PHYSICOCHEMICAL AND BIOCHEMICAL CHARACTERIZATION OF ENZYMES IMMOBILIZED ON INORGANIC MATRICES

Thesis submitted to the

Cochin University of Science and Technology

In partial fulfilment of the requirements for the degree of

Doctor of philosophy

in

Chemistry

Under the faculty of Science

By

RESHMI.R

DEPARTMENT OF APPLIED CHEMISTRY

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

KOCHI-682 022, KERALA, INDIA

September 2009

Physicochemical and Biochemical Characterization of Enzymes Immobilized on Inorganic Matrices

Ph.D Thesis in the field of Catalysis

Author:

Reshmi. R

Research Scholar,
Department of applied Chemistry
Cochin University of Science and Technology,
Cochin-682022, Kerala, India
Email: rreshmi@cusat.ac.in, reshmimythri@gmail.com

Supervising guide:

Dr.S.Sugunan

Professor,
Department of Applied Chemistry,
Cochin University of Science and Technology,
Cochin-682022 ,Kerala, India
Email: ssg@cusat.ac.in

Department of Applied Chemistry
Cochin University of Science and Technology
Kochi, Kerala, India – 682 022

September, 2009

Dedicated to

my family.....

Dr. S.Sugunan
Professor
Department of Applied Chemistry
Cochin University of Science and Technology
Kochi-682 022
Kerala, India

Certificate

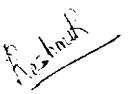
Certified that the thesis entitled "**Physicochemical and Biochemical Characterization of Enzymes Immobilized on Inorganic Matrices** ", submitted by Ms. Reshmi.R is an authentic record of research work carried out by her under my supervision at the Department of Applied Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry of the Cochin University of Science and Technology and the work embodied in this thesis has not been included in any other thesis submitted previously for the award of any other degree.

Kochi-22
14-09-2009

Dr. S. Sugunan
(Supervising guide)

Declaration

I hereby declare that the thesis entitled "**Physicochemical and Biochemical Characterization of Enzymes Immobilized on Inorganic Matrices**" submitted for the award of Ph.D. the degree of Doctor of Philosophy in Chemistry of the Cochin University of Science and Technology is based on the original work done by me under the guidance of Prof. Dr. S. Sugunan, Professor, Department of Applied Chemistry, Cochin University of Science and Technology, Kochi-682 022 and this work has not been included in any other thesis submitted previously for the award of any other degree.



Kochi-682 022
14-09-2009

Reshmi.R

Acknowledgement

I have worked with a great number of people whose contribution in assorted ways to the research and the making of the thesis deserved special mention. It is a pleasure to convey my gratitude to them in my humble acknowledgment.

With great pleasure and sincere respect, I owe my deepest gratitude to my research guide, Dr. S. Sugunan, for his invaluable guidance, supervision, advices and unending support through out this research effort. I am grateful to him for giving me liberty to carry out my research work independently throughout the course of this programme. My deepest personal regards are due for him forever. I have been extremely fortunate to work under his guidance, which has always been a source of inspiration to me and have enriched my growth as a student, researcher and a scientist want to be.

I am extremely grateful to Dr. K. Girish Kumar, Head, Department of Applied Chemistry for providing me all facilities required for my work. I would also like to thank former head of the department, Dr. Prathapachandra Kurup for extending all possible help during my initial stages of work. I am deeply grateful to Dr. K. K. Mohammed Yusuff, member of the doctoral committee for all his help in my official matters. I thank all the faculty members in the Department of Chemistry, who have made my academic experience rich and memorable. I express my sincere thanks to the librarian for providing me the necessary reference books which has helped me a lot during my work. I owe my special thanks to all the non-teaching staffs for their help, hospitality and cooperation given to me in completing my research work successfully.

I also take this golden opportunity to convey my earnest respect to my teachers, Dr. K. Remadevi, N. S. S Training College and K.R Hariharan, N. S. S Hindu College for their extraordinary way of teaching that build up my research career in science. They have been a constant source of strength and inspiration for me.

I am grateful to Dr. Krishnaswamy Raja, Department of Chemistry, City University of New York and his students Sukanata dolai and Manik Mandal for analyzing samples by TEM in the midst of their busy schedule. I sincerely acknowledge Mr. Gurulinga, IISC, Bangalore and Dr. Parvathy Amarasetti, Shivaji University, Maharashtra for SEM and contact angle measurements and for their valuable help in every possible way through fruitful discussions. I warmly thank Mr. Nilesh Kulkarni, TIFR, Mumbai for the XRD analysis and SIF, IISC, Bangalore for the NMR results. I am deeply

indebted to STIC, CUSAT for providing timely help with all the analytical facilities. I am thankful to Dr. P. R. Rajamohan, NCL, Pune for the NMR results. I warmly thank Mr. Kashmiri and Mr .Jose of USIC, CUSAT for all the technical assistances.

I am very grateful to Dr. S. Shylesh, Post doctoral Fellow, Fachbereich Chemie, Germany for his valuable discussions that has helped me a lot to understand better about the characteristics of different mesoporous materials. I am also extremely thankful to Bineesh K.V, Research Fellow, Korea for helping me out with my queries related to clays and also for all the literature survey. Hamza and Sunil, NCL, Pune had taken a lot of pain to get my NMR samples done and I am really thankful to them. I would convey special thanks to Jissa and Sooraj, Department of Biotechnolog, CUSAT for kindly helping me in clarifying all my doubts and to carry out sonication of my samples.

My lab was the place where I could always find someone to share with the successes and frustrations of the lab work. These rooms have also been a nest for friendships. That is why a big word of thank is dedicated to my colleagues who created a wonderful working environment, very appealing to me. I would like to extend my thanks to Dr. Sanjay Gopinath, for introducing me this interesting topic of research. I was very lucky to have Sunaja, Bejoy, Suja, Fincy, Shali, Binitha, Rani, Ramanathan and Shalini as my seniors. I learnt a lot by talking to them and they have been very cooperative and kind to me and extended all possible help for the completion of this work. Radhika is like an elder sister to me who is so caring and with her gentle love have given me all the moral support throughout. Maya and Radhika had always being willing to teach me all the basics of computer and the finer points of running the GC instrument. I am really obliged to them for their help and will always cherish the moments we had in our lab.

I wish to express my warm and sincere thanks to Ajitha miss, Joyes miss and Rose miss who have generously shared their time with lively discussions and advices. I am deeply indebted to my friend, Reni George for the help in proofreading and giving me various suggestions on how to improve the writing of this thesis. My heartfelt thanks for her company and encouragement. I would like to thank Mrs. Bolie Therattil for being my friend throughout the highs and the lows of these four years. Good luck with defending your own thesis and with the fulfillment of your dreams! Thank you for your support whenever I needed it, for your humor and friendship. I was very fortunate to have my juniors: Rajesh, Ambili, Dhanya, Temy, Cimi, Nissam, Mothi and hridya for their indispensable help and support and the enjoyable and memorable days.

I have been blessed with a friendly and cheerful group of students: Manju, Nancy, Vijutha and Geetha who have been spreading happiness with their jokes during those

scientifically dark days. I would especially like to thank my loving sisters, Dhanya N.N and Asha for their love and support which have been invaluable during all my days of hardship. Varsha's witty character has always alleviated my tensions to a great extent and I really enjoyed those moments with her during my hectic days of work. Thanks for giving me a pleasant time. I could also contribute some papers in reputed journals from the work done with Murukesh .P and for this I am extremely thankful to him.

Through the wonders of e-mail and internet, I was able to correspond with numerous individuals around the world whose gracious provided me with references/information used in this thesis: Christy George, Gigi, Pramod and Sumesh. Collective and individual acknowledgments are also owed to all my colleagues in the department whose presence somehow perpetually refreshed and was helpful, and memorable. They all have given me a nice company and lovely atmosphere in CUSAT. I would like to thank all the people who have directly or indirectly helped me in completing this thesis.

This work would not have been possible without the contributions of many, many people, which, if all listed here, could possibly need another hundred pages. So if you do not find your name below but feel you deserve my appreciation – just take it without hesitatation! I express my apology that I could not mention personally one by one. The constant help and support rendered by all will always be remembered with appreciation and sincere thanks.

I have had many uncles, aunts, cousins, and friends who have encouraged me all these years; their prayers and support have carried me through this process and is very much appreciated.

I cannot end without thanking my family for instilling in me confidence and a drive for pursuing my Ph.D. They deserve special mention for the inseparable support and prayers.

All praise, glory and thanks to almighty God for His amazing Grace and blessings undeservingly bestowed upon me that led me throughout the whole process of completing this research.

Reshmi.R

Preface

Biotechnology is currently considered as a useful alternative to conventional process technology in industrial and catalytic fields. The increasing awareness of the need to create green and sustainable production processes in all fields of chemistry has stimulated materials scientists to search for innovative catalysts supports. Immobilization of enzymes in inorganic matrices is very useful in practical applications due to the preserved stability and catalytic activity of the immobilized enzymes under extreme conditions. Nanostructured inorganic, organic or hybrid organic-inorganic nanocomposites present paramount advantages to facilitate integration and miniaturization of the devices (nanotechnologies), thus affording a direct connection between the inorganic, organic and biological worlds. Mesoporous materials not only have highly ordered pore structures with uniform pore diameters and large surface areas, but they also are liable to chemical modification and functionalization. These properties, combined with good chemical stability, make them competent candidates for designed biocatalysts, protein-separation devices, drug delivery systems, and biosensors Aluminosilicate clays and layered double hydroxides, displaying, respectively, cation and anion exchange properties, were found to be attractive materials for immobilization because of their hydrophilic, swelling and porosity properties, as well as their mechanical and thermal stability.

In this venture, various inorganic supports with different physicochemical characteristics were evaluated for the immobilization of the enzyme, Candida rugosa lipase and to select the most suitable biocatalyst for various biotransformations. The aim of this study is the replacement of inorganic catalysts by immobilized lipases to obtain purer and healthier products. The reaction kinetics and mechanism were investigated. Kinetic studies were carried out to select the best reaction conditions (water activity, temperature, time) to maximize activity and operational stability. Synthesized mesocellular silica foams, montmorillonite

K-10 clay as well as the immobilized supports were characterized by various physicochemical techniques. To obtain a better understanding of the structure-property relationship, the activity of the immobilized enzymes is correlated with the pore sizes and functionalities of different silica and clay matrices. The aim of this project is to develop new enzyme-immobilized systems and their use in adequate environments favoring the desired bioconversions.

With the development of methods like sol-gel, template method, polymer pyrolysis for the synthesis of oxides with unusual surface properties, the scenario of enzyme immobilization can be expanded to definite areas. Thus there are interesting possibilities within the field of immobilized enzymes and it is imminent that in the future many applications will be replaced by immobilized enzymes and many more new systems will become technically as well as commercially feasible.

.....ooo.....

Contents

Chapter 1

Chapter 2

Chapter 1

GENERAL INTRODUCTION – LITERATURE SURVEY, OBJECTIVES AND SCOPE

Contents

Biotechnology is currently considered as a useful alternative to conventional process technology in industrial and analytical fields. Immobilized enzymes are used in a wide variety of commercial processes, including chemical conversion of intermediates for environmental, agricultural and pharmaceutical applications. Biocatalysis could gain an enhanced breakthrough with the introduction of novel mesoporous silica materials as enzyme carriers: the dimensions of most of the enzymes are within the pore size range of these new silica materials and a broad spectrum of enzymes can be immobilized. Lipases are the workhorses of biocatalysis because of their availability, stability and their capability of resolving more than 1000 different substrates and have come a long way in establishing themselves as an important synthetic tool for bio-organic researchers. Close working relationship between materials scientists, chemists and biologists are crucial to the success of the promising future of silica–biomolecule hybrid materials. With the realization of the biocatalytic potential of microbial lipases in both aqueous and nonaqueous media in the last one and a half decades, industrial fronts have shifted towards utilizing this enzyme for a variety of reactions of immense importance. Two enzymes: lipase from Candida rugosa and β-glucosidase has been chosen for the present work in which lipase has been immobilized via adsorption and chemical binding on siliceous silica and nature's clay while β-glucosidase have been crosslinked onto siliceous silica. The catalytic activity of immobilized enzymes has been assayed and correlated with the microstructures of the host silica materials. This research provides an essential understanding of the relationship between support (clay and silica) characteristics and enzyme loading and activity.

1.1 General Background

Catalysis is of crucial importance for the chemical industry, the number of catalysts applied in industry is very large and catalysts come in many different forms, from heterogeneous catalysts in the form of porous solids over homogeneous catalysts dissolved in the liquid reaction mixture to biological catalysts in the form of enzymes

Biocatalysis has many attractive features in the context of green chemistry: mild reaction conditions (physiological pH and temperature), an environmentally compatible catalyst (an enzyme) and solvent (often water) combined with high activities and chemo-, regio- and stereoselectivities in multifunctional molecules.

1.2 Enzymes as catalysts

The enzymes offer a distinct advantage due to their specificity and stereoselectivity, catalytic efficiency under mild conditions, biodegradability and limitation of side-products formation. Some criteria must be met for an enzyme to be a viable industrial catalyst. The enzyme must be both process compatible and stable. Conformational changes as well as chemical processes at the level of the polypeptide chains may be induced, leading to the enzyme inactivation. Unfortunately, functional stability of an enzyme often limits practical application of enzymes in medicinal and biotechnological processes. Research over the last four decades has focused on understanding the modes of enzymatic deactivation, as well as developing methods to overcome this shortcoming of the biocatalytic approach. A first approach to prevent deactivation consists of changing the enzyme environment, for example, by means of soluble additives such as metals, surfactants, polyols, PEGs and sugars. Stabilizing effects by reducing irreversible unfolding and aggregation is their immobilization onto a suitable carrier or entrapment within a solid matrix. Other strategies rely on the alteration of the enzyme primary structure using one of the following methods: chemical modification; covalent immobilization; protein engineering; or directed evolution.

1.3 Immobilized Enzymes: Bona Fide Heterogeneous Catalysis

Immobilization refers to the preparation of insoluble biocatalytic derivatives and involves the coupling of enzymes to solid supports that are either organic or inorganic. There are many advantages to the immobilization of proteins on a solid support [1, 2].

- Improved enzyme shelf-life (half-life);
- Improved stability in adverse reaction conditions;
- Improved stability in the presence of organic solvents..
- Separation from product stream;
- Allows for continuous flow operations and repetitive usage; and
- Increased enzymatic activity, in few cases.

The main disadvantage is that immobilization creates an extra diffusion barrier, loss of activity and changes in properties (selectivity).

1.4 Binding Enzymes to Solid Supports

Binding enzymes to solid supports can be achieved via covalent bonds, ionic interactions, or physical adsorption, although the last two options are prone to leaching. There are three basic methods for enzyme immobilization: entrapment, cross-linking and carrier binding [3]. Entrapment is the trapping of the enzyme within a cage-like, porous network that allows the substrate to diffuse through. Cross-linking is achieved through intermolecular bonding between enzyme molecules. 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 often proceeds by the reaction of specific functionalities at the support surface with amino acid side chains that are readily available on the enzyme surface. The covalent coupling may induce drastic changes in the enzymatic kinetics especially

when it occurs near the active site. The different methods of immobilization are depicted in Fig 1.1.

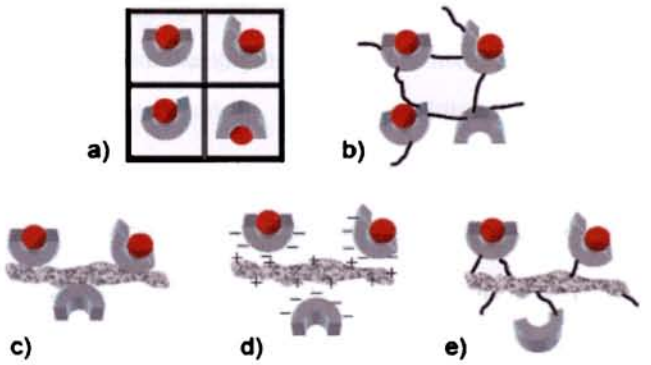


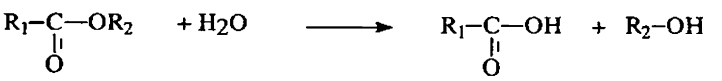
Fig.1.1 Methods of enzyme immobilization includes **(a)** Entrapment **(b)** Crosslinking, and physical attachment, by **(c)** adsorption, **(d)** ionic interaction, and **(e)** covalent binding

1.5 Lipases

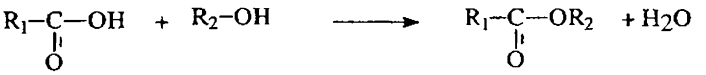
Hydrolases are the most frequently used enzymes due to their broad substrate spectrum and considerable stability. Lipases catalyze the hydrolysis/synthesis of a wide range of soluble or insoluble carboxylic acid esters and amides catalyse various other types of reactions such as esterification, transesterification, polymerization and lactonization [4, 5]. Their broad substrate specificity as well as high regio- and stereospecificity causes their various application: hydrolysis of oils and fats, synthesis of fatty acid esters as cosmetic ingredients, surfactants and biofuel, and production of intermediates for organic synthesis [6, 7, 8]. The stability of most lipases in organic solvents paves the way for their exploitation in organic synthesis: in esterification, transesterification, aminolysis and oximolysis reactions [9]. Such properties make lipases key players in the industrial enzyme sector [10, 11]. The main reason for the use of lipases is the growing interest and demand for products prepared by natural and environmentally compatible means.

The three main areas of lipase-catalaysed reactions in organic solvents are (Scheme 1.2): i) **Hydrolysis**: occurs in aqueous media when there is large excess of water, ester hydrolysis is the dominant reaction; ii) **Esterification**: under low water conditions such as in nearly anhydrous solvents, esterification can be achieved (improved product yields can be obtained if the water content of the medium is controlled); iii) **Transesterification**: the acid moiety of an ester is exchanged with another one (if the acyl donor is a free acid the reaction is called acidolysis, whereas the reaction is called interesterification if the acyl donor is an ester; in alcoholysis, the nucleophile alcohol acts as an acyl acceptor).

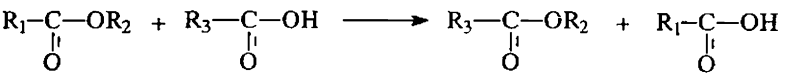
Hydrolysis :



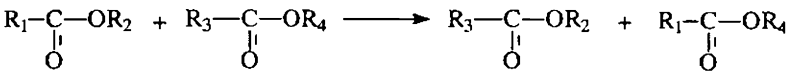
Ester synthesis :



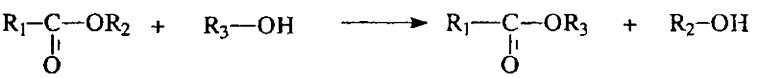
Acidolysis :



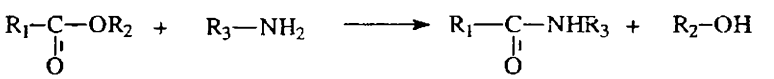
Interesterification :



Alcoholysis :



Aminolysis :



Scheme 1.2 Various lipase mediated reactions

1.6 Determinants of Lipase Specificity

Two structural elements came into focus as being major determinants of lipase specificity: the substrate binding site and the lid.

(a) The Substrate Binding Site

The active site in lipases is buried within the protein structure and substrate access to it is through a binding site located in a pocket on the top of the central β-sheet (Fig 1.3).

(b) The Lid: The Concept of Interfacial Activation

Lipases display a peculiar mechanism of action, "interfacial activation" [12]. Lipases may exist in two different forms. One of them, where the active centre of the lipase is secluded from the reaction medium by a polypeptide chain called "lid", is considered to be inactive (closed form). The other one, presenting the lid displaced and the active centre exposed to the reaction medium, is considered to be active (open form) [12, 13].

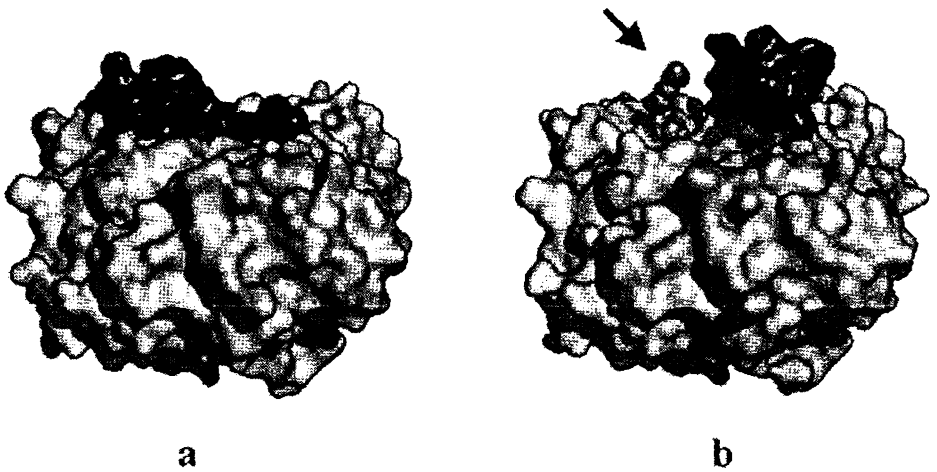


Fig.1.3 Lipase from *Candida rugosa* represented in the closed **(a)** and open conformation **(b)** with the lid depicted in black. In the active conformation **(b)** the enzyme active site is accessible to substrates, here represented by an inhibitor (dark grey) and highlighted by the arrow

Lipase form Candida Rugosa

The success of a lipase in industrial applications is due to its specific properties and price, which depends on its source. Commercial available lipases are from pancreas of mammalian or microbial sources [14], the latter easily produced on large scale by growing fungi or bacteria in a bioreactor. Among the microbial lipases, lipase from Candida rugosa is one of the most attractive commercially available lipases for the complete hydrolysis of tryglycerols due to its ability to liberate all types of acyl chains, regardless of their position in the triacylglycerols [15]. It has also been widely used for several stereoselective esterification reaction in organic medium under mild reaction conditions. *Candida rugosa* stands out due to its wide application in oil hydrolysis, transesterification, esterification and enantioselective biotransformation [16]. This lipase is fast becoming one of the most industrially used enzymes due to its high activity and low price [17, 18].

1.7 Ideal Attributes of an Enzyme Carrier

A variety of immobilization materials are available for use in both laboratories and industrial processes with many of these materials based on organic polymers (e.g.agarose, dextran, etc.) which fall under the category of "soft gels". The major drawback of soft gels is their low mechanical strength and in some cases low thermal and chemical stability [19, 20]. Low mechanical strength materials can only sustain a small pressure drop and thus they are limited to low solution flow rates when operated as a packed bed reactor (PBR). This can be a major drawback in industrial processes.

The appropriate immobilization matrix is chosen based on several different properties which affect the production process [3].

- Surface area and porosity: It is desirable to have materials with high surface areas (> 100 m^2/g), for high enzyme loadings, and high porosity to provide enzyme access for the substrate. Pore sizes>30 nm are ideal for the diffusion of enzymes during the immobilization process.
- Surface functional groups

- Mechanical and chemical stability
- Size and shape:
- Microbial resistance:
- Hydrophobic/hydrophilic nature

Inorganic carrier materials including silica gels, alumina and zeolite possess high thermal and mechanical stability, non-toxicity, and high resistance for microbial attack and organic solvents.

1.8 Mesoporous silica supports

Porous materials are used as adsorbents, catalysts and catalyst supports owing to their high surface areas and large pore volumes. An important characteristic of porous materials is the diameter of the pores Depending on the predominant pore size, he porous materials are classified by IUPAC into three classes: (1) microporous, having pore sizes below 2.0 nm, (2) macroporous, with pore sizes exceeding 50.0 nm, and (3) mesoporous, with intermediate pore sizes between 2.0 and 50.0 nm [21].Well known members of the microporous materials are the zeolites which have the pore sizes in the range of 0.2-1.0 nm, they provide excellent catalytic properties by virtue of their crystalline aluminosilicate network. But their small pore sizes are not suitable for catalyst immobilization, especially for large catalysts like enzymes. Traditional porous materials such as silica gels possess a wide range of pore sizes and also being amorphous, thus limiting their applications in some cases, e.g. shape selective catalysis.

Mesoporous silicas are in between these two materials, both in pore diameter and in distribution of pore diameters. Thus, mesoporous materials with larger pores and well defined pore structure remain an active and demanding research area. Well ordered mesoporous silica materials are promising supports for catalysts because they have pores that are large enough to immobilize molecular catalysts (e.g. metal complexes) and still the uniformly sized tunable pores have the potential to induce shape selectivity or hydrophobicity in the local environment of the active center.

It was not until extra large pore mesoporous solids, such as SBA-15 (pore size up to 8–10 nm), were prepared [22] that more molecules of biological interest really came into range. At the same time, routes to the organic functionalization of mesoporous solids were developed, giving a family of large pore molecular sieves with great chemical versatility [23]. According to theoretical calculations maximum stabilization of proteins can be obtained in spherical cages with a diameter of 2–6 times the diameter of the native protein. Since the average diameter of a hydrated protein is within the range of 10nm, it is concluded that the use of meso to nanoporous materials [24] will provide the maximum stabilization for the proteins.

The use of silica in enzyme immobilization can provide several benefits:

- **High surface areas** (500 – 1000 m^2/g) provide the possibility of high enzyme loadings on the matrix. This would increase the rate of reaction, measured as the product produced over time.
- **Greater mechanical strength** would allow the use of column reactors at higher flow rates without matrix distortion.
- **The silica matrix consists of surface hydroxyl groups** that can be readily used for a variety of surface modifications.
- The **hydrophilic silica surface** would minimize fouling problems often encountered with the hydrophobic "soft gels".
- The **open pore morphology** of silica gels allows substrates to quickly move into the interior regions of the particle.
- Solvents used in the processing of silica materials are **environmentally benign.**

1.9 Mesoporous molecular sieves for bioadsorption/ immobilization and biocatalysis

(a) Physisorption of enzyme onto silicas

Ordered mesoporous silicas (OMMs) provide excellent opportunities for the immobilization of both homogeneous and enzyme catalysts via covalent binding because

of the availability of well defined silanol groups [25, 26]. In the early 1990s, OMMs with uniform pore sizes in the mesopore range (2-50 nm), high surface area (~1000 m^2/g), and large pore volume (~1 cm^3/g) were reported [27, 28]. Among the OMMs, FSM-16 [27], MCM-41 [28], MCM-48 [28], SBA-15 [29], and MCFs [30] have been extensively studied for catalyst immobilization. The results of the studies carried out by Takimoto et al. indicated that the relative pore size of SBA-15 affects the location of cellulase within the pores and that the enzymatic activity of encapsulated cellulase was highly retained within SBA-15, which has a pore size slightly smaller than that of cellulose [31]. Vinu et al. [32] and Deere et al. [33] observe adsorption loadings as high as ~500 mg/g for cytochrome c on SBA-15 materials. The work of Takahashi et al. [34] indicates the importance of a close match between the pore size of a support and the molecular size of the enzyme. Han and co-workers have found that, during the immobilization of Fe Heme chloroperoxidase (CPO), the pore size of mesocellularfoam (MCF), MCM-48, SBA-16, or SBA-15 evidently influences the activity of the immobilized CPO [35]. Very recently, Deere et al. [36] reported the adsorption and activity of cytochromec (cyt c) on mesoporous materials with different pore diameters. They found that cyt c is only adsorbed in the mesopores of MCM-41 with large pore diameter. The rate of adsorption of the enzyme alpha amylase on ordered SBA-15 with different pore diameters synthesized by post synthesis treatment (PST) hydrothermally after reaction at 40°C of various pore sizes revealed the influence of morphology, pore diameter, pore volume and pH in the work done by Ajitha et al. [37].

(b) Adsorption and binding onto functionalized solids

In efforts to overcome the problems of leaching without the subsequent deactivation by addition of reactive siloxanes, it has been found that organic modification of the silica surface can strengthen the binding of the enzyme on the surface *via* either electrostatic or covalent mechanisms. For enzyme or protein immobilization, the most potentially useful surface functional groups are thiols, carboxylic acids, alkyl chlorides and amines. Other functional groups such as alkyl,

phenyl and vinyl can be added to modify the enzyme's environment by increasing the hydrophobicity of the surface.

Wang and coworkers [38] observed that α-chymotrypsin immobilized on mesoporous silicas functionalized with trimethoxysilyl propanal exhibited >1000 folder higher half life than the native enzyme, both in aqueous solution and organic solvents. Yiu et al. [39] employed SBA-15 materials with different surface functionalities (–SH, -Ph, Cl, NH_2, and –COOH) to immobilize trypsin. Leaching of the enzyme was largely solved by using SBA-15 functionalized with –SH, –Cl, and –COOH. PGA physically adsorbed onto the pores of SBA-15 silica retains up to 97% of the activity of free PGA, while PGA covalently attached onto the pores of oxirane-grafted SBA-15 retains only 60% of the activity. Hudson et al. [40] measured the adsorption properties of cytochrome c and xylanase on pure silica SBA-15 and organo-functionalized SBA-15 carriers. They concluded that electrostatic forces dominate the interaction between the enzymes and pure silica SBA-15, while weak hydrophobic forces provide the major interaction between the proteins and organofunctionalized SBA-15.

1.10 Nature's Clay as Immobilization Support

Of all the clay minerals, smectites are the most chemically interesting for modification and application. The use of Layered double hydroxides (LDH's) has recently extended to applications in biotechnology such as host materials, pesticides waste carriers and supports for enzymes. Smectites are the only group of clay minerals that have the ability to swell, i.e. they can absorb water molecules between the layers, thereby increasing the layer thickness. Among the various inorganic supports used for enzyme immobilization, clays and related materials have potentially interesting properties such as hydrophobic/hydrophilic behavior, electrostatic interactions, and mechanical, chemical and bacterial resistance. Another advantage is the presence of silanol groups that, after activation by different functional groups [41], act as attachment sites for bioactive species. Clays possess high specific surface available between 200 and 800m^2/g. The facility of water dispersion- recuperation has three

kinds of entities: (i) the neutral siloxane surface, (ii) reactive OH groups and (iii) permanent charged sites resulting from isomorphic cationic substitutions. The non-polar portion of larger biological molecules can efficiently bind to this type of surface through Van der Waals forces.

Clays are amorphous, layered (alumino)silicates in which the basic building blocks – SiO_4 tetrahedra and MO_6 octahedra ($M=Al^{3+}$, Mg^{2+}, Fe^{3+}, Fe^{2+}, etc.) – polymerize to form two–dimensional sheets [42]. One of the most commonly used clays is montmorillonite in which each layer is composed of an octahedral sheet sandwiched between two tetrahedral silicate sheets (Fig. 1.4). Typically, the octahedral sheet comprises oxygens attached to Al^{3+} and some lower valence cations such as Mg^{2+}. The overall layer has a net negative charge which is compensated by hydrated cations occupying the interlamellar spaces. Natural montmorillonite (MMT) consisted of layered silicates carrying negative charges that formed ionic bonds with metal cations in interlayer of the clay [43].

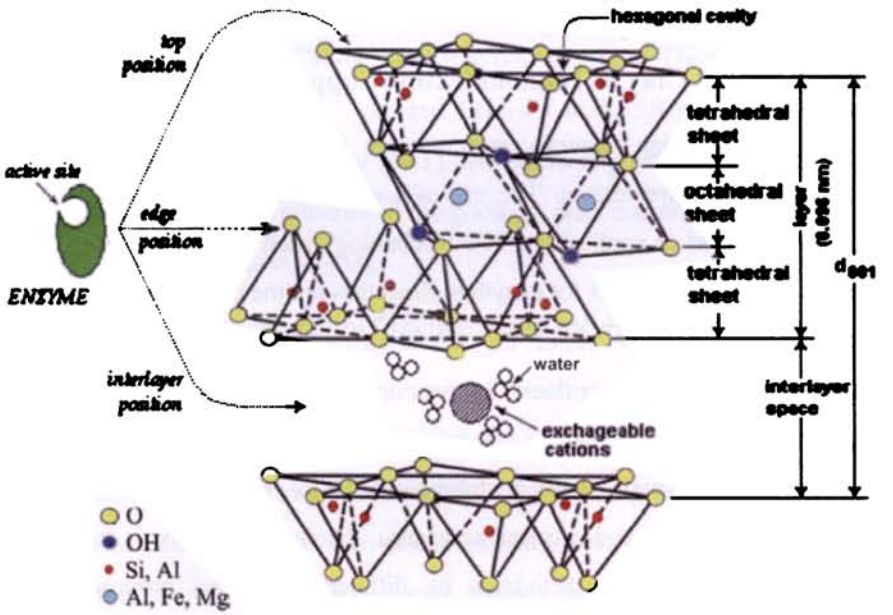


Fig.1.4 Structure of Montmorillonite K-10 clay

Expandable clays such as smectites have a high affinity for protein adsorption. The entrapment of biomolecules within clay matrices constitutes an inexpensive, fast and easy method for the elaboration of enzyme biosensors [44]. Horseradish peroxidase had been successfully immobilized on the Al-PILC and the immobilized enzyme could be applied over a broader range of pH from 4.5 to 9.3 and had better storage stability. However, the reusability of the immobilized enzyme was not very satisfactory [45]. Montmorillonite clay and clay extracted from Elledge Lake basins oil were combined with alkaline phosphatase, glucosidase, protease, and xylosidase solutions to assess adsorption and the effect of this adsorption on enzyme activity [46]. Naidja and Huang [47] found that the large molecules of aspartase (MW 180,000) were intercalated within the montmorillonite layers. Garwood et al. [48] observed an expansion of 35Å for the complex glucoseoxidase (MW 153,000)–Na-montmorillonite. On the otherhand, Harter and Stotzky [49] reported that the adsorption of catalase onto Ca-montmorillonite did not result in expansion of the mineral structure. They concluded that the adsorption is entirely external. It is reported that a high percentage of laccase activity is maintained after its immobilization on clays (kaolinite, montmorillonite) [50]. Fusi et al. [51] reported that montmorillonite may adsorb enzyme molecules on both external and internal surfaces.

In the continuous operation, the covalently bound glucoamylase on montmorillonite clay (K-10) could be used without any loss in activity for 100 h while the adsorbed form lost 5% activity after 84 h [52]. In another work done by Sanjay et al, it is proposed from surface area measurements, that the enzymes α-amylase, glucoamylase and invertase are situated at the periphery of the clay mineral particles whereas the side chains of different amino acid residues penetrate between the layers [53]. The covalently bound invertase on Montmorillonite K-10 resisted leaching even after 15 cycles at higher loadings due to the stronger bond between the grafted groups and the enzyme while the adsorbed systems were prone to leaching [54].

1.11 The immobilization of lipases on mesoporous silica and clay

Immobilized lipases are generally used to perform biotransformations of most interesting industrial applications. Hyperactivation of most lipases is achieved by the hydrophobic surface of the matrix which resembles the interface that induces the conformational change on lipases necessary to enable free access of substrates to their active centers. The majority of the functionalization compounds are hydrophobic organic molecules, which will determine an increase of the hydrophobicity of support surface [55].

Duan et al. [56] achieved the first immobilization of the Porcine Pancreatic lipase (PPL) in the channels of MCM-41 supports, and to prevent leaching of the weakly bound lipase, the mouths of the channels were subsequently reduced by covalent coupling with an organic siloxane. Porcine pancreatic lipase (PPL) has been successfully immobilized in the mesoporous channels of SBA-15 with different pore diameters (6.7, 8.0, and 13.0 nm). The amount of enzyme adsorbed in the channel of the supports is found to be related to the pore diameters of SBA-15 [57]. Two different immobilization techniques were used: physical and chemical adsorption for the immobilization of lipase on SBA-15 and it was found that chemical adsorption was suitable in aqueous solvents, while physical immobilization is sufficient in organic solvents [58]. Lipase from *C. antarctica* B was easily adsorbed onto the hydrophobic surface of silica activated with octyl groups which achieved thermal stabilization and an excellent operational stability shown by the cycles of esterification reactions [59]. Shaker et al. reported the preference of organosilica mesoporous materials compared to pure silica as supports of lipases in tranesterification reaction by immobilizing Rhizopus oryzae lipase (ROL) onto SBA-15 (a pure silica) and PMO (an organosilica with ethane bridging groups) with different structural chemical composition [60]. A conversion of 68% with 100% selectivity for p-chlorobenzyl acetate (transesterification of p-chlorobenzyl alcohol with vinyl acetate) was obtained in 120 min, with CALB being pre-immobilized on Hexagonal mesoporous silica (HMS) and encapsulated

using CA (CALB/HMS/Encap) [61]. Higher activities were obtained for CALB lipase immobilized on the hydrophobized supports of MCM41 (MCM41UM3 (Chlorotrimethoxy silane), PrMCM41 (Propyltrimethoxy silane)) in comparison to CALB covalently retained on amino functionalised MCM41which were evaluated in the alcoholysis of ethyl acetate with two alcohols (1-hexanol and 1-butanol) [62]. He et al. [63] reported marked improvement in the activity of lipase immobilized on vinyl grafted SBA-15 in the hydrolysis of triacetin reaction (an aqueous system) as compared to the unfunctionalized support.

When clay minerals are used as supports for enzymes, different types of binding mechanisms are possible, including ion exchange, Van derWaals interactions, hydrogen bonding, and ion-dipole interactions with metal exchanged ions on the clay surfaces. Concerning the positions of immobilization, the enzymes can be anchored on the external surfaces and the edges of the clay sheets, or intercalated within interlayer space. The hydrophobic properties of clays can be improved by various treatments [64]. The enzymatic activities resulting from the adsorption of *Rhizomucor miehei* lipase and *Candida cylindrace* lipase onto three different phyllosilicates (sepiolite,palygorskite and montmorillonite) were determined [65]. From the studies it was concluded that sepiolite and palygorskite would be useful as supports for immobilisation for proteins of relatively low molecular weight such as Rhizomucor *miehei lipase* for further use in biotransformations, while for *C. cylindracea lipase* the immobilisation onto duolite rendered a derivative specially active in the hydrolysis of ethyl formiate esterasic activity. Immobilization of *Candida rugosa* lipase onto modified and unmodified bentonites was carried out and the effect of hydrophilic or hydrophobic nature of the support, the reuse efficiency, and kinetic behavior of immobilized lipase were studied [66]. The immobilized enzyme exhibited an activity comparable to the free enzyme after storage at 30°C. Natural kaolin was evaluated as a support for the immobilization of lipase from Candida rugosa as biocatalyst for effective esterification [67]. Kaolin immobilized lipase exhibited activities higher by four folds than the native lipase after thermal stability test at 70^0C and was found to be

stable in hexane at room temperature up to 12 days. The catalytic efficiency of lipases, the lipase B from Candida antarctica and lipase from Burkholderia cepacia on beidellite supports and the influence of the Si/Al ratio were studied [68]. Lipase was immobilized onto three different modified palygorskite supports which were modified by acid treating, with 3-aminopropyltriethoxysilane and treating with octodecyl trimethyl ammonium chloride. The P_{APTES} showed the highest enzyme activity and activity recovery in the hydrolysis of olive oil. The enzyme activity and the activity recovery of lipase immobilized on P_{APTES} was 27.24 U/g and 19.43%, respectively [69].

