy at 600 nm.

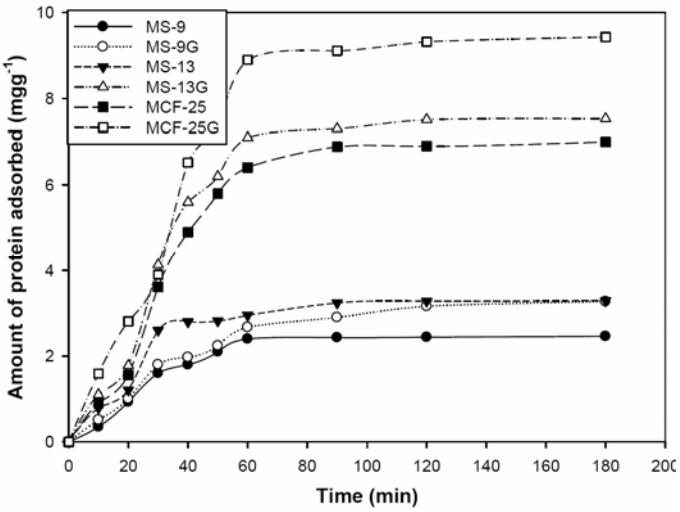

Fig. 8.1. Kinetics of protein adsorption on different mesoporous materials at 30°C

Adsorption isotherms are shown in the Fig.8.1. The lipase binding capacity for the various supports is in the order MCF-25G>MS-13G> MCF-25 > MS-13> MS-9G> MS-9. The grade of affinity of the enzyme molecules for each carrier was evaluated from the adsorption isotherms of lipase on MS-9, MS-13 and mesocellular silica foams. Surface area and porosity plays an important role in enzyme adsorption. MCF-25G yields the highest adsorption capacity and MS-9 showed the lowest adsorption capacity.

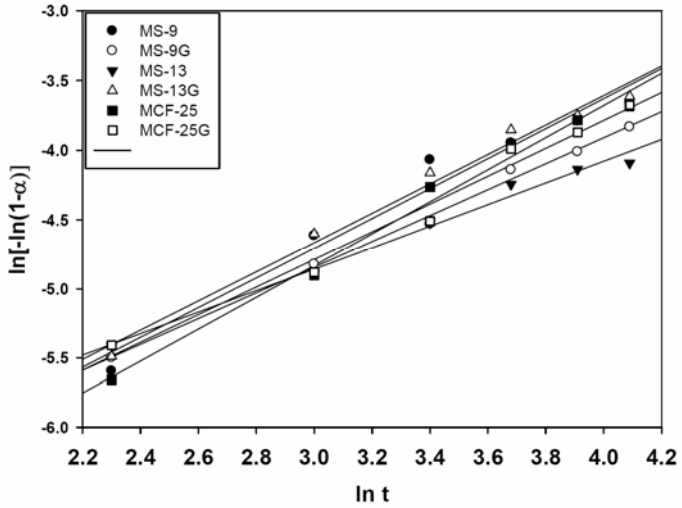

Fig. 8.2. KEKAM plots ln [ln (1-α)] against ln t for different mesoporous silica at 30°C

Table 8.1 The values of the specific kinetic parameters from KEKAM equation and amount of adsorption at equilibrium for different mesoporous materials.

Sample	n	K (μ s)	q_e	R^2
MS-9	1.07	359	3.00	0.98
MS-9G	0.67	112	4.77	0.90
MS-13	0.78	752	3.51	0.98
MS-13G	0.91	624	8.25	0.92
MCF-25	1.15	203	8.27	0.98
MCF-25G	1.00	415	10.54	0.97

From the Table 8.1 it is observed that n value is different for different support. If n >1 suggests that the process is surface reaction limited and diffusion of enzyme is faster. Low n value (0.2-0.8) suggests that distribution of enzyme to the support is homogenous and adsorption does not occur with constant growth rate. If the value of n = 1 indicates that the protein binding is instantaneous. MS-9G, MS-13 and MS-13G n values are less than 1. MS-9 and MCF-25 shows n value higher than one where as MCF-25G shows n value equal to 1.