1.12 Mesocellular foams (MCF) and related materials as carriers

The pore size of mesoporous silicas is limited by the dimensions of the micelle templates and, to date, the largest pore size claimed for a well ordered mesoporous molecular sieves is around 10 nm [70]. With this restriction, only relatively small enzymes can be immobilized inside the mesoporous channels of the molecular sieves. The recent discoveries of various mesoporous silicate materials, such as SBA-15 [71] (pore size ca. 50–130 Å) and mesocellular foam [72, 73] (MCF, pore size ca.150–400 Å), provide new avenues for encapsulation/immobilization processes.

The discovery of mesocellular foam (MCF) materials in 1999 allows a much wider choice of enzymes to be studied in this area of research. These materials are prepared using emulsions as templates and the pore size varies from 15 to 40 nm [72]. Unlike MCM-41 and SBA-15, which have two-dimensional mesopore structures, mesocellular silica foams (MCF) is a new class of three-dimensional (3D) hydrothermally robust materials with ultra-large mesopores (up to 50 nm) [72, 73, 74]. In terms of the textural and framework structure, the MCF materials resemble aerogels and are composed of uniform spherical cells interconnected by windows with a narrow size distribution [73]. Especially given its continuous 3D mesopore system with ultralarge pore diameters and interconnected windows, MCF materials have the advantage over their more ordered counterparts such as

MCM-41 or SBA-15 of better diffusion of reactants and products and thus overcome internal mass transfer limitations [75]. These mesopores are large enough to host enzymes and allow an easy transport of substrates into its active site, and importantly, create an environment most favourable for the expression of enzyme activity [76, 77]. A schematic of the strut like structure, given in Figure 1.5, shows the cells of the MCF structure framed by the silica struts.

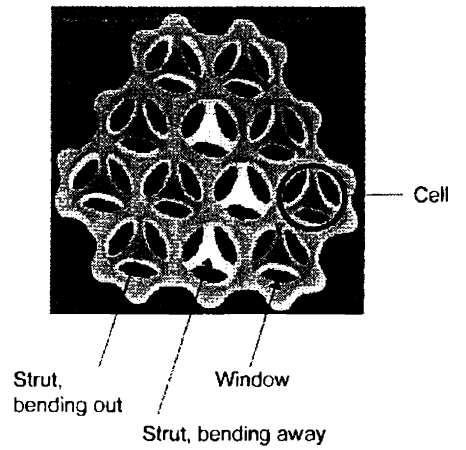


Fig 1.5 Schematic cross section of the strutlike structure exhibited by MCFs.

The epoxy-functionalized mesoporous cellular foams (G-MCFs) with high specific surface area (~400 m^2/g) and large-size mesopores (~17 nm) with mesoporous cellular foams (MCFs) and were used as the support for immobilization of penicillin G acylase (PGA) in the work done by Xue et al.[78]. The immobilized supports could retain about 91.4% of its initial activity after the 10th cycle reuse. Lipase from Candida Antarctica B (CALB) was successfully entrapped in the cage like pores of siliceous mesocellular foam (MCF) using a pressure-driven method [79]. In the work done by Shakeri et al. Rhizopus oryzae lipase (ROL) was immobilized on MCF and alkyl-functionalized MCF (alkyl-MCF) by physical adsorption and found that the transesterification reaction activity of (S)-glycidoland vinyl n-butyrate using ROL immobilized on alkyl-MCF increased with increase in alkyl chain length [80]. The application of siliceous mesostructured cellular foams (MCF) with functionalisation using different

organosilanes to immobilize covalently invertase and glucoamylase was studied by Szyman´ska et al. The glutaraldehyde (GLA)-amino linkage formed by organosilanes with two amino groups was the most effective system for MCF-bound invertase and that formed by APTS in the covalent immobilization of glucoamylase. Activity of MCF-based biocatalysts was significantly higher than of the silica gel and Eupergit C based counterparts [81]. The clear advantage of using functionalized MCFs as supports for enzymes was first demonstrated for organophosphorous hydrolase [82] and most recently confirmed in the hydrolysis of starch using α-amylase [83] and glucose oxidation with glucose oxidase [84].

1.13 Synthesis of mesoporous molecular sieves

(a) Principles of synthesis

The understanding about the synthesis of these materials and the corresponding mechanism has opened up a new era of molecular engineering. The most outstanding feature of the preparation of these materials is the role of the templating agents. The template molecules used are not single solvated organic cations as used in zeolite synthesis, but a self assembled surfactant molecular array around which the main structure is built up. Surfactants are large organic molecules having a long hydrophobic tail of variable length and a hydrophilic head.

(b) Interaction between surfactants and silicate species.

Solubilization of nonionic poly(alkylene oxide) surfactants and block copolymers in aqueous media is due to the association of water molecules with the alkylene oxide moieties through hydrogen bonding [85]. This should be enhanced in acid media where hydronium ions, instead of water molecules, are associated with the alkylene oxygen atoms, thus adding long-range coulombic interactions to the coassembly process. If carried out below the aqueous isoelectric point of silica, cationic silica species will be present as precursors, and the assembly might be expected to proceed through an intermediate of the form $(S^0H^+)(X^-I^+)$. The anion may be coordinated directly to the silicon atom through expansion of the silicon atom's coordination sphere. Our goal in this research was to use this structure directing route

to create highly ordered structures with low cost, non toxic, and biodegradable nonionic organics under relatively dilute aqueous conditions. In the present work, inexpensive sodium silicate has been used to synthesize mesoporous silica instead of expensive teraalkoxy silane. In order to make a comparison, mesoporous silica were also prepared via sol-gel route using tetra ethoxy ortho silane (TEOS), the pore size of which was increased by using o-xylene as a hydrophobic swelling agent.

Pinnavaia et al. [86] used nonionic surfactants to synthesize disordered, worm-like mesoporous silica and alumina under neutral pH conditions, and proposed an S^0I^0 mechanism involving hydrogen-bonding interactions between the surfactant and siloxane species. On the basis of these results, we postulate that the assembly of the mesoporous silica organized by nonionic alkyl-ethyleneoxide surfactants or poly (alkylene oxide) triblock copolymer species in acid media occurs through an $(S^0H^+)(X^-I^+)$ pathway. In the synthesis there are three kinds of charged species in solution: Silica species (I^-/I^+), non-ionic surfactant (S^0) and its counter ion (X^-). Their interactions depend on the silicate oligomeric species present because their charge density is different in the degree of oligomerisation. First, alkoxysilane species are hydrolyzed which is followed by partial oligomerization at the silica. The EO moieties of the surfactant in strong acid media associate with hydronium ions,

$$Si(OEt)_4 + nH_3O^+ \xrightarrow[pH<2]{\text{hydrolysis}} Si(OEt)_{4-n}(OH_2^+) + nEtOH$$

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

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

These charge-associated EO units and the cationic silica species are assembled together by a combination of electrostatic, hydrogen bonding, and van der Waals interactions $REO_{m-y}[(EO),H_3O^-]_y...yX^-...I^-$, which can be designated as $(S^0H^+)(X^-I^+)$. Coordination sphere expansion around the silicon atom by anion (e.g., Cl^-) coordination of the form $X^-.Si\text{-}OH_2^-$ may play an important role. During

the hydrolysis and condensation of the silica species, intermediate mesophases, such as hexagonal, cubic, or lamellar mesostructures, are sometimes observed. Further condensation of the silica species and 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 [87, 88].

Alkaline synthesis of mesoporous silica

In alkaline surfactant-silicate assembly equilibrium can be shifted by the presence of other ionic species in solution. This mean that the salt can effect the screening of the surfactant charge and hence the structure of the assembly. In the synthesis mixture, silicate polymerization can occur either at the surfactant/water interface or in solution. The competition with the X^-anions to bind with micelles depends on the relative binding ability of silicate anion species with respect to counter ion.

Acidic synthesis of mesoporous silica

In acidic media (pH<2), the positively charged silica oligomers cannot combine directly with positive surfactant and for charge balance, there must exist a bridge counter ion (X^-) at the interface of silicas and surfactants. In acidic synthesis, the silica is usually less condensed and the structure order is thus softer and often leads to rich morphologies (such as film, fiber, and gyroid) [89, 90]. In acidic synthesis one can involve the surfactant mesophase first and then use the structure as a template. Because of the weaker surfactant-silicate interaction the original liquid crystalline phase can be preserved when mixing with silica sources.

One of the most unique and useful features of M41S family materials is the ability to tailor the pore diameter (15–100Å). This can be achieved in three different ways; (i) by varying the chain length of alkyl groups (from 8 to 22 atoms) in surfactant molecules [91] (ii) by adding auxiliary chemicals such as 1, 3, 5 trimethylbenzene [91, 87, 92] which dissolve in the hydrophobic region of the micelles, thus increasing their size, or (iii) by aging a sample prepared at a lower temperature in its mother liquor at a higher temperature for different periods of time [93].

1.14 Synthesis of Mesostructured Cellular Foams.

The Mesocellular foams (MCFs) are formed at a pH below the aqueous isoelectric point of silica [88]. The acid catalyzed synthesis of nonordered silica has been known to introduce a large number of residual surface hydroxyl groups [94], which may facilitate further functionalization of the MCFs. The oil-in-water microemulsions [73, 95] templating method has been adopted for the synthesis of MCFs.

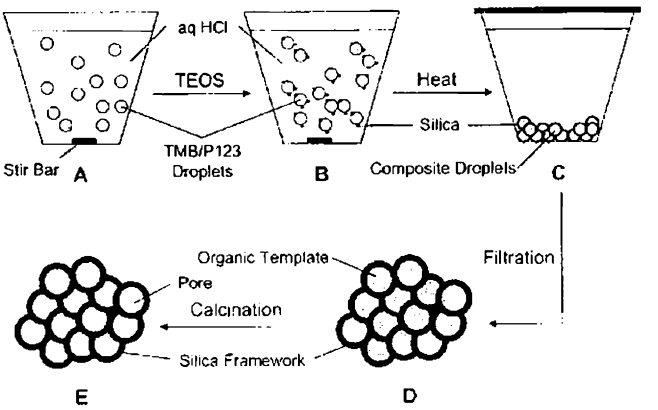


Fig. 1.6 Schematic of the suggested stages of the MCF formation

The easily prepared microemulsion templates consist of water, Pluronic P123 (EO_{20}-PO_{70}-EO_{20}), and 1, 3, 5-trimethylbenzene (TMB) as the organic cosolvent (oil). The nonionic block copolymer surfactant P123 forms expandable aggregates that template periodic SBA-15-type mesoporous silica in acidic media [96], and is well suited for stabilizing oil-in-water and oil-in-formamide emulsions and oil in-water microemulsions [97]. To the oil-in-water microemulsions is added tetraethyl orthosilicate (TEOS) as the source of both silica and ethanol (which acts as a cosurfactant [97]. The cationic silica species may begin to condense and form a "soft" silica coating through hydrogen bonding to the P123-coated TMB droplets around the hydrophobic templates to give composite droplets (Fig 1.6). During aging at elevated temperatures in an autoclave, agglomeration and packing of the composite droplets may occur while the cationic silica species continue to condense. The precipitated

aggregates of composite droplets are filtered, dried, and calcined to burn off the organic templates, thus producing the porous MCF material.

1.15 Formation mechanism of mesocellular silica foams: Hexagonal to Mesocellular Foam Phase Transition

SBA-15 and MCF materials are synthesized with polymer templates and are related by a phase transition from a hexagonally ordered cylindrical mesoporous structure to the mesocellular foam structure. Stucky and co-workers achieved the assembly of mesostructured cellular foams of silica (denoted MCF silicas) with cell dimensions in the range of 24-44 nm and pore volumes of 1.0-2.4 cm^3/g, depending on the composition of the microemulsion template and the reaction conditions [73]. In the low oil concentration regime, the pores were modeled as long straight cylinders (Figure 1.7a) [97, 98]. (Figures 1.7 a-c) show a change in pore morphology in the oil-polymer range of 0.2-0.3. The walls of the one-dimensional cylindrical pores begin to buckle with approximately the same periodicity as the pore diameter, forming spherical nodes down the length of the pores as illustrated in Figure 1.7b. At this point, microemulsion templating takes over, and spherical, TMB-swollen P123 micelles template the formation of the MCFs.

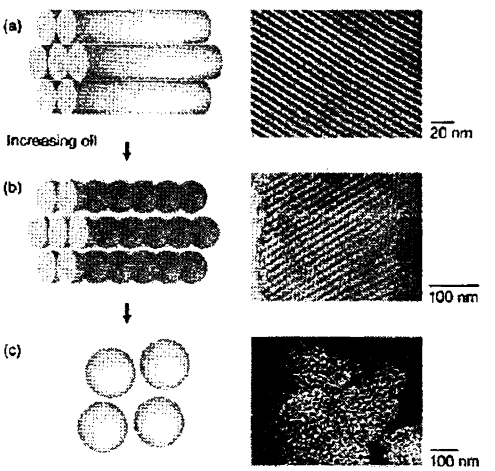


Fig. 1.7 Progression of the morphological transition in P123-templated materials swollen by TMB. The proposed schemes of formation and TEM micrographs of the mesoporous silicas synthesized at oil-polymer mass ratios of **(a)** 0.00, **(b)** 0.21, and **(c)** 0.50 are illustrated

Adding a sufficiently large amount of TMB leads to a phase transformation from the highly ordered *p6mm* mesostructure of SBA-15 type mesoporous silicas to disordered MCFs [95]. SBA-15-type silicas are obtained at TMB/P123 < 0.2, mixed phase silica consisting of domains of SBA-15 and MCF is found at TMB/P123 = 0.2-0.3, and MCFs are synthesized with TMB/P123 > 0.3. The concentration of TMB plays the major role in determining the ultimate structures of the mesoporous silicas obtained from P123/TMB templates. For 0.3 <TMB/P123 ≤ 2.5, the size of the spherical cells can be controlled continuously from 24 to 42 nm by adjusting the amount of TMB added.

1.16 Chemical modification of the carriers

Organic-inorganic composites combine the rigidity of the silica network with the reactivity of organic functional groups. The silanol groups on the surface can react with silane compounds with leaving groups. It is expected that the increase in the density of functional groups while retaining the open space would result in a higher activity. Therefore, high loading of functional groups in mesoporous silicas has received much attention, being reported abundantly [99, 100]. Organic functionalization of silicates permits precise control over the surface properties, modification of the hydrophilic/hydrophobic of the surface, alteration of the surface reactivity, protection of the surface from attack, modification of the bulk properties of the materials and at the same time stabilizing the materials towards hydrolysis.

The organo-functionalization of mesoporous silica can be accomplished by two general routes: (1) the post-modification of silica through the reaction of organosilanes with surface silanol groups, and (2) the co-condensation of organosilanes with the silica precursor during gel formation [101, 102].

In the post-synthesis grafting method, the surface of the pre-fabricated inorganic mesoporous materials is reacted with organosilane coupling reagents through the surface silanol groups [103]. All amino-organic moieties

introduced into the silica are anchored on the surface silanol group by covalent bonds.

Disadvantages of the classical two step method are:

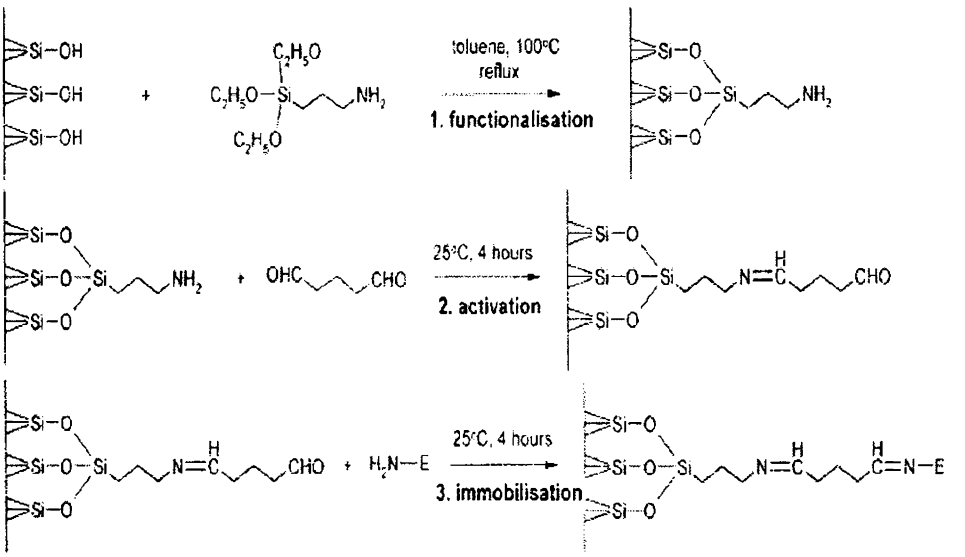
- It is more elaborate because it involves more reaction steps.
- The pore diameter is further decreased during functionalization.
- Advantages of the classical two step method are:
- The templating micelles of the mesoporous material can be removed by calcination. During a calcination already functionalized organic groups would be decomposed and the silica framework becomes more stable.
- Only the surface of the mesoporous material is functionalized.

The co-condensation method, also called "one-pot" synthesis, is currently the most common and direct synthesis route to the introduction of the organic groups into the silica network [104]. The co-condensation approach is preferred because it requires fewer processing steps and the material obtained has a more uniform, controlled distribution of the functional group [105]. Although both synthesis routes have been used to prepare ordered, mesoporous silicagels, the co-condensation process has generally been shown to yield higher degrees of surface modification.

To enhance electrostatic interactions with negatively charged adsorbents, amine functionalization will introduce positive charge to the silica surface which would otherwise be positive. Han et al. [106] showed that the post-synthetic modification of SBA-15 using 3-aminopropyltriethoxysilane could be used to adsorb anionic proteins at pH 7 whereas a carboxy-functionalized surface, which would give a negative surface charge, readily adsorbed proteins that were cationic at the same pH. Subsequent modification of the electrostatic interaction, either by changing pH or strongly increasing the ionic strength, can result in release of the protein back into solution.

The inclusion of functional groups also opens up the possibility to tether molecules covalently. For proteins this can take the form of reactions with amine, carboxylic acid or disulfide bridges. Such immobilization procedures have been developed for protein immobilization [107]. A charged surface can bind proteins with opposite charge and release the adsorbants by changing the pH. For permanent binding, the formation of covalent bonds is the obvious choice. Since, in our view, the advantages of the classical two step method (in particular its higher flexibility) outweigh the disadvantages, this method was chosen for all the functionalization reactions performed in this study.

Immobilization of enzyme was carried out using three-step process [108] as shown in Scheme 1.8. In the first step, alkylamine, 3-aminopropyl triethoxysilane (APTS), was covalently bonded to silanol groups presenton the surface of mesoporous silica using organic silanization technique. In the second step, glutaraldehyde was coupled with amino group of alkylamine through one of the –CHO groups using derivatives of alkylamines and coupling technique. There still will be one –CHO group in glutaraldehyde free to chemically react with $-NH_2$ groups present in enzyme molecules.



Scheme 1.8. Three steps for immobilization of enzyme on mesoporous silicas.

1.17 Immobilization in Organic Solvents

Even today, enzyme immobilization is predominantly performed in aqueous media in which the enzyme is completely soluble. In recent decades there has been interest in developing non-aqueous enzyme immobilization techniques. Early in 1970, Brown and co-workers used poly (4-iodobutyl methacrylate) [109], prepared by halogen exchange of poly (4-chlorobutyl methacrylate) with sodium iodide, to immobilize urease. During this work it was discovered that enzymes could be immobilized not only in buffer but also in organic solvents such as dioxane. The feasibility of immobilizing enzymes in organic solvents such as DMF (hydrophilic or hydrophobic) was studied by the same group [109, 110, 111]. Consequently, it was further established that fixing of enzymes to a mineral, organic, or organomineral support could be performed in anhydrous organic liquids at temperatures from 60 to 120°C. Stark and Holmberg found that the medium used for the immobilization of lipase could have a large effect on the activity of the lipase in organic solvent but not in aqueous reaction medium. In their study, *Rhizopus sp* lipase was immobilized on two types of Celite-based carrier activated with tresylate,i.e. with/or without PEG as spacer, and in three types of media – aqueous, hexane, or micro emulsion of hexane-buffer. Lipase immobilized in buffer had no transesterification activity in the organic solvents. In contrast, lipase immobilized in organic solvent was not only fully active in aqueous medium but also in organic solvent, suggesting that the immobilization chemistry was different in two cases [112]. The activity of lipase immobilized in organic solvents is probably ascribed to enlargement of the active site by binding of the amino acids residues near the active site. The immobilization of lipases in organic solvents can be explained as a result of opening of the lid of the lipases in organic solvents during the immobilization. In subsequent coupling of the enzyme to the carrier, the conformation with the opened lid could be restricted to this open state by binding of the buried amino acids near the active site. In contrast, the lipase immobilized in aqueous buffer via the lysine groups

exposed on the protein surface might have difficulty opening the lid in organic solvents. Moreover, it was demonstrated that introduction of a spacer significantly improved enzyme activity and stability. Immobilization of enzyme in organic solvents also has several other obvious advantages, for example:

- many types of reaction that are not favourable for the binding enzyme in aqueous medium can be used;
- modulation of enzyme activity and structure is possible; and
- polymers that are not water soluble can be dissolved in organic solvent for binding to the enzyme, and such immobilized enzymes can also be used in aqueous media.

1.18 Adsorption of Enzyme on to the Carrier in Organic Solvents

It has recently been reported that *Candida rugosa* lipase (CRL) has been immobilized on poly (styrene–divinylbenzene) in heptane with higher enzyme loading than in aqueous medium. CRL immobilized in organic solvent not only had higher activity in both aqueous media (for hydrolysis) and organic solvent (for synthesis) but also had higher operational stability in organic solvents [113]. Similarly, CRL has been immobilized by physical adsorption on several inorganic supports using hexane as coupling medium [114]. Lipases from Candida cylindracea, Aspergillus niger, and Pseudomonas fluorescens were immobilized by adsorption on anion-exchange resin and diatomaceous earth using buffer or hexane as a reaction medium. Immobilized C. cylindracea preparations were more active when hexane was used as the reaction medium [115]. The solvent used in immobilization has been shown to have a great effect on the applicability of a covalently immobilized *Rhizopus*lipase. Hexane has been used as a medium in the immobilization of *Rhizopus* lipases on Celite [116].

1.19 Objectives and scope of the present work

One of the important areas of nanotechnology is the development of reliable processes for the synthesis of nanomaterials over a range of sizes (with good monodispersity) and chemical composition. The use of layered double hydroxides

has recently extended to applications in biotechnology such as host materials, pesticides wastecarriers and supports for enzymes. Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) are versatile enzymes that can catalyze numerous reactions of interest for several food, chemical and pharmaceutical industries. The major goal of this research is to immobilize industrially important enzymes (lipase and glucosidase) on silica and clay and for further applications of these systems in synthetic organic reactions. Mesostructured cellular foam (MCF) silica with large pores in the range of 20–50 nm has been successfully synthesized using a PEO-PPO-PEO triblock copolymer (Pluronic P123), tetraethyl orthosilicate, and 1, 3, 5-trimethylbenzene (TMB) as an organic template, a silica source, and a swelling agent hydrothermally and via room temperature method. In order to use mesoporous silica as a supporting material in catalysis, mesoporous silica has been modified using organic silanes such as 3-aminopropyl-triethoxysilane (APTES) with a terminal amine group ($-NH_2$) and further with glutaraldehyde.

Our hypothesis was that organically modified mesoporous materials would be a better host than conventional mesoporous sol-gels for lipophilic biomolecules. The aim of this study was to compare the hydrolytic and synthetic activities, biochemical properties and stability of free and immobilized lipase from *Candida rugosa* and compared with commercially available immobilized lipases in silica sol-gel and alumina. The simple but powerful approach of Crosslinked enzyme aggregrates employed for the immobilization of β-glucosidase in mesoporous media opens up a new possibility for enzyme stabilization and will contribute to a variety of enzyme applications in biosensors, bioremediation, and bioconversion. The advent of large pore mesoporous solids, together with the development of routes to functionalize their surfaces to a high degree of sophistication, has suggested these materials as suitably well defined candidates for enzyme supports. The effect of the pore size and volume on the activity and stability of the enzyme were investigated in order to obtain a better understanding of the structure-property relationship. The major objectives of the present work are summarised as,

- To template mesocellular silica foams (MCF) by oil-in-water microemulsion method hydrothermally and via room temperature.
- To immobilize Candida Rugosa lipase onto Montmorillonite K-10 and mesocellular siliceous silica foams (MCF) via two independent techniques namely simple adsorption and covalent binding after functionalization with aminopropyl triethoxy silane and using glutaraldehyde spacer.
- To characterize the pure supports and immobilized enzymes via various physico-chemical techniques like XRD, FTIR, NMR, CHN, thermal analysis, surface area measurements, SEM, TEM and contact angle measurements.
- To synthesize butyl isobutyrate from n-butanol and isobutyric acid including the screening of various immobilized lipases and optimization of reaction conditions such as catalyst loading, effect of mole ratio and temperature.
- To investigate the enzymatic transesterification of ethyl butyrate to butyl butyrate in non-aqueous media using free and immobilized lipases
- To determine the kinetic parameters for esterification and transesterification reaction using the Ping-Pong Bi-Bi mechanism with substrate inhibition and to represent it by the Lineweaver and Burk plot.
- To assay and compare the properties of the free and the immobilized lipases using the hydrolysis reaction of p-nitrophenyl palmitate in aqueous and organic media. To study the effects of protein concentration, pH, temperature, activity and stability of the immobilized lipases.
- To compare the immobilized forms with respect to its catalytic properties and also with conventional supports.
- To study the reusability and storage stability characteristics of these immobilized systems with respect to free enzyme.

- To develop crosslinked linked enzyme aggregrates (CLEA's) of β-glucosidase in mesocellular silica foams (MCF) and to evaluate the reusability and stability of these systems with the adsorbed and covalently bound ones.

References

[1] P. Monsan, D. Combes, Methods Enzymol., 137 (1988) 584.

[2] C. Mateo, R. Torres, Biomacromol., 4(3) (2003) 772.

[3] W. Tischer, F. Wedekind "Immobilized enzymes: Methods and applications. Biocatalysis - from Discovery to Application (1999) 200, 95.

[4] D. Bezbradica, D. Mijin, S. Siler-Marinkovic, Z. Knezevic, J. Mol. Catal.B: Enzym., 38 (2006) 11.

[5] M. I. Kim, H.O. Ham, S. D. Oh, H. G. Park, H. N. Chang, S. H. Choi, J. Mol. Catal. B: Enzymatic., 39 (2006) 62.

[6] K. Drauz, H. Waldmann, editors. Enzyme catalysis in organic synthesis. A comprehensive handbook, vol. 1. Weinheim: VCH (1995).

[7] Basso, L. De Martin, C. Ebert, L. Cardossi, P. Linda, V. Zlatev, J Mol. Catal. B: Enzym., 11 (2001) 851.

[8] D. B Sarney, E. N. Vulfson, TIBTECH., 11 (1995) 64. O. Kirk, T. V. Borchert, C.C. Fuglsang, Curr. Opin. Biotechnol., 13 (2002) 345.

[9] O. Kirk, T.V. Borchert, C.C. Fuglsand, Curr. Opin. Biotechnol., 13 (2002) 345.

[10] K. Lohith, S. Divakar, J. Biotechnol., 117 (2005) 49.

[11] K. E. Jaeger, B. W. Dijkstra, M. T. Reetz, Ann. Rev. Microbiol., 53 (1999) 315.

[12] L. Wilson, J. M. Palomo, G. Fernandez-Lorente, A. Illanes, J. M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol., 39 (2006) 259.

[13] Z. S. Derewenda, U. Derewenda, J. Mol. Biol., 227 (1992) 818.

[14] K. Fabber, Biotransformations in Organic Chemistry: AText book, Springer Productions-Gesellschaft, Berlin (1997) (Chapters 1, 2 and 3).

[15] M. D. Virto, I. Agud, S. Montero, A. Blanco, R. Solozabal, J. M. Lascary, Enzyme Microb. Technol., 16 (1994) 61.

[16] G. Kunkova, J. Szilva, J. Hetflejs, S. Sabata, J. Sol–Gel Sci. Technol., 26 (2003) 1183.

[17] J. Vakhlu, A. Kour, Eletronica J. Biotechnol., 9 (2006) 69.

[18] D. Bezbradica, D. Mijin, S. Siler-Marinkovic, Z. Knezevic, J. Mol. Catal.B: Enzym., 38 (2006) 11.

[19] S. A. Camperi, M. Grasselli, Process Biochem., 39 (8) (2004) 1017.

[20] J. Tharakan, F. Highsmith, J. Chromatogr., 595 (1-2) (1992) 103.

[21] K. S. W. Sing, D. H. Everett, R. H. W Haul, L. Moscou, R. A Pierotti, J. Rouquerol, T. Siemieniewska, Pure Appl. Chem., 57 (1985) 603.

[22] D. Y. Zhao, Q. S. Huo, J. L. Feng, B. F. Chmelka, G. D. Stucky, J. Am. Chem. Soc., 120 (1998) 6024.

[23] D. J. Macquarrie, D. B. Jackson, J. E. G. Mdoe, J. H. Clark, New J. Chem., 23 (1999) 539.

[24] Y. Wie, J. Xu, Q. Feng, H. Dong, M. Lin, Mater. Lett., 44 (2000) 6.

[25] X. S. Zhao, G. Q. Lu, A. K. Whittaker, G. J. Millar, H. Y. Zhu, J. Phys. Chem. B., 101 (1997) 6525.

[26] X. S. Zhao, G. Q. Lu, J. Phys. Chem. B., 102 (1998) 1556.

[27] T. Yanagisawa, Bull. Chem. Soc. Jpn., 63 (1990) 988.

[28] T. Kresge, Nature, 359 (1992) 710.

[29] Zhao, Q. Huo, J. Feng, B. F. Chmelka, G. D. Stucky, J. Am. Chem. Soc., 120 (1998) 6024.

[30] P. Schmidt-Winkel, W. W. Lukens, Jr., P. Yang, D. I. Margolese, J. S. Lettow, J. Y. Ying, G. D. Stucky, Chem. Mater., 12 (2000) 686.

[31] Takimoto, T. Shiomi, K. Ino, T. Tsunoda, A. Kawai, F. Mizukami, K. Sakaguchi, Microporous Mesoporous Mater., 116 (2008) 601.

[32] Vinu, V. Murugesan, O. Tangermann, M. Hartmann, Chem. Mater., 16 (2004) 3056.

[33] Deere, E. Magner, J. Gerard Wall, B. K. Hodnett, J. Phys. Chem. B., 106 (2002) 7340.

[34] H. Takahashi, B. Li, T. Sasaki, C. Miyazaki, T. Kajino, S. Inagaki, Chem. Mater.,12 (2000) 3301.

[35] Y. Han, J. T. Watson, G. D. Stucky, A. Butler, J. Mol. Catal. B: Enzym., 17 (2002) 1.

[36] J .Deere, E. Magner, J. G. Wall, B. K. Hodnett, Catal. Lett., 85 (2003) 19.

[37] S. Ajitha, S. Sugunan, Tuning mesoporous molecular sieve SBA-15 for the immobilization of α-amylase, J. Porous Mater (In press).

[38] P. Wang, S. Dai, S. D. Waezsada, Alice Y. Tsao, B. H. Davison., Biotechnol. Bioeng., 74 (2001) 249.

[39] H. H. P. Yiu, P. A. Wright, Nigel P. Botting., J. Mol. Catal B: Enzym.,. 15 (2001) 81.

[40] S. Hudson, E. Magner, J. Cooney, B. Kieran Hodnett, J. Phys. Chem. B., 109 (2005) 19496.

[41] J. C. Dai, J.T. Huang, Appl. Clay Sci., 15 (1999) 51.

[42] P. Laszlo, Science, (1987) 235.

[43] M. Ogawa, Y. Takizawa, Chem. Mater., 11 (1999) 30.

[44] S. Poyard, N. Jaffrezic-Renault, C. Martelet, J. L. Besombes, S. Cosnier, P. Labbe, Sens. Actuators B., 33 (1996) 44.

[45] J. Cheng, S. Ming Yu, P Zuo, WAT E R RE S E A R C H, 40 (2006) 283.

[46] T. Tietjen, G. W. Robert, Aquat. Ecol., 37 (2003) 331.

[47] Naidja, P. M. Huang, J. Mol. Catal. A: Chem., 106 (1996) 255.

[48] G. A. Garwood, M. M. Mortland, T. J. Pinnavaia, J. Mol. Catal., 22 (1983) 153.

[49] R. D. Harter, G. Stotzky, Soil Sci. Soc. Am. Proc., 37 (1973) 116.

[50] E. Dodor, H. M. Hwang, S. I. N. Erkunwe, Enzyme Microb. Technol., 35 (2004) 210.

[51] P. Fusi, G. G. Ristori, L. Calamai, G. Stotzky, Soil Biol. Biochem., 21 (1989) 911.

[52] G. Sanjay, S. Sugunan, Catal. Commun., 6 (2005) 525.

[53] S. Gopinath, S. Sugunan, Applied Clay Sci., 35 (2007) 67.

[54] G. Sanjay, S. Sugunan, Catal. Commun., 6 (2005) 81.

[55] J. P. Lindsay, R. F. Wormsbecher, J. S. Dordick, Surface hydrophobicity of silica supports effects activity of immobilized enzymes in organic reactions, The 225th ACS National Meeting, New Orleans, LA (2003) March 23–27.

[56] H. Ma, J. He, D. G. Evans, X. Duan, J. Mol. Catal. B: Enzym., 30 (2004) 209.

[57] Y. Kang, J. He, X. Guo, Xin Guo, Z. Song, Ind. Eng. Chem. Res., 46 (2007) 4474.

[58] Salis, D. Meloni, S. Ligas, M. F. Casula, M. Monduzzi, V. Solinas, E. Dumitriu, Langmuir, 21 (2005) 5511.

[59] R. M. Blanco, P. Terreros, M. Fernández-Pérez, C. Otero, G. D´ıaz-González, J. Mol. Catal. B: Enzym., 30 (2004) 83.

[60] M. Shakeri, K. Kawakami, Catal. Commun., 10 (2008) 165.

[61] G. D. Yadav, S. R. Jadhav, Microporous Mesoporous Mater., 86 (2005) 215.

[62] Dragoi, E. Dumitriu, Acta Chim. Slov., 55 (2008) 277.

[63] J. He, Y. Xu, H. Ma, D.G. Evans, Z. Wang, X. Duan, Microporous Mesoporous Mater., 94 (2006) 29.

[64] S. A. Boyd, M. M. Mortland, J. Mol. Cat., 34 (1986) l.

[65] E. de Fuentes, C. A.Viseras, D. Ubiali, M. Terreni, A. R. Alcantara, J. Mol. Catal. B: Enzym., 11 (2001) 657.

[66] Y. Yesiloglu, Process Biochem., 40 (2005) 2155.

[67] M. B. Abdul Rahman, S. M. Tajudin, M. Z. Hussein, R. N. Z. R. Abdul Rahman, A. B. Salleh, M. Basri, Appl. Clay Sci., 29 (2005) 111.

[68] Secundo, J. Miehe´-Brendle´, C. Chelaru, E. E. Ferrandi, E. Dumitriu, Micropor. Mesopor. Mater., 109 (2008) 350.

[69] Huang, Y. Liu, X. Wang, J. Mol. Catal. B: Enzym., 55 (2008) 49.

[70] M. Kruk, M. Jaroniec, J. Phys. Chem. B., 106 (2002) 4732.

[71] Flodstrom, C. V. Teixeira, H. Amenitsch, V. Alfredsson, M. Linden, Langmuir, 20 (2004) 4885.

[72] J. S. Lettow, Y. H. Han, P. Schmide-Winkel, P. Yang, D. Zhao, G. D. Stucky, J. Y. Ying, Langmuir, 16 (2000) 8291.

[73] P. Schmidt-Winkel, P. P. Lukens, D. Zhao, P. Yang, B. F. Chmelka, G. D. Stucky, J. Am. Chem. Soc., 121 (1999) 254.

[74] D.T. On, S. Kaliaguine, J. Am. Chem. Soc. 125 (2003) 618.

[75] Y. M. Liu, Y. Cao, K. K Zhu, S. R Yan, W. L. Dai, H. Y He, K. N. Fan, Chem. Commun., (2002) 2832.

[76] Lei, Y. Shin, J. Liu, E. J. Ackerman, J. Am. Chem. Soc., 124 (2002) 11242.

[77] K. Szymanska, J. Bryjak, J. Mrowiec-Białon, A. B. Jarzebski, Micropor.Mesopor. Mater., 99 (2007) 167.

[78] P. Xue, F. Xu, L. Xu, Appl. Surf. Sci., 255 (2008) 1625.

[79] Y. Han, S. S. Lee, J.Y. Ying, Chem. Mater., 18 (2006) 643.

[80] M. Shakeri, K. Kawakami, Micropor.Mesopor. Mater., 118 (2008) 115.

[81] K. Szymanska, J. Bryjak, J. Mrowiec-Białon', A. B. Jarze, Micropor. Mesopor. Mater., 99 (2007) 167.

[82] C. Lei, Y. Shin, J. Liu, E. J. Ackerman, J. Am. Chem. Soc., 124 (2002) 11242.

[83] H. Pandya, R. V. Jasra, B. L. Newalkar, P. N. Bhatt, Microporous Mesoporous Mater., 77 (2005) 67.

[84] X. Zhang, R. F. Guan, D. Q. Wu, K. Y. Chan, J. Mol. Catal. B., 33 (2005) 43.

[85] B. Chu, Z. Zhou, 'In Nonionic Surfactants: Polyoxyalkylene Block Copolymers; Nace', V. M., Ed.; Surface Science Series Vol. 60; Marcel Dekker, New York (1996).

[86] Prouzet, T. J. Pinnavaia, Angew. Chem., Int. Ed. Engl., 36 (1997) 516.

[87] Q. Huo, D. I. Margolese, U .Ciesla, D.G. Demuth, P. Feng, T. E. Gier, P.Sieger, A. Firouzi, B. Chmelka. F, Schuth F, G. D. Stucky, Chem. Mater., 6 (1994) 1176.

[88] Q. Huo, D. Margolese, P. Ciesiafeng, T. E. Gier, P. Sieger, R. Leon, P. M. Petroff, F. Schuth, G. D. Stucky, Nature, 368 (1994) 317.

[89] P. J. Bruinsma, A.Y. Kim, J. Liu, S. Baskaran, Chem. Mater., 9 (1996) 589.

[90] H.Yang, N. Coombs, I. Sokolov, Nature, 38 (1996) 589.

[91] C. T. Kresge, M. E. Leonowicz, W. J. Roth, J. C. Vartuli, U.S. Patent, 5098, 684 (1992).

[92] J.S. Beck, U.S. Patent, 5. 057, 296 (1991).

[93] D. Khushalani, A. Kuperman, G. A. Ozin, K. Tanaka, J. Garcés, M. M. Olken, N. Coombs, Adv. Mater., 7 (1995) 842.

[94] C. J. Brinker, G. W. Scherer, In Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing; Academic Press: San Diego (1990).

[95] P. Schmidt-Winkel, C. J. Glinka, G. D. Stucky, Langmuir, 16 (2) (2000) 356.

[96] D. Zhao, P. Yang, N. Melosh, J.Feng, B. F. Chmelka, G. D. Stucky,. Adv. Mater., 10 (1998) 1380.

[97] W. W. Lukens, Jr.; P. Schmidt-Winkel, D. Zhao, J. Feng, G. D. Stucky,. Langmuir, 15 (1999) 5403.

[98] J. C. P. Broekhoff, J. H. de Boer, J. Catal., 9 (1967) 8.

[99] L. Beaudet, K. Z. Hossain, L. Mercier, Chem. Mater., 15 (2003) 327.

[100] Y. Mori, T. J. Pinnavaia, Chem. Mater., 13 (2001) 2173.

[101] A.B. Bourlinos, T. Karakostas, D. Petridis, J. Phys. Chem. B., 107 (2003) 920.

[102] A. Munoz, A. Ramila, J. Pe´rez-Pariente, I. Dı´az , M. Vallet-Regı´, Chem. Mater., 15 (2003) 500.

[103] X. Feng, G. E. Fryxell, L. Q. Wang, A. Y. Kim, J. Liu, K. M. Kemner, Science, 276 (1997) 5314.

[104] M. Bambrough, R. C. Slade, R. T. Williams, S. L. Burkett, S. D. Sims, S. J. Mann, Colloid Interface Sci., 201 (1998) 220.

[105] S. L. Burkett, S. D. Sims, Chem. Comm., 11 (1996) 1367.

[106] Y. J. Han, G. D. Stucky, A. Butler, J. Am. Chem. Soc., 121 (1999) 9897.

[107] H. H. Weetall, Appl. Biochem. Biotechnol., 41 (1993) 157.

[108] K. Mosbach, Methods in Enzymology, vol. XLIV, (1976) p. 134.

[109] E. Brown, A. Racois, H. Gueniffey, Tetrahedron Lett., 25 (1970) 2139.

[110] J. Bartling, H. D. Brown, S. K. Chattopadhyay, Nature, 243 (1973) 342.

[111] J. Bartling, H. D. Brown, S. K. Chattopadhyay, Biotechnol Bioeng., 14 (1974) 361.

[112] B. Stark, K. Holmberg, Biotechnol Bioeng., 34 (1989) 942.

[113] P. C. De Oliveira, G. M. Alves, H. F. de Castro, Biochem Eng J., 5 (2000) 63.

[114] F. Castro, M. L. C. P. Silva, G. L. J. P. Silva, Brazilian. J. Chem. Eng., 17 (2000) 849.

[115] A. Mustranta, P. Forssell, K. Poutanen, Enzyme Microb. Technol., 15 (1993) 133.

[116] B. Stark, K. Holmberg, Biotechnol. Bioeng., 34 (1989) 942.

Chapter 2

EXPERIMENTAL

Contents

Catalysis is still very much a 'black box'. Discipline and catalyst characterization tools help us look inside this box. Work in surface science has led to the development of novel concepts and spectroscopic techniques which have had some impact in catalysis. Characterization is done on several levels: On the first, the macroscopic level (the reactor level), engineers search for the optimal formulation and operating conditions of the catalytic process. The second, the mesoscopic level, includes surface analysis and temperature-programmed techniques (in heterogeneous catalysis as well as kinetic studies, with the aim of finding composition/activity and structure/activity relationships. Depending on the nature of the material being investigated, a suite of techniques may be utilized to assess its structure and properties. Whereas some techniques are qualitative, such as providing an image of a surface, others yield quantitative information such as the relative concentrations of atoms that comprise the material. Recent technological advances have allowed materials scientists to accomplish something that was once thought to be impossible: to obtain actual two-dimensional/three dimensional images of atomic positions in a solid, in real time.

2.1 Introduction

Characterization is an important field in catalysis. Spectroscopy and microscopy is the fastest-growing area in catalyst characterization. Using a host of methods (and acronyms), samples are bombarded with photons, electrons, and ions, giving chemists large amounts of data on what is happening at the catalyst surface. Three principal types of problems may be distinguished in the application of molecular spectroscopic techniques in surface chemistry, namely (i) the characterization of the surface, (ii) the estimation of the type and structures of surface compounds, and (iii) the obtaining of information required to understand the mechanisms of the processes proceeding on the surface of a solid. The primary objective of this chapter is to provide a practical description of the methods used to characterize a broad range of materials.

Physicochemical Characterization

The materials can be characterized by various techniques, which provide important information for the understanding of different physicochemical features. The most extensively used techniques can be categorized into the following:

a) X-ray diffraction (XRD)

b) Nitrogen adsorption measurements

c) Nuclear magnetic resonance spectroscopy (NMR)

d) Scanning electron microscopy (SEM)

e) Transmission electron microscopy (TEM)

f) Fourier transform infrared (FTIR) spectroscopy

g) Thermal Analysis

2.2 Chemicals and reagents used

All chemicals and reagents were used as received without further purification.

Chemicals	Company
Tetra Ethyl Ortho Silicate	Sigma Aldrich Chemicals, Bangalore
Aminopropyl triethoxy silane, Glutaraldehyde	Sigma Aldrich Chemicals, Bangalore
Poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide), Pluronic P123	Sigma Aldrich Chemicals, Bangalore
Trimethyl benzene	Sigma Aldrich Chemicals
Cetyl trimethyl ammonium bromide (CTMABr)	Merck
Disodium hydrogen phosphate, sodium potassium tartarate	Merck
Sodium acetate, sodium carbonate	SRL Chemicals
Commercial C. rugosa lipase (Type VII)	Sigma Aldrich Chemicals, Bangalore
p-nitrophenyl palmitate (p-NPP)	Sigma Aldrich Chemicals, Bangalore
Gum arabic and Triton X-100	Sigma Aldrich Chemicals, Bangalore
β-glucosidase from almonds (3.4 Unit/mg),	Sigma Aldrich Chemicals, Bangalore
p-nitrophenylβ-D-glucopyranoside (PNPG)	Sigma Aldrich Chemicals, Bangalore
Isobutyric acid, ethyl butyrate, n-heptane	Sigma Aldrich Chemicals, Bangalore
LiCl, $MgCl_2$, $Mg(NO_3)_2$, NaCl and KNO_3, calcium chloride ($CaCl_2$),	SD fine chemicals, Mumbai
Magnesium chloride ($MgCl_2$), Manganese chloride ($MnCl_2$), Ethylenediamine tetraacetic acid (EDTA), mercury chloride ($HgCl_2$), cobalt chloride ($CoCl_2$)	Sisco Research Labs Ltd., Mumbai
Iso-octane, toluene, dioxane, molecular sieve MS 4A, methanol, dimethyl sulfoxide, Acetone, ethanol, benzene	S.d. Fine Chemicals Ltd., Mumbai.
n-butanol, hexane, 2-propanol	Merck
Hydrochloric acid	Merck

2.3 Preparation of Silica Supports

2.3.1 One pot room temperature synthesis of mesocellular silica foams (MCF) by emulsion mediated method

An aqueous solution was used as a continuous phase and mesitylene or a monodisperse polystyrene suspension was used as a dispersed phase [1]. Cetyl trimethylammonium bromide (CTAB) was used as surfactant and tetraethyl orthosilicate (TEOS) as a silica source.

First, CTAB was dissolved in water under stirring, and then the required amount of hydrochloric acid was added to this stirred solution to give a colourless solution. Finally, a mixture of mesitylene and tetraethyl orthosilicate was added slowly for a period of 30 min under stirring at a fixed rpm. During addition, the mixture turned opaque. The whole mixture was then stirred for 1 h using a mechanical stirrer. The mixture was kept at room temperature over night. The separated solids were washed with de-ionised water several times and dried at 40°C for 6 h. These dried materials were calcined at 650°C for 10 h in air.

2.3.2 Mesostructured cellular foam (MCF) prepared via microemulsion templating route (hydrothermal synthesis)

The synthesis of MCF was done by following a protocol of Winkel et al. [2]. The MCFs are prepared in aqueous hydrochloric acid using dilute solutions of the nonionic block copolymer surfactant Pluronic P123 (poly (ethylene oxide)-block-poly (propylene oxide)-block-poly (ethylene oxide), EO_{20}-PO_{70}-EO_{20}, M_{av}=5800) with 1, 3 ,5-trimethylbenzene (TMB)as the organic swelling agent. To control the pore sizes in the MCFs, the TMB concentration is varied while the amount of P123 is kept constant. In a typical preparation, P123 (2.0g, 0.4 mmol) is dissolved in 1.6 M HCl (75 mL, 120 mmol HCl) at room temperature while being stirred in a beaker covered with a watch glass. TMB (2.0g, 17 mmol) and the mixture is heated to 37-40°C. Following 0-65min of stirring, tetraethyl orthosilicate (TEOS: 4.4g, 21 mmol) is added. After 20h at 37-40°C, the milky reaction mixture is transferred to an autoclave and aged at 100 or 120°C for 24 h under static conditions. The

mixture is then allowed to cool to room temperature, and the white precipitate is isolated by filtration, dried in air for at least 2 days, and calcined at 500°C for 8 h in air to produce the MCF materials.

2.3.3 Functionalisation of the supports

The surface of silica and clay samples was amino functionalised by reacting 1 g of solid with 50ml of a 2.2 mmol solution of Amino propyltriethoxy silane (APTES) in molecular sieve 4A dried toluene (v/v) at 373K for 6h with stirring [3]. Products were separated by filtration, washed with dry toluene and methanol. It was also washed with dichloromethane by Soxhlet extraction for 18h and dried at ambient temperature. Following this, 1g of amino functionalized support was reacted with 25ml of a 2.5% solution of glutaraldehyde in phosphate buffer (pH = 6.62) for 4h at room temperature. The product was washed exhaustively with distilled water till all excess glutaraldehyde was removed which was tested by Tollen's reagent method and then dried at room temperature.

2.3.4 Lipase immobilization by adsorption

(a) In non-aqueous media for synthetic reactions

Lipase was immobilised by adsorption onto silica and clay using aqueous and non-aqueous coupling media. The method followed by Oliveira et al. was adopted for the immobilization in heptane [4].

In non-aqueous environment, 1 g of silica and clay were mixed with 250 and 170 mg of powder lipase. The coupling took place under low stirring for 16 h at room temperature. Both the derivatives were filtered and washed with sodium phosphate buffer (20 ml, pH 7.0) in order to assay the non-adsorbed lipase and dried at room temperature and kept at 4°C for further use.

(b) In aqueous medium

In aqueous environment, the enzyme was dissolved in 100 mM sodium phosphate buffer (4mg/ml, pH 7.0) and mixed with 100mg of the support. The immobilization process was carried out at 4°C in a shaking water bath for 16h

under low stirring at room temperature. After this, the supports were thoroughly washed with PBS (0.1M, pH 7), dried at room temperature and held at 4°C for further use.

Protein assay

Protein was determined according to Lowry et al. [5] using bovine serum albumin (BSA) as a standard. The amount of bound protein was determined indirectly by the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein in the filtrates. The immobilization capacity of the protein on the support was defined as the amount of protein (mg) per gram of the support.

2.3.5 Activity yield, Immobilization Yield, Catalytic efficiency and effectiveness factor

Immobilization yield (IY) was calculated by measuring the difference between protein concentration on the supernatant before (Ct_0) and after (Ct_f) immobilization [6], according to the following equation:

$$IY(\%) = \frac{Ct_0 - Ct_f}{Ct_0}$$

The amount of protein in the enzyme solution and in the elution solutions was determined by the Lowry method [5], and the amount of protein (p) bound on the carriers was calculated from the formula

$$p = \frac{(C_i - C_f)V}{W}$$

where p is the amount of bound enzyme onto carriers (mg/g), C_i and C_f are the concentrations of the enzyme protein initial and final in the reaction medium (mg/ml), V is the volume of the reaction medium (ml), W is the weight of the carriers (g).

The catalytic efficiency of an enzyme is determined by maximum specific constant K_{cat}/K_m ($M^{-1}s^{-1}$).The values ranges from 10^5 $M^{-1}s^{-1}$ to 10^9 $M^{-1}s^{-1}$ and cluster

around $10^7 s^{-1}$ which is a common factor for most hydroxylases. It reflects both the affinity and catalytic ability for comparing different enzymes against each other.

The effectiveness factor (EF) was used as a comparison parameter for the immobilized system. The formula for 'EF' [7, 8] is given by

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

If the value of 'EF'$\leq$ 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 [9].

The activity yield (AY%), immobilization yield (IY%) and active units per gram of support (U/g) are defined as follows: $AY\% = U_{ads}/U_{eq} \times 100$; $IY\% = U_{act}/U_{ads} \times 100$, where U_{eq} are the equilibrated enzyme units, U_{ads} are the adsorbed units measured as the difference between U_{eq} and units remaining in the supernatant, and U_{act} is the active adsorbed activity.

The relative activity (R_a) of the free and immobilized lipase was calculated according to the formula:

$$R_a(\%) = \frac{A}{A_m} \times 100$$

where R_a is the relative activity of CRL (%); A is the activity of the immobilized enzyme (Ug^{-1}); A_m is the activity of the free enzyme in solution ($U\ g^{-1}$).

Activity assays of free and immobilized lipase

2.3.6 Effect of pH on the activity and stability of free and immobilized lipase

The activity of free and the immobilized enzymes was investigated within the pH range of 6.0–10.0 with the corresponding substrate as described in the activity studies. The effect of pH on lipase (β-glucosidase) stability was determined by incubating the lipase fraction in various buffer solutions ranging from 4.0 to 9.0 for 15, 30, 60 and 120 min at 25°C. After the incubation period, enzyme activity

was assayed spectrophotometrically at 410 nm using *p*-NPP (1.65mM and pH 8.0 in the case of lipase and 0.9ml of 5mM *p*-nitrophenyl β-D-glucopyranoside substrate (PNPG) pH 4.8 in the case of β-glucosidase) as substrate.

2.3.7 Effect of temperature on the activity and stability of lipase

The temperature dependence of enzyme activity was measured by equilibrating the substrate at 20°C, 25°C, 30°C, 35°, 40°C, 45° C, 55°C, 60°C, 65°C, and 70°C for 30 min. The thermostability of the free and immobilized lipase was studied by incubating the enzyme extract at 50 and 55°C for 30, or 60 and 120 min. At the end of the incubation period the enzyme was rapidly cooled, and the remaining enzyme activity was assayed.

2.3.8 Effect of various metal ions on enzyme activity and stability

Aliquots of the enzyme were incubated with equal volumes of the various metal ions at various concentrations at 25°C for 30 min. Appropriate blanks and control were performed for each chemical tested. The residual lipase activity after the incubation period was assayed with the corresponding substrate and the results were expressed as percentage of the activity obtained without the chemical agent.

2.3.9 Enzyme activity and stability in organic solvents

The activity and stability of free and immobilized enzymes in various organic solvents were determined using a modified version of the method by Sztajer et al. [10]. One hundred microliter aliquots of the enzyme was absorbed on a 3mm disc paper, and incubated at 25°C for 1 h in the presence of 1.4 ml of the following water immiscible organic solvents, benzene (log P = 2.0), toluene (log P = 2.5), hexane (log P = 3.5), n-heptane (log P = 4.0) or isooctane (log P = 4.5). After the incubation period the filter papers were removed, the solvents were evaporated off, and the filter papers were incubated in 25mM phosphate (pH 7.8) overnight at 4°C on a shaker. For the water-miscible organic solvents, i.e., dimethyl sulfoxide (log P =−1.35), methanol (log P =−0.76), iso-propanol (log P =−0.28), and acetone (log P =−0.23) the extract was incubated directly with the solvent at 25°C for 30 min. The residual activity was

measured spectrophotometrically using *p*-NPP (1.65mM and pH 8.0 in the case of lipase and 0.9ml of 5mM *p*-nitrophenyl β-D-glucopyranoside substrate (PNPG) pH 4.8) as substrate.

2.3.10. Kinetics analysis

Michaelis–Menten (M–M) model (Eq. (1)) was used to describe the dependence of enzyme activity on substrate concentration for the immobilized lipases.

$$V = \frac{v_{max} S}{K_m + S} \qquad \text{.....(1)}$$

where V_{max} is the highest possible specific lipase activity (U/mg protein), and K_m is the half-saturation constant (mM) determined from substrate concentration that gives a specific lipase activity of ½ V_{max}. The two kinetic parameters (V_{max} and K_m) of M–M model were evaluated using the Lineweaver–Burk plot by plotting double reciprocals of initial velocity versus their corresponding substrate concentrations and linear regression with Graph Pad prism software. The catalytic efficiency (V_{max}/K_m) for the hydrolysis of the *p*-NPP or PNPG substrate was also determined. The assay of kinetic study was carried out using different concentrations of *p*-NPP (0.2-1.5mM) and *p*-nitrophenyl β-D-glucopyranoside (0.25–2.5mM).

2.3.11 Reusability stability

The reusability stability was tested by repeated batch experiments using the method for activity determination. The initial activity of freshly prepared support in the first operation was defined as 100% activity.

2.3.12 Storage stability

This experiment was conducted to determine the stabilities of free and immobilized enzyme preparations after storage in phosphate buffer (100mM, pH 7.0 in the case of lipase /0.1M pH 4.8 for β-glucosidase) at 4°C for 40 days. The residual activities were then determined and activity of each preparation was expressed as a percentage of its residual activity compared to the initial activity.

2.4 Notation of Catalysts synthesized

The pure silica as well as the functionalized supports synthesized for the present work is noted below.

Notation	Catalyst
MCF160	Hydrothermally synthesized silica
MHSG	Covalently bound silica
MCF-35	Silica prepared via room temperature
MTSG	Covalently bound silica
K-10G	Covalently bound clay
MHI	Enzyme adsorbed on MCF-160
MTI	Enzyme adsorbed on MCF-35
KI-10	Enzyme adsorbed on montmorillonite

2.5 Material Characterization

Spectroscopy, microscopy, diffraction and methods based on adsorption and desorption or bulk reactions (reduction, oxidation) all offer tools to investigate the nature of an active catalyst. Depending on the nature of the material being investigated, a suite of techniques may be utilized to assess its structure and properties. A host of characterization techniques have been employed in the present study to determine the structural characteristics of the as synthesized supports, functionalized ones as well as the enzyme immobilized supports which provide further support for the difference in activity observed for the supports chosen.

2.5.1 X-ray Diffraction

X-ray diffraction is a very important technique that has long been used to determine the crystal structure of solids, including lattice constants and geometry, identification of unknown materials, orientation of single crystals, defects, etc [11].

X-ray diffraction (XRD) is used for identifying the crystalline phases and estimating particle sizes. When there is constructive interference from X rays scattered by the atomic planes in a crystal, a diffraction peak is observed. Bragg's

equation relates the distance between two hkl planes (d) and the angle of diffraction (2θ) as: $n\lambda = 2d\sin\theta$, where, λ = wavelength of X-rays, n = an integer known as the order of reflection (h, k and l represent Miller indices of the indices of the respective planes) [12].

The intensity of the diffracted X-rays is plotted as a function of the diffraction angle and the sample orientation. From the diffraction patterns, the uniqueness of mesoporous structure [12], phase purity [13], degree of crystallinity [14] and unit cell parameters [13] of the semicrystalline hybrid materials can be determined.

Powder X-ray diffraction (XRD) can readily provide direct information of the pore architecture and symmetry of mesoporous materials. Microporous solids show peaks in the 2θ range of 5-50° whereas the mesoporous materials exhibit characteristic peaks in the low angle region between 1.5-10° (2θ). XRD analysis becomes the primary methodology in identifying different mesophases, because the different mesophases exhibit distinct "finger print" XRD diffraction patterns.

The unit cell parameters for Ordered mesoporous materials (OMS) are related to the distance between the repeating arrangement of the pores (i.e. hexagonal, cubic, etc.). The diffraction pattern from amorphous materials (including many polymers) is devoid of the sharp peaks characteristic of crystals and consists of broad features or halos. Quantitative analysis of XRD data from amorphous materials is complicated but provides important information on the local atomic structure (short range order), including the bond lengths, the number of neighbours, and the extent of atomic range It has been concluded that the pore walls are mainly amorphous. Thus, XRD is in general not particularly suitable for the quantitative phase composition determination for most ordered surfactant-templated materials because of noncrystallinity of their frameworks [15]. Conventional mesoporous materials like MCM-41, MCM- 48 or SBA-15 are amorphous. Nevertheless reflexes are observed in X-ray powder patterns at low 2θ angles ($0.5° < 2\theta < 10°$). These reflexes are due to the long-range order induced by

the very regular arrangement of the pores. Because d spacings are rather big for the mesopores, the reflexes appear at low angles.

The X-rays used are typically from bombarding either copper (Cu) or molybdenum (Mo) with high energy electrons. The emitted X-rays are then filtered or passed through a monochromater to produce K_α radiation from the respective metal. CuKα possesses a wavelength of 1.541 Å and MoK_α possesses a wavelength of 0.709 Å. Once the X-rays impinge on the sample, they diffract off of each of the exposed planes and a detector rotates around the sample recording the intensities of diffraction at each angle. The angles at which the peaks are observed can be used to determine the interplanar spacing of atoms (periodicity) or d-spacing for each crystal plane using Bragg's Law. For OMS, these values can be used to estimate the unit cell dimensions of the pore arrangements.

XRD is noncontact and nondestructive, which makes it ideal for in situ studies. One of the disadvantages of XRD, compared to electron diffraction, is the low intensity of diffracted X- rays, particularly for low-Z materials. Compared to TEM, XRD has poor area resolution capability.

Small 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-1.5406 Å) within the 2θ range 1-10° at a speed of 1°/minute.

2.5.2 IR spectroscopy

The most common application of infrared spectroscopy in catalysis is to identify adsorbed species and to study the way in which these species are chemisorbed on the surface of the catalyst [16, 17].

The infrared spectrum is commonly plotted in one of three formats: as transmittance, reflectance, or absorbance. The energy corresponding to these frequencies correspond to the infrared region (4000–400cm^{-1}) of the electromagnetic spectrum. The term Fourier transform (FT) refers to a recent development in the

manner in which the data are collected and converted from an interference pattern to an infrared absorption spectrum that is like a molecular "fingerprint" [18]. The absorption wavelengths represent excitations of vibrations of the chemical bonds and are specific to the type of bond and the group of atoms involved in the vibration. The vibrational frequencies of surface groups and probe molecules are identified by comparing their fingerprints with literature databases. Much of the FTIR work with silica has been directed to studies of the chemistry of high-surface-area gels and fumed powders [19, 20, 21]. The high surface area of these silicas enables ease in detection of surface functionalities from bulk features. The dried powder or fiber is then usually dispersed with a suitable salt such as KBr and pressed into a disk for transmission or recorded as a powder using DRIFT.

Reflection spectroscopy

Diffuse reflection infrared spectroscopy (DRIRS)

If the sample is inappropriate for a transmission experiment, for instance if the supporting substrate is opaque, a reflectance configuration will often be employed. Numerous oxide systems exist as opaque powdered samples from which light is reflected nor transmitted. Reflected radiation, in this case, consists of two components: the specular component reflected from the surface without transmission (mirror or specular reflection), and the diffuse component which is absorbed into the sample and reappears at the surface after multiple scattering [22]. In this technique, diffuse scattered radiation is collected by the sphere or an ellipsoidal mirror and focused on the detector with the infrared absorption spectrum being described by the Kubelka–Munk function [23, 24]. The relationship between the intensity of the band due to an adsorbed substance and its concentration is as follows

$$F(R) = I\,[\,(1/(R-R_{\infty})] = 2\varepsilon/S \qquad \text{.....(2)}$$

Theoretical studies indicate that diffuse reflection spectroscopy is more sensitive than transmission spectroscopy if the effectively scattering components are investigated [25]. This method allows the direct investigation of powdered materials contained in cells of small volume. Therefore, it has

significantly simplified sample preparation, as well as pretreatment techniques and the obtaining off the spectra, particular at low temperatures.

FTIR is a sensitive technique to probe into the secondary structure of proteins in the 1400–1700 cm^{-1} region which provides information about the presence of –CO- and –NH- groups [26, 27].

The IR spectrum of the samples was obtained using a JASCO FTIR spectrometer. The resolution and acquisition applied were 4 cm^{-1} and 60 scans respectively. The spectra were obtained in the wavelength range from 400 to 4000 cm^{-1} for evaluation of the immobilization procedures, in accordance with Soares et al. [28].

2.5.3 Nitrogen Adsorption

The most widely used gas adsorption measurement for the characterizing of the surfaces of solid materials is nitrogen physisorption. To obtain accurate adsorption measurements the amount of adsorbed gas should be high and the temperature constant. Therefore nitrogen physisorption measurements are often performed at liquid nitrogen temperature (-195.8°C = 77.35 K). In this technique, the amount of gas adsorbed onto the solid surface is directly correlated to the material's surface area and pore structure [29].

In ordered mesoporous materials the multilayer adsorption reaches a state where additional adsorptive molecules do not interact with a flat layer of adsorbate any more but with a curved surface. The adsorption on curved surfaces is more favorable. That can be expressed mathematically with the so-called Kelvin equation (eq. 3, P/P_0 is the relative vapor 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 vapor pressure, P, in the pore

$$\frac{P}{po} = \exp\left(-\frac{2\gamma\gamma^{1}}{rK * RT}\right) \qquad \text{..... (3)}$$

Isotherms

To facilitate the interpretation of the isotherms the shape of six physisorption isotherms was defined by IUPAC (Fig. 2.1) [30].

The reversible *Type I* isotherms are given by microporous solids having relatively small external surfaces. The reversible *Type II* isotherms is the normal form of obtained with a non-porous or macroporous adsorbent. Characteristic features of the *Type IV* isotherm are its hysteresis loop, which is associated with capillary condensation taking place in mesopores. The *Type V* isotherm is uncommon, and is obtained with certain porous adsorbents. The *Type VI* isotherm, in which the sharpness of the steps depends on the system and the temperature, represents stepwise multiplayer adsorption on a uniform non-porous surface.

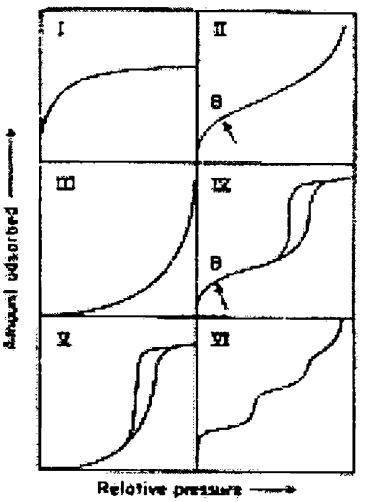


Fig 2.1 Type of physisorption isotherms. Point B, the beginning of the almost linear middle section of the isotherm, is taken to indicate the stage at which monolayer coverage is complete and multilayer adsorption is about to begin [31].

Hysteresis appearing in the multiplayer range of physisorption isotherms is usually associated with capillary condensation in mesopore structures. IUPAC has classified the various hysteresis loops that are observed experimentally as types H1, H2, H3 and H4 as shown in Figure 2.2 and their interpretation is given in the Table 2.1

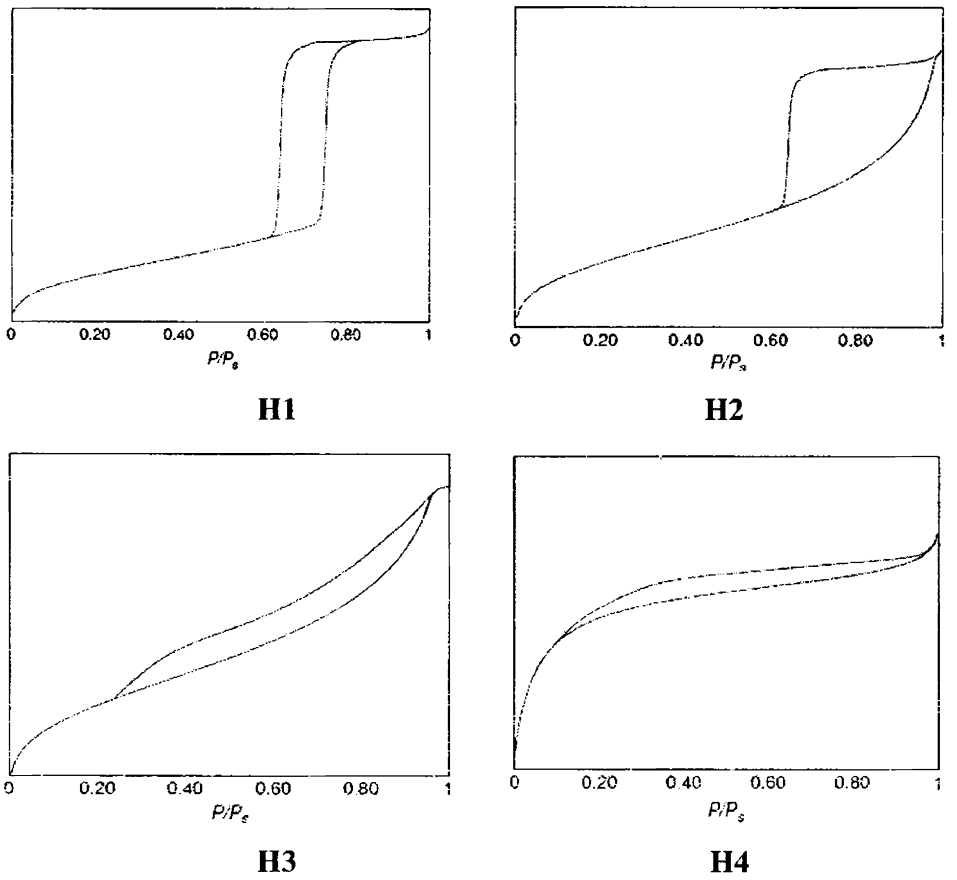


Fig 2.2 IUPAC classification of hysteresis loops

Table 2.1 Characteristics and interpretation of hysteresis loop types

Type	Characteristics	Usual interpretation
H1	Nearly vertical and parallel adsorption and desorption branches	Regular even pores without interconnecting channels
H2	Sloping adsorption branch and nearly vertical desorption branch	Pores with narrow and wide sections and possible interconnecting channels
H3	Sloping adsorption and desorption branches covering a large range of P/P_s with underlying type II isotherm	Slit-like pores for which adsorbent–adsorbate pair which would yield a type II isotherm without pores
H4	Underlying type I isotherm with large range for the hysteresis loop	Slit-like pore for the type I adsorbent–adsorbate pair

Hysteresis loops do also occur in mesoporous materials with pore geometries other than cylinders. Then the pore geometry can also influence the shape (i.e. the type of hysteresis loops) or the difference between adsorption and desorption branch.

Specific surface area (BET method)

The method was established by Brunauer, Emmet and Teller [32]. In the BET method the isotherm is linearized with the so called BET equation (eq. 4, V_a is the adsorbed volume at a P/P_0, V_m is the volume adsorbed in a monolayer and C is an empirical constant that is related to the difference between the heat of adsorption on the naked surface (E_1) and on the following layers (E_L).

$$\frac{p}{V_A(po-p)} = \frac{1}{V_m * C} + \frac{C-1}{V_m * C} * \frac{p}{po} \qquad \text{.....(4)}$$

$$C \approx e^{\frac{E_1 - E_L}{RT}}$$

As long as only multilayer adsorption occurs the BET plot is linear. From the slope ($s = (C-1)/(V_m \times C)$) and the intercept ($i = 1/(V_m \times C)$ of this straight line V_m and C can be derived. With V_m and the area of a single adsorbate particle on a certain surface (16.2nm^2 with N_2 as adsorptive on silica surfaces) the specific BET surface area can be calculated. The weight of the degassed sample was used in the surface area calculations. To obtain the surface area, the results were fitted into the equation, $S_{BET} = V_m \times N \times A_m$ where, V_m is the monolayer volume, N is the Avogadro number and A_m is the crosssectional area of the adsorbent.

Specific pore volumes

The specific pore volumes were determined with two different methods. In the first it is assumed that all pores are filled at a relative pressure $P/P_0 = 0.97$. From the volume adsorbed at that pressure the pore volume was calculated directly. This very simple method gives correct results if (1) the investigated material has a low external surface area and (2) no macropores are present that cause condensation in this pressure range. Since both requirements are fulfilled in ordered

mesoporous materials this method was mostly used. Alternatively, a more sophisticated approach based on the so called t-plot method [33] was used.

This method was developed for the characterization of the porosity of a material. In the so called t-plot the measured adsorbed volumes are plotted against the statistical layer thickness t.

Pore diameter distributions and average pore diameters

BJH Method

The pore size distribution and pore diameters were calculated using the Barret-Joyner-Halenda (BJH) model based on the Kelvin equation, corrected for multilayer adsorption [31]. The above model was chosen since it has been derived for nonintersecting cylindrical pores (as in MCM-41 type materials) [34]. The emptying of the filled pores with decreasing relative pressure is incrementally evaluated to obtain a pore diameter distribution. From this value and the volume difference before and after the increment the volume of emptied pores in that increment is calculated. The pore size is divided into three categories based on diameter: micropores (< 2 nm), mesopores (2 – 50 nm), and macropores (> 50 nm). Assuming cylindrical pores, which is the case for most OMS (Ordered mesoporous silica), and using parameters developed in 1997 by Jaroniec et al. [35] the pore size distribution can be estimated. Mesopores of size >30 nm are ideal for materials used in enzyme immobilization due to improved access of the interior surfaces [36].

Overall Pore Size Distribution

The mesopores size distribution is usually expressed as a plot of $\Delta Vp/\Delta rp$ versus rp, where Vp = mesopore volume, and rp = pore radius. It is assumed that the mesopores volume is completely filled at high p/p_0. This plot will represent the relative abundance of the pores of various radii in the solid. Specific surface areas S_{BET} were determined by the BET method in the 0.05–0.35 relative pressure range, single-point total pore volume Vp was determined at $p/p_0 = 0.99$ and average pore diameter Dp was calculated on the basis of 4 $Vp/SBET$ [31, 34].

A Micromeritics 2000 surface area analyzer was used to measure the nitrogen adsorption isotherms of the samples at liquid nitrogen temperature. Prior to the measurement the silica samples were degassed at 200°C and the immobilized samples were degassed at room temperature overnight in nitrogen flow. Functionalized materials with organic material bound to the surface of the silica were degassed at 80°C.

Solid-State Nuclear Magnetic Resonance Spectroscopy (SS-NMR)

2.5.4 Magic Angle Spinning Nuclear Magnetic Resonance (MAS NMR)

NMR technique reflects the short-range ordering of structure and is very sensitive to the local environment of nuclei. Among characterization spectroscopies, magnetic resonances are liable to produce specific analyses of bulk and interface of solid powders, fibers, or composites. Such methods need the presence of atoms with a magnetic moment or electrons belonging to a probe molecule (spin label). The same is true for ^{13}C (1.1 percent), which is a component of the organic molecules adsorbed or grafted on the surface.

NMR instrumentation consists of three chief components: a magnet, a spectrometer console, and a probe. The sample is in the magnetic field, and is placed inside an inductor of a radiofrequency circuit tuned to the resonance frequency of the nucleus under observation. The magnetization present at time is then detected by applying a short, intense (100-1000 W) radiofrequency pulse (typically 1-10 ps) in a direction perpendicular to $\boldsymbol{B_0}$. The oscillating magnetic component of the radiofrequency pulse stimulates transitions between the magnetic states and tips *Mo* into the plane perpendicular to the direction of the magnetic field (90° pulse). It thereby induces an ac voltage signal in a coil, which is amplified, digitized, and acquired over a typical period of several ms. Fourier transformation of this free induction decay (FID) signal then results in the NMR spectrum, a plot of absorption intensity versus frequency. The area under a spectral peak is directly proportional to the number of nuclei contributing to the resonance, and can be used for quantification purposes.

Averaging does not occur in the solid state, so that spectra are normally more complex, but also contain more information. The use of a combination of two techniques can however remove or decrease these interactions to such an extent that NMR spectroscopy of solid samples becomes possible. These two techniques are called **cross polarization (CP)** and **magic angle spinning (MAS)**: in combination, these are thus called **CP-MAS**. Magic angle spinning (MAS) is a useful line-narrowing technique in solid state NMR spectroscopy, in which the sample is mechanically rotated rapidly on an axis that makes an angle of 54.7° relative to the direction of the static magnetic field. Consequently, MAS converts broad powder patterns into highly resolved sharp resonances that can be straightforwardly assigned to individual sites [37]. In the cross-polarization (CP) approach, spin polarization from a more abundant spin set that has a larger nuclear magnetic moment (^{1}H) is transferred via a double-resonance method to a less-abundant spin set that has a smaller nuclear magnetic moment (^{13}C) [38].

Therefore, the sensitivity of ^{13}C will increase. Modern high-resolution solid-state NMR spectroscopy allows to elucidate the chemical and structural environment of several atoms (e.g. ^{13}C, ^{27}Al, ^{29}Si, ^{31}P, ^{51}V etc.) in a solid matrix like that of porous materials [39].

Sufficiently rapid MAS bring about the coherent averaging of inhomogeneous line broadening effects, such as the chemical shift anisotropy and inhomogeneous magnetic dipole-dipole interactions. With solid state NMR, the characteristic time of cross polarization was analyzed as a function of the position of the carbon along an alkyl chain.

Solid State ^{13}C NMR

The ^{13}C CPMAS NMR spectra of as-synthesized molecular sieves 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 surfactant organization in Si-MCM-48 [40]. The situation for carbon-13

is however completely different. Here spectra are normally recorded with complete proton decoupling, so that a series of single lines are obtained; in addition the chemical shift range is around 200 ppm. Magnetization transfer from protons to carbon-13 leads to an increase of signal intensity by a factor of up to 4.

Solid-State ^{29}Si NMR

Lippama et al. showed that ^{29}Si MAS NMR spectra are very sensitive to the nature and chemical environment of the atoms [41]. ^{29}Si solid-state NMR spectroscopy has been used extensively to elucidate the molecular environment in silicate materials [42].