In the enzyme binding q_e denotes the amount adsorbed at equilibrium. q_e can be obtained from the slope of the plot of (t/q_t) against time (typical graph are shown Fig 8.3). From graphical data MCF-25G showed highest amount of adsorption at equilibrium which is 10.54 mg g^{-1}. For MS-25 and MS-13G the q_e values are almost similar 8.27 and 8.27 mg g^{-1} respectively.

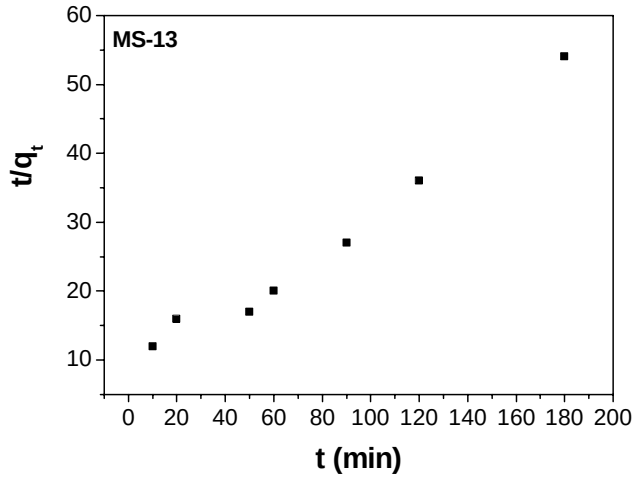

Fig. 8.3 Plot of (t/q_t) against t

8.3.2 Effect of temperature

To investigate the effect of temperature on the adsorption of lipase on mesoporous silica the mixtures were incubated with stirring at different

temperatures (20, 30 and 40°C). For this study MCF-25 material were selected because of their high capacity to bind enzyme. Adsorption to the mesoporous silica surface was followed by removing small aliquots at various times measuring the protein content in the filtrate of the suspension. The protein content was measured by the Lowry method with BSA as a standard at 600 nm. Bovine serum albumin (BSA) was used as standard to construct the calibration curve. The amount of adsorbed protein on the membranes was calculated from the protein mass balance among the initial and final lipase solutions, and the washings.

The kinetic parameters n and k were determined from the slope and intercept of the extrapolated straight line at different temperatures (Fig 8.4). Data are given in Table 8.2. As seen in Table 8.2**,** the values of the *n* parameter are higher than one in all cases. This suggests that the process may be surface reaction limited i.e., protein transport to the interface is not rate limiting. The most important factor in the Avrami kinetic model is the constant *n*, the values of which can be used to verify possible alterations of the adsorption mechanisms in relation to the contact time and the temperature. From the results it was observed that value of the n parameter decreases with temperature. This result suggests that the temperature increase may cause the change in lipase binding mechanism from surface controlled reaction to diffusion controlled reaction. The higher value of n suggests that diffusion of the enzyme is faster than mass transfer process and it seems to be a temperature-dependent phenomenon. In this study the Avrami kinetic model suggests temperature-dependent adsorption mechanisms, for which the parameter n presents very similar values (i.e. greater than 1). Data show a satisfactory fit with correlation coefficients of 0.97.

Table 8. 2 The values of the specific kinetic parameters from KEKAM equation

Temperature (°C)	k (μs)	n	Correlation Coefficient(R^2)
20	255.29	1.29	0.97
30	203.46	1.15	0.98
40	152.86	1.11	0.97

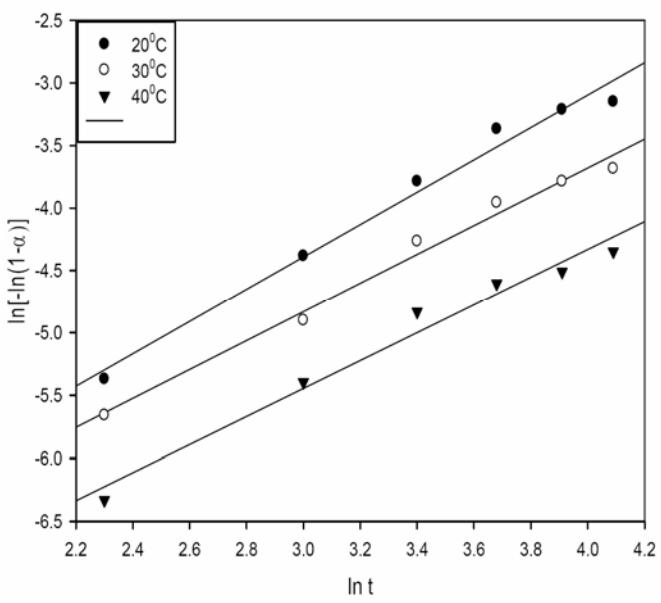

Fig.8.4 KEKAM plots ln [ln (1-α)] against ln t for lipase on MCF-25 at three different temperatures 20°C, 30°C and 40°C

Kinetic parameter k changes with temperature according to the Arrhenius equation:

$$k = A \exp(-E_a/RT)$$

Where A is pre-exponential factor, E_a is activation energy, R is the gas constant, and T is temperature in Kelvin. The effect of the temperature on affinity of lipase towards MCF-25 can be seen in Arrhenius plot (Fig 8.5)

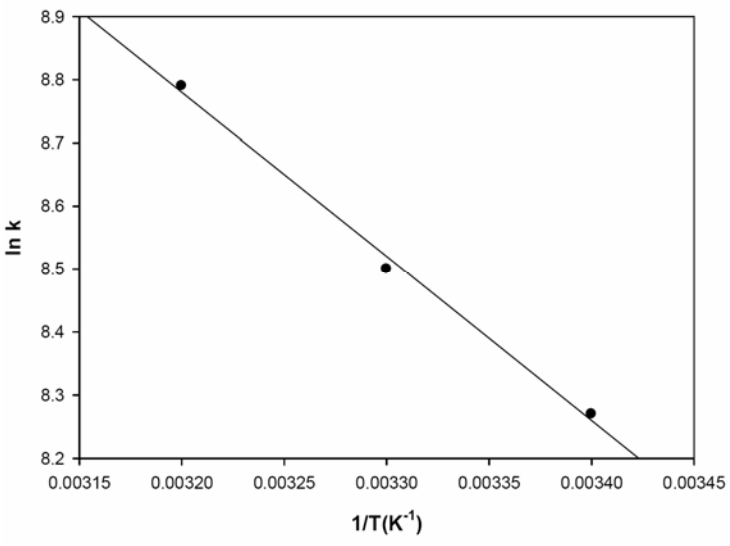

Fig. 8.5 Arrhenius plot ln k against 1/T for lipase-MCF-25 system

From these data, activation energy for lipase adsorption on MCF-25 of around 49 kJmol^{-1} was calculated between 20-40°C. This value of E_a approximately corresponds to only two hydrogen bonds per molecule. Since enzyme molecules in solution are surrounded with water molecules, it may be supposed that for its binding to silica, these hydrogen bonds between enzyme and water molecules should be broken [20]. It may be concluded from the E_a value as well as rate constants k**,** the enzyme has a great affinity toward the MCF-25 active sites. It was concluded that the physical interaction between the surface and lipase may be quite effective for the lipase immobilization.

8.4 Leaching studies

The leaching studies of adsorbed enzymes were investigated in the batch reactor. The immobilized enzyme was suspended in 0.1M phosphate buffer and vigorously shaken for 2h in a covered Erlenmeyer flask to prevent evaporation. It was centrifuged and the centrifugate was estimated for protein. The process was repeated continuously for 6 cycles at 30°C temperature and

the influence of repeated use on leaching was analyzed. The extent of leaching of the immobilized enzyme can be determined by the enzyme content of the supernatant liquid at different time intervals.