^{29}Si solid-state NMR spectra of the sol–gel solids show signals representative of various substructures of the T_n and Q_n silane moities. Spectra displaying Qn (n < 4) peaks indicate that the condensation of the silicon alkoxide precursor is incomplete [42]. As the degree of condensation increases, the T_n and Qn peaks are shifted to higher field in the NMR spectrum. T_n peaks are representative of silicon atoms in the sol–gel matrix directly bonded to an organic species. An increase in the Tn species, the number of organic species bonded to a silicon atom decreases.

Solid State MAS NMR experiments were carried out over a Bruker DSX-300 spectrometer at a resonance frequency of 75.4MHz. For all measurements a standard 4 mm double- bearing Bruker MAS probe was used. The number of scans collected was 1024. The ^{27}Al spectra were externally referenced with respect to a dilute solution of $AlCl_3$ and recorded at room temperature. XWIN NMR software was employed to acquire and retrieve data.

2.5.5 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) is the measure of change in sample weight with increasing temperature. This measurement allows us to determine the degree of surface modification through comparison of the percent weight loss. A derivative weight loss curve can be used to tell the point at which weight loss is most apparent.

DTA and DTG study are useful in evaluating the thermal properties of molecular sieves [43].

The shape and splitting of the endotherms (low temperature) helps to identify the location of water molecules and also helps in studying the kinetics of dehydration.TGA is commonly employed in research and testing to determine characteristics of materials such as polymers, to determine degradation temperatures, absorbed moisture content of materials, the level of inorganic and organic components in materials, decomposition points of explosives, and solvent residues. The technique can analyze materials that exhibit either mass loss or gain due to decomposition, oxidation or loss of volatiles (such as moisture).

The analyzer usually consists of a high-precision balance with a pan (generally platinum) loaded with the sample. The pan is placed in a small electrically heated oven with a thermocouple to accurately measure the temperature. The atmosphere may be purged with an inert gas to prevent oxidation or other undesired reactions. A computer is used to control the instrument.

The thermoanalytical technique has been widely used to get information on the thermal stability of microporous and mesoporous molecular sieves. Both TGA and DTA provide important information about the following: (i) temperature programmed desorption (TPD) and removal of physisorbed water below 150°C, (ii) oxidative decomposition of the occluded organic materials, accompanied by one or several exotherms within 150°C and 600°C, and (iii) dehydroxylation occurring from condensation of adjacent silanol groups to form siloxane bonds at or above 600°C [44]. Further, DTA can also detect phase transitions.

TGA/DTG was done on a TA INSTRUMENTS Q50, thermal analysis instrument at a heating rate of 10°C/min from room temperature to 800°C using nitrogen as the purge gas.

Electron microscopy

It is used for determining the size, shape, and composition of supported particles [45]. The transmission mode measures the intensity from an electron source after it has passed through a transparent sample. In contrast, SEM features the scanning of an electron beam over selected regions of an opaque sample. The interaction of the primary beam with the sample provides a wealth of information on morphology, crystallography and chemical composition.

2.5.6 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is one of the most widely used techniques for characterization of nanomaterials and nanostructures. This technique provides not only topographical information like optical microscopes do, but also information of chemical composition near the surface.

A scanning electron microscope can generate an electron beam scanning back and forth over a solid sample. The electron beam is produced by an electrongun, often based upon a lanthanum hexaboride filament. The beam is condensed by the first condenser lens and then focused by the second condenser lens into a thin, coherent beam. A set of coils then scans or "sweeps" the beam in a grid-like fashion. A fraction of these electrons will be completely backscattered, reemerging from the incident surface of the sample. These electrons, known as back-scattered primaries, give information on the surface topography, and on the average atomic number in the scanned area. The secondary electrons are detected by attracting them onto a phosphor screen, and measuring the light intensity with a photomultiplier. The microscope records the secondary and back-scattered electrons as a function of the beams position. The scan generator is connected to other components, the magnification module and the cathode ray tube, displaying the final image. Since the scattering angle depends on the atomic number of the nucleus, the primary electrons arriving at a given detector position can be used to produce images containing topological and compositional information [46, 47].

This image is highly magnified and usually has the look of a "a traditional" microscopic image but with a much greater depth of field. Because the secondary electrons originate mainly from the surface, whereas the back-scattered electrons arise from the bulk, SEM gives a 3D image of the catalyst, albeit at a lower resolution than TEM. SEM images have a characteristic 3-D appearance and are therefore useful for judging the surface structure of the sample. Beside the emitted electrons, X-rays are also produced by the interaction of electrons with the sample. These can be detected in a SEM equipped for energy dispersive X-ray (EDX) spectroscopy [48].

Generally the resolution goes down to 20nm to 1 nm, which is much lower than that of transmission electron microscopy (TEM) but SEM has some advantages compared with TEM. For example a quite large area of the specimen can be imaged, bulk materials can be used as samples and a variety of analytical modes are available for measuring the composition and nature of the specimen.

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 dusted on a metal stab and coated with a layer of gold to minimize charge effects.

2.5.7 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is typically used for high resolution imaging of thin films of a solid sample for microstructural and compositional analysis. The technique involves: (i) irradiation of a very thin sample by a high-energy electron beam, which is diffracted by the lattices of a crystalline or semicrystalline material and propagated along different directions, (ii) imaging and angular distribution analysis of the forward scattered electrons (unlike SEM where backscattered electrons are detected), and (iii) energy analysis of the emitted X-rays [49]. The topographic information obtained by TEM in the vicinity of atomic resolution can be utilized for structural

characterization and identification of various phases of mesoporous materials, *viz.*, hexagonal, cubic or lamellar [50]. TEM also provides real space image on the atomic distribution in the bulk and surface of a nanocrystal [51]. Using transmission electron microscopy to make a projection of the sample density is a routine way to study particle sizes in catalysts.

A transmission electron microscope (TEM) is like an optical microscope in which the optical lenses have been replaced by electromagnetic ones .In Transmission Electron Microscopy (TEM) a thin solid specimen (200 nm thick) is bombarded in vacuum with a highly-focused, monoenergetic beam of electrons. The beam is of sufficient energy to propagate through the specimen. When an electron beam is impinged upon a sample, a black and white TEM image is formed from the passage of some electrons through the sample untouched, alongside the combination of interactions between other electrons and sample atoms (*e.g.*, inelastic/elastic scattering, diffraction. The optics bring the scattered electrons from the same point in the sample to the same point in the image (the so-called bright-field image), in contrast, selection of strongly diffracting regions of the sample, which would appear brighter than the transmitted beam, is known as dark-field imaging. A series of electromagnetic lenses then magnifies this transmitted electron signal. Diffracted electrons are observed in the form of a diffraction pattern beneath the specimen. The image is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD camera. The latter has the advantage that the image may be displayed in real time on a monitor or computer. This information is used to determine the atomic structure of the material in the sample.

Transmitted electrons form images from small regions of sample that contain contrast, due to several scattering mechanisms associated with interactions between electrons and the atomic constituents of the sample. Analysis of transmitted electron images yields information both about atomic structure and about defects present in the material.

One of the major limitations of TEM is the extensive sample preparation, which makes TEM analysis a relatively time consuming process with a low throughput of samples.

TEM images were recorded on a FEI Tecnai G2 Twin microscope operated at 60 kV. The sample was dispersed in toluene through sonication and then drop casted on a carbon-coated copper grid

2.5.8 C H N contents in solid materials

Organic elemental analysis (C, H, 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.

2.5.9 Contact angle measurements

The wetting of a surface by a liquid and the ultimate extent of spreading of that liquid are very important aspects of practical surface chemistry. Of more practical and widespread importance is the contact angle of a liquid directly on a solid. The contact angle may be geometrically defined as the angle formed by the intersection of the two planes tangent to the liquid and solid surfaces at the perimeter of contact between the two phases and the third surrounding phase. Typically, the third phase will be air or vapor, although systems in which it is a second liquid essentially immiscible with the first are of great practical importance. The extent of movement will be determined by the competition between solid-liquid adhesion forces and liquid-liquid cohesive forces [52].

Measurement of the contact angle

(a) Experimental methods

The most common direct methods include the sessile drop (*a*), the captive bubble (*b*), the sessile bubble (*c*), and the tilting plate.

The most common method of measuring the contact angle is to observe a **sessile drop** with a telescope or microscope. A light source is positioned behind the drop, which then appears dark. The contact angle is either determined directly with a goniometer or the image is recorded by a video system and the contour is fitted by a computer by the Laplace equation.

Young's equation

Young's equation is the basis for a quantitative description of wetting phenomena. If a drop of a liquid is placed on a solid surface there are two possibilities: the liquid spreads on the surface completely (contact angle $\theta = 0°$) or a finite contact angle is established. In the second case a three-phase contact line — also called wetting line — is formed. At this line three phases are in contact: the solid, the liquid, and the vapor (Fig. 2.3). Young's equation relates the contact angle to the interfacial tensions γS, γL, and γSL [53]:

$$\gamma L \text{Cos}\theta = \gamma S - \gamma SL \quad \text{.....(5)}$$

If the interfacial tension of the bare solid surface is higher than that of the solid–liquid interface ($\gamma S > \gamma SL$), the right hand side of Young's equation is positive. Then $\cos\theta$ has to be positive and the contact angle is smaller than 90°; the liquid partially wets the solid.

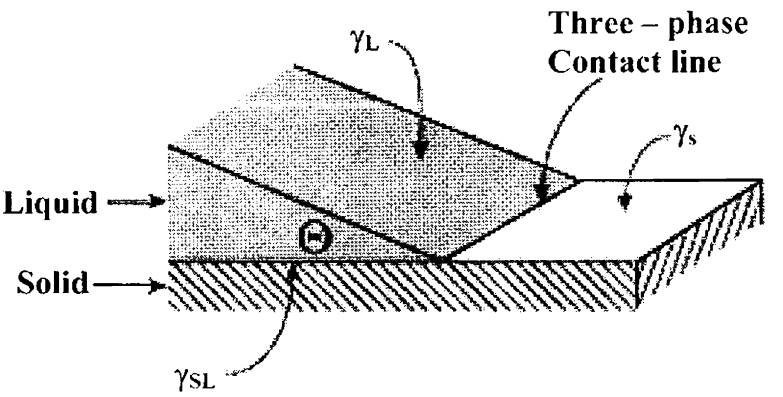


Fig 2.3 Rim of a liquid drop with a contact angle θ on a solid surface.

If the solid–liquid interface is energetically less favorable than the bare solid surface ($\gamma S < \gamma SL$), the contact angle will exceed 90° because cosθ has to be negative. If the contact angle (θ) is zero and the Young's equation for such a surface would be:

$$\gamma SL = \gamma S - \gamma L \quad \text{.....(6)}$$

In this case, the liquid is fully absorbed by the material

The contact angle that the droplet edge makes with the solid surface is often used as a comparative measure of a material's wettability. Some degree of hydrophilicity will lead to a contact angle <90°. If the material surface is hydrophobic, an energy penalty is associated with the liquid spreading over the surface. In this case droplet formation occurs, but with an obtuse contact angle [53].

The as produced silica without any organic surface chemical modification (i.e., without attaching alkyl, aryl or vinyl groups) has a high energy surface and hence absorbs moisture. But, the surface chemical modification of the silica results in a low energy surface which repels water. Proteins adsorbed to solid surfaces alter the original interfacial properties [54, 55]. Evidence of immobilization of lipase is confirmed by contact angle studies.

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.

References

[1] T. Sen, G. J. T. Tiddy, J.L. Casci, M.W. Anderson, Microporous Mesoporous Mater., 78 (2005) 255.

[2] P . S. Winkel, W. W. Lukens, Jr., P. Yang, D. I. Margolese, J. S. Lettow, J. Y. Ying, G. D. Stucky, Chem. Mater., 12 (2000) 686.

[3] S. Shylesh, A. P. Singh, J. Catal., 244 (2006) 52.

[4] P. C. de Oliveira, G. M. Alves, H. F. de Castro, Biochem, Eng. J., 5 (2000) 63.

[5] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. J Randall, J.Biol. Chem., 193 (1951) 265.

[6] D. S. Rodrigues, A. A. Mendesa, W. S. Adriano, L. R. B. Goncalves, R. L. C. Giordano, J. Mol. Catal. B: Enz., 51 (2008) 100.

[7] S. K. Dahodwala, A. E Humphrey, Biotechnol. Bioeng., 18 (1976) 987.

[8] M. Shuler, F. Kargi, Bioprocess Engineering: Basic Concepts, 2nd ed. Prentice-Hall, Upper Sadle River, NJ (2002).

[9] E. Taqieddin, M. Amiji, Biomater., 25 (2004) 1937.

[10] H. Sztajer, Lunsdorf, H. Erdmann, U. Menge, R. Schmid, Biochim BiophysActa., 124 (1992) 253.

[11] Z. L. Wang, in: Characterization of Nanophase Materials, Ed: Z. L. Wang, Wiley-VCH, Weinheim (2000) Ch 2.

[12] W. H. Bragg, W. L. Bragg, The Crystalline State, McMillan, New York (1949) Vol. 1.

[13] S. Biz, M. Occelli, Catal. Rev. Sci. Eng., 40 (1998) 329.

[14] G. Bergeret, in: Handbook of Heterogeneous Catalysis, Vol. 2, Eds: G. Ertl, H.Knozinger, J. Weitkamp, Wiley-VCH, Weinheim (1997) 464–475.

[15] F. Schüth, U. Ciesla, Microporous Mesoporous Mater., 27 (1999) 131.

[16] J. B. Peri, in Catalysis: Science and Technology (Anderson, J. R. and Boudart,M., Eds.), Springer-Verlag: Berlin (1984) Vol. 5, 169.

[17] J. W. Niemantsverdriet, Spectroscopy in Catalysis: an Introduction; VCH:Weinheim (1993).

[18] J. A. Griffiths, De Haseth, Fourier Transform Infrared Spectrometry, John Wiley and Sons Inc., New York (1986).

[19] A.V. Kiselev, V. I. Lygin, Infrared Spectra of Surface Compounds, Hallstead, New York (1975).

[20] M. L. Hair, Infrared Spectroscopy in Surface Chemistry, Marcel Dekker, NewYork (1967).

[21] B. A. Morrow, Surface groups on oxides, In Spectroscopic Analysis of Heterogeneous Catalysts, Part A: Methods of Surface Analysis, J. L. Fierro, Ed., Elsevier, Amsterdam (1990).

[22] W. Wendlandt, H. G. Hecht, "Reflectance Spectroscopy", Interscience Publishers/John Wiley (1966) FHI: 22 E 13.

[23] P. Kubelka, F. Munk, Z. Tech. Phys., 12 (1931) 593.

[24] P. Kubelka, J. Opt. Soc. Am., 38 (1948) 448.

[25] P. R. Griffiths, J. A. de Haseth, Fourier Transform Infrared spectrometry, John Wiley & Sons, New York (1986).

[26] C. N. Banwell, E. M. McCash, Fundamentals of Molecular Spectroscopy, McGraw-Hill, United Kingdom (1996) Ch. 3, 55.

[27] J. K. Kauppinen, D. J. Moffate, H. H. Mantsch, D. G. Lameron, Appl. Spectrosc., 35 (1981) 271.

[28] C. M. F. Soares, O. A. dos Santos, J. E. Olivo, H. F. de Castro, F. F. deMoraes, G. M. Zanin, J. Mol. Catal. B: Enzym., 29 (2004) 69.

[29] J. C. Groen, L. A. A. Peffer, Microporous and Mesoporous Mater., 60 (1-3) (2003) 1.

[30] K. S. W. Sing, D. H. Everett, R. A. W. Haul, L. Moscou, R. A. Pierotti, J. Rouquerol, T. Siemieniewska, Pure and Appl. Chem., 57(4) (1985) 603.

[31] E. P. Joyner, P. P. Halenda, J. Am. Chem. Soc., 73 (1951) 373.

[32] P. Brunauer, P. H. Emmett, E. Teller, J. Am. Chem. Soc., 60(2) (1938) 309.

[33] B. C. Lippens, J. H. de Boer, J. Catal., 4 (1965) 319.

[34] D. J. C. Yates, J Phys. Chem., 70 (1966) 3693.

[35] M. Kruk, M. Jaroniec, A. Sayari, Langmuir, 13 (1997) 6267.

[36] W. Tischer, F. Wedekind. "Immobilized enzymes: Methods and applications." Biocatalysis - from Discovery to Application, (1999) 95-126.

[37] G. Engelhardt, in: Handbook of Heterogeneous Catalysis, Vol. 2, Eds: G. Ertl, H.Knozinger, J. Weitkamp, Wiley-VCH, Weinheim (1997) 525–539.

[38] E. R. Andrew, A. Bradbury, R. G. Eades, Nature, 182 (1958)1659.

[39] G. Engelhardt, D. Michel, High-Resolution Solid-State NMR of Silicates and Zeolites, John Wiley and Sons Ltd, Chichester (1987).

[40] D. W. Sindorf, G. E. Maciel, J. Am. Chem. Soc., 105 (1983b) 1848.

[41] E. Lippama, M. Magi, A. Samoson, M. Tarmak, G. Engelhardt, J. Am. Chem. Soc., 103 (1981) 4992.

[42] B. Gorlach, S. S. Hellriegel, H. Yiksel, K. Albert, E. Plies, M. Hanack, J. Mater. Chem., 11 (2001) 3317.

[43] R. M. Barrer, D.A. Langley, J. Chem. Soc., 3804 (1958) 3811.

[44] C. Y. Chen, H. X. Li, M. E. Davis, Microporous Mater., 2 (1993) 17.

[45] Amelinckx, D. van Dyck, J. van Landuyt, G. vanTendeloo, Handbook of Microscopy VCH, Weinheim (1997).

[46] D. E. Newbury, D. C. Joy, P. Echlin, C. E. Fiori, J. I. Goldstein, Advanced Scanning Electron Microscopy and X-Ray Microanalysis, Plenum Press, New York (1986).

[47] G. Lawes, Scanning Electron Microscopy and X-ray Microanalysis, John Wiley and Sons Ltd, Chichester (1987).

[48] J. I. Goldstein, Dale E. Newbury, l. Echlin, D. C. Joy, C. Fiori, E. Lifshi, Scanning Microscopy and X-Ray Microanalysis. Plenum Press, New York (1981).

[49] J. R. Fryer, Chemical Applications of Transmission Electron Microscopy, Academic Press, San Diego (1979).

[50] J. M. Thomas, O. Terasaki, P. L. Gai, W. Zhou, J. Gonzalez-Calbet, Acc. Chem. Re., 34. (2001) 583.

[51] Z. L. Wang, in: Characterization of Nanophase Materials, Ed: Z. L. Wang, Wiley-VCH, Weinheim (2000) Chapter 3, 37–80.

[52] R. E. Johnson, R. H. Dettre, In Wettability; Berg, J. C., Ed.; Marcel Dekker: New York (1993) Vol. 49.

[53] A.W. Adamson, Physical Chemistry of Surfaces, John Wiley, New York (1982) 338.

[54] R. J. Lee, S. W. Kim, J. Biomed. Mater. Res., 8 (1974) 251.

[55] H. S. Lee, E. Ruckenstein, J. Colloid Interface Sci., 125 (1988) 365.

Chapter 3

RESULTS AND DISCUSSION

Contents

The synthesis and characterization of new materials with tailor-made properties is one of the most important areas in science today. Physicochemical characterization requires the selection of analytical technologies that are quantitative, specific and sensitive. Insight in the physico-chemical processes that play a role during the preparation of catalyst extricates its essential to design catalysts in a more controlled way. The main biomaterial related research carried out is the synthesis, functionalization and immobilization of enzymes and detailed characterization of inorganic or hybrid siliceous silica and clay supports and their structural characterization by various physicochemical techniques. The overall aim is to establish a rational link between the physicochemical and structural properties of the materials and their biological activity and response to external parameters such as temperature and pH. Immobilized biocatalysts have been developed for a variety of applications in the laboratory, for analytical purposes and in enzyme technology. In order to allow for their reproducible use, and to select a optimum biocatalyst for specific application, they must be well characterized- that is, their essential physicochemical (including mechanical), kinetic properties, and their stability under process conditions must be determined in standard procedures. The characterization of the immobilized biocatalysts-and especially comparative studies with the same enzyme immobilized in different supports used for the same enzyme process-can provide useful information on the properties of the supports that are important to improve the process.

3.1 Physico-Chemical Characterization

A number of techniques are used to characterize mesoporous molecular sieves. Among these, X-ray powder diffraction, transmission electron microscopy (TEM) and adsorption measurements are the essential ones to identify the structure of the mesoporous molecular sieves. Infrared spectroscopy, ultra violet spectroscopy, nuclear magnetic resonance spectroscopy and electron spin resonance spectroscopy have also been applied to obtain additional structural information about molecular sieves. A complete characterization of molecular sieves requires information from a number of physical, chemical and spectroscopic techniques. A combination of X-ray diffraction, sorption studies and TEM of mesoporous molecular sieves provides a reasonable idea about the structure of these materials. The applications of these techniques have been discussed in Chapter I. This chapter summarizes the results of the physicochemical characterization of montmorillonte K-10 clay and mesoporous silica foams. The discussion is focused on XRD, thermal analysis (TG/DTG), N_2-adsorption-desorption, transmission electron microscopy (TEM), scanning electron microscopy (SEM), Fourier Transform infrared spectroscopy (FTIR), CHN measurements, CPMAS Nuclear magnetic resonance spectroscopy (NMR) and Contact angle measurements of these materials. The present results are the full characterization research and structural comparison between the functionalized/immobilized samples with the pure supports.

3.2 Fourier Transform Infrared Spectroscopy

The most common application of infrared spectroscopy in catalysis is to identify adsorbed species and to study the way in which these species are chemisorbed on the surface of the catalyst. FTIR is a sensitive technique to probe into the secondary structure of proteins. The interaction between the supports and lipase molecules can be studied by comparing the IR spectra of the pure silica and clay, pure lipase, and lipase immobilized on silica and clay.

The infrared spectra of the calcined MCF samples are present in Fig. 3.1. The spectra of the pure siliceous MCF showed narrow vibration band at 3476 cm^{-1} belonging to isolated terminal silanol groups. The corresponding –OH bending mode around 1634 cm^{-1} correlate very well with the water adsorption property (hydrophilic property) of the catalysts. The peaks between 500 and 1200 cm^{-1} are assigned to framework vibrations. The intense peaks at 1038cm^{-1} with a shoulder at 1220 cm^{-1} are due to internal and external asymmetric Si–O stretching modes. The bands around at 800 and 471 cm^{-1} are assigned to symmetric Si–O stretching and tetrahedral Si–O bending modes. The MCF 35 silica samples (Fig 3.2A) display bands due to the abundant free OH groups at 3704cm^{-1}. The bands at 806cm^{-1} is due to the symmetric stretching vibrations of Si-O-Si bonds belonging to ring structures. The peak at 968cm^{-1} is due to the stretching vibration of Si-O (H) bonds. Figure 3.1 are spectra obtained for unmodified MCF silica, immobilized lipase, silane binded (MTS) and Glutaraldehyde binded (MTSG). Free lipase showed a typical spectrum of proteins, with the absorption band associated with their characteristic amide groups (CONH). Between the wave number range from 1600 to 1700 cm^{-1}, there is the amide I band, due to the double bond CO stretching, the CN stretching and NH bending [1]. The amide II band (1600–1500 cm^{-1}) results from a combination of N–H in-plane bending and C–N stretching of the peptide groups.

The band position of lipase MCF is slightly shifted in comparison with that of lipase which is attributed to the weak interactions of MCF with lipase molecule. After loading on the MCF160, the two absorption flexible peaks shift to 1634 and 1550 cm^{-1}, which may be due to the strong electrostatic interactions between the charged amino acid residues on the surfaces of the protein molecules and the silanol groups on the mesoporous structure; these shifts may also arise from hydrophobic interactions between protein molecules and the supports. This was further evidenced from an intense OH stretching band in the range of 3451cm^{-1} after enzyme binding depicting the presence of

enzyme on the surface of the particles. The -CH stretching band due to the enzyme groups occurs at 2942cm^{-1}. These results suggest that lipase immobilized in these matrices retains its native structure and biological activity. The same observation is observed with MCF 35 and KI-10.

The presence of APTES applied in the functionalization of MCFs was identified by methylene stretching bands of the propyl chain in the region of 2929 cm^{-1} (Fig. 3.1B and 3.2B), which cannot be observed in the FT-IR spectra of bare MCFs. The corresponding simple bending vibrations occur at 1407 cm^{-1} for the silane modified supports. At 693 cm^{-1}, a weak peak was observed, which was due to the bending of N–H bonds. A strong band around 1550 cm^{-1} associated with the –NH bending vibrations indicate the presence of aminopropyl functional group in all the silane binded samples [2, 3].

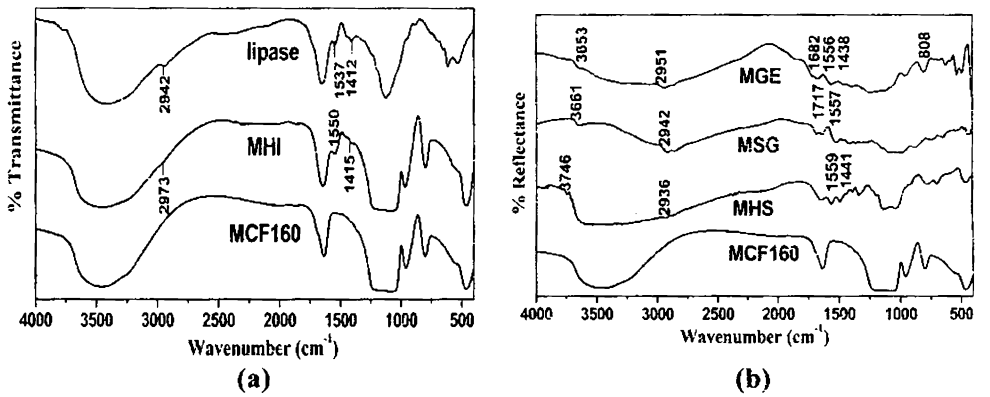


Fig 3.1 IR spectra of **(a)** MCF 160 and immobilized sample **(b)** silane, glutaraldehyde and enzyme binded samples

As expected, C-H stretching vibration frequency is seen at 2936 cm^{-1} for all spectra except the pure silica and clay supports with contributions from the organosilane, glutaraldehyde, and the enzyme [4]. The presence of aldehyde in MHSG and MTSG is clearly evident from the peak at 1720cm^{-1} (Fig 3.1B and 3.2B). The amine-glutaraldehyde reaction produces an imine N=C bond, Schiff-base, seen at 1647 cm^{-1} while an ethylenic C=C bond formed by resonance stabilization of the imine appears at 1563 cm^{-1}[5]. A peak at 794cm^{-1}

is due to the Si-C bond which demonstrates the incooporation of alkyl groups into silica.

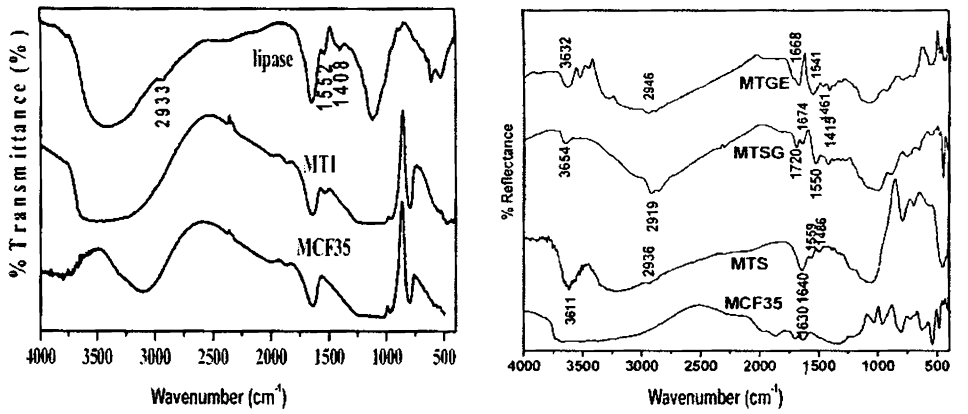


Fig 3.2 IR spectra of **(a)** MCF 35 and immobilized sample **(b)** silane, glutaraldehyde and enzyme binded samples

The IR spectra of Montmorillonite K-10 clay (Fig 3.3A) have two characteristic regions: (i) 4000–3000 cm^{-1} and (ii) 1200–400 cm^{-1}. The transmission bands in the first region, which correspond to (O–H), were assigned to the free surface hydroxyl groups of the layer as well as to the adsorbed water which indicated that there were an abundance of free hydroxyl groups on the surface of K-10. The band at 3638cm^{-1} is attributed to the stretching vibration of OH groups bonded to Al or Mg. The bands in the region 1200 to 400 cm^{-1} give more information about the structural characteristics of clay minerals and are attributed to lattice vibration [6, 7]. Bands at 1048, 521, and 688cm^{-1} originate from the clay lattice Si–O stretching, Al–O–Si vibrations and coupled Al-O and Si-O out of plane vibrations. The band at 931 cm^{-1} is due to Al-Al-OH deformation.

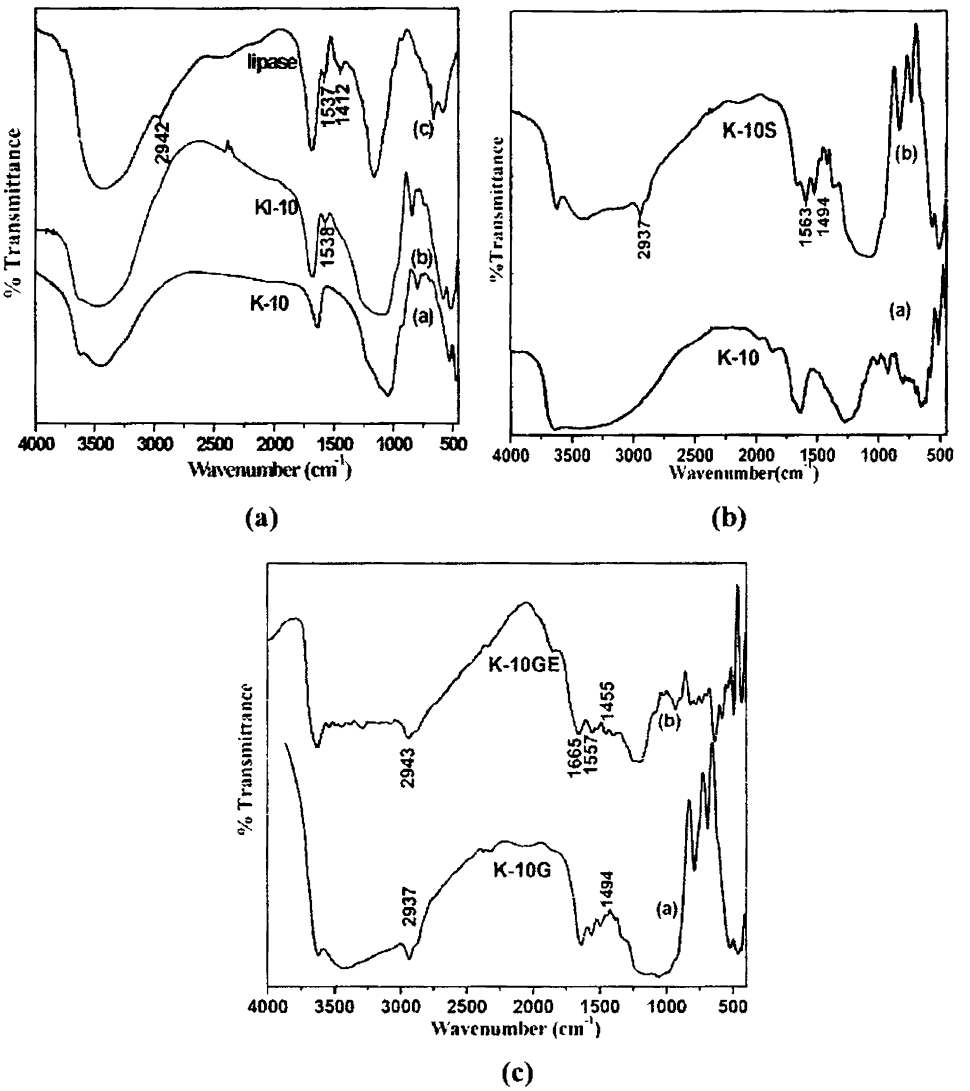


Fig 3.3 IR spectra of **(a)** K-10 and immobilized sample **(b)** silane binded samples **(c)** Glutaraldehyde binded sample (K-10G) and enzyme adsorbed on K-10G (K-10GE)

After immobilization of lipase (Fig 3.3A) there was a shift in the position of adsorption suggesting that there might be intermolecular interaction between enzyme and some specific sites of matrix, and apparently the montmorillonite K-10 was a good immobilization matrix for enzyme loading.

The successful grafting of silane moieties onto clay is evident in the infrared spectra as shown in Fig 3.3B. A new peak at 2964 cm^{-1} was attributed to antisymmetric stretching of ethyl group of silane, indicating the existence of silane in the grafted products. In the region 1400–1720 cm^{-1}, the spectrum of the silane binded clay exhibits a strong band around 1595 cm^{-1} associated with the $-NH_2$ bending vibrations. In addition a number of bands at lower frequencies attributed to bending vibrations of CH_2 and CH_3 units are resolved. The strong band at 1637 cm^{-1} is due to adsorbed water deformation.

3.3 Thermogravimetric analysis

The thermal stability of enzymes is one of the important criteria for long-term and commercial application. The activity of immobilized enzyme is known to be more resistant against heat than that native state. Thermal gravimetric analysis (TG) provides an important tool for thermal stability studies of macromolecules [8]. This technique has enabled us to determine the temperature range at which a heated sample undergoes a major conformational change by means of monitoring the thermal weight loss profile. In the case of free lipase and immobilized lipase derivatives such temperature range can be related to the protein unfolding and thus to the enzyme denaturation.

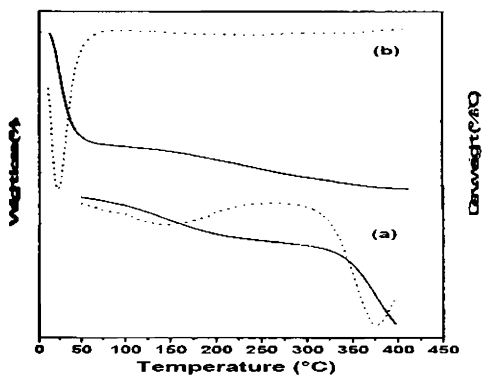


Fig 3.4 TG/DTG spectra of
(a) MCF160 (uncal),
(b) MCF160 (calc)

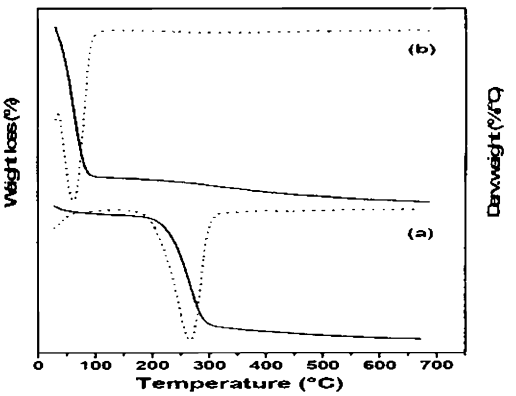


Fig 3.5 TG/DTG spectra of
(a) MCF35 (uncal)
(b) MCF35 (calc)

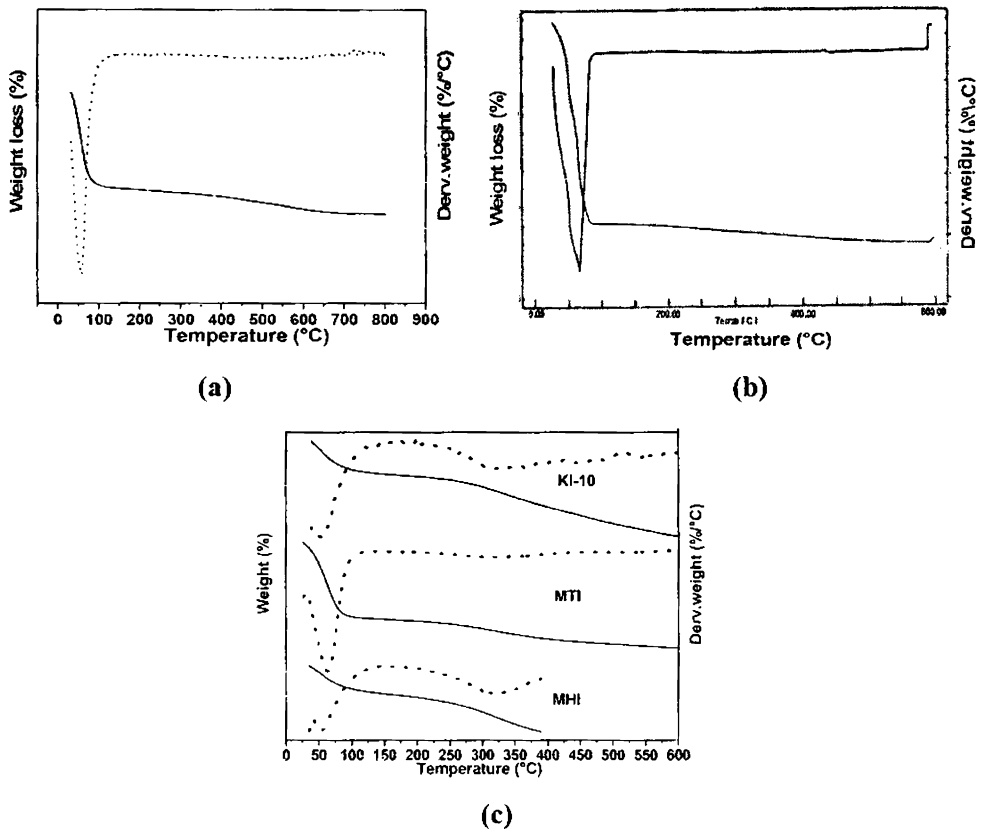


Fig 3.6 TG/DTG spectra of **(a)** K-10 **(b)** Free lipase **(c)** Immobilized lipases

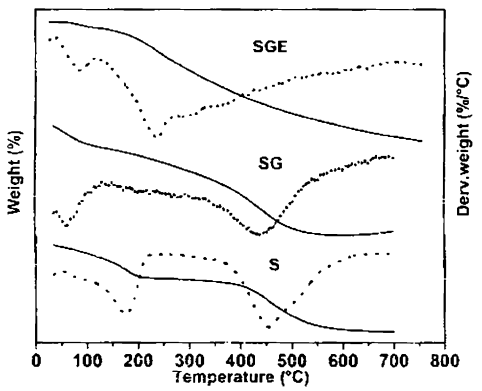


Fig 3.7 TG/DTG spectra of functionalized samples of MCF160

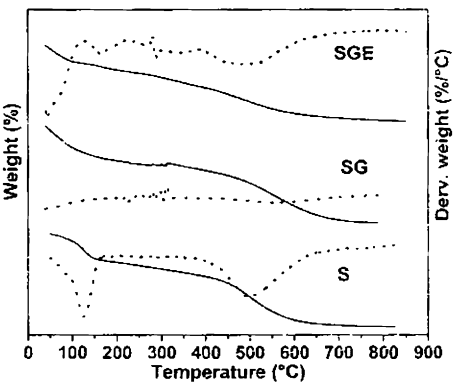


Fig 3.8 TG/DTG spectra of functionalized samples of MCF35

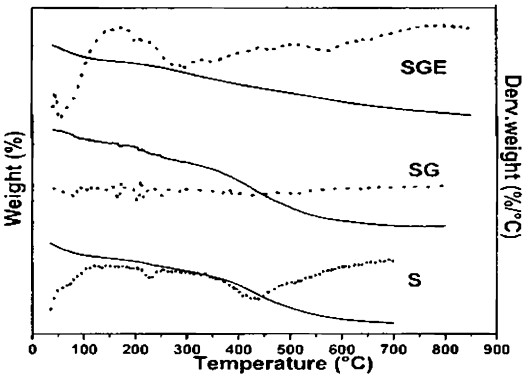


Fig 3.9 TG/DTG spectra of functionalized samples of K-10

In order to optimise the calcination condition, TG-DTA analysis (Fig.3.4. and 3.5) for silica synthesized via hydrothermal and room temperature method was carried out. The presence of peak at 100°C in both samples is probably due to the loss of adsorbed water. The sample MCF35 displayed a weight loss: at 280°C. 0There is virtually no weight loss above 480°C. The low temperature weight loss is partly due to loss of mesitylene and CTAB. The steady TG pattern is observed above 550°C. MCF160 displayed two processes: a weight loss at 50-150 °C due to the desorption of water and another weight loss from 180 to 370°C is due to the decomposition of P123 and other organics. The results from TGA/DTA demonstrate that P123 and TMB are readily removed from the MCFs under mild conditions.