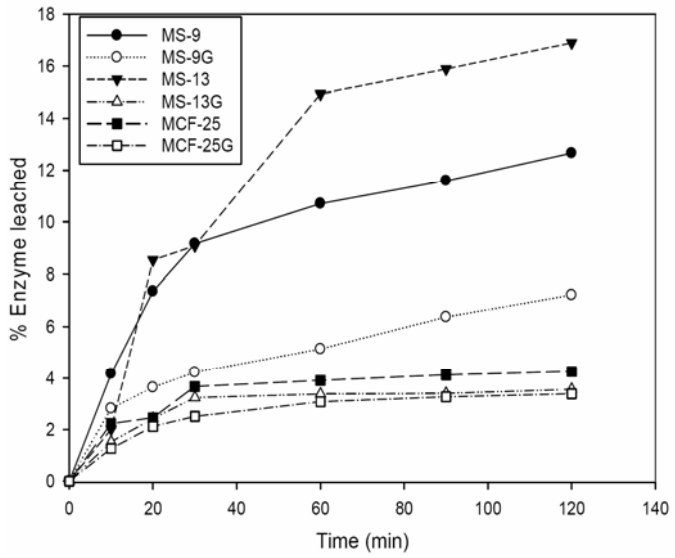

Fig. 8.6 Enzyme leaching from the support at different time intervals

Fig 8.6 shows the profile of enzyme leaching with time for all support. The enzyme immobilized on functionalized support showed very low amount (less than 6%) of leaching. It was observed that the amount of enzyme leached out from covalently bound enzyme (MCF-25G and MS-13G) was always less than 3% after vigorous shaking for 120 min. MS-13 showed about 17% leaching of enzyme. Covalent binding leads to a very strong bond between the enzyme and support and hence leaching occurs to a negligible extent. The leaching is due to weak interaction between lipase molecule and silanol groups of MS-13. From Fig.8.6 it is clear that enzyme leaching decreases and stabilizes after 20min. The weakly bound enzyme leaches out first. Hydrophobicity of the material plays a vital role in lipase binding. Lipase has better affinity towards

hydrophobic supports and hydrophobic materials are good hosts for enzyme immobilization [21- 24]. MCF-25 and MS-13 have highest hydrophobic character than other supports and they showed the highest stability without much loss in enzyme after 120 min of shaking.

Gopinath & Sugunan argued that the adsorbed enzyme was prone to more leaching, whereas the covalently bound enzyme resisted leaching [25]. MCM-41and MCM-40 entrapped trypsin using 3-aminopropyl triethoxy silane (3-APTES) did not leak into solution when the material was immersed in pH 9 buffer which would release ~90% of enzyme after 24h [13]. More than 70% of immobilized enzyme can be desorbed when the sample is dispersed /immersed in solution. Such leaching can be attributed to relatively weak physical adsorption between the enzyme and siliceous surface [26]. Wang *et al* showed the combination of using porous particles for enzyme immobilization and coating with nanoscale multilayer shells to overcome enzyme desorption encountered with the direct immobilization of enzyme on mesosilica [27, 28]. Serra *et al* studied the loss of the adsorbed lipase and found that nearly 30% of the enzyme was leached from the support within 2 h [29]. Yiu *et al* also reported that 35-72% of the adsorbed trypsin on MCM-41, MCM-48 and SBA-15 leached from the support after stirring in the buffer solution for 2 h [21]. Results show less than 17% leaching of enzyme from the support so our t is better than literature reports.

8.5 Elution of enzyme

Complete elution of enzyme was achieved by shaking the immobilized support with increasing concentrations of NaCl (0.05M to 1M) in optimum 0.01M phosphate buffer pH. Increasing the concentration of NaCl the strongly bound enzyme leached out and it was measured by Folin reagent using a

standard curve. Increasing values of ionic strength were sequentially applied to the supports containg immobilized catalase. The catalase immobilized on MS-9, MS-13 and MCF-25 was used for elution study [30]. Sequential elution of enzyme from support is demonstrated in Table 8.3 and the total enzyme recovered was calculated and summarized in Table 8.4. The total amount of enzyme adsorbed on MS-9, MS-13 and MS-25 were determined using standard curve 58.2, 82.8 and 124 mg respectively.

Table 8.3 Sequential elution of enzyme from support

NaCl [M]	MS-9		MS-13		MCF-25	
	Protein eluted (mg)	Recovery of protein (%)	Protein eluted (mg)	Recovery of protein (%)	Protein eluted (mg)	Recovery of protein (%)
0.05	0.80	1.37	7.50	9.06	16.79	13.54
0.10	8.00	13.75	17.00	20.53	26.00	20.97
0.25	22.10	37.97	31.20	37.68	42.61	34.36
0.50	11.00	18.90	19.52	23.57	28.40	22.90
0.60	10.10	17.35	6.30	7.61	4.82	3.89
0.75	1.90	3.26	0.20	0.24	0	0
1.00	0	0	0	0	0	0

Table 8.4.Amount of Protein recovered from supports

	Immobilized enzyme (mg)	Total protein eluted (mg)	Recovered protein (%)
MS-9	58.2	53.90	92.61
MS-13	82.8	81.72	98.70
MCF-25	124.0	118.62	95.66

Table 8.3 showed that as the concentration of NaCl increases leaching also increases. High concentration of NaCl required to elute strongly adsorbed enzyme molecules from the support. The total protein recovered is shown in Table 8.4. Above 90% enzymes were eluted from the support and the proteins retain their activity after elution.

8.6 Maximum loading capacity of supports

Balkus *et al*. suggested that the loading efficiency depends on the size of the enzyme and the pore size of the adsorbent, and indicated that the immobilization process takes place in the mesopores [13, 31]. Fan *et al* reported that very rapid and high capacity (533 mg g^{-1}) immobilization of enzymes within mesoporous silica has been achieved by finely tuning their morphologies [32]. The immobilization of lipase on several kinds of mesoporous silica such as MCM-41, MCM-48, MCF, SBA-15, has been well investigated [33-36].

The lipases have inherent affinity towards hydrophobic media, and the hydrophilic/hydrophobic nature of the supports could provide a proper micro-environment for lipase [37]. Gao *et al* investigated immobilization efficiency of Lipase in five kinds of SBA-15 with different pore-sizes (6.8 nm, 9.1 nm, 13.2 nm, 15.6 nm and 22.4 nm) and the one with pore diameter of 15.6 nm was proved to be a more suitable immobilization support than the others, and the loading amount reached 343.6 mg/g support [38]. Duri *et al* showed that the capacity of SI-P1 resin for lipase from C. cytindracea was 78 mg lipase/g support and it was less than the calculated value and the difference may be due to diffusional and sterric effect [39]. When lipase was immobilized onto a macroporous copolymer support under optimum conditions the maximum amount of protein bound was 15.4 mgg^{-1} and the

immobilization efficiency was 62% [40] but lipase immobilized on zeolite support achieved only 8mg/g [20]. Gum Arabic coated magnetic Fe_3O_4 nanoparticles (GAMNP) showed the capacity of 69 mg lipase/ (100 mg carrier) [41]. A facile method of lipase immobilization was developed by the use of polydopamine coated magnetic nanoparticles (PD-MNPs) under optimal conditions 73.9% of the available lipase was immobilized on polydopamine coated magnetic nanoparticles, yielding a lipase loading capacity as high as 429 mgg^{-1}.[42]. 67.42mgg^{-1} for methyl modified aero gel [43], 200 mg protein/g of octyl silica [44] and 34 mgg^{-1} for amino functionalized magnetic supports [45] are other literature reports about lipase binding capacity on various supports.

Table 8.5 Maximum lipase loading capacity of different mesosilica supports

Samples	Lipase(mgg^{-1})
MS-9	94.20
MS-9G	124.80
MS-13	178.05
MS-13G	267.20
MCF-25	258.30
MCF-25G	356.25

It can be observed in Table 8.5 that the bound protein is higher for MCF-25G due to caged pore structure and higher hydrophobicity of the material. All the prepared mesoporous materials show better binding capacity than the literature values.