Figure 3.6 (b) shows the TG curve for free lipase which was characterized by two weight loss peaks. In the first one, at temperature range from 30 to 180°C, characterized by a low weight loss due to the dehydration of the interstitial water containing in the free lipase sample. From 180 to 600°C, continuous weight loss was observed indicating a complete decomposition of the organic structure of lipase [9].

After adsorption of lipase (Fig 3.6 (c)), a weight loss is observed at 300° C due to the decomposition of enzyme moieties which confirms the adsoption of enzyme on MHl as these peaks are not observed in the case of calcined silica. The lower values obtained for the weight loss associated with the lipase encapsulated derivatives is the result of an increased matrix thermal stability suggesting that a strong interaction

between enzyme and all tested supports occurred which enhanced the conformation stability of the native form. Montero et al. [10] have observed similar behaviour after immobilization of C. cylindracea lipase in poly(propylene). When TG curves of both lipase adsorbed on MCF160 and MCF35 are compared a greater amount of enzyme decomposition is seen on MCFA.

All the functionalized samples exhibited weight loss at 60°-90°C due to the loss of adsorbed water. In the case of MHS (Fig 3.7), the weight loss which extends from 190 to 500 °C was substantial, and it is attributed primarily to the removal of water by dehydroxylation and some loss of organic constituents (C, H, O and N) in the form of volatiles either present or formed by the beginning of organics decomposition of silane moieties. This indicates that the silane binded samples are thermally stable at temperatures below 450°C. Above 500°C, little weight loss occurred for the immobilised derivatives on functionalized samples. The weight loss in this region is associated with final dehydroxylation reactions and definitive carbonisation of organic compounds, including the lipase. A greater weight loss due to the adsorbed water is observed at 100°C in the case of lipase immobilized on MHSG. The weight loss which occurs due to the decomposition of the enzyme at 237°C is not observed in any of the functionalized samples which confirm the strong adsorption of lipase via covalent bonding. In the case of MCF35 (Fig 3.8), the same peaks were observed for the functionalized samples and the strong adsorption via hydrophobic bonding of the lipase is evident from the peak around 500°C.

The DTG curve of MK-10 (Fig 3.6 (a)) displayed peak at 56°C, assigned to the loss of the physically adsorbed water. There is a weight loss in the region 100-300°C which is due to the decomposition of the adsorbed enzyme (Fig 3.6 c). The peaks at 69°, 225°C and 429°C are attributed to the loss of the physically adsorbed water and due to the decomposition of the intercalated silane while the peak at 533°C is corresponding to the decomposition of the grafted silane as illustrated in the Fig 3.9 [11]. An additional peak was recorded at 225°C which was due to the desorption from the external surfaces.

A weight loss is observed at 437°C in the case of K-10SG (Fig 3.9) which is due to the decomposition of silane and glutaraldehyde moieties from the functionalized surfaces. In the case of chemical adsorption of lipase on functionalized surfaces, a single weight loss is observed at 233°C in the case of MHGE, at 276°C in the case of MTGE and at 297°C in the case of K-10GE which is assigned to the decomposition of amino acid residues of the enzyme as well as the organic groups (silane and glutaraldehyde). Two stages of weight loss were observed for MTGE at 289°C and 485°C while only a single weight loss was observed in the case of MHGE which indicated the enhanced thermal stability of these systems. The weight loss peak is shifted to lower temperature which depicts the enhanced thermal stability of the covalently bound enzyme systems. A much greater loss of water is observed in the case of MHG and K-10G after enzyme adsorption than MTG which also proves the higher amount of loading of enzyme on these supports.

3.4 Small angle X-ray diffraction

XRD studies of MCF160 (Fig 3.11 (a)) exhibits one strong primary peak in the range of $2\theta = 0.88°$ due to the presence of mesopores. Unlike highly ordered mesoporous silica materials such as SBA-15 and MCM-41 [12], a certain plane or space group (e.g., p6mm) or to a lamellar diffraction pattern, cannot be observed in MCF silica which confirmed that the cells in the MCF materials are spherical and quite uniform in size. The appearance of single XRD peak in these samples is well consistent with a previous report [13].

After functionalisation (Fig 3.11 (b and c)) and immobilization (Fig 3.10b) in MCF160 there is a decrease in d spacing (Table 3.1) and intensity of the peaks due to the packing of lipase molecules inside the mesopores without affecting the structural integrity of the adsorbent. The XRD patterns of MCF160 after immobilization are similar to that before the immobilization, which shows that the regular mesoporous structure is retained even after immobilization and functionalisation. The decrease in peak intensities after enzyme binding and functionalisation may be due to the surface binding of the enzyme as well as the

functional groups suggesting the indirect evidence for enzyme incorporation in these particles. The decrease in intensity is probably not due to lower structural order but to the larger contrast in density between the silica walls and the empty pores relative to that between the silica walls and the pores filled with lipase molecules as observed by Marler et al. [14].

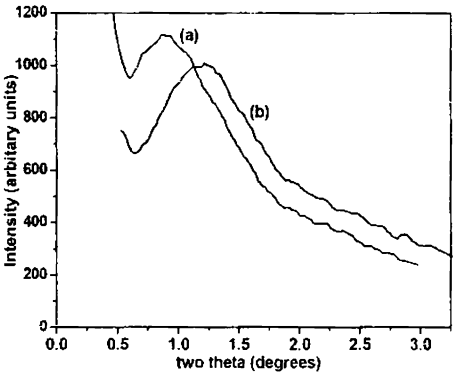


Fig 3.10 X-ray diffraction patterns of **(a)** MCF-160 **(b)** MHl

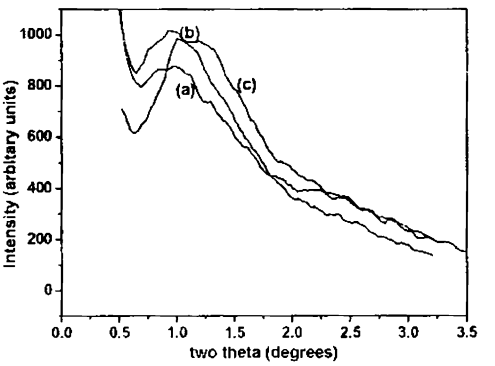


Fig 3.11 X-ray diffraction patterns of **(a)** MCF-160C **(b)** MHS **(c)** MHSG

Surface functionalized materials exhibit XRD patterns at the low-angle region, as well. As Sayari [15] also observed, the overall intensity of the XRD peaks decreased after this treatment. In our study, little structural changing was observed by post-synthesis treatment. Thus, sample functionalized with APTES (MHS) shows larger peaks reflecting a smaller ordered pore structure.

X-ray diffractograms of MCF35 are shown in Fig. 3.12 (a) & (b). They all exhibit low angle reflections characteristic of ordering at the mesoscale. Broad reflections in the low angle region is characteristic of a mesoporous lamellar silicate phase [16]. After calcination (Fig 3.12 b) the silicate phase displays one reflection with a slight contraction of d-spacing. This suggests that whilst ordering remains at the mesoscale this ordering is less than that of a lamellar phase. In other words, the mesophase becomes more disordered upon calcination. The increase in d spacing (Table 3.1) after immobilization and functionalisation probably may be due to the disordering of the pores.

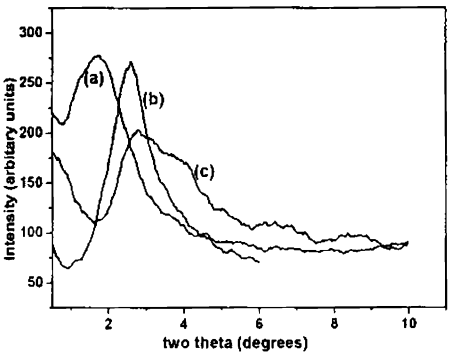


Fig 3.12 X-ray diffraction patterns of **(a)** MCF 35 (uncalcined), **(b)** MCF-35 (calcined) **(c)** MTI

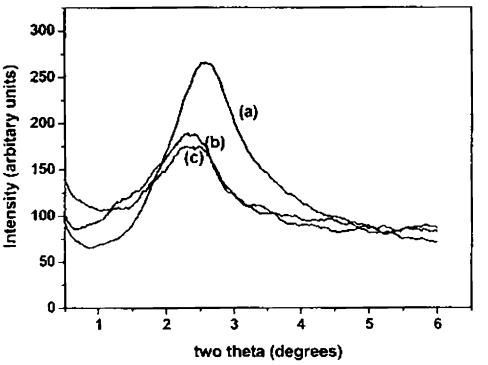


Fig 3.13 X-ray diffraction patterns of **(a)** MCF-30C **(b)** MTS **(c)** MTSG

Due to the immobilization of Porcine pancreatic lipase onto the mesoporous channels of SBA-15, a similar widening and broadening of the peaks as well as shift to higher angle was observed [17]. Retention of the hexagonal mesoporous structure of all SBA-15 as well as C_{16}-MCM41 adsorbents after cytc adsorption at different solution pHs while decrease in intensity of both the lower and higher order peaks with increasing cyt c concentration were observed in the work done by Vinu et al. [18].

Table 3.1 d-spacing values of the pure, functionalized and immobilized samples of silica and clay

Sample	d spacing (A°)
MCF35	32
MTI	40
MTS	39
MTSG	36
MCF160	100
MHI	74
MHS	96
MHSG	89
K-10	9.95
KI-10	9.95
K-10S	9.97
K-10SG	9.94

XRD is the usual method for measuring the structure of layered silicates. The Bragg equation based on XRD patterns is generally used to detect layer expansion of montmorillonites. The basal distances for total dehydrated clays are expected to be between 9.6 and 10 Å [19, 20, 21].

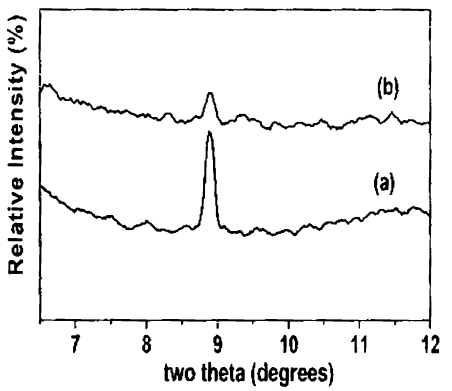


Fig 3.14 X-ray diffraction patterns of **(a)** MK-10 **(b)** KI-10

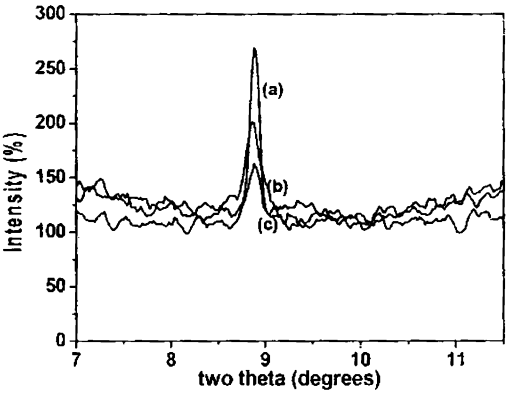


Fig 3.15 X-ray diffraction patterns of **(a)** MK-10 **(b)** K-10S **(c)** K-10SG

The parent montmorillonite clay gives a distinct peak around 2θ equal to 8.88°, which corresponds to a basal spacing of 9.95Å (Fig 3.14 (a)). After grafting with 3-aminopropyltriethoxysilane and glutaraldehyde, (Fig 3.15 b & c) the d spacing for the same was 9.97Å and 9.94Å (Table 3.1). Upon immobilization of lipase (Fig 3.14 b) there was no change in d-spacing which confirmed that the texture of MK-10 clay is maintained and the modification takes place only at the external surface, further supported by surface area measurements. Thus, the enzyme did not enter the interlayer space. Mostly lipase inhabited on the external surfaces and at the edges of the interlayer sheets through hydrogen bonding, Van Der Waals and electrostatic force interactions with hydrophilic residues of the enzyme. According to the results of XRD, FTIR and thermal analysis, it can be concluded that the texture of the montmorillonite is maintained and the 3-aminopropyltriethoxysilane is only connected with the surface of clay. After immobilization of Horseradish peroxidase enzyme on aluminum-pillared interlayered clay (Al-PILC), no change appeared in the XRD pattern. The rough dimensions of Horseradish peroxidase were far larger than the basal spacing of the

support, which made HRP diffuse into the Al-PILC galleries impossible [22]. There was not much change in the basal spacing when lipase was adsorbed onto modified and unmodified bentonite [23]. There was not much change in the d spacing after APTES functionalisation with palygorskite due to functionalisation only on the external surface [24]. Naidja and Huang [25] found that large molecules of aspartase (MW 180,000) were intercalated between the montmorillonite layers and expanded the d-spacing of Ca-montmorillonite from 14.7 to 26.5 Å.

3.5 C H N Analysis

Elemental microanalysis results of the enzymatic prepared systems give an indication of the presence of nitrogen, with respect to the pure support. In order to illustrate the superiority of enzyme-support complex obtained from heptane, two set of experiments in aqueous and in heptane were carried out. The carbon, hydrogen and nitrogen levels of the support, free lipase and immobilized derivatives (in both aqueous and organic medium) are presented in Table 3.2. The nitrogen content of the various immobilized supports in heptane increases in the order MCF160>KI-10>MCF35. By comparing the results, the nitrogen incorporation on the immobilized derivative obtained from buffer solution was much lower than the one measured for the preparation obtained from heptane, which is in agreement with the catalytic activities.

Table 3.2 Elemental (CHN) analysis data of the samples immobilized in buffer and heptane

Sample name	%C	%H	%N
Free lipase	26.2	5.6	1.8
MT-Buffer	2.84	3.22	0.46
MT- Heptane	3.10	3.67	0.57
MH-Buffer	2.54	0.36	0.52
MH-Heptane	2.90	0.56	0.64
K-10-buffer	3.06	0.64	0.51
K-10-heptane	3.52	0.81	0.58

Functionalisation of the mesocellular silica foams (MCF160 and MCF35) was carried out in two different solvents (acetone and toluene) in order to find the better solvent in which maximum functionalization took place. As expected, calcined silica has no C, H and N. However, on APTS loading, % of C, % of H and % of N is observed to increase. On further binding of glutaradehyde to amino functionalised silica, %C and %N are expected to increase whereas overall %N is expected to decrease which is evident form the CHN data given in Table 3.3.

These data show that loading of APTS and glutaraldehyde increases in the order MCF160 > K-10 > MCF35. This data along with FTIR, TG, NMR and surface area results confirmed the incorporation of the amine and the glutaraldehyde moieties on to silica and clay. It was confirmed from the CHN results that acetone was a better solvent for fuctionalization of MCF-160 since the % of nitrogen incorporated is much greater in this medium. In the case of MCF-35, toluene was chosen as the medium for functionalisation from the CHN results. In the case of K-10 functionalisation was carried out in acetone.

Table 3.3 Elemental (CHN) analysis data of various samples after functionalisation with APTES and glutaraldehyde

Sample name	%C	%H	%N
MHS-A	24.7	7.1	8.1
MHSG-A	40.3	7.5	5.2
MHS-T	21.5	5.5	6.9
MHSG-T	31.1	6.0	5.1
MTS-A	14.9	4.4	5.1
MTSG-A	18.8	4.7	3.7
MTS-T	18.5	4.8	5.7
MTSG-T	32.8	6.2	4.4
K-10SG-A	29.3	5.2	5.3

A-acetone, T-toluene

3.6 Nitrogen Adsorption Measurements

Fig. 3.16 shows the N_2 adsorption isotherm of MCF160. The material exhibits a type IV adsorption isotherm with a type-H1 hysteresis loop, which is the characteristic of mesoporous material [26]. The isotherms show large hystereses, which suggests that the MCFs possess ink-bottle-type pores in which large cells are connected by narrower windows [27]. The nitrogen adsorption/desorption isotherms exhibited steep hysteresis of type H1 at high relative pressures which is typical for mesoporous materials that exhibit capillary condensation and evaporation and have large pore sizes with narrow size distributions. The as-prepared MCF material has high pore volume and large pore diameter. The sharp rise at high relative pressures (P/P_0 near 1) indicates the existence of large mesopores in this material. These unique characteristics of MCF demonstrate that it is a favorable host matrix for immobilization of protein.

To know whether the lipase molecules are adsorbed inside the mesopores of MCF, the adsorbent was characterized by nitrogen adsorption after lipase adsorption. Nitrogen adsorption measurements were carried out on samples immobilized in aqueous (pH-7) (Fig 3.17b) and organic medium (heptane) (Fig 3.17c). The amount of nitrogen adsorbed in MCF160 loaded with lipase from n-heptane was higher as compared to those from aqueous medium. There is a shift in the P/P_0 to lower value which shows the encapsulation of enzymes inside the pore in MCF's, leading to the occlusion of most pores. Second, the increasing weight of silica after adsorption of CRL can also reduce the pore volume and specific surface area. Third, the tight packing of CRL molecules instead of adsorption in the mesopores may also decrease the pore volume and specific surface area. The shape of the adsorption isotherm curves for MCF160 (Figs. 3.2b & c) are very similar before and after encapsulating lipase.

The specific pore volume of MCF 160 after immobilization in n-heptane decreases from 2.49 to 0.99 while in aqueous medium the pore volume

decreases from 2.49 to 1.25. There is a much greater decrease in pore diameter for heptane immobilized samples than the aqueous ones which is evident from the pore size distribution curves [Fig 3.18 (b) & (c)]. The BJH pore size distributions of MCF160 and MHI are relatively narrow. Moreover, the monotonic decrease of specific surface area and pore volume confirms the tight packing of the lipase molecule inside the mesopores of MCF 160 silica (Table 3.3). Table 3.3 summarizes the textural properties of the adsorbents before and after lipase immobilization in aqueous and organic medium. The sharp peaks in the pore size distribution plots confirm the narrow size distributions of both the cells and the windows.

Here, the larger pore size in MCF160 undoubtedly enhanced lipase physical adsorption and functionalisation. The nitrogen adsorption/desorption isotherms as well as the pore size distribution (PSD) of the parent MCF unambiguously reveal the characteristic three-dimensional mesocellular structure of the MCF support.

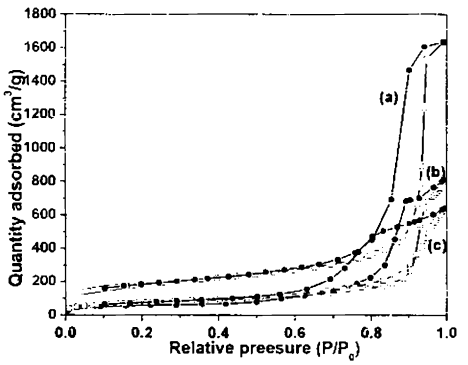


Fig 3.16 Nitrogen adsorption isotherms of **(a)** MCF160, **(b)** MHI (pH-7) **(c)** MHI (heptane)

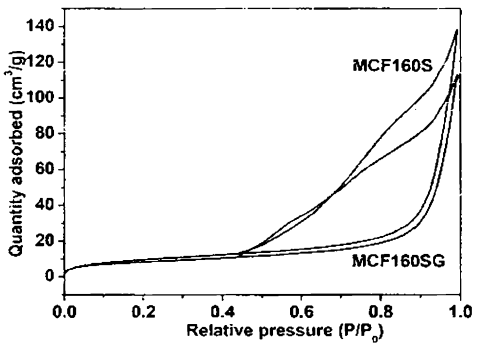


Fig 3.17 Nitrogen adsorption isotherms of functionalized samples of MCF160

After functionalisation (Fig 3.17 (a) & (b)) the shape of the hysteresis shifted from H1 to a H3. A slight pore disordering takes place due to the strain arising in the pore channels due to the high content organic groups and such features are common in mesoporous silicas after organic modifications. These

features are prominent for MCF like silicas due to the flexibility in the pore walls and to their typical morphological structure. The capillary nitrogen condensation was shifted gradually to lower relative pressures, and thus indicating the diminishing of pores size. The surface area decreases from 595 m^2/g to 266 m^2/g after glutaraldehyde binding (Table 3.5). The decrease in surface area and pore volume is not supposed to result from the structure collapse caused by the immobilization of lipase and the following silylation. The pore diameter decreases from 161 to 126m^2/g for silane binded samples and to 98m^2/g for glutaraldehyde samples. The pore size distribution curves of the silane and glutaraldehyde samples are shown in Fig 3.19 (b) & (c).

The amount of nitrogen adsorbed decreases markedly in the rod-like silica (from 687 $cm^3\ g^{-1}$ to 472 $cm^3\ g^{-1}$) and the vesicle-like silica (from 1028 $cm^3\ g^{-1}$ to 703 $cm^3\ g^{-1}$) upon CRL adsorption [28]. Similar results have been reported for the adsorption of lysozyme and cytochrome c onto MCM-41 and SBA-15 [18, 29].

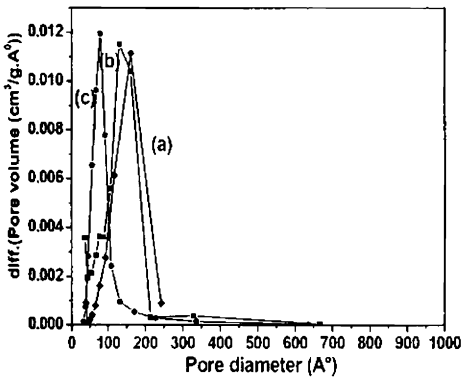


Fig 3.18 Pore size distribution of **(a)** MCF160 **(b)** MHI (pH-7) **(c)** MHI (heptane

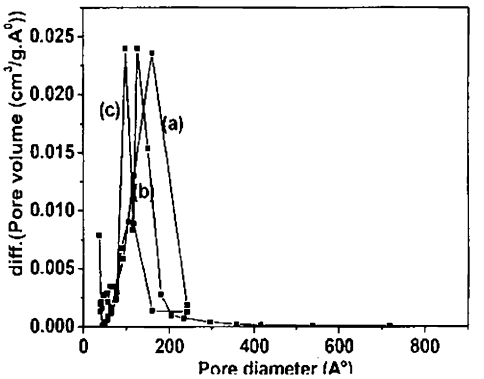


Fig 3.19 Pore size distribution of **(a)** MCF160 **(b)** MHS **(c)** MHSG

The N_2 adsorption/desorption isotherms curves of MCF 35 are shown in Fig. 3.20 & 3.21). The MCF35 materials prepared via room temperature method exhibits a nitrogen adsorption isotherm of type IV with a hysteresis in the region of $0.4 < p/p_0 < 1$, which is a direct evidence of the presence of mesopores. MCF35

exhibited a weak hysteresis indicating a small mesopores. The meso cellular foams exhibit H2 hysteresis. Type H2 hysteresis is attributed to a difference between the condensation and evaporation processes occurring in pores with narrow necks and wide bodies (''ink-bottle'' pores). This is consistent with the previous report [30] on meso-cellular foams formed with windows and cells. After two step functionalization [Fig 3.21 (a) & (b)] the material gets completely distorted due to the less stability of the room temperature synthesized sample and thus the insertion of high content of organic groups inside the pore channels can meaningfully distort its pore structure. The mesoporosity is gradually getting reduced due to the functionalisation step. Thus, the materials synthesized under room temperature conditions were not stable enough for functionalisation while the mesocellular silica foams synthesized hydrothermally are stable enough for further modification. As a large number of silanol groups are present on MCF35 compared to MCF160 a higher degree of functionalisation takes place on MCF35. But due to the instability of the material disordered mesostructures are obtained. The surface area decreases from 914m^2/g to 506 m^2/g after immobilization of lipase in heptane while the surface area of aldehyde functionalized sample decreases from 914 to 148 m^2/g (Table 3.5).

Table 3.4 Textural parameters of catalysts immobilized in aqueous and organic media

SAMPLE	Surface area (m^2/g)	Pore diameter (A^0)	Pore volume (cm^3/g)
MCF160	595	161	2.49
MHI(pH-7)	266	131	1.25
MHI (heptane)	246	78	0.99
MCF35	914	35	0.81
MTI (pH-7)	514	34	0.47
MTI (heptane)	506	33	0.44
MK-10	246	36	0.42
KI-10 (heptane)	73	34.7	0.17

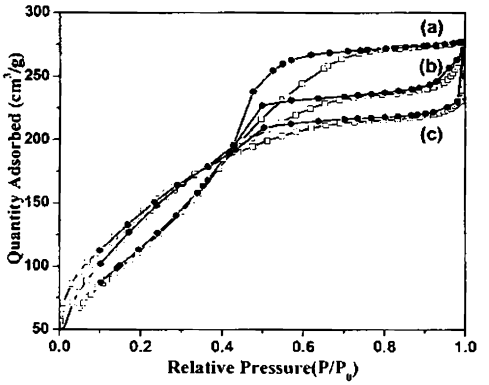


Fig 3.20 Nitrogen adsorption isotherms of **(a)** MCF35 **(b)** MTI (pH-7) **(c)** heptane

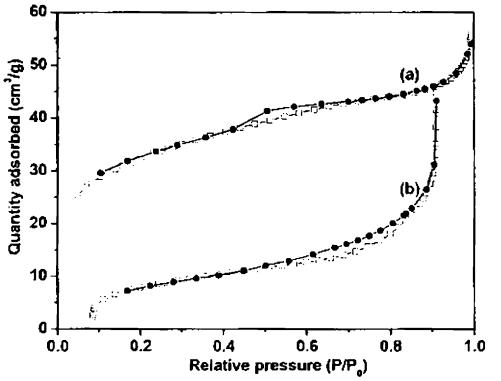


Fig 3.21 Nitrogen adsorption isotherms of **(a)** MTS **(b)** MTSG

MCF35 presented a wider pore size distribution (Fig 3.22 a) with a lower pore volume due to its more disordered nature compared to MF160. The physical adsorption of lipase in MCF35 did not significantly change the main pore size, which remained around 34Å [Fig 3.22 (a) and (b)]. Nonetheless, the surface area of lipase loaded MCF35 decreased from 914 to 512 m^2/g for heptane immobilized sample and 514 m^2/g for pH-7 immobilized sample. The maintenance of the pore size in MCF35 suggests that lipase molecules should mainly occupy the external surface of the particles.

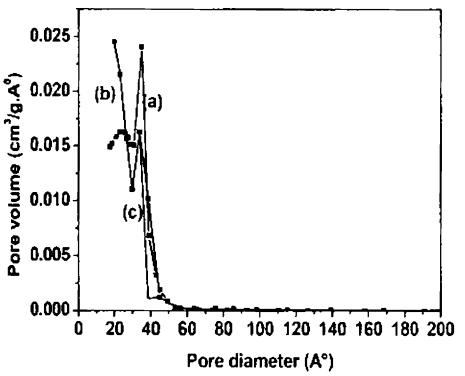


Fig 3.22 Pore size distribution of **(a)** MCF35 **(b)** MTI (pH-7) **(c)** heptane

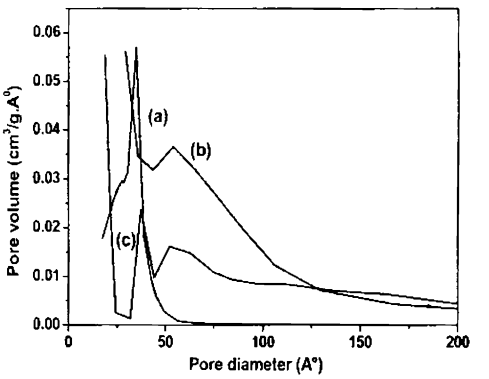


Fig 3.23 Pore size distribution of **(a)** MCF35 **(b)** MTS **(c)** MTSG

The pore size distribution shifts to higher value after functionalisation with silane and glutaraldehyde (Fig 3.23 (a) and (b)) which clearly shows that the walls of MCF35 are very thin and hence gets easily disrupted to form larger pores. This observation is also supported by XRD studies in which an increase in d spacing is observed. Table 3.5 summarizes the textural properties of pure silica and the functionalized samples.

Table 3.5 Textural parameters of functionalized samples

SAMPLE	Surface area (m^2/g)	Pore diameter (Å)	Pore volume (cm^3/g)
MCF160	595	161	2.49
MHS	309	126	0.21
MHSG	266	98	0.05
MCF35	914	35	0.81
MTS	339	55	0.24
MTSG	148	39	0.07
MK-10	246	36	0.42
K-10S	186	36	0.36
K-10SG	133	35	0.30

Natural smectite clays display type IV isotherms with a H3 or H4 hysteresis loops. There is neither a high uptake at high P/P_0 values nor a definitive mesoporous region. The pore size distribution is shifted to smaller pore sizes, and indeed the assignment of aggregrates of platelet like particles giving rise to slit shaped pores applies to smectites. The phenomenon arises due to the complexity of capillary condensation in pore networks with pore blocking effects. H4 hysteresis loops arise in systems containing microporous slit shaped pores. Fig. 3.24 (a) & (b) shows the N_2 adsorption/desorption isotherms of parent montmorillonite and the immobilized sample. The steep increase in adsorbed volume at low partial pressure is attributed to microporous (pore radius ~10 Å) condensation, while the hysteresis occurring at high partial pressures is attributed to the mesoporous structures (pore radius of 18-200 Å) [31].

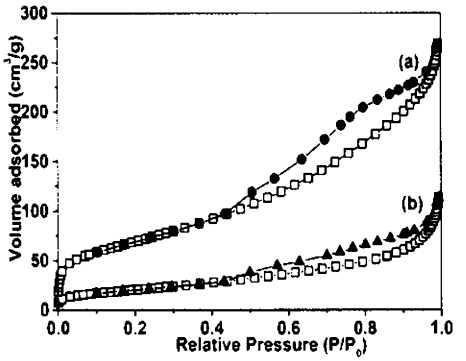


Fig 3.24 Nitrogen adsorption isotherms of **(a)** MK-10 **(b)** K1-10 (heptane)

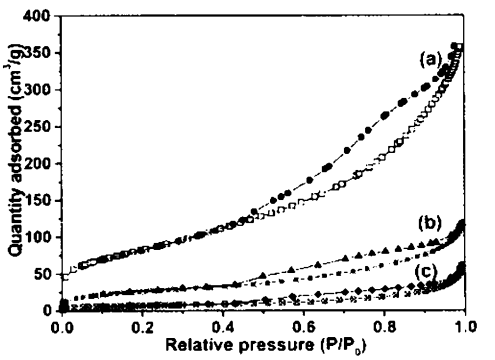


Fig 3.25 Nitrogen adsorption isotherms of **(a)** K-10 **(b)** K-10S **(c)** K-10 SG

Adsorption isotherm of montmorillonite K-10 clay belongs to the type II in the Brunauer, Deming, Deming and Teller (BDDT) classification [32], characteristic of nitrogen adsorption on macroporous adsorbents (with less or no porosity). Furthermore, the hysteresis loops of these isotherms are assigned to type H4 in the IUPAC classification [33], which is representative of the slit-shaped pores in layered materials. The pore size curves Fig 3.26 (a) shows a wide pore size distribution.

After adsorption of lipase in heptane the amount of N_2 adsorbed decreases while there is not much change in the P/P_0 value as shown in the Fig 3.24 (b), which shows that the adsorption is entirely external and no intercalation is taking place in the clay which is evident form the XRD results. The surface area of pure montmorillonte K-10 is 246m^2/g which decreases to 73 m^2/g (Table 3.5) after lipase adsorption while there is not much shift in the pore size distribution curves (Fig 3.26 (b)). The results of the textural characterization for the K10 clay are similar to those reported in the literature. Kawi and Yao [34] obtained a surface area of 197 m^2/g for this clay. The K-10 clay has a low microporosity.

After functionalisation with silane and glutaraldehyde, the surface area decreases from 246 to 133m^2/g with no appreciable decrease in the pore diameter and pore volume.

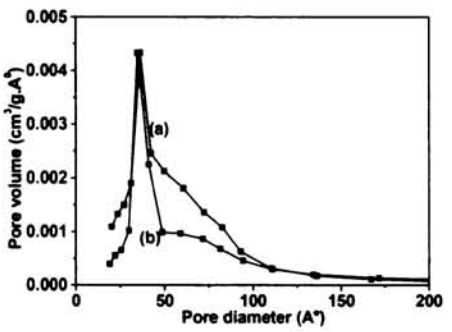


Fig 3.26 Pore size distribution of **(a)** K-10 **(b)** KI-10

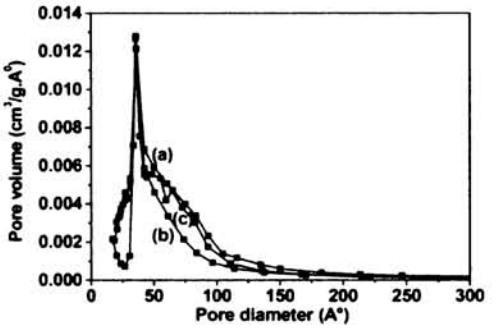


Fig 3.27 Pore size distribution of **(a)** K-10 **(b)** K-10S **(c)** K-10SG

The pore size distributions of the pure clay and the functionalized samples are shown in Fig 3.27 (a) and (b). There is no intercalation taking place upon functionalisation which depicts that the binding of organic groups is entirely external.

3.7 Transmission electron microscopy

TEM image (Fig. 3.28) of MCF 35 sample reveals a disordered array of silica struts comprising of uniformly sized spherical cells (20–34 nm) interconnected by windows with a narrow size distribution, which is characteristic structural feature of the MCF materials. The strut-like structure resembles that of aerogels [30, 35]. TEM images of this material exhibited disordered mesopores with wormhole like structure. Uniformly sized cellular pores is evident from the TEM image.

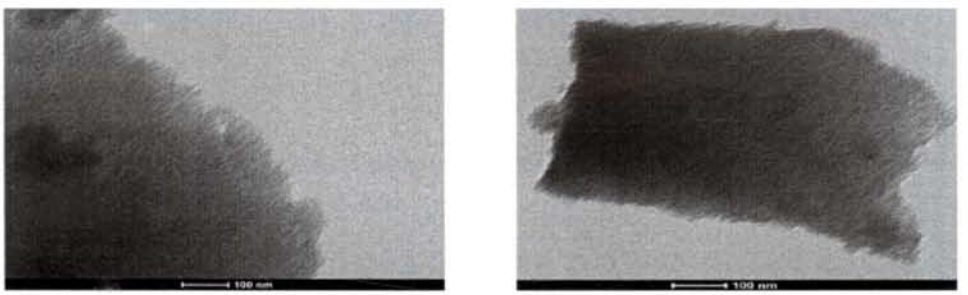


Fig 3.28 Transmission electron micrographs of MCF 35 at two different magnifications

Transmission electron micrographs of MCF160 are shown in Fig. 3.29. It can be easily seen that the hydrothermally synthesized MCF catalysts present the typical three-dimensional and ultralarge pore structures of the pure MCFs.

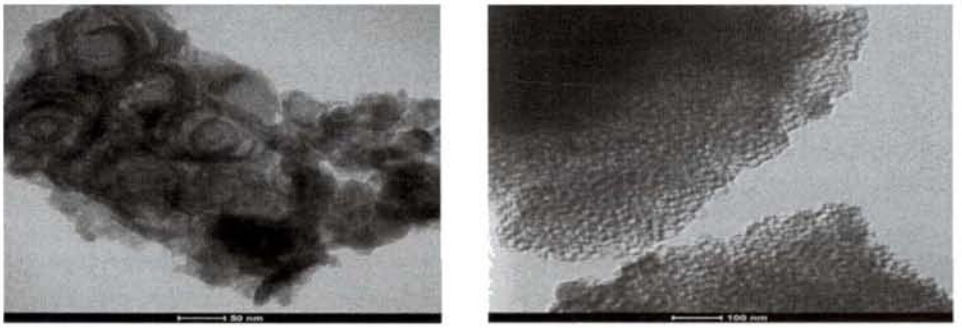


Fig 3.29 Transmission electron micrographs of MCF 160 at two different magnifications

The TEM image illustrates that MCF has a three dimensional mesocellular arrangement of the MCF frameworks. Mesopore cells in the sample could be observed from the image.

3.8 Scanning Electron Microscopy

Since an enzyme is a highly polymeric material, immobilization on a solid matrix can change the morphology.

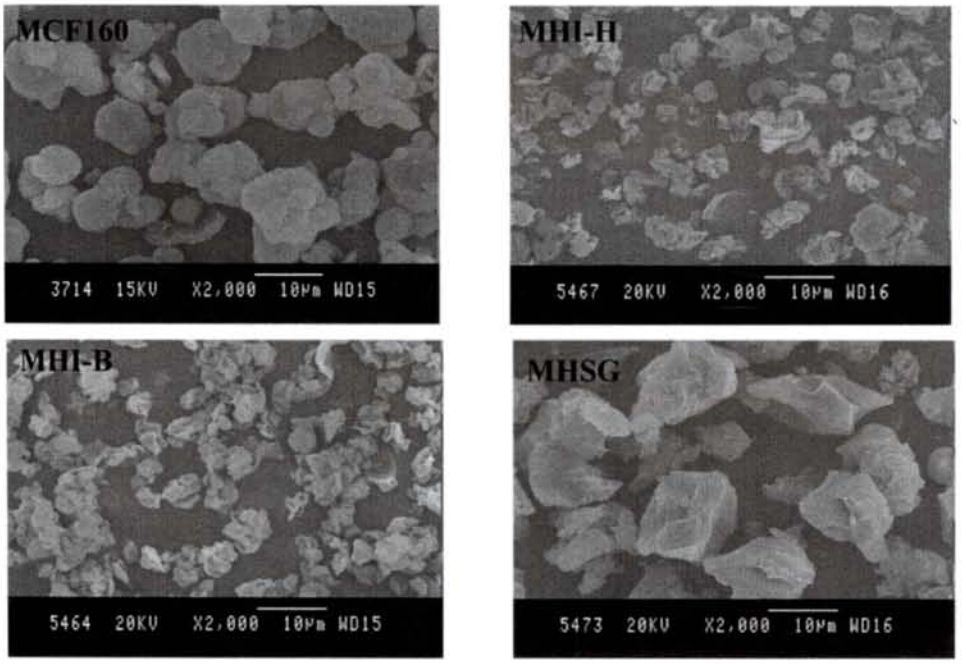


Fig 3.30 Scanning electron micrographs of MCF160

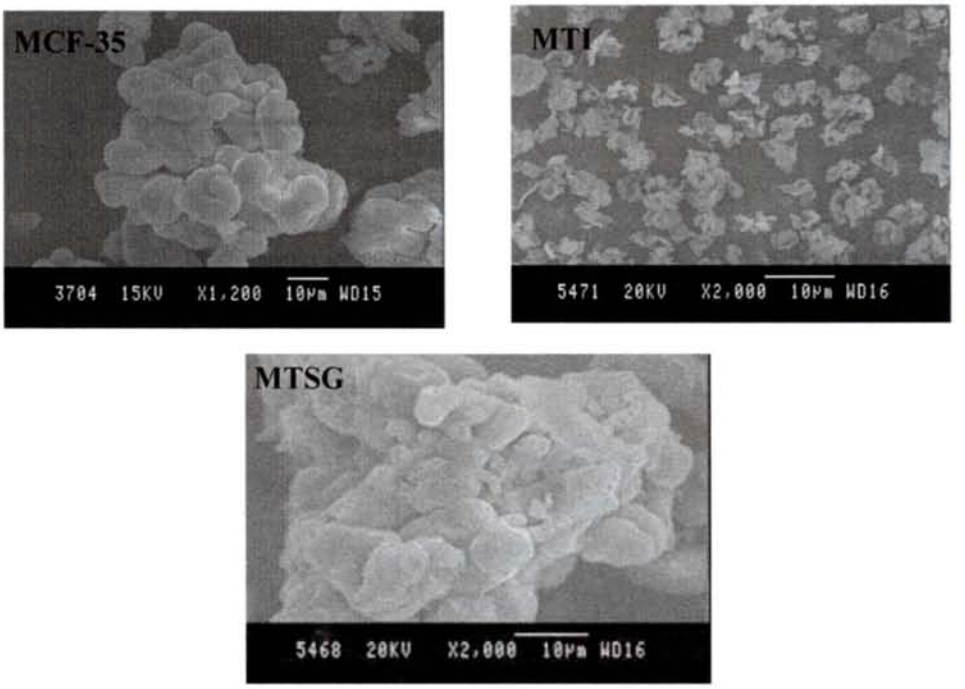


Fig 3.31 Scanning electron micrographs of MCF35

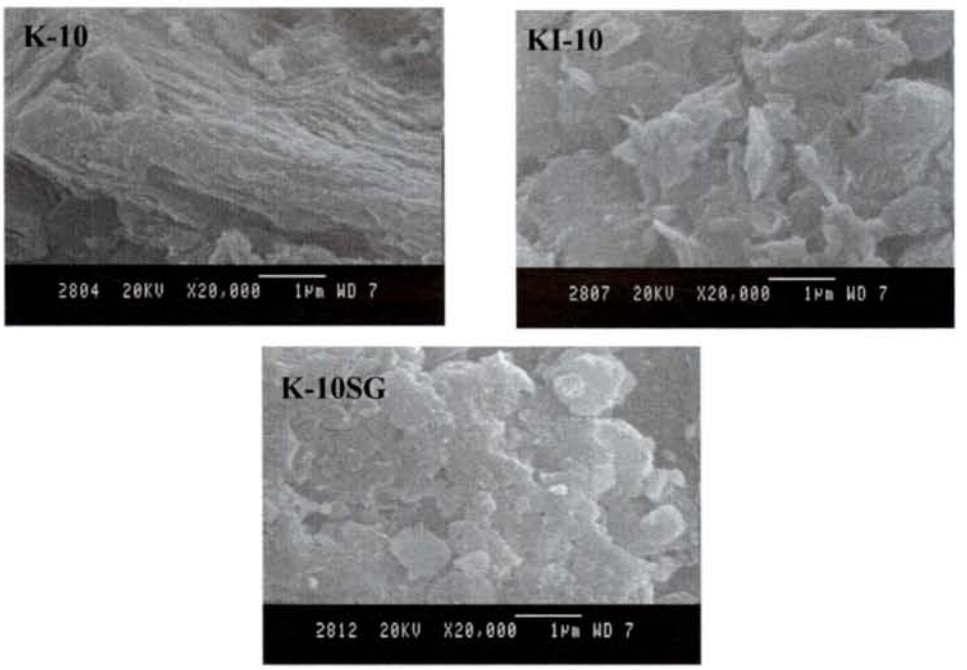


Fig 3.32 Scanning electron micrographs of K-10

Fig. 3.30 and 3.31shows the SEM images of MCFs prepared via hydrothermal route and room temperature method. MCFs appear to be bigger particles due to aggregation. The MCFs exhibit cauliflower-type morphology. After immobilization (MHI, MTI), the surface was filled by the rounded structure, which is presumably protein aggregates. Similar enzyme aggregrates of chymotrypsin were also observed on polystyrene/polystyrenecomaleic anhydride) NFM after covalent binding [36]. After functionalisation with glutaraldehyde the particles appear to be bigger due to aggregation.

The morphologies of montmorillonite K-10 are displayed in SEM photographs (Figure 3.32). Generally, both morphologies shown were uniformly layered structures with a flaky aspect on a smooth surface. The appearance of boulder like structures may be due to the presence of enzyme and an increase in particle size after immobilization is observed. A slight porous nature is observed after functionalization with glutaraldehyde. The polyhedral particles of KIT-6 samples lost their individual nature after lipase immobilization due to aggregration into larger entities [37].

3.9 CPMAS Nuclear magnetic resonance spectroscopy

The ^{29}Si MAS spectrum of the parent MCF 160 (Fig 3.33) exhibits two broad resonances at -112.3 ppm for a Q^4 environment and at -102.6 ppm for a Q^3 environment together with a shoulder at -93.1 ppm ascribable to Q^2 species. After immobilization of lipase (MHI), the intensity of Q^2 and Q^3 sites decreased (Fig 3.33). The Q^3 peak shifted to -104.1ppm which showed that the enzyme is interacting with Q^3 and Q^2 sites than Q^4. From specific surface area and ^{29}Si NMR data, the presence of interactions between the enzyme and the silica network is evident. The solid-state ^{29}Si CP-MAS NMR spectra of MCF 35 silica showed two chemical shifts at d = -102.5 (Q^3), and -92.5 (Q^2) ppm due to the different surroundings (Fig 3.34). There was no Q^4 peak detected which means that there are decreased crosslinked silanol groups or else due to the increased content of non-condensed silanol groups. The explanation is that Q^2+Q^3/Q^4 ratio of the unclacined form of MCF35 is 0.09 and it is 1.31 for MCF's. Upon calcinations this ratio remains the same in the case of MCF35 but it decreases to 0.39 for MCF's. Hence the walls of the MCF's

are more strained due to the transformation of Q^3 to Q^4 by calcination which accounts for the lower stability of MCF 35.

After functionalisation with aminopropyl triethoxysilane (APTES), MHS showed a distinctive chemical shift at d =-65 ppm (T^3), which was attributed to APTES grafted on the MCF silica (Fig 3.33). The peak intensity ratio of Q^3/Q^4 for MCF 160 silica was higher than that for MAS and Q^2 peak observed in the MCF silica undergoes a shift from -92 to -85ppm. A decrease in intensity of the Q^2 peak is observed. These results indicate that hydroxyl groups of MCF silica reacted with APTES by covalent bonding. In the case of MTS, in addition to the peaks at -101ppm (Q^3) and -111ppm (Q^4) due to the $(Si\text{-}O)_3$ -SiOH and $(Si\text{-}O)_4$ Si moieties of silica surface, there appears two peaks at -59 and -67ppm identified with the silane silicons that have two Si-O-Si attachments to the silica surface (or to other silicons) and R-Si slicons with three Si-O-Si linkages [Fig 3.34 (b)]. The presence of ^{29}Si peaks due to the specified types of silane silicons attached indicates the grafting of silane on the surface of silica. The peaks at -60ppm and -67 ppm are attributed to T^2 [R-Si)$OSi)_2$-OH] and T^3 silicons [R-Si $(OSi)_3$].

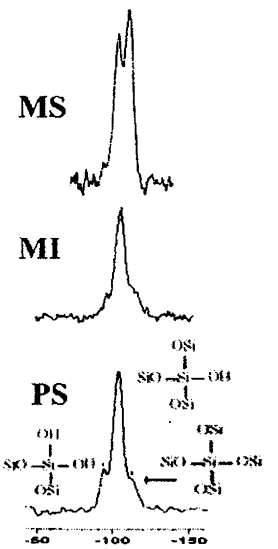


Fig 3.33. ^{29}Si NMR spectra of MCF160, MHI and MHS

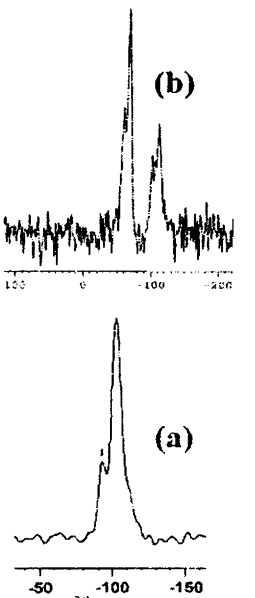


Fig 3.34. ^{29}Si NMR spectra of **(a)** MCF35 **(b)** MTS

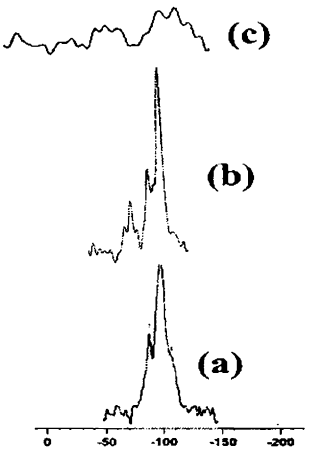


Fig 3.35 ^{29}Si NMR spectra of (a) K-10 (b) K-10S (c) K-10SG

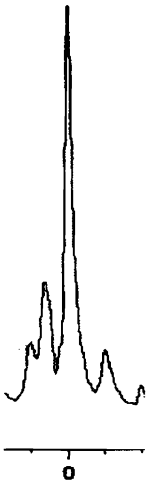


Fig 3.36 ^{27}Al NMR spectra of K-10

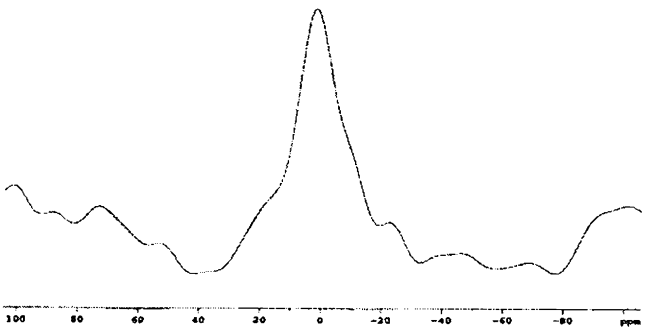


Fig 3.37 ^{27}Al NMR spectra of K-10 S

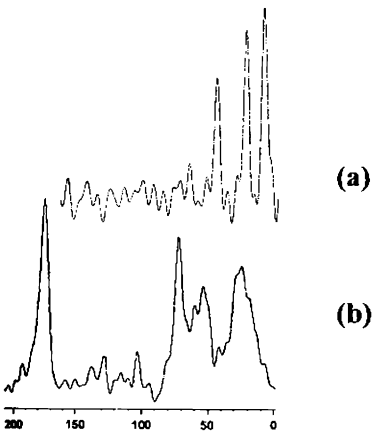


Fig3.38 ^{13}C NMR spectra of (a) MHI (b) MHS

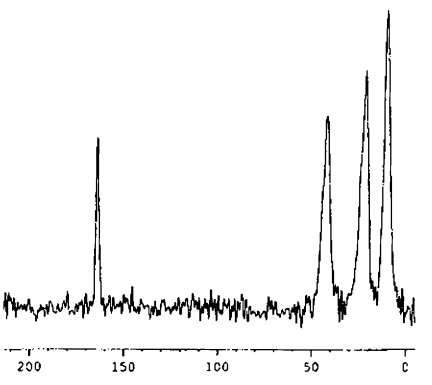


Fig 3.39 ^{13}C NMR spectra of MTS

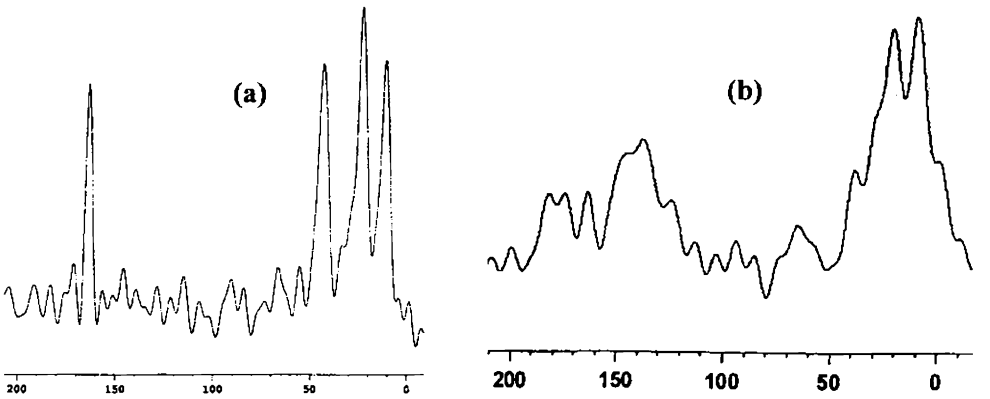


Fig 3.40 ^{13}C NMR spectra of **(a)** K-10S **(b)** K-10SG

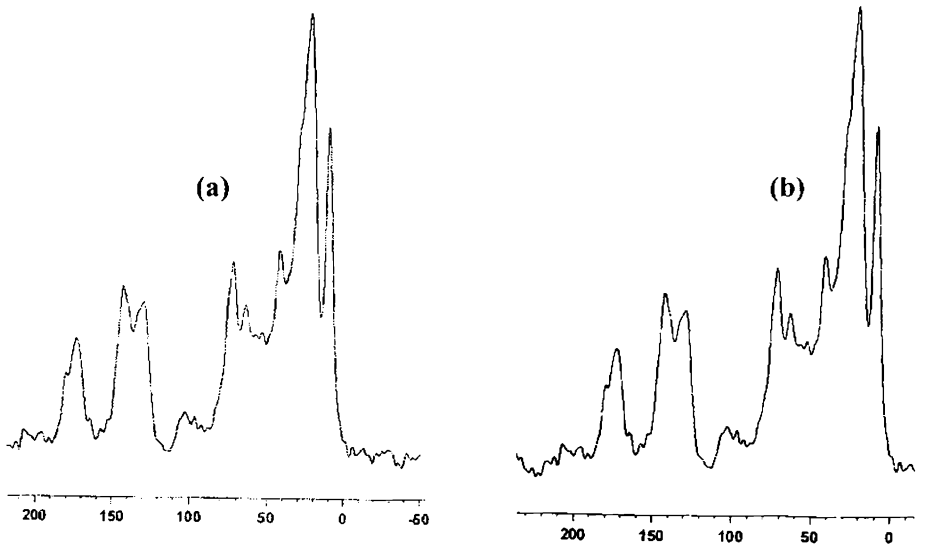


Fig 3.41 ^{13}C NMR spectra of **(a)** MCF160G **(b)** MCF160GE

The ^{13}C NMR spectrum of MHS showed three chemical shifts at d = 9 (C1), 24 (C2), and 43 (C3) ppm, while MCF silica exhibited no resonance peak (Fig 3.38 (b)). The three resonance peaks observed in the MHS were attributed to different carbon atoms (C1, C2, and C3) in the APTES. This result indicates that aminopropyl functional groups were successfully grafted on the MCF silica. In the case of MTS also, ^{29} Si NMR gave characteristic peaks due to the three type of C atoms of APTES at C1 (10.4ppm), C2 (21.7ppm) and C3

(42.3ppm), which confirmed the grafting of silane onto MCF35 silica. After immobilization (MAI) (Fig 3.38 (a)) peaks due to CH_2 groups, C-N bond, aromatic C, amide and carboxylic acid groups of various amino acids in enzyme appeared in the spectrum which gives further support for the information obtained from IR, TG, surface area and CHN results. After binding with glutaraldehyde (Fig 3.41 (a)(MHG), peaks due to alkane C atom attached to imine appears at 8, 22, 42, 66, 73 and 145 ppm. The peak due to the imine groups formed between enzyme and glutaraldehyde appears at 175 ppm and the aldehydic carbon of glutaraldehyde appears at 214 ppm.

In the case of lipase binded covalently to the functionalized samples (MHGE) (Fig 3.41 (b)), the ^{13}C spectra gives peaks due to alkane C of lysine in enzyme at 10, 11.8, 40.7 ppm. Peaks due to C-O and C-N groups present in tyrosine and cysteine appears at 61 and 72 ppm. Aromatic C atoms in tyrosine appear at 99 ppm. The imine group formed between the enzyme and aldehyde appears at 174 ppm and also the peaks due to the carboxylic acid groups in cysteine and lysine appears at 180 ppm.

Clays are aluminosilicates and hence there is ample scope for NMR analysis due to the presence of two NMR nuclei- ^{27}Al and ^{29}Si. Changes in the chemical shift environment of Al can be easily visualized with the help of ^{27}Al NMR. This technique is of particular interest in studying octahedral and tetrahedral sites in the Al framework and so can be easily applied for the study of enzyme immobilization on montmorillonite.

^{27}Al NMR spectra of Montmorillonite K-10 clay (Fig 3.36) showed two resonances around 0 and 63ppm representing Al in octahedral and tetrahedral coordination [38, 39, 40]. The ^{27}Al of K-10S (Fig 3.37) appears much broader, due to the tight binding of organic moieties with the surface of clay. The octahedral resonance undergoes a shift from 0ppm to 2ppm and the tetrahedral resonance undergoes a similar shift from 63.8ppm to 73.2ppm. These shifts

confirmed that both the octahedral and tetrahedral sites are involved in binding with silane.

The ^{29}Si MAS NMR spectra of montmorillonite K-10 (Figure 3.35 (a)) show a broad resonance line showing several shoulders. This line is centered at -103 ppm, but ranges from -90 to -113 ppm. This is the chemical shift region characteristic of Si atoms surrounded by zero, one, two, three, and four Al atoms. For ^{29} Si NMR, two peaks originate at -93 and -103 ppm that is assigned to Si in Q^3 and Q^4 states respectively. Additional peaks due to the presence of APTES were observed at -63ppm due to the T^2 species (Fig 3.35 (b). The Q^3 peak disappeared after silanisation which means that the isolating groups are interacting with clay surface. The Q^4 intensity increases after silanisation which may be due to the interaction of silane groups of APTES. After glutaraldehyde binding the intensity of the peaks decreased with not much change in the peak position.

Incorporation of silane groups onto clay was confirmed by the presence of peaks at 10.7, 22.6, 43ppm in the ^{13}C NMR spectrum of K-10S (Fig 3.40 (a)). The incorporation of glutaraldehyde (K-10SG) is evident from the peak at 165ppm and 145ppm due to the imine and C attached to CH=N group [Fig 3.40 (b)].

3.10 Contact angle measurements

Proteins adsorbed to solid surfaces alter the original interfacial properties [41, 42]. To show that the modified supports are hydrophobic in nature, we have carried out the contact angle measurement with the unmodified and modified surfaces at room temperature (25°C).

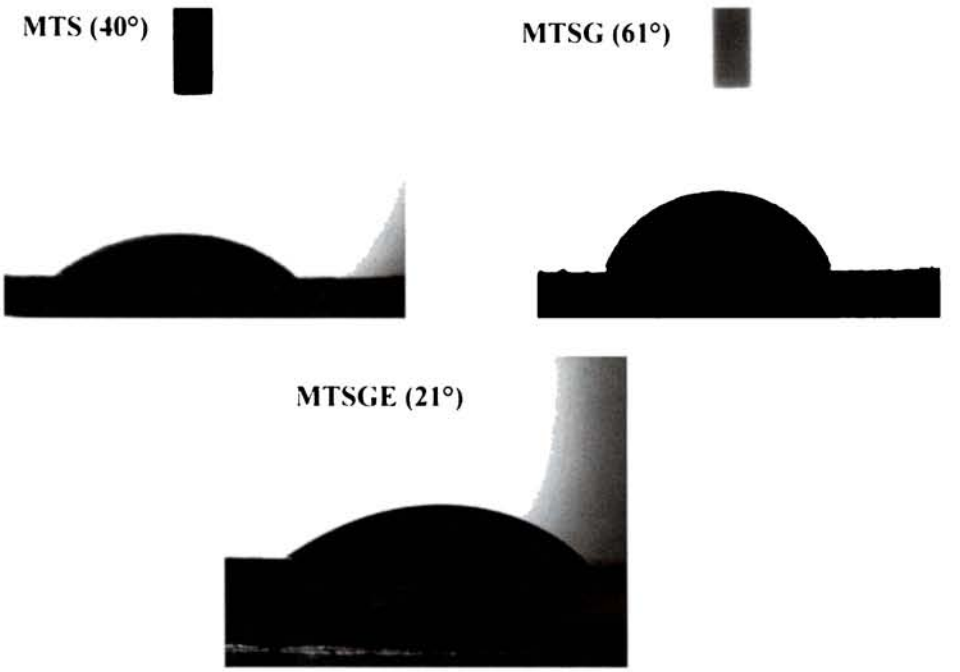


Fig 3.42 Contact angle photographs of functionalised samples of MCF35

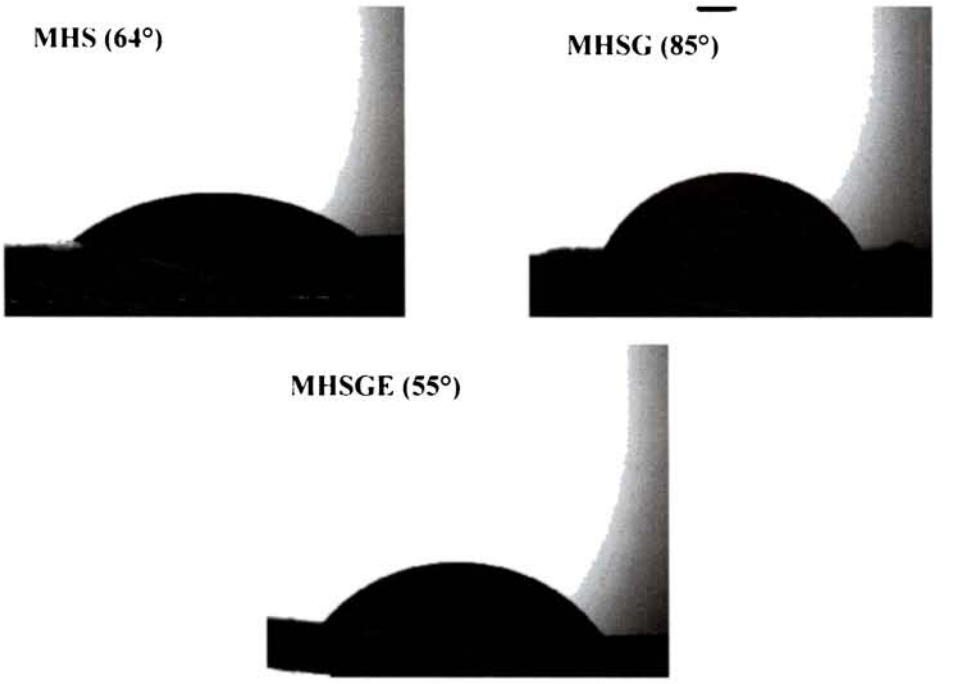


Fig 3.43 Contact angle photographs of functionalised samples of MCF160

Fig. 3.42, 3.43 and 3.44 shows the contact angle of the functionalized surfaces of all the supports. The contact angles of pure supports MCF-160, MCF-35, KI-10 as well as the adsorbed ones remained 0° (water drops spread instantly when placed on the surface of the substrate) indicating that the surfaces are hydrophilic in nature. This is due to the hydroxyl groups of the different supports that make it superhydrophilic.

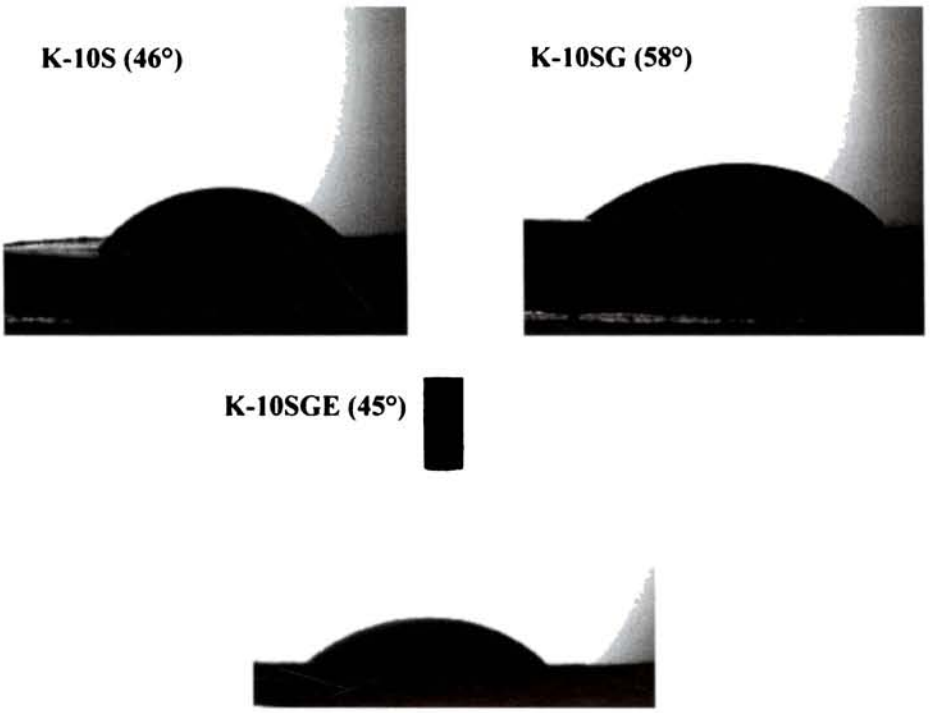


Fig 3.44 Contact angle photographs of the functionalised samples of K-10

The contact angle of MCF-160 increases from 64° to 85° after glutaraldehyde treatment. Adsorption occurring via favorable electrostatic attraction would orient hydrophobic regions of the molecule away from the surface. Indeed, the presence of these hydrophobic domains leads to a higher water contact angle than that measured on bare hydrophilic surfaces [42]. The same increase in pattern of the contact angle values was observed for MTS (40°) to MTSG (61°) and also in the case of K-10S (46°) to K-10SG (58°). The increase in the contact angle [43]

indicated an increase in the hydrophobicity of the chemically modified surfaces and also the successful incorporation of the amine and glutaraldehyde moieties on to the surface. The lower contact angle of MCF-35G compared to other supports features its wettability nature and results in lower immobilization.

Table 3.6 Contact angles of the pure, immobilized and functionalized samples

SAMPLE	CONTACT ANGLES (°)
MCF160	0
MHS	64
MHSG	85
MHSGE	55
MHI	0
MCF35	0
MTS	40
MTSG	61
MTSGE	21
MTI	0
K-10	0
KI-10	0
K-10S	46
K-10SG	58
K-10SGE	45

The higher contact angle of MHSG signifies the relative hydrophobic surface with respect to MTSG and K-10G.The surface modification of carriers resulted in the variation of the contact angle (Table 3.5). From the static contact angle measurements, the water contact angle of the modified MHSG is about 85° and value decreased to 55° after lipase immobilization, indicating that surface immobilization has changed the surface property of the support to a more hydrophilic one which will be beneficial for carrying out the hydrolysis reaction. The surfaces became more hydrophilic after lipase adsorption in the case of MTSG and K-10G. The order of hydrophilicity being is MTSG>MHSG >K-10G which is also a proof for the higher amount of lipase adsorption. But probably due to the tight packing of enzyme molecules and also the steric hindrances

imparted by the organic groups, only on the surface rather than inside the pores the activity is much less than the other covalently bound immobilized systems in nonaqueous medium which favors a hydrophobic surface.

Yang et al. [44] used contact angle methods to measure the change in hydrophilic-hydrophobic balance exhibited by a number of different materials following adsorption of protein. They found that adsorption of protein rendered hydrophilic surfaces more hydrophobic and hydrophobic surfaces more hydrophilic.

3.11 Conclusions

Mesocellular silica foams were successfully prepared via hydrothermal route and via room temperature. The resulting carriers were evaluated for the immobilization of Candida rugosa lipase via adsorption and chemical bonding. Various physico chemical characterization techniques were employed for the characterization of the pure silica supports and clay and also for the study of functionalized as well as immobilized samples. The important conclusions from the characterization studies are:

- Mesocellular silica foams were synthesized by oil-in-water microemulsion templating route and were functionalized with silane and glutaraldehyde.
- The experimental results from IR spectroscopy and elemental analysis demonstrated the presence of immobilized lipase and also functionalisation with silane and glutaraldehyde on the supports.
- A slight broadening and lowering of dspacing values after immobilization and modification was observed in the case of MCF160 and MCF35 but there was no change in the d-spacing in the case of K-10 which showed that the enzymes are adsorbed only on the external surface.
- The surface area, pore diameter and pore volume decreased drastically in the case of MCF160 due to the incorporation of the organic groups into

the mesopores. In the case of MCF35 a disordered nature was observed while in the case of K-10 there was only a decrease in the amount of nitrogen adsorbed after functionalisation without any change in pore diameter revealing that the functionalisation took place on the external surface in conformity with the XRD results.

- The thermal stability of the systems improved after immobilization and functionalisation when compared to the pure supports.
- SEM micrographs showed aggreration due to protein immobilization.
- TEM micrographs revealed the mesocellular structure of silica foams.
- ^{29}Si NMR of K-10 clay shows broadening after functionalisation which shows the strain imparted by the organic groups.
- ^{29}Si NMR and ^{13}C studies revealed the incorporation of the organic groups onto the supports which was further confirmed from the CHN results. There was an increase in contact angle with surface functionalisation in all the supports which can be correlated with the increased activity observed for the covalently bound systems. These studies illustrated that hydrophobicity could explain the difference of enzyme activity of the functionalized samples.

References

[1] B. Stuart, W. O. George, P. S. Mcintyre, in: D. J. Ando (Ed.), Modern Infrared Spectroscopy, John Wiley & Sons, England (1996), pp. 117.

[2] X. Wang, K. S. K. Lin, J. C. C. Chan, S. Cheng, J. Phys. Chem. B., 109 (2005) 1763.

[3] A. S. M. Chong, X. S. Zhao, J. Phys. Chem. B., 107 (2003) 12650.

[4] O. A. C. Monteiro. Jr, C. Airoldi, 26 (1999) 119.

[5] C. Airoldi, O. A. C. Jr. Monteiro, Polymer Bulletin, 50 (2003) 61.

[6] J. Ravinchandran, B. Sivasankar, Clays Clay Miner., 45 (1997) 854.

[7] V. C. Farmer, "The Infrared Spectra of Minerals, "Mineralogical Soc., London (1974) pp. 331–363.

[8] C. Airoldi, S. F. de Oliveira, J. Braz. Chem. Soc., 3 (1992) 47.

[9] H. F. Castro, M. L. C. P. Silva, G. L. J. P Silva, Braz. J. Chem. Eng., 17 (2000) 4.

[10] S. Montero, A. Blanco, M. D. Virto, L. C. Landeta, I. Agud, R. Solozabal, J. M. Lascaray, M. de Renobales, M. J. Llama, J. L. Serra, Enzyme Microb Technol., 15 (1993) 239.

[11] H. P. He, J. Duchet, J. Galy, J. F. Gerard, J. Colloid Interface Sci., 288 (2005) 171.

[12] L. A. Feigin, D. I. Svergun, Structure Analysis by Small-Angle X-ray and Neutron Scattering; Plenum Press: New York (1987) Chapter-3.

[13] J. Lee, K. Sohn, T. Hyeon, J. Am. Chem. Soc., 123 (2001) 5146.

[14] B. Marler, U. Oberhagemann, S. Vortmann, H. Gies, Microporous Mesoporous Mater., 6 (1996) 375.

[15] A. Sayari, S. Hamoudi, Chem. Mater., 13 (2001) 3151.

[16] T. Sen , G. J. T. Tiddy, J. L. Casci, M. W. Anderson, Microporous Mesoporous Mater., 78 (2005) 255.

[17] Y. Kang, J. He, X. Guo, X. Guo, Z. Song, Ind. Eng. Chem. Res,. 46 (2007) 4474.

[18] A. Vinu, V. Murugesan, O. Tangermann, M. Hartmann, Chem. Mater., 16 (2004) 3056.

[19] T. J. Pinnavaia, Scienc, 220 (1983) 365.

[20] T. J. Pinnavaia, R. Raythatha, J. G. S.Lee, L. J. Halloran, J. Hoffman, J. Am. Chem. Soc., 101 (1979) 6891.

[21] P. Laszlo, Science, 1473 (1987). 235.

[22] J. Cheng, S. Ming Yu, P. Zuo, WAT E R RE S E A R C H, 40 (2006) 283.

[23] M. Ghiaci, H. Aghaei, S. Soleimanian, M. E. Sedaghat, Applied Clay Sci., 43 (2008) 289.

[24] J. Huang, Y. Liu, X. Wang, J. Mol. Catal. B: Enzym., 55 (2008) 49.

[25] A. Naidja, P. M. Huang, J. Mol. Catal. A: Chem., 106 (1996) 255.

[26] S. J. Gregg, K. S. W. Sing, In Adsorption, Surface Area and Porosity; Academic Press: New York (1982).

[27] K. S. W. Sing, D. H. Everett, R. A. W. Haul, Pure Appl. Chem., 57 (1985) 603.

[28] G. Zhou, Y. Chen, S. Yang, Microporous Mesoporous Mater., 119 (2009) 223.

[29] A. Vinu, V. Murugesan, M. Hartmann, J. Phys. Chem. B., 108 (2004) 7323.

[30] P. S. Winkel, W. W. Lukens, P. Yang, D. I. Margolese, J. S. Lettow, Y. J. Ying, G. D. Stucky, Chem. Mater., 12 (2000) 686.

[31] C. N. Satterfield, Heterogenous Catalysis in Industrial Practice, McGraw-Hill, New York, (1991) p. 39.

[32] S. Brunauer, L. S. Deming, W. S. Deming, W. J. Teller, J. Am. Chem. Soc., 62 (1940)1723.

[33] J. H. de Boer, B. G. Linsen, Th. van der Plas, G. J. Zondervan, J. Catal., 4 (1965) 649.

[34] S. Kawi, Y. Z. Yao, Micropor. Mesopor. Mater., 28 (1999) 25.

[35] P. Schmidt-Winkel, W. W. Lukens Jr., D. Zhao, P. Yang, B. F. Chmelka, G. D. Stucky,J. Am. Chem. Soc., 121 (1999) 254.

[36] B. C. Kim, S. Nair, J.Kim. D.Li, J. H. Kwak, Naotechnol., 16 (2005) S382.

[37] E. Serra , A. l. Mayoral, Y. Sakamoto, R.M. Blanco, I. Dıaz, Microporous Mesoporous Mater., 174. (2008) 369.

[38] S. E. Ashbrook, J. McManus, K. J. D. MacKenzie, S. Wimperis, J. Phys. Chem., 104 (2000) 6408.

[39] Q. Liu, D.A. Spears, Q. Liu, Appl. Clay Sci., 19 (2001) 89.

[40] R. Jelink, B. F. Chmelka, A. Stein, G. A. Ozin, J. Phys. Chem., 96 (1992) 6744.

[41] J. Schakenraad, H. Busscher, Colloids Surf., 42 (1989) 331.

[42] Y. Tamada, Y. Ikada, J. Colloid Interface Sci., 155 (1993) 334.

[43] K. S. Kim, K. H. Lee, J. Membr. Sci., 199 (2002) 135.

[44] J. Yang, J. McGuire, E. Kolbe, J. Food Prot., 54 (1991) 879.

.....ooo.....

Chapter 4

LIPASE CATALYZED ESTERIFICATION REACTION IN NON-AQUEOUS MEDIA FOR THE SYNTHESIS OF BUTYLISOBUTYRATE- A FLAVOR ESTER

Contents

Flavor compounds synthesis by biotechnological processes plays nowadays an increasing role in the food industry. Non-aqueous enzymology has emerged as a major area of biotechnology research and development, offering valuable production of chemicals using reactions that are not feasible in aqueous media. Esters serve as precursors and/or additives for a variety of perfumes and flavours, pharmaceuticals, agrochemicals, plasticizers and polymers, and also as solvents. Lipases, as a class of enzymes, are stable and extremely valuable catalysts for many practical/industrial applications. In view of the current high costs of lipases, the possibility of regenerating and reusing, the search for an inexpensive support has motivated our group to undertake this work dealing with the selection of potential inorganic matrices (mesoporous silica and clay) which can be recognized by lipases, at molecular level, as solid surfaces would be an attractive feature of biocatalysis. Short chain aliphatic esters have immense applications as flavors in fruit juices, cheeses, baked goods, candies, beverages and ice creams. To understand the phenomena which lead to enzyme inactivation in these systems, we have turned to various thermodynamic approaches to characterize the supports and understand fundamentals aspects of enzyme stability by carrying out the esterification of isobutyric acid with n-butanol for the synthesis of butyl isobutyrate, which is an important flavor ester. In this work we have demonstrated that cheap metal inorganic supports are biocompatible with lipases rendering immobilized derivatives with similar or better characteristics than those obtained with controlled porous silica.