8.7 Conclusions

The main conclusions from the present study are summarized below:

- The kinetic studies indicated that the temperature of protein binding was of significant importance in relation to the determination of the intrinsic constants.
- The binding interactions between enzyme and support showed a good fitting to the Avrami kinetic model.
- The value of kinetic parameter n showed that, the adsorption process is controlled by surface kinetics. The activation energy for lipase adsorption on mesoporous silica was 49 $kJmol^{-1}$. The Ea, value and rate constants k for the enzyme show a rather high affinity toward the mesoporous silica active sites.
- Temperature increase could cause the change in enzyme binding mechanism.
- The nature of attachment of enzyme to the support plays a very significant role in leaching. Adsorbed enzymes were more prone to leaching while covalently bound enzyme resisted leaching.
- Less than 4% enzyme leaching was observed for MCF-25G and MS-13G. It was due to the hydrophobic interaction between lipase and support.

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Chapter 9

CONCLUSION AND FUTURE OUTLOOK

Contents

Research progress in the field of enzyme modification enables the opportunity to tune them for industrial application. Immobilization has been used to impart novel characteristics to an enzyme, there by intentionally modifying its basic catalytic behavior. The discovery of mesoporous material led the immobilization research into a new level. Higher activity can often be obtained by the use of carriers with bigger pore sizes. Three commercially important enzymes catalase, glucoamylase and lipase have been immobilized into mesoporous material. It can be further modified by different techniques and used for different applications. This chapter covers the summary of the present work, scope and relevance of further research in this field.

9.1 Introduction

Biocatalysts are the key players in various industrial processes. Constant efforts are being made to improve the enzyme activity, efficiency, reproducibility and stability during industrial processes. Different supports successfully utilized for biosensors. Currently, keen efforts are being taken for increasing the stability of enzymes. Immobilization of enzymes into nanocavities showed significant results. Synthesis of different commercially useful compounds have been facilitated by immobilized enzymes, a greener alternative to conventional catalysts .With the advent of nanotechnology, silica nanoparticles with immobilized enzymes

have been applied for elimination of micropollutants from wastewater. Increasing environmental concerns have led to the use of immobilized biocatalysts for biodiesel production. The different factors influencing enzyme immobilization and the possible modifications for their enhancement in activity have been studied by several researchers. Selection of the right support for enzyme catalysis is of the utmost importance, as enzyme efficiency depends largely on the support and its linkage to it. The selected support should have a well-developed internal structure, a large surface area, high porosity and a reasonable pore size distribution. The support should have high affinity (or capacity) for enzymes and a suitable chemical structure to provide maximum enzyme activity and enzyme-substrate contact. The support also should be thermally stable, chemically durable, resistant to contamination, and available at a reasonable cost.

Nowadays siliceous mesostructured cellular foams show great potentials as carrier materials for enzyme immobilization to give very active biocatalysts, and are far superior to other carriers. With the vast array of research on enzyme immobilization, we can conclude that it is one of the most promising techniques for highly efficient and economically competent biotechnological processes in the field of environmental monitoring, biotransformation, diagnostics, pharmaceutical and food industries. Enzyme based strategies are increasingly replacing conventional chemical methods in both laboratories and industries which attributes like efficiency, quicker performance and multifarious use. However, commercialization of immobilized enzymes is still at a lower pace because of their costs and storage problems.

9.2 Summary

The thesis is divided into nine chapters; a brief chapter wise summary is given below.

Chapter 1 deals with general introduction, literature review of enzyme, immobilized enzyme and mesoporous materials. The general mechanisms for the synthesis of mesoporous materials were outlined and the major objectives of the present work have been summarized in the first chapter.

Chapter 2 focuses the experimental procedures adopted for the synthesis of mesoporous silica supports. The detailed procedures used for the immobilization, physicochemical characterization and methodology used to perform the catalytic activity studies were also discussed.