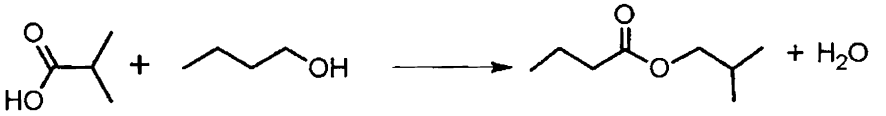
4.1 Use of Lipases in the Industrial production of esters

The use of lipases for a variety of biotechnological applications is rapidly and steadily increasing. The use of immobilized enzymes, in particular lipases, in organic media rather than aqueous media has several advantages, such as the shift in thermodynamic equilibria in favor of the product over the hydrolysis reaction, increased solubility of nonpolar substrate, elimination of side reactions, ease of enzyme and product recovery, and increased enzyme thermostability [1, 2]. The lipases are specific towards the ester bond and hence the formation of undesirable by-products is eliminated [3].

Although ester synthesis can be done chemically with acid or base catalysis, the use of enzyme technology offers the advantages of mild conditions, reduced side reactions, and specificity. Short-chain esters are compounds with a great application mainly in food and cosmetic industries. The use of homogeneous chemical catalysts leads to several problems such as corrosion of equipment, hazards of handling of the corrosive acids which are not reused, neutralisation of the resulting reaction mass leading to generation of large quantities of dilute dissolved salts, loss of conversion, yield and selectivity. Esters of short-chain fatty acids and alcohols are extremely important aroma compounds, which serve as precursors and/or additives for a variety of perfumes and flavours, pharmaceuticals, agrochemicals, plasticizers and also as solvents. Currently most of the flavour and fragrance components are produced by traditional methods which include chemical synthesis and extraction from natural sources [3]. Heterogeneous catalysts such as zeolites and other acid treated metal oxides, acid treated clays, cation exchange resins, etc. are exploited to develop green processes.

Butyl isobutyrate is one such important ester [4, 5] used as a modifier or fixative in flavour industry. Isobutyric acid has a very bad smell but when it is converted in an ester, it possess a fruity odour. Butyl isobutyrate is used as reminiscent of apple, banana and pineapple flavour. It is occasionally used as a fruity modifier in lipstick perfumes, in flavour compositions for imitation apple, apricot,

banana, butter, cherry, ginger, etc [6, 7]. The chemical method for synthesis of butyl isobutyrate generally encounters the use of excess quantity of n-butanol over isobutyric acid and a mineral acid catalyst requiring high temperature and corrosive conditions [7]. Oteral et al. have claimed the synthesis of butyl isobutyrate in presence of distannoxane as catalysts [8]. The use of enzymes to catalyse such reactions has become relatively popular because enzymes offer high catalytic efficiency, specificity and selectivity, which result in the development of purer and unique product [5]. The reaction is represented by the following equation:



Isobutyric acid **n-butanol** **Butylisobutyrate**

For flavour applications, it is desirable to use milder conditions and thus there is a tremendous incentive to apply bio-catalysis for the synthesis of butyl isobutyrate by using immobilised enzymes, which would lead to a clean and green process. Therefore, on account of its hydrolytic and synthetic activities, it is becoming increasingly useful for chemical and pharmaceutical industries involving hydrolysis of oil and fats, esters [9], synthesis of fatty acid esters as cosmetic ingredients [10], flavor esters [11, 12], amino acid esters [13], biodiesel [14, 15].

4.2 Factors affecting lipase-mediated esterification

4.2.1 Nature of Substrate

Steric hindrance (branching, unsaturation and chain length) and electronic effects of the substrates are the two major factors that determine selectivity. In esterification reactions, many lipases display high selectivity for long and medium chain fatty acids rather than short chain or branched ones [16]. Substrate molar ratio plays an important role in the esterification reaction. The latter can be improved by increasing the amount of either alcohol or acid present but in most cases alcohols may be inhibitory and acids may cause acidification of the microaqueous interface resulting in inactivation of lipases [17].

4.2.2 Nature of Solvent

When enzymes are directly dispersed in organic solvents they exhibit remarkable changes in their properties. Differences in enzyme activity in different solvents could be due to variable degrees of enzyme hydration imposed by the solvents rather than a direct effect on the enzyme or substrate. Laane et al. [18] quantified solvent polarity on the basis of log P values. The log P value of a solvent is defined as the logarithm of the partition coefficient of the solvent in an n-octanol/water two-phase system. Generally, biocatalysis is low in solvents of log P<2, is moderate in solvents with a log P value between 2 and 4 and high in non-polar solvents of log P>4. Hence, polar solvents may remove the essential water from the enzyme and disrupt the active confirmation [19]. Solvents of log P>2 dissolve to a lesser degree in water, leaving the enzyme suitably hydrated in its active conformation and hence are able to support product synthesis [20].

4.2.3 Role of Water in Lipase-mediated Catalysis

Water plays a crucial role in the reversible reaction catalysed by lipase [21]. While a critical amount of water is necessary for maintaining the active conformation of the enzyme, excess water facilitates hydrolysis [22]. Bound water is very important in stabilizing the conformation of a lipase in nonaqueous media.

4.3 Use of lipases in organic synthesis: Esterification processes

The *Pseudomonas cepacia* lipase (PS-30) immobilized on a phyllosilicate sol-gel matrix was found to be more active than the lipases of *Candida antarctica* and *Thermomyces lanuginosa* immobilized on granulated silica. *Thermomyces lanuginosa* and *Pseudomonas cepacia* lipases immobilized on a phyllosilicate sol-gel matrix were shown to catalyse ester formation (80–90% yield) from greases containing a range of free fatty acids from 2.6 to 36% [23]. The choice of support seems to influence the methanolysis of triolein in n-hexane. *Pseudomonas fluorescens* lipase was significantly more active when immobilized on polypropylene EP100 compared to celite. A conversion of 72.4% was achieved in the former case but only 1.5% in the latter [24]. It should be pointed out that these two supports have

very different morphological features in terms of surface area, pore size distribution and chemical nature. CRL immobilised on Accurel with smaller particles and smaller pore diameters gave higher enantiomeric ratio in the esterification of racemic 2-methylhexanoic acid with 1-decanol in organic solvent at a constant water activity (a_w) of 0.76 [25]. In the work done by Dave et al. a ten time increase in thermal stability was found upon immobilization of Candida rugosa lipase was immobilized in the polymer of polyvinyl alcohol (PVA), alginate and boric acid. The beads showed nearly complete retention of activity in reuse upto 10 cycles and also higher esterification ability as compared to its free counterpart [26].

Although several publications have appeared on enzyme catalyzed reactions in non-aqueous media, there is a dearth of quantitative information on kinetics and modeling of some of the industrially relevant reactions. The aim of this work was to conduct a kinetic study of the enzyme synthesis of butyl isobutyrate in *n*-hexane by using immobilised lipases on mesoporous silica and clays with the mechanism of the reaction. Mesocellular siliceous foams as well as clays were employed as supports for the immobilization of lipases via physical adsorption and covalent bonding. The present work also includes the screening of various immobilized lipases and optimization of reaction conditions such as catalyst loading, effect of mole ratio and temperature. Degrees of immobilization in aqueous and organic medium and catalytic properties of immobilized lipases were investigated. Among different lipases, MCF160G was found to be the best catalyst with 100% conversion in 7 h at 30°C. The kinetic data appeared to proceed via a Ping Pong bi–bi mechanism with 1-butanol inhibition. The aim of the present work was to produce an immobilized form of Candida rugosa lipase with advantageous catalytic properties and stability.

4.4 (a) Experimental procedure

The synthesis of butyl isobutyrate was carried out according to the procedure carried out by Yadav et al. [27]. A typical reaction mixture consisted of 0.01 mol of *n*-butanol and 0.01 mol isobutyric acid diluted up to the volume of 15 ml with heptane as a solvent. The reaction mixture was agitated at 30°C for 15 min and the required

amount of enzyme was then added to initiate the reaction. Samples were withdrawn periodically and analysed by using gas chromatography (GC).

Analytical method

The analysis was performed on a Chemito Gas Chromatograph equipped with flame ionisation detector. A 2m ×3.2mm stainless steel column packed with 10% SE-30 on chromosorb W was used for the analysis. Quantification of data was done by calibration with standard samples. The unit of enzyme activity is defined as μmol of isobutyric acid consumed (in an esterification reaction with butyl alcohol) per min per mg of the enzyme [28]:

$$\text{Specific enzymatic activity (mmol converted acid /h.g)} \cong \frac{(N_{FA})^0 \times (X_{ac})_t}{t \times w_0}$$

where $(N_{FA})^0$ is the initial mmol of acid (mmol of isobutyric acid); $(X_{ac})_t$ is the conversion of isobutyric acid at time t (in hours); and w_0 is the total biocatalyst mass (g). Total biocatalyst mass is the support plus the adsorbed CRL in the case of immobilized lipase and mass of lipase when CRL is used. The yield per gram of

$$\text{the biocatalysts (mmol converted acid/g)} \cong \frac{(N_{FA})^0 \times (X_{ac})_t}{w_0}$$

4.5 Physicochemical characterization of the free and the immobilized biocatalyst

4.5.1 Effect of immobilization medium

Microbial lipase from Candida rugosa immobilised by physical adsorption onto six different supports via two different coupling media were evaluated: aqueous solution (sodium phosphate buffer) and organic solvent (heptane). The choice of the immobilisation medium had a large effect on the synthetic lipase activities as well as on the immobilized ones. The reaction medium is believed to govern both, the conformation of protein and the relative reactivity of their functional groups, thus influencing the immobilization pattern. Table 4.1 highlights the activity parameters as well as the bound protein of the immobilizates in aqueous and organic medium.

The role of the coupling media was investigated in terms of the coupling yield and esterification activity of the bound lipases. The qualitative evaluation of the immobilization procedure was carried out by carbon, hydrogen and nitrogen contents of the free enzyme, support and immobilised lipase derivatives in buffer and heptane as coupling media, the results of which confirmed the higher incorporation of enzyme in organic medium. According to our data, the immobilised lipases prepared from heptane were highly superior to the corresponding preparation in buffer. This may be explained by the conformation of the enzyme molecule which changes according to the medium polarity.

Table 4.1 Activity parameters of the immobilizates

Sample	**Immobilization Medium**	**Bound protein (%)**	**Coupling yield (%)**	**Esterification activity (mmol/hg)**	**Yield (mmol/g)**
Free enzyme	-	-	-		91
H_{MCF}	buffer	77.3	85.8	0.77	
	heptane	88.4	93.6	1.88	13.2
R_{MCF}	buffer	51.8	69.9	0.29	
	heptane	72.3	80.4	0.98	6.86
KI-10	buffer	63.7	76.3	0.42	
	heptane	81.6	84.5	1.5	10.4
HG_{MCF}	buffer	82.8	93.2	3.8	
	heptane	92.9	96.1	6.1	42.5
RG_{MCF}	buffer	73.5	80.6	2.2	
	heptane	85.3	89.8	5.2	37
K-10G	buffer	75.6	82.8	2.8	
	heptane	88.3	93.7	5.5	39.1

Notations- H_{MCF}- lipase adsorbed on hydrothermally synthesized silica, R_{MCF}- lipase adsorbed on MCF silica prepared via room temperature, KI-10- enzyme adsorbed on montmorillonite, HG_{MCF}- lipase covalently bound to MCF silica (hydrothermal), RG_{MCF}- lipase covalently bound to MCF silica (room temperature), K-10G- lipase covalently bounded to clay.

Whereas in buffer solution the enzyme tends to fold so that the more hydrophilic amino acids become exposed to the coupling solution. In a medium of low polarity the enzyme should attain a different conformation with the more hydrophobic amino acids at the surface. Thus it appears that besides the support hydrophobicity, the use of a solvent which has low polarity (heptane, log P=4.0) was able to create around the enzyme a specific microenvironment that might enhance its activity.

The amount of protein adsorbed on each support in aqueous medium was in the range of 51 to 88% while it was 72 to 92% in the case of heptane immobilized systems. The best result with respect to protein (93%) and coupling yield (96%) recovered after immobilization was obtained with HG_{MCF} in the case of covalently bound system while it was H_{MCF} in the case of adsorbed systems. Higher amount of protein adsorbed of 88.4% on MCF160 was due to the larger surface area and pore diameter as compared to only 72.3% of protein adsorbed on R_{MCF}. It is common to compare the esterification activity for an immobilised enzyme, to that for the hydrolytic activity. The enzyme activity per mg enzyme was different in each case. Smaller pore sizes restrict mass transfer and pore penetration of the protein, which limit the protein interaction with the total surface area of the R_{MCF} silica. In addition for a successful immobilization by adsorption, an electrostatic interaction is needed between the enzyme and support. The coupling yield, esterification activity and yield was higher in the case of heptane immobilized samples with HG_{MCF} showing the highest among the covalently bound ones and H_{MCF} in the case of adsorbed systems. The effect of pore size was noted on the esterification activity that significantly increased for support having larger pores. Free enzyme gave a yield of 91% while R_{MCF} gave the lowest yield per gram of the biocatalyst among the immobilized supports.

4.5.2 Effect of various catalyst on esterification

Various catalysts were tested were for the determination of activity of the synthetic reaction (Table 4.2). The highest conversion was obtained using HG_{MCF} while

among adsorbed systems R_{MCF} led to poor conversions (44%) in 7h at 30°C. The lipase molecules may generally be immobilized on the surface and within the support as the protein molecules of lipase replacing the molecules of water in the case of H_{MCF}.

Table 4.2 Butanol conversion with respect to the various catalysts

Carrier	Conversion (%) after 7h
H_{MCF}	58
R_{MCF}	44
KI-10	48
HG_{MCF}	99
RG_{MCF}	97
K-10G	98

It is clear that hydrophobic interaction between enzyme and the support makes enzyme more efficient probably by adoption of a more favourable catalytic active site. Higher values obtained for the covalently bound systems during the catalytic reaction indicate the hydrophobicity as a key factor of lipase activity. Immobilization of lipase, especially onto hydrophobized supports, increases the catalytic efficiency of lipase. Similar results were obtained by Dumitriu et al. for CALB lipase immobilized on MCM-36 micro-mesoporous material [29]. The activity of the immobilized enzyme was better than that for the free CALB.

4.5.3 Effect of solvents

Enzyme activity and stability are known to be a function of the properties of the organic solvent. In aqueous medium, it acts as hydrolysing enzyme whereas in organic it favours the esterification reaction. Table 4.3 indicates that the free and the immobilized lipases showed higher activity in more hydrophobic solvents except in the case of isooctane. Different solvents such as toluene, heptane, iso-octane, 1, 4-dioxane and hexane were used.

Table 4.3 Effect of various solvents on the activity of fee and immobilized lipases

Solvent	Sample						
	Free lipase	H_{MCF}	R_{MCF}	KI-10	HG_{MCF}	RG_{MCF}	K-10G
	% conversion						
n-heptane	98	99	94	95	100	97	99
Isooctane	73	88	85	86	98	95	98
Hexane	100	97	90	78	99	92	96
1,4-dioxan	68	83	75	79	89	79	90
DMSO	73	78	65	72	94	78	86
Toluene	89	94	88	60	90	84	88

After 7 h of reaction time, almost 95% isobutyric acid conversion was observed for both n-heptane and n-hexane as opposed to only 68% and 73% for 1,4-dioxan and DMSO respectively. Therefore, all further experiments were carried out in heptane.

The high surface area and pore diameter accounted for the higher conversion obtained in the case of H_{MCF} while the overcrowding of lipase molecules on the surface of R_{MCF} lead to denaturation and subsequent loss in activity. Lipase attached via adsorption onto montmorilonte K-10 could attain a favourable conformation for its activity due to the negative charge, surface hydroxyl groups and the attachment via edges and on the external surface. Clay provides good distribution of lipase on mass transfer and prevented lipase particles from aggregation which facilitated dispersion of lipase in the reaction media than R_{MCF}. In comparison, native lipase was not easily dispersed as they tend to aggregate, thus causing a decrease in their activity.

The lower enzyme activity in polar solvents might be attributed to the stronger ability of the polar solvents to strip the essential monolayer of water

surrounding enzyme molecules, which is required for an enzyme to be catalytically active [30]. Enzymes are more stable when suspended in non-polar solvents that have low solubility in water [31]. Apolar solvents with high log *P* do not distort the essential water layer around the particle, thereby leaving the enzyme in an active state [32]. These trends are in accordance with the results by Gandhi et al. [33] on the esterification of lauric acid and oleic acid (with n-propanol, n-butanol, iso-amylalcohol ,n-hexanol, n-octanol, 2-ethyl-1-hexanol, n-decanol and lauryl alcohol) using an immobilized lipase in a solvent free system and the data reported by Yadav et al. [34] on the transesterification of vinyl acetate (with n-decanol, 2-ethyl-1-hexanol, benzyl alcohol, cinamyl alcohol, 1-phenylethyl alcohol and 2-phenylethyl alcohol) using an immobilized lipase in n-heptane. In both studies straight chain alcohols give higher conversions than aromatic and branched alcohols and optima for the activity as a function of the carbon length of the straight chain alcohols were observed. 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 [35]. 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.

4.5.4 Effect of catalyst loading

The effect of enzyme amount on synthesis of flavor esters was studied by varying the free enzyme concentration from 50mg to 150mg. The results are illustrated in Fig. 4.1. It can be seen that there is a strong correlation between the concentration of the biocatalyst and both the initial rate of ester synthesis and the yield of ester after 7 h, which might be due to the increase in the concentration of catalyst above the substrate concentration. Upon increasing the enzyme amount further, the reaction rate did not increase

significantly which may be due to the lack of substrate to access the active site of enzyme, and/or difficulty in maintaining uniform suspension of the biocatalysts at higher enzyme concentration [36]. In the case of 15% (w/w) catalyst-loading conversion did not increase after 5 h, which, in addition, could have been due to the attainment of equilibrium. All further experiments were carried out with 10% (w/w) of lipase from C. rugosa in order to reach maximum initial rate and utilization of enzyme activity.

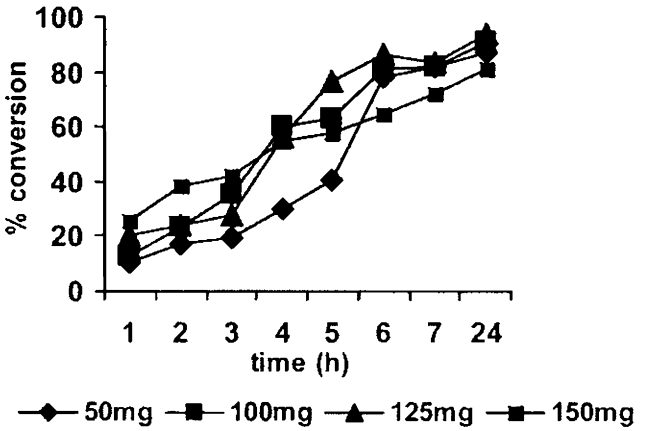


Fig 4.1 Effect of catalyst loading.

According to Bosley and Peilow [37], at low enzyme loadings, the lipase attempts to maximize its contact with the surface, which results in a loss of conformation and, consequently, in a reduced activity. As loading increased, less area is available for the lipase to spread itself, more of its active conformation is retained, and the loss in activity is reduced. According to these authors, the enzyme from Candida rugosa has a structure that is much more difficult to distort, making it less likely to spread on the support surface.

4.5.5 Effect of temperature on the activity of lipase

The effect of temperature on the initial esterification reaction rate is shown in Fig. 4.2. In most cases, rate of reaction increases with temperature, while the stability of enzymes declines [38, 39].

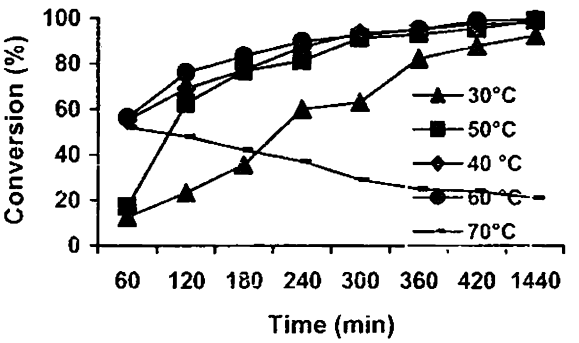


Fig 4.2 Effect of temperature.

The effect of temperature on free lipase was studied at 30, 40, 50, 60 and 70°C and the final conversions after 7 h were as 87, 94, 97, 99 and 24%, respectively. With an increase in temperature from 30 to 50°C, the rate of the reaction and conversion increased from 87 to 97% in 7 h. Further increase in the temperature to 70°C, resulted in a decrease in conversion due to thermal degradation of the enzyme [40]. Temperature affects the enzyme stability [40], and thus the reaction thermodynamic equilibrium and kinetics. These factors may interact leading to the observed trend. Therefore, all further experiments with free and immobilized lipases were carried out at 30°C.

4.5.6 Effect of mole ratio

One of the most frequently utilized ways of increasing the yield of esterification is performing the reaction with the excess of nucleophile. A drawback of this strategy is that it is often followed with a decrease of the initial rate. In order to investigate the influence of the excess of one substrate on the initial rate of butyl isobutyrate synthesis, reaction was performed at various substrate molar ratios.

The quantity of isobutyric acid was kept constant at 0.01 mol. The quantity of *n*-butanol was varied as 0.01, 0.02 and 0.03 mol and the total liquid volume was kept the same with heptane (Fig 4.3). The conversions of isobutyric acid were 82, 69 and 41% at 7 h, respectively. The rate of reaction was found to decrease

marginally with increasing concentration of *n*-butanol, which could be attributed to the formation of dead end complex between enzyme and excesses of n-butanol. It could be due to the substrate getting strongly adsorbed on the enzyme active site or inhibition of enzyme due to excess substrate. Therefore, all further reactions were carried out by using 0.01 mol of n-butanol. The results imply that in the synthesis of butyl isobutyrate by the lipase from C.rugosa nucleophilic substrate (butanol) acted as inhibitor. Inhibitory effect of alcohol has been previously reported in several studies, and proposed mechanism of inhibition includes formation of non-reactive dead-end complex between enzyme and alcohol [27].

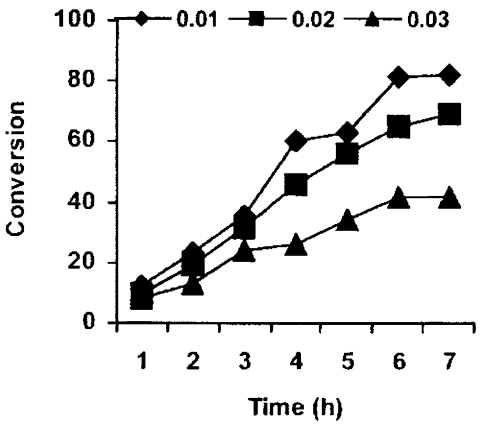


Fig 4.3 Effect of concentration of n-butanol. Isobutyric acid, 0.01 mol;heptane to make total volume to 15 ml; CRL lipase 100 mg; temperature,30 °C; speed,. n-butanol(♦) 0.01 mol; (■) 0.02 mol; (▲) 0.03 mol.

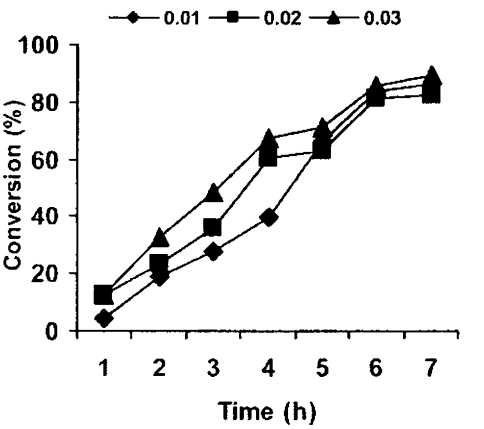


Fig 4.4 Effect of concentration of isobutyric acid. *n*-Butanol, 0.01 mol;heptane to make total volume to 15 ml; temperature, 30 °C; CRL lipase, 100 mg; isobutyric acid-(♦) 0.01 mol; (■) 0.02 mol; (▲) 0.03 mol.

In another set of experiments, n-butanol was kept constant at 0.01 mol and the quantity of isobutyric acid was varied as 0.01, 0.02 and 0.03 mol. Once again a constant liquid phase volume was made by using heptane as a solvent. The conversions were 84, 87 and 89% at 7 h, respectively. It was found that there was an increase in the rate of reaction with an increase in quantity of isobutyric acid (Fig. 4.4). Thus, it was found that 0.01 mol of n-butanol and 0.01 mol of isobutyric acid are the optimum quantities at 30°C using heptane as solvent. In the study by Garcia et al. [41], the effect of substrate molar

ratio on initial rate of cetyl oleate synthesis was investigated and it was found that, an excess of alcohol accelerated ester synthesis. The highest rates were observed at acid/alcohol ratio 1:10. A majority of studies on molar substrate ratio was concentrated on its effect on yield of esterification. In a study of synthesis of amylacetate, excess of acid drastically decreased yield of esters, while excess of alcohol was beneficial up to acid/alcohol ratio 1:2 [42]. Similar results were obtained in a study of synthesis of isoamyl isovalerate, in which optimum acid/alcohol ratio was 1:1.5 [43].

4.5.7 Effect of addition of water

Water has immense importance in lipase-mediated reactions both for the maintenance of three-dimensional structural integrity and also for optimal catalytic activity of the enzyme [44]. Water is thought to increase protein flexibility by forming multiple hydrogen bonds with enzyme molecule in organic solvents. There appears to be a critical amount of water necessary for enhancing activity.

To study the effect of water on the rate of reaction, the free enzyme as well as the immobilized lipases was dried at 30°C for 6 h under vacuum. Both the reactants and the solvent were dried with molecular sieve 4A prior to use and then employed for the reaction. The effect of water addition to the medium was studied from 100 to 250µl under otherwise similar conditions (Fig 4.5). With increasing water concentration conversion increased from 78 to 85%. However, further increase in water concentration up to 250µl resulted in decrease in the rate of reaction and conversion decreased to 65% which could be attributed to shifting the equilibrium towards the reactant (onset of the reverse reaction) [45]. This implies that the amount of water initially adsorbed on the silica is sufficient for the enzyme to exhibit highest activity. When 0.01ml of water was added to the free enzyme, probably a monolayer of water was placed around the enzyme and consequently the initial rate obtained was much higher while the conversion percentage was lower (77%). These results suggest that as the initial addition of water increased, the hydrolysis was accelerated and hence reduced the conversion percentage gradually. With addition of water beyond a critical point the silica support as well as clay gets strongly hydrated and the thickness of water

layer around the enzyme molecule increases. This results in problem in diffusion of the substrates to the active site of the enzyme disfavoring the esterification reaction [46]. Similar results were reported by Ghamgui etal. [47] for the enzymatic synthesis of butyl oleate. Mukherjee [48] suggested that low water content favors ester synthesis over hydrolysis. Lipases from different Rhizopus species have been reported previously to express maximal activity at relatively low water activity [49]. A similar effect was observed in the transesterification reaction of triolein with the propanol by P. fluorescens lipase [50].

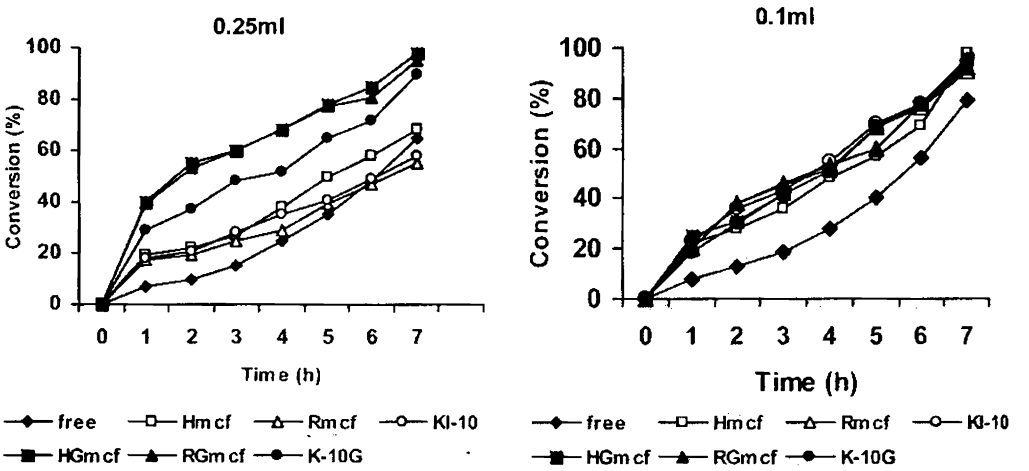


Fig 4.5 Effect of addition of water on free and immobilized lipases

Thus, the activity of the enzyme can depend on induced inactivation or partition of components between bulk solvent and microenvironment of enzyme. Another factor which may influence the conversion percentage is the affinity of the support for water when the enzyme is immobilized. The high hydrophobicity of the covalently bound systems could explain why the conversion percentage is high without addition of water. In fact, when the water was added, the support was not strongly hydrated and so was the microenvironment of the immobilized lipase and hence a higher conversion was observed with increase in water addition. In the case of adsorbed couter parts, the addition of water causes much hydration at the walls than the pores and as a result the esterification reaction is disfavored in the case of

KI-10 and R_{MCF} samples while the enzyme perturbed inside the pores in the case of H_{MCF} is not much hydrated and hence could esterify the reactants more than KI-10 and R_{MCF}.

4.5.8 Thermal stability

At high temperature, enzyme undergoes partial unfolding by heat-induced destruction of non-covalent interactions. According to Fagain [51], lipases are easily denatured at high temperature where the peptide bonds and amino acid side chains are reactive and can participate in deleterious reactions at high temperature.

In order to assess the thermal stability of the immobilized lipases, the free and immobilized enzyme were incubated at two temperatures, 50°C and 60°C for different time intervals and the residual activity determined (Fig. 4.6). Free enzyme in solution lost more than 80% of its activity after 2h incubation at 60°C. Free enzyme actually decays with time at faster rate as compared to immobilized enzyme. The heat stability of the lipase entrapped in silica and clay matrices is much better than that of the corresponding free enzyme.

Mostly lipase molecules inhabited on sites on the external surfaces, inside the pores and at the edges of the interlayer sheets through hydrogen bonding, Van der waals and electrostatic force interactions in the case of KI-10. In the case of KI-10 the adsorption mechanism must be produced through positive charges (proto-nated lysines) of the enzymes. For K-10G, an interaction between the enzymatic amino and the aldehyde group of the support as well as hydrophobic interactions must be responsible for the adsorption which accounts for the higher thermal resistance compared to the KI-10. The lower thermal stability retention in the case of R_{MCF} and RG_{MCF} samples is likely ascribed to steric impediments (as result of the high content of immobilized enzyme) and to enzyme conformation changes (as result of the multipoint attachment of the enzyme to support). The other explanation

for the lower activity of RG_{MCF} could be the interference between the first layer lipase adsorbed on the support and the second layer [52].

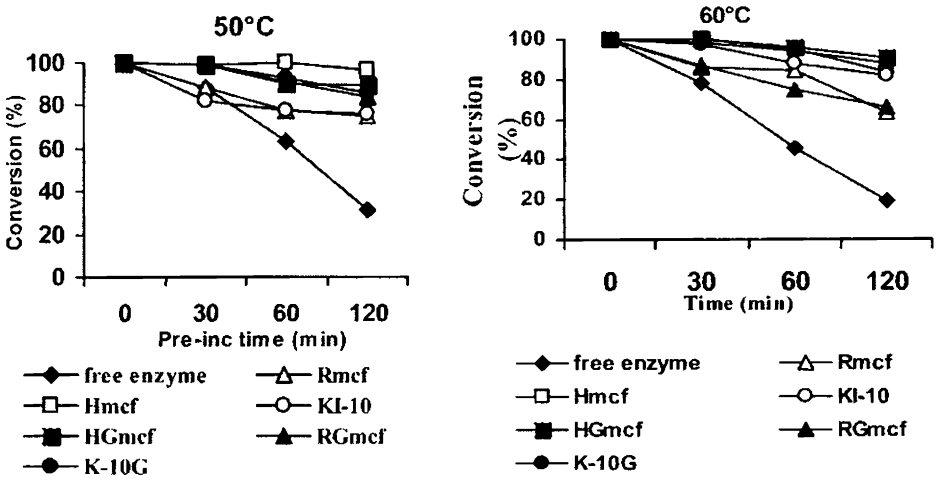


Fig 4.6 Thermal stability of the free and immobilized lipases at 50° and 60°C

After 2 h, the immobilized lipase, in presence of hexane, did not show thermal deactivation. The thermal stabilization in the case of H_{MCF} could be explained by the lipase location inside the mesopores of the support where the enzyme is protected against alterations of the microenvironment. So, the inactivation observed with the enzyme in solution should be considered as a result of lipase denaturation only. Lipase immobilised onto K-10G showed higher thermal stability by retaining 88% of its activity at 60°C. Although heat considerably reduces conformational flexibility of native and immobilized lipase, immobilised lipase is still capable of performing its vibrational and more complex movement required for efficient catalytic activity. These include the prevention of auto-proteolysis due to a restriction of the intermolecular contact in the immobilized enzymes and the protection of the enzymes from structural rearrangement, due to the likely multipoint attachments to the support [53]. The increase in thermal stability of the covalently bound systems compared to adsorbed ones could be attributed to

an improved physical and mechanical stability of the lipase arising from the covalent bonding of lipase on the carriers.

The immobilized lipase, therefore, could be said to have a greater potential for biotechnological applications than the free one. The ability to retain enzyme activity at higher temperature expands the range of conditions suitable for the enzyme function and provides a number of processing advantages such as reduced risk of contamination, lower viscosity, improved transfer rates and improved substrate solubility [54]. These data confirm previous findings stipulating that the interaction between the support and the enzyme improves the enzyme stability [55, 56, 57].

4.5.9 Reusability of catalyst

For any application based on immobilized lipases, the feasibility of regeneration of the lipase activity (and consequent reuse of the support) provided clear economic benefits for its industrial use and facilitates product recovery without contamination.

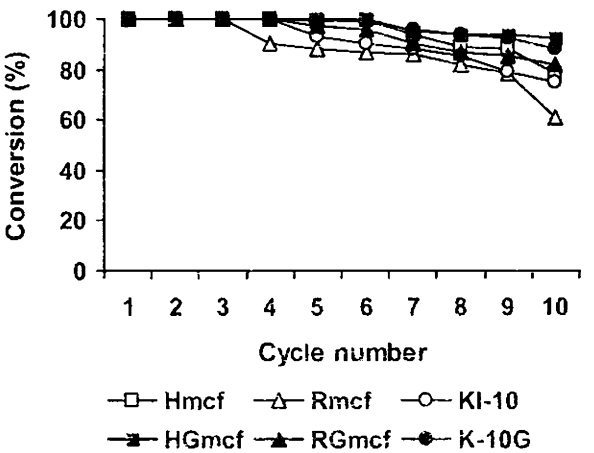


Fig 4.7 Reusability characteristics of the free and the immobilized lipases

The activity of native lipase began to decrease dramatically from 100% to 40 % after it was reused five times (Fig 4.7). The immobilized lipases

could retain more than 75% activity even after 10 reuses except in the case of R_{MCF} which retains only 60% activity. This may be also due to the fact that the butanol (polar substrate) may be accumulated in the aqueous microenvironment that surrounds the enzyme, reaching a concentration level sufficient to cause a denaturation of the protein. The covalently bound supports retain higher activity than the adsorbed ones due to the spacer arm group attached which prevents the inhibition by alcohol and also the hydrophobic activation as a result of the binding functionalities which maintains the lipase's open state. The decrease in the conversion after several runs were due to the turnover of large quantities of substrates that resulted in the production of substantial quantities of water (as co-product) and also due to the loss of some catalyst during filtration [26].

It is evident that HG_{MCF} retained its high activity after ten cycles, and K-10G showed the highest operational stability. The higher activity retention shown by H_{MCF} and HG_{MCF} confirmed that the enzyme is occluded in the mesopores and not on the external surface and hence reduced leaching. These are the main properties that industrial biocatalysts should fulfill (including a support of low cost), which make the prospects of these new derivatives very promising.

The same phenomenon was observed during the repeated use of porcine liver esterase adsorbed onto QAE-sephadex for the enantioselective production of levofloxacin [58]. Crude Rhizopus oryzae lipase immobilized on $CaCO_3$ could be repeatedly used for six cycles without a decrease of synthetic activity in the work done by Ghamgui et al. [59].

4.5.10 Storage stability

The storage stability of the immobilized enzymes without appreciable loss of enzyme activity is important for the economic viability of a biosynthetic process [60].

Table 4.4 Storage stability of the free lipase and the immobilizates

Carrier	Storage stability (4^0C) Activity (%)	
	After 4 days	After 30 days
Free CRL	67	33
H_{MCF}	100	99
R_{MCF}	85	98
KI-10	95	89
HG_{MCF}	100	100
RG_{MCF}	95	89
K-10G	98	92

The enzymatic activity of various lipase preparations determined after storing for 30 days at 4°C were summarized in Table 4.4. All lipases showed full catalytic activity after storing them at −20°C as commonly practiced in laboratory. However, only immobilized lipases retained their full catalytic activity when stored at 4°C as at very low temperatures, lipase is probably locked in its native situation, catalytically active conformation. Immobilized lipases after 60 days storage still showed good storage stability up to 100% compared to native lipase. A total reduction of 70% in the relative activity of native lipase was observed after storage for 60 days but only a 10% reduction for all the adsorbed and covalently bound systems. The stabilization at this temperature in immobilized enzyme may be due to multipoint attachment of the enzymes to the supports, creating a more rigid enzyme molecule which prevents any intermolecular reaction such as aggregation and proteolysis. Hence, disruption of the active center becomes less likely to occur [61]. HG_{MCF} retained 100% catalytic activity when stored at 4°C even after 60 days storage.

4.5.11 Kinetics and mechanism

The thorough knowledge of kinetics is of great importance not only in order to elucidate mechanism of this reaction, but because 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. The kinetics of the esterification is scarce. A range of initial substrate concentrations was examined and the initial rates of

conversion were determined. The kinetics were determined for the industrial free Candida rugosa lipase and for the immobilized HG_{MCF}.