Chapter 3 describes the results obtained from various characterization techniques of the pure supports as well as the immobilized ones. The structural changes were confirmed by various techniques such as nitrogen adsorption measurements, TG/DTG, NMR, XPS, TEM, SEM, FT-IR, CHN analysis and contact angle measurements.

Chapter 4 illustrates the immobilization studies of glucoamylase in starch conversion to glucose. Activity and stability studies of immobilized glucoamylase for starch hydrolysis were studied. The kinetic constants were studied and compared with that of free enzyme.

Chapter 5 outlines the biodegradation of phenol using free and immobilized bovine liver catalase.

Chapter 6 deals the synthesis of ethyl valerate and amyl isobutyrate with free and immobilized Candida rugosa lipases and dependence of initial water and molecular sieves on activity. The kinetic parameters have been derived from Ping Pong Bi-Bi mechanism with inhibition by alcohol.

Chapter 7 presents biochemical characterization of the free and immobilized lipase in hydrolysis of esters. The influence of different

chemicals and surfactants were also studied. The kinetics of the reaction was studied and compared with that of free lipase

Chapter 8 illustrates the kinetics of adsorption studies of enzymes onto mesoporous silica and evaluation of Avrami model.

Each chapter is provided with an abstract in the beginning and a reference list at the end. Chapter 3-8 have the conclusion of the work towards the end

Chapter 9 describes the summary and the main conclusion of the work. The benifits and relevance of immobilization were highlighted in this chapter.

9.3 Conclusions

The major conclusions of the present work are outlined below:

- Ordered mesoporous silica having different pore size were synthesized via hydrothermal method and functionalized by co-condensation of 3-APTES and glutaraldehyde.
- Meso cellular foams having ultra large pore diameter of 241 Å were synthesized using TMB as swelling agent.
- Catalase, glucoamylase and lipase were successfully immobilized on the support by adsorption and covalent binding.
- The synthesized samples were analyzed by various characterization techniques. such as Low angle XRD, N_2 adsorption studies and TEM analysis confirmed the mesoporosity of the prepared supports.
- ^{29}Si, ^{13}C CP MAS NMR and XPS studies revealed the incorporation of the organic groups onto the supports which was further confirmed

by FT-IR results. There was an increase in the contact angle with surface modification with silane and glutaraldehyde.

- Catalytic activities of the immobilized enzymes were evaluated for various reactions and kinetic parameters were determined by Michaelis Menten & Lineweaver – Burk plots.
- Thermal and storage stabilities of enzymes were enhanced by immobilization and highest storage and reusability results were obtained for MCF- 25G.
- The kinetic parameters V_{max} decreased and K_m increased after immobilization and the altered kinetic parameters are due to diffusional limitations and blocking of active sites during immobilization.
- Leaching was minimized by covalent binding of the enzyme and MCF-25G showed negligible amount of enzyme leaching.
- Among the supports screened for immobilization of enzyme, MCF-25G showed maximum enzyme loading capacity and retained higher activity than other supports.
- The kinetic parameters of enzyme adsorption proved that it is controlled by surface kinetics and confirmed by experimental studies.

9.4 Futuristic approach

Research should be focused to overcome the current limitations related to immobilization techniques, so as to expand the horizon for all-round application. The topic of enzyme immobilization continues to attract great interest in the industry sector because it requires stable and robust immobilized enzymes to withstand necessary harsh conditions of operation. The lack of

guidelines that could govern the selection of the immobilization method is being replaced by a rational design of immobilized derivatives, in which the protein 3D structure (and reactivity) as well as the textural properties of the support are important parameters. An important feature is that the carrier not only functions as a scaffold for the protein molecules but also alters the enzyme properties. Various analysis may help to establish the optimal immobilization conditions and to understand the behaviour of immobilized enzymes. One of the major challenges currently facing material scientists is the development of tailor-made carriers with specific physical and chemical properties such as suitable geometry and binding properties, which can be used in different reactor configurations and bio-nanodevices. Novel concepts such as the application of ordered mesoporous (organo) silicas as enzyme adsorbents or the entrapment of enzymes in a spatially restricted sol-gel matrix promise exciting research and advances in the next few years. In addition, a combination of different immobilization techniques, which provide high enzyme loading and high retention of activity will increasingly be used.