(a) Kinetics mechanism of the free Candida rugosa lipase

It is well known that Michaelis constant K_m can be used to characterize approximately the affinity between enzyme and substrate at the beginning of a reaction or at low conversion phase. Therefore, the concentration–time profiles were determined to calculate the initial reaction rates from the linear portion of the concentration–time profiles, respectively; and then analyzed the plots of substrate concentration versus the initial rate.

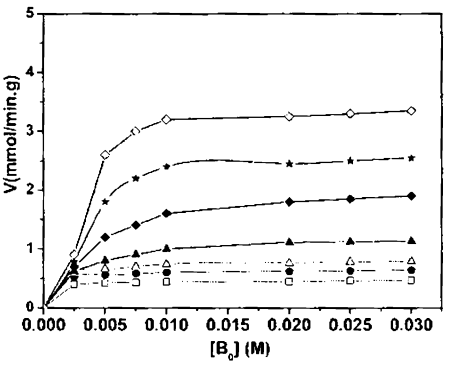


Fig 4.8 Initial conversion rates as a function of the mole number of n-butanol for free CRL lipase Isobutyric acid-(□)-0.0025M, (●)-0.005M,(Δ)-0.0075M, (▲)-0.01M,(♦)-0.02M, (★)-0.025M,(◊)-0.03M

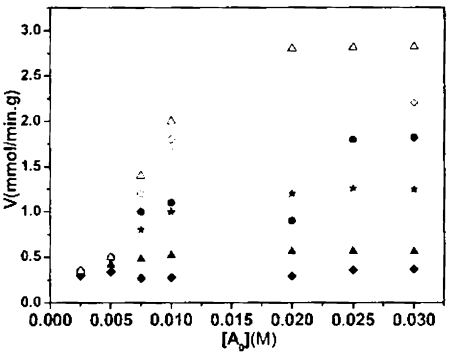


Fig 4.9 Initial conversion rates as a function of the mole number of isobutyric acid for free CRL lipase n-butanol-(♦)-0.0025M,(▲) 0.05M, (★)-0.0075M, (●)-0.02M, (◊)-0.025M, (Δ)-0.03M

The kinetics of the industrial CRL powder was studied at 30°C in 15ml of n-heptane and in a batch reactor with isobutyric acid and n-butanol as substrates. Hence, for the study on the esterification kinetics mechanism, approximately 100 mg of industrial CRL powder was used. The mole number of the substrates: isobutyric acid and n-butanol were 0.0025M, 0.005M, 0.075M, 0.01M, 0.02M, and 0.03M and all possible combinations between these concentrations for the two substrates were examined. 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 results on the initial conversion rates of n-butanol as a function of the mole number of n-butanol or isobutyric acid with free CRL lipase are presented in Fig 4.8 and Fig 4.9.

The effect of concentration of both the substrates on the rate of reaction was investigated. It was found that when the concentration of *n*-butanol (B) was increased, the rate of reaction increased and reached a maximum at a critical concentration. A subsequent increase in n-butanol concentration decreased the initial rate. Therefore, it may be concluded that n-butanol at higher concentration reacts with the enzyme to form dead end inhibitory complex. There was no evidence of inhibition by isobutyric acid (A) at any concentrations tested [62]. 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.

Mechanism of the reaction

In this reaction the lipase may react with n-butanol to yield a dead end enzyme n-butanol complex or it may react with isobutyric acid to yield the effective lipase isobutyric acid complex. Then the lipase isobutyric acid complex is transferred to an enzyme–acyl intermediate and water is released. This is followed by the interaction of the enzyme–acyl complex with n-butanol to form another binary complex, which then yields the ester and free lipase. The reaction sequence may be given as follows:

$$E + A \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} EA$$

$$EA \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} E^1 + P$$

$$E^1 + B \underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}} E^1B$$

$$E^1B \underset{K_{-4}}{\overset{K_4}{\rightleftharpoons}} E + Q$$

$$E + B \underset{K_{-5}}{\overset{K_5}{\rightleftharpoons}} EB$$

where A is isobutyric acid, E free enzyme, EA enzyme–isobutyric acid complex, E1- enzyme–acyl complex, B-n-butanol, E1B- binary complex of acyl enzyme and *n*-butanol, P- water and Q is butyl isobutyrate. The shape of the graphs in the Fig 4.11 was found to qualitatively correspond to a Bi-Bi Ping-Pong model with inhibition by n-butanol, according to the various kinetics models detailed by Segel [63]. 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 n-butanol and iosbutyric acid) as a function of the mole number of n-butanol (Fig. 4.9). The fitting of these experimental points with a theoretical model function were achieved with the data-processing software Sigmaplot 8.0 from SSPS. The equation of the function corresponding to the Bi-Bi Ping-Pong model with inhibition by one of the substrates [63] 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 for which initial rate data and concentration are 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 n-butanol (mmol/l), $K_{m(A)}$ is the Michaelis constant of isobutyric acid, $K_{m(B)}$ the Michaelis constant of *n*-butanol, K_i the inhibition constant of *n*-butanol, r_0 the initial rate of the reaction, r_{max} is the maximum rate of the reaction. Most of the kinetic studies on the lipase-catalyzed synthesis of esters have considered the direct esterification of alcohols with acids, and have described a Ping-Pong Bi-Bi kinetic mechanism with inhibition by both or one of the reactants [64, 65].

Chulalaksananukulet al. carried out ester synthesis using lipase from Mucor miehei and proposed ping–pong bi–bi model with dead-end inhibition by alcohol [66]. The same model was proposed by Hazarika etal. for synthesis of ethyl oleate with porcine pancreatic lipase [67]. 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 [68]. 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 [69]. Similar inhibition effect was also observed for the Mucor miehei catalysed synthesis of ethyl oleate [70] using immobilised lipase in n-hexane and in supercritical CO_2.

The inverse initial conversion rate of n-butanol is presented as a function of the inverse mole number of isobutyric acid or n-butanol in Fig. 4.10 and 4.11. These graphs show typical characteristics of the above equations, as described by Segel [63]. Fig 4.10 illustrates that, when the mole number of isobutyric acid increased from 0.0025 to 0.03M, all the plots of $1/V_i$ as a function of $1/n_{butanol}$ ($1/B_0$) converged towards the same lower curve.

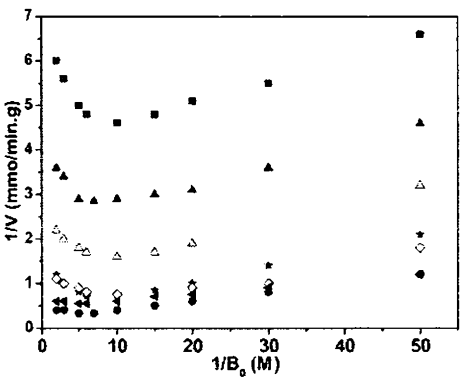


Fig 4.10. Lineweaver-Burk plots 1/ υ versus 1/[But] obtained with free CRL lipase at seven fixed concentrations of isobutyric acid.(■)-0.0025M, (▲)-0.005M, (Δ)-0.0075M (★)-0.01M, (◊)-0.02M, (◄)-0.025M, (●)-0.03M

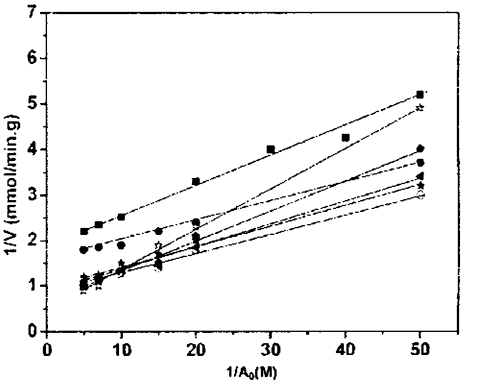


Fig 4.11 Lineweaver-Burk plots 1/ υ versus 1/[Is buty] obtained with free CRL lipase at seven fixed concentrations of n-butanol (○)-0.0025M, (★)-0.005M, (◄)-0.0075M, (●)-0.01M, (⬟)-0.02M, (☆)-0.025M, (■)-0.03M

On the other hand when $1/Vi$ was plotted as a function of $1/n_{isobuty}(1/A_0)$ (Fig. 4.11), the plots progressively transformed from quasi parallel straight lines at low n-butanol to lines secant near the vertical axis at high n-butanol.

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 4.5. Based on the calculated kinetic parameters, affinity of the enzyme towards n-butanol seems to be greater than of iosbutyric acid, since $K_{m,ol}$ was lower than $K_{misobut}$. In other words, the relatively large value of $K_{misobut}$ 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.

(b) Kinetics mechanism of HG_{MCF}

The kinetics of the same reaction catalyzed by HG_{MCF} was followed in the same solvent mixture and at the same temperature. The initial rates were calculated from aliquots taken after 10 min of reaction.

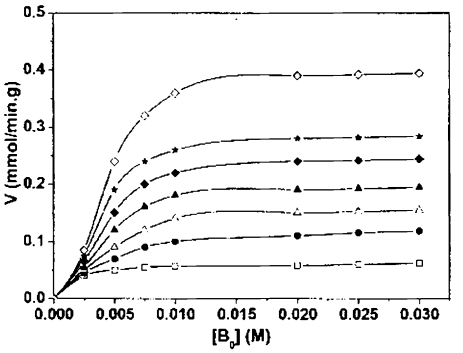


Fig4.12. Initial conversion rates as a function of the mole number of n-butanol for HG_{MCF} Isobutyric acid - (□)-0.0025M, (●)-0.005M, (Δ)-0.0075M, (▲)-0.01M, (-0.02M, (★)-0.025M, (◊)-0.03M

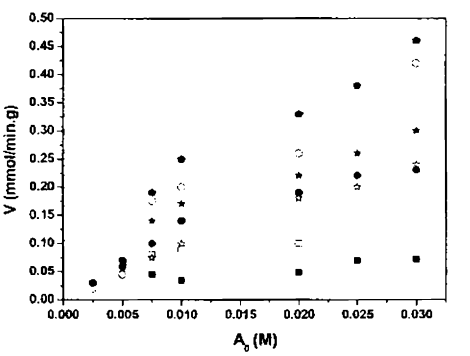


Fig 4.13. Initial conversion rates as a function of the mole number of isobutyric acid for HG_{MCF} n-butanol -(■)-0.0025M, (□)-0.005M, (●)-0.0075M, (☆)-0.01M, (★)-0.02M, (○)-0.025M,(⬟)-0.03M

The mole number of the substrates isobutyric acid and n-butane were 0.0025M, 0.005M, 0.075M, 0.01M, 0.02M, and 0.03M and all possible combinations between these concentrations for the two substrates were examined. As it can be seen from Fig. 4.12 and Fig 4.13, initial rates were higher than in the experiment with equimolar conditions, but rapid decrease of rate at high concentrations of substrates indicates that certain inhibition occurs. Since the initial rates are considerably lower at excess of alcohol, it is plausible inhibition was caused by n-butanol.

The concentration of one of the two substrates was maintained constant while the full series of concentration for the second one was investigated. The inverse initial rate, plotted as a function of the inverse mole numbers of the two substrates, is presented in Fig. 4.14 and Fig 4.15. The shape of the graphs again suggested that the same kinetics mechanism was operating, that is to say the Bi-Bi Ping-Pong mechanism with inhibition by n-butanol.

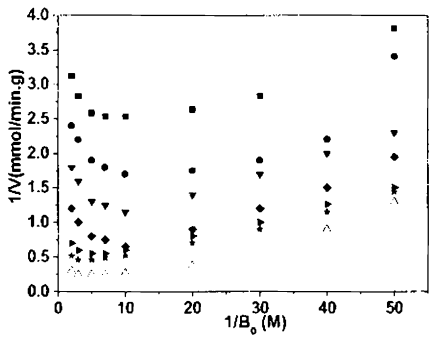


Fig 4.14. Lineweaver-Burk plots 1/ υ versus 1/[But] obtained with HG_{MCF} at seven fixed concentrations of isobutyric acid . (■)-0.0025M, (●)-0.005M, (▼)-0.0075M (♦)-0.01M, (►)-0.02M, (★)-0.025M, (Δ)-0.03M

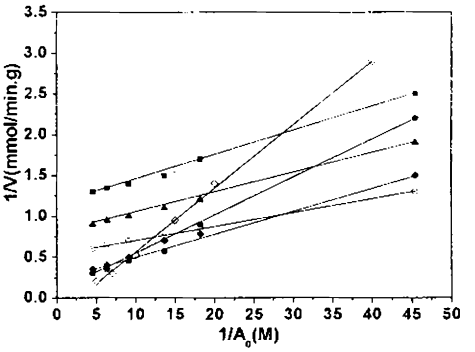


Fig 4.15. Lineweaver-Burk plots 1/ υ versus 1/[Is buty] obtained with HG_{MCF} at seven fixed concentrations of n-butanol (○)-0.0025M, (♦)-0.005M, (▲)-0.0075M, (●)-0.01M, (■)-0.02M, (□)-0.03M

Curve fitting with the theoretical kinetics function provided the following values for the kinetics constant (Table 4.5). It appears that the kinetics constants $K_{m.ol}$ and $K_{m.isbutyric}$ were increased after immobilization except for the inhibition

constant by n-butanol ($K_{i,ol}$) and V_{max} which decreased. $K_{m,ol}$ was also higher when compared to free enzyme but lower than $K_{m,isbutyric}$. The apparent Michealis–Menten constant for CRL lipase was increased after immobilization.

Table 4.5 Kinetic parameters for the synthesis of butyl isobutyrate by reaction of n-butanol with isobutyric acid using Candida rugosa lipase

Sample	$K_{m,ol}$(M)	$K_{m,isbutyric}$(M)	V_{max} (mmolmin^{-1}g^{-1})	$K_{i,ol}$(M)
CRL LIPASE	0.53	1.32	32.29	1.95
HG_{MCF}	3.18	3.57	23.72	1.05

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 once an enzyme has been immobilized indicates that the immobilized enzyme has an apparent lower affinity for its substrate than that of the native enzyme, which 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 HG_{MCF} were lower than that of the free enzyme indicating a lowering of activity of enzymes on account of immobilization. This decrease may have been due to steric hindrances imposed by the support on the macromolecular substrate or to chemical modification [71]. 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.

4.6 Conclusions

The present work is a comprehensive study on enzymatic synthesis of butyl isobutyrate through esterification reaction using lipase immobilized onto mesocellular siliceous foams and montmorillonite K-10 via adsorption and

covalent binding. Moreover, the immobilization does not modify the nature of the kinetic mechanism proposed which is of the Bi-Bi Ping-Pong type with inhibition by n-butanol. The immobilized biocatalyst can be commercially exploited for the synthesis of other short chain flavor esters. The main inferences from the present study are outlined as:

- The use of a solvent which has low polarity (heptane, log P =4.0) was able to enhance immobilization in organic medium than that in the aqueous medium. The activity yield, coupling yield, activity and yield per gram of the biocatalysts were higher for heptane immobilized systems.
- All the covalently bound systems gave higher conversion than the adsorbed systems due to the increased hydrphobicity of the supports. The most efficient systems (H_{MCF} and HG_{MCF}) appear to be worthy of further application in biocatalysis.
- Increase in water concentration up to 250µl resulted in decrease in the rate of reaction and conversion decreased to 65% which could be attributed to shifting the equilibrium towards the reactant (onset of the reverse reaction).
- The thermal stability of the covalently bound systems were higher compared to the adsorbed and free ones as more than 85% activity was retained even after 2 h incubation at 55 and 60°C.
- HG_{MCF} could retain high activity even after 10 reuses. All the derivatives achieved an excellent operational stability.
- All the immobilized lipases could retain more than 90% activity even after 60 days storage at 4°C.
- The rate of reaction was found to decrease marginally with increasing concentration of n-butanol, which could be attributed to the formation of dead end complex between enzyme and excesses of n-butanol.

- A model based on Ping Pong Bi Bi with inhibition by n-butanol was found to fit the initial rate data in the kinetics of lipase-catalyzed esterification in n-butanol.
- $K_{m,ol}$ was found to be lower in than $K_{m,isbutyric}$ which showed greater affinity for the alcohol in both the native and immobilized lipase. The V_{max} as well as the $K_{i,ol}$ were reduced after immobilization.
- Siliceous mesostructured cellular foams show great potentials as carrier materials for lipase immobilization to give very active biocatalysts, and in that they are far superior to other carriers.
- Natural montmorillonite K-10 showed a promising future of applying natural resource as support for biocatalyst for various organic syntheses as it allows easy immobilization of lipase from Candida rugosa through a simple and inexpensive method.

References

[1] A. M. Klibanov, Chemtech., 16 (1986) 354.

[2] C. C. Akoh, L. N. Yee, J. Mol. Catal. B: Enzym., 4 (1998) 149.

[3] E. Santaniello, P. Ferraboschi, P. Grisenti, Enzyme Microb. Technol., 15 (1993) 367.

[4] S. Ztatanos, A. Sagredos, V. Papageorgiou, J. Am. Oil Chem. Soc., 62 (1985) 1575.

[5] W. Linfield, R. Barauskas, L. Sivien, S. Serota, R. Stevenson, J. Am.Oil Chem. Soc., 61 (1984) 191.

[6] K. Bauer, D. Garbe, Common Fragrance and Flavour Materials,VCH, Berlin (1985).

[7] S. Arctander, Perfume and Flavor Chemicals, vol. I, Stefan Arctander Publishing Co., Montclair Co., NJ, USA (1969) p. 413.

[8] N. Oteral, D. Oh, H. Nozaki, J. Org. Chem., 56 (1997) 5307.

[9] S. Zhu, Y.Wu, Z. Yu, J. Biotechnol., 116 (2005) 397.

[10] S. Chang, J. Shaw, C. Yang, C. Shieh, Process Biochem., 42 (2007) 1362.

[11] A. Guvenc¸ N. Kapucu, U. Mehmetoglu, Process Biochem., 38 (2002) 379.

[12] F. W. Welsh, R. E. Williams, K. H. Dawson, J. Food Sci., 55 (6) (1990) 1679.

[13] K. Lohith, S. Divakar, J. Biotechnol., 117 (2005) 49.

[14] H. Noureddini, X. Gao, R. S. Philkana, Bioresource Technol., 96 (2005) 769.

[15] C. Lai, S. Zullaikah, S. R. Vali, Y. Ju, J. Chem. Technol. Biotechnol., 80 (2005) 331.

[16] S. Alhir, S. Markajis, R. Chandan, J. Agric. Food Chem., 38 (1990) 598.

[17] N. Dörmö, K. Bélafi-Bakó, L. Bartha, U. Ehrensteinm, L. Gubicza, Biochem. Eng. J., 21 (2004) 229.

[18] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng., 30 (1987) 81.

[19] S. Adachi, T. Kobayashi, J. Biosci. Bioeng., 99(2) (2005) 87.

[20] E. Soo, A. B. Salleh, M. Basri, Rahman, K. Kamaruddin, J. Biosci.Bioeng., 95 (2003) 361.

[21] S. Gayot, X. Santarelli, D. Coulon, J. Biotechnol., 101 (2003) 29.

[22] P. A. Cameron, B. H. Davison, P. D. Frymier, J. W. Barton, Biotechnol. Bioeng., 78 (2002) 251.

[23] A. F. Hsu, K. C. Jones, T. A. Foglia, W. N. Marmer., Biotechnol. lett., 26 (2004) 917.

[24] M. M. Soumanou, U. T. Bornscheuer, Eur.J. Lipid Sci. Technol., 105 (2003) 656.

[25] S. Sabbani, E. Hedenstroma, O. Nordin, J. Mol. Catal. B: Enzym., 42 (2006) 1.

[26] R. Dave, D. Madamwar, Process Biochem., 41 (2006) 951.

[27] G. D. Yadav, P. S. Lathi, Biochem. Eng. J., 16 (2003) 245.

[28] M. L. Foresti, M. L. Ferreira, Anal Bioanal Chem., 381 (2005) 1408.

[29] E. Dumitriu, F. Secundo, J. Patarin, I. Fechete, J. Mol. Catal.B: Enzym., 22 (2003) 119.

[30] M. Basri, W. M. Z. W. Yunus, W. S. Yoong, K. Ampon, C. N. A Razak, A. B. Salleh, J. Chem. Technol. Biotechnol., 66 (1996) 169.

[31] J. A. Arcos, C. G. Hill Jr, C. Otero, Biotechnol. Bioeng., 73 (2001) 104.

[32] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng., 30 (1987) 81.

[33] N. N. Gandhi, S. B. Sawant, J. B. Joshi, Biotechnol. Prog., 11 (1995) 282.

[34] G. D. Yadav, A. H. Trivedi, Enzyme Microbial Technol., 32 (2003) 783.

[35] P. Aldercreutz, A. O. Triantafyllou, B. Mattiasson, J. Mol. Catal. B: Enzym., (1992) 167.

[36] D. Wei, C. Gu, Q. Song, W. Su, Enzyme Microb. Technol., 33 (2003) 508.

[37] J. A. Bosley, A. D. Peilow, J. Am. Oil Chem. Soc., 74 (1997) 107.

[38] T. Garcia, N. Sanchez, J. Martinez, J. Aracil, Enzyme Microb. Technol., 25 (1999) 584.

[39] M. L. Foresti, M. L. Ferreira, Enzyme Microb. Technol., 40 (2007) 769.

[40] K. Naoe, T. Ohsa, M. Kawagoe, M. Imai, Biochem.Eng. J., 9 (2001) 67.

[41] T. Garcia, A. Coteron, M. Martinez, J. Aracil, Chem. Eng. Sci., 55 (8) (2000) 1411.

[42] S. Hari Krishna, S. Divakar, S. G. Prapulla, N. G. Karanth, J. Biotechnol., 87 (2001) 193.

[43] G. V. Chowdary, M. N. Ramesh, S. G. Prapulla, Process Biochem., 36 (2000) 331.

[44] A. M. Klibanov, Trends Biotechnol., 15 (1997) 97.

[45] R. Goddard, J. Bosley, B. Al-Duri, J. Supercrit. Fluids., 18 (2000) 121.

[46] V. Dossat, D. Combes, A. Marty, Enzyme Microb. Technol., 30 (2002) 90.

[47] H. Ghamgui, M. Karra-châabouni, Y. Gargouri, Enzyme Microb. Technol., 35 (2004) 355.

[48] K. D. Mukherjee, Biocatalysis., 3 (1990) 277.

[49] G. Koller, M. J. Aris, Z. Ujang, A. M. Vaidya, Biocatal. Biotransform., 19 (2001) 37.

[50] M. Iso, B. Chen, M. Eguchi, T. Kudo, S. Shrestha, J. Mol. Catal. B: Enzym., 16 (2001) 53.

[51] C. O. Fagain, Manipulating protein stability, in: Stabilizing Protein Functions, Springer, Berlin (1997) pp. 67–191.

[52] J. M. Palomo, C. Ortiz, G. Fern´andez-Lorente, M. Fuentes, J. M. Guis´an, R. Fern´andez-Lafuente, Enzyme Microb. Technol., 36 (2005) 447.

[53] M. D. Trevan, Immobilized Enzymes—an Introduction and Applications in Biotechnology, Wiley, Chichester (1980).

[54] H. R. Luckarift, J. C. Spain, R. R. Naik, M. O. Stone, Nat Biotechnol., 22 (2004) 211.

[55] V. V. Mozhaev, N. S. Melik-Nubarov, M. V. Sergeeva, V. Siksnis, K. Martinek, Biocatalysis, 3 (1990) 179.

[56] H. Ogino, H. Ishikawa, J Biosc. Bioeng., 91 (2001) 109.

[57] M. B. A. Rahman, M. Basri, M. Z. Husein, R. N. Z. Rahman, A. B. Salleh, Catal Today., 93 (2004) 401.

[58] S. Y. Lee, B. H. Min, S. H. Hwang, Y. M. Koo, C. K. Lee, Biotechnol Lett., 23 (2001) 1033.

[59] H. Ghamguia, M. Karra-Chaabouni, Y. Gargouri, Enzyme Microb. Technol., 35 (2004) 355.

[60] E. Y. Park, M. Sato, S. Kojima, Enzyme Microb.Technol., 39 (2006) 889.

[61] M. Basri, K. Ampon, W. M. Z. Wan Yunus, C. N. A. Razak, A. B. Salleh, J.Mol. Catal. B: Enzym., 3 (1997) 171.

[62] G. D. Yadav, P. S. Lathi, Biochem. Eng. J., 16 (2003) 245.

[63] H. Segel, Enzyme kinetics, John Wiley & Sons., Inc., New York (1975).

[64] A. Zaidi, J. L. Gainer, G. Carta, A. Mrani, T. Kadiri, Y. Belarbi, A. Mir, J. Biotechnol., 93 (2002) 209.

[65] V. Dossat, D. Combes, A. Marty, Enzyme Microb. Technol., 30 (2002) 90.

[66] W. Chulalaksananukul, J. S. Condoret, D. Combes, Enzyme Microb. Technol., 14 (1992) 293.

[67] S. Hazarika, P. Goswami, N. N. Dutta, A. K. Hazarika, Chem. Eng. J., 85 (2002) 61.

[68] G. D. Yadav, P. S. Lathi, J. Mol. Catal. B: Enzym., 27 (2004) 109.

[69] T. Garcia, A. Coteron, M. Martinez, J. Aracil, Chem. Eng. Sci., 55 (2000) 1411.

[70] A. Marty, W. Chulalaksananukul, R. M. Willemot, J. S. Condoret, Biotechnol. Bioeng., 39 (1992) 273.

[71] P. G. PiVeri, M. Tramontini, A. Malacarne, Biotechnol. Bioeng., 33 (10) (1989) 1258.

.....ꕥ.....

Chapter 5

TRANSESTERIFICATION REACTION: SYNTHESIS OF BUTYL BUTYRATE WITH FREE AND IMMOBILIZED LIPASES

Contents

Enzymatic catalysis has gained considerable attention in recent years as an efficient tool in the preparation of natural products, pharmaceuticals, fine chemicals, and food ingredients. Due to the overwhelming interest in natural products, biotechnology should be attractive to produce flavors of natural aroma from natural precursors. A great attraction of using organic solvents rather than water as a reaction solvent is the ability to perform synthetic transformations with relatively inexpensive hydrolytic enzymes. Lipases have attracted much attention of organic chemists because of their high stability in organic solvents, and now they are usually treated in organic media as catalysts for asymmetric transesterifications. Lipases have been successfully used to catalyse esterification and interesterification reactions aimed at the production of flavouring esters for food, pharmaceutical and cosmetics purposes. Transesterification (acidolysis or solvolysis) is preferable to esterification because inhibition by the acid or the alcohol does not occur. Organic esters are employed as solvents, fragrance, flavors, and precursors in a variety of industries. The synthesis of butyl butyrate (used as a pineapple flavor) by green chemistry is of immense importance to the food industry. Transesterification of n-butanol with ethyl butyrate for the synthesis of butyl butyrate was studied at 30°C as a model reaction by employing Candida rugosa lipase and the lipase immobilized on mesocellular silica foams and montmorillonite K-10 including kinetic modeling. The influence of water activity with different salt hydrates on the synthesis of butyl butyrate were also investigated and it was found that the ping-pong bi–bi mechanism with inhibition by the substrate n-butanol fits the data for transesterification.

5.1 Introduction

Lipase-catalyzed acylation is an enzymatic process that has recently gained wide popularity in organic synthesis, because the reaction can be carried out efficiently in organic solvents and often proceeds with a high degree of regiocontrol and stereocontrol. Several acylation procedures such as esterification, transesterification have been reported in the literature, but, from a preparative point of view, the transesterification of alcohols is by far the most useful and applied method [1].

The term transesterification refers to the process of exchanging acyl radicals between an ester and an acid-acidolysis, an ester and another ester-interesterification, or an ester and an alcohol-alcoholysis. Transesterification is accomplished industrially by heating a mixture of the anhydrous ester and another reactant species at relatively high temperatures. Alternatively, alkali metals or alkali alkylates may be used at lower temperatures. However, the application of lipases for the modification of fats and oils by transesterification offers again the advantages of mild conditions, reduced side reactions, and specificity [2-4]. One example is the production of cocoa butter analogues from cheaper feedstocks. Enzymatic transesterification processes are more widely used than esterification reactions in resolution or desymmetrization processes, and the acylation of alcohols using lipases is currently the most frequently used process in biocatalysis.

Organic esters are employed as solvents, fragrance, flavors and precursors in a variety of industries. Particularly, aliphatic esters are greatly used in flavor industry, mainly as fixatives and modifiers, and aromatic esters in fragrance compositions. The direct extraction of these esters from plant materials involves rather expensive and low yield processes [5]. However, low yields, decreasing and variable availability of certain natural sources due to climatic instability have stimulated large investigations to produce flavors in controlled biotechnological processes. Esters are produced by a variety of methods among which esterification and transesterification with acid catalysts under reflux conditions are prominent. The use of biocatalysts provides an opportunity for carrying out reactions under milder conditions leading to better

quality products suitable in fragrance and flavor industry. Also, the product can be labelled as "natural" [6, 7]. In nonhydrolytic reactions it is often advantageous to use immobilized lipases. The texture of the carrier also influences the biocatalytic reaction. The hydrophilic/hydrophobic character of the support is also an important parameter to consider. Many hydrophobic materials have been used to immobilize lipases since the large hydrophobic pocket surrounding the catalytic site of lipases can easily interact with hydrophobic solid supports [8]. Such adsorbed lipases have been shown to display a hyperactivation due to interfacial activation promoted by the hydrophobic surface of the support [9].

5.2 Why is water activity control important?

The catalytic activity of an enzyme in organic medium depends on its hydration state. It is important to remember that hydrolytic reactions can occur as a side reaction when hydrolytic enzymes are used for transferase reactions (e.g., transesterification reactions) for biosynthetic reactions. To minimize this, reaction conditions have to be such that hydrolysis becomes negligible which is generally obtained in media where the thermodynamic activity of water (a_w) is adjusted to a value that is as low as possible but high enough to keep the enzyme in a hydrated state that allows its activity [10, 11]. A small variation in water activity can have a significant influence on the catalytic activity and selectivity of lipase [12].

The thermodynamic water activity (a_w) is the parameter that best reflects enzyme hydration [13]. a_w may be fixed by pre-equilibrating reaction components with saturated salt solutions through the gas phase [14], a method that does not prevent a_w from changing when water itself is a reactant or a product of the reaction, by using salt hydrate pairs in situ [15] and very conveniently in the case of polar solvents, by direct addition of water to the reaction medium [16].

When the biocatalysts are immobilized, the influence exerted by the support on the availability of water to the biocatalysts to maintain its catalytic activity becomes more crucial [17]. Apart from water content, water activity (a_w), the intrinsic water

content or the water layer around the enzyme molecule, is considered as a good measure of the amount of water [18]. There was a clear correlation between the aquaphilicity of the support material and the enzymatic activity. Support with low aquaphilicity gave the highest activity. The result can be interpreted as a competition between the enzyme and the support material for the water. Supports with high aquaphilicity adsorb a lot of water, so that the enzyme is insufficiently hydrated. When support materials with low aquaphilicity are used, the enzyme can compete successfully for the water and therefore show high activity.

5.3 Saturated salt solutions for water activity control

Saturated salt solutions have fixed water activity (at fixed temperature). Such solutions have been used extensively for water-activity control of enzymatic reactions in organic media. Generally, it is advantageous to use the water activity (a_w) instead of water content or water concentration to characterize the water effects in the reaction mixture [14]. One of the most effective activation methods demonstrated to date involves the inclusion of simple salts during lyophilization, or freeze-drying, of the enzyme prior to use of the enzyme powder as a suspension in an organic solvent. Khmelnitsky et al. showed that including excess KCl in an aqueous enzyme solution prior to lyophilization increased the catalytic efficiency of subtilisin catalyzed transesterification by 3750-fold [19]. Triantafyllou et al. demonstrated that adding buffer salts or KCl also increased the catalytic activity of lyophilized *Candida antarctica* lipase four fold over that with no added salt [20]. Awang et al. [21] have reported that water activity ranging from 0.09 to 0.96 did not have a marked influence on the yields of the synthesized ester in the esterification of dihydroxy stearic acid with octanol catalyzed by Rhizomucor miehei and Novozyme 435lipases. The effect of water activity on the synthesis of dodecyl dodecanoate catalyzed by various lipases has also been reported [22].

5.4 Fixing water activity by equilibration

Equilibration with a saturated solution of an appropriate salt is a convenient method of obtaining vapor phases of known water partial pressure. The saturated

solution is normally best used in the form of a solid rich slush. In other words, the appearance should be of wet solid crystals. The lowest water activity that can be obtained using salt solutions is around 0.05. The water activity of these saturated solutions is usually fairly weakly temperature dependent. A selection of salts is shown in Table 5.1, with water-activity values for 25°C.

Table 5.1 Saturated salt water activities at 25°C

LiCl	0.113	KI	0.689
KAc	0.225	NaCl	0.753
$MgCl_2$	0.328	KCl	0.843
K_2CO_3	0.432	KNO_3	0.936
$Mg(NO_3)_2$	0.529	K_2SO_4	0.973
NaBr	0.576		

5.5 Pre-equilibration of reaction mixtures using saturated salt solutions

Reaction mixture components can be pre-equilibrated by keeping them in a closed vessel with the vapor phase in contact with the appropriate saturated salt solution [23, 24] (Fig 5.1). Wide-mouth screw-cap jars are commonly used. The salt solutions lush is placed in the bottom of the jar. This particular method of equilibration was adopted in our work for the attainment of different water activity levels with Candida rugosa.

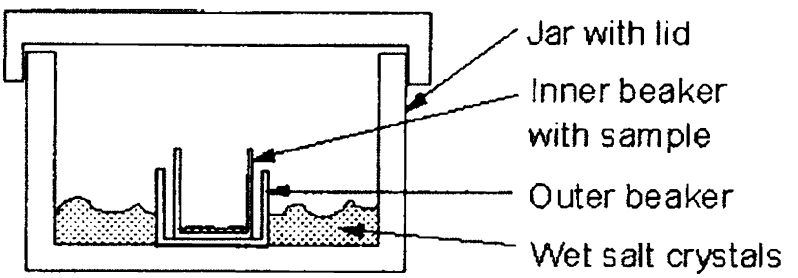


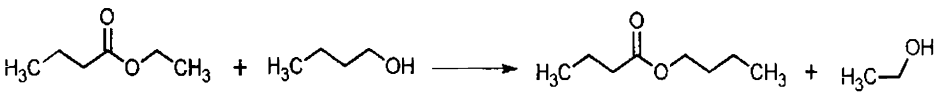
Fig 5.1 Equilibration of samples using saturated salt solutions.

5.6 Use of lipases in transesterification reaction

Lipase-catalyzed transesterification of cis- and trans-4-methylcyclohexanols with vinyl acetate in various organic solvents and the effect of solvent on activity and

stereoselectivity of lipase from Pseudomonas sp. has been investigated in the work by Nakamura et al. [25]. The composition of the AOT-based microemulsion used to immobilize enzymes in gels affects the rate of the enzyme catalysed reaction for transesterification between vinyl butyrate and racemic 2-octanol and between vinyl butyrate and racemic menthol with Candida cylindacea lipase [26]. Pseudomonas cepaciae lipase adsorbed onto carbon fiber supports was used to catalyze the transesterification reactions in the gas phase between vinyl acetate and n-propanol. The reaction yield of the transesterification carried out at 60°C was higher at 0% relative humidity as compared to 11% [27]. Highly porous silica aerogels with differing balances of hydrophobic and hydrophilic functionalities were studied as a new immobilization medium for two types of lipases from Candida rugosa and Burkholderia cepacia and activities were compared in a simple transesterification reaction of vinyl laurate by 1-octanol [28]. Very few studies have reported the kinetics of lipase-catalyzed synthesis of butyl butyrate. Santos et al. [29] used factorial design to optimize the lipase catalyzed synthesis of butyl butyrate in heptane, while Lozano et al. [30] studied the continuous synthesis of butyl butyrate using an active membrane coated with lipase. Other studies [31, 32, 33] used synthesis of butyl butyrate synthesis as a model for enzymatic activity and stability of specially treated lipase.

Butyl butyrate has a pleasant aroma. Butyl butyrate is an important flavor in high demand as a component of pineapple flavor in the food and beverage industry and/or manufacturing of fragrance and flavor concentrates of all types. The reaction is represented by the following equation:



A transesterification reaction was chosen for the aim of comparing the activity of the free and the immobilized enzyme in the presence and absence of water activity. Heptane was used as a medium in the immobilization of Candida Rugosa lipase onto mesocellular silica foams and clay via adsorption and covalent binding. The

preparations obtained were used for the transesterification reaction between ethyl butyrate and n-butanol for the synthesis of butyl butyrate. The kinetic parameters for the transesterification reaction were determined using the Ping-Pong Bi-Bi mechanism with n-butanol inhibition and represented by the Lineweaver Burk plot.

The influence of water activity on the synthesis of butyl butyrate by transesterification reaction (alcoholysis) using free and immobilized lipases was studied. The influence of initial n-butanol concentration and amount of enzyme in the reaction system was analyzed. The transesterification activity in the presence and absence of water activity and the influence of different water activity levels on the transesterification reaction was carried out.

5.7 Experimental procedure

(a) Water activity equilibration and transesterification reaction

The substrate, lipases and organic solvent were separately pre-equilibrated to known a_w in vapour phase in a vacuum-sealed jar. The equilibration process was carried out for 3 days using salt hydrates of known water activities as described by Halling [34]: LiCl, (a_w: 0.12); $MgCl_2 .7H_2O$ (a_w: 0.33); $Mg(NO_3)_2$ (a_w: 0.54); NaCl (a_w: 0.75) and KNO_3 (a_w: 0.96).

Transesterification was carried out in stoppered conical flasks (25 ml) containing 0.01M butanol and 0.01M ethyl butyrate in 10 ml of hexane with known quantity of enzyme (100mg free enzyme, 500mg of H_{MCF}, R_{MCF}, KI-10 and 200mg of HG_{MCF}, RG_{MCF}, K-10SG). Flasks were incubated on an orbital shaker at 40°C for 24 h. Samples were withdrawn at regular intervals and ester concentrations monitored by Gas Chromatographic analysis [35]. Quantification of data was done by calibration with standard samples.

(b) Analytical method

The analysis was performed on a Chemito Gas Chromatograph equipped with flame ionisation detector. A 2m×3.2mm stainless steel column packed with 10% SE-30 on chromosorb W was used for the analysis. Quantification of data was done by

calibration with standard samples. The unit of enzyme activity is defined as μmol of ethyl butyrate consumed (reaction with butyl alcohol) per min per mg of the enzyme.

$$\text{Specific enzymatic activity (mmol converted ethyl but/h.g)} \cong \frac{(N_{FA})^0 \times (X_{ac})_t}{t \times w_0}$$

where $(N_{FA})^0$ is the initial mmol of ethyl butyrate (mmol of ethyl butyrate); $(X_{ac})_t$ is the conversion