The immobilized catalase, glucoamylase and lipase were highly stable in mesoporous materials could be promisingly used for various biotechnological applications and biomedical fields. The discovery of ordered mesoporous materials has opened great opportunities for new applications in heterogeneous catalysis. The better support has many disadvantages and research should be focused to overcome the current limitation so it still remains as a fascinating challenge for biotechnology researchers. Adopting logistic and sensible immobilization techniques along with innovatively modified supports to improve the state of enzyme immobilization is an important goal for the coming period and provides new perspectives to the industrial sector.

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RESEARCH PUBLICATIONS

- "Improved stabilities of immobilized glucoamylase on functionalized mesoporous silica synthesized using decane as swelling agent", Reni George and S. Sugunan, Bulletin of chemical engineering and catalysis. (In Press)
- "Biodegradation of phenol using bovine liver catalase immobilized on SBA-15" ,Journal of Hazardous Materials (communicated)
- "Kinetics of adsorption of lipase onto Mesocellular foams – Evaluation of Avrami model", Enzyme And Microbial Technology (communicated)
- "Synthesis of green apple ester ethyl valerate by Candida rugosa lipase immobilized on SBA-15", Food chemistry (communicated)
- "Kinetics and thermodynamic studies of starch hydrolysis by immobilized glucoamylase on SBA-15", Reaction Kinetics and catalysis letters (communicated)

CONFERENCE PRESENTATIONS

- Bio-degradation of phenol by bovine liver catalase immobilized on SBA-15, National conference on advances in physical and theoretical chemistry**,** Reni George, S. Sugunan Poster presentation to be delivered at University of Calicut, March 2012
- Synthesis of green apple ester ethyl valerate by Candida rugosa lipase immobilized on SBA-15, Current trends in chemistry, Department of Applied Chemistry, CUSAT, KOCHI, Jan2012
- Immobilization of Lipase from Candida rugosa on ordered mesoporous silica for esterification reaction ,Current trends in chemistry, Department of Applied Chemistry ,CUSAT, KOCHI, March-2011
- Comparative catalytic efficiency between adsorbed and covalently bound glucoamylase on mesoporous silica, ICAS2010,International congress onanalyticalscience2010, Kochi, Nov-2010
- Catalytic Activity of Glucoamylase Immobilized on Silane modified ordered mesoporous silica by covalent Binding" at 22nd Kerala Science Congress, KSCTEC, Trissur, Jan-2010

- Bio-chemical and catalytic characterization of Glucoamylase immobilized on ordered mesoporous silica, Mat-Con2010, Department of Applied Chemistry, Cochin University of Science and Technology, Jan-2010
- Immobilized bovine liver catalase in mesoporous silica materials for bio-degradation of phenol , Reni George and S. Sugunan, Catalysis symposium, NCCR, IIT-Chennai, Dec-2010

ACHIEVEMENTS

- The best poster award was won for the paper entitled "Catalytic Activity of Glucoamylase Immobilized on Silane Modified Ordered Mesoporous Silica by Covalent Binding" on 22nd Kerala Science Congress held by KSCTEC, Trissur, Jan-2010
- The best poster award, National seminar on nanoscience and Technology, Sree Narayana Gurukulam College of Engineering, Kolenchery ,Feb-2013

WORKSHOPS ATTENDED

- Orientation Programme in catalysis Research, IIT Chennai, 17th- Nov-8th- Dec 2008
- Workshop on applications of mass spectrometry and related hypernated techniques in analytical science, Indian society of analytical scientists [ISAS], 25th- April 2009
- Workshop on applications of X-ray fluorescence spectrometry in analytical science and technology, Department of Physics, CUSAT/Kochi/Kerala, 21st-Sept 2009
- Workshop on molecular probes – technology and applications, ISAS/Kochi, 24th- November 2010

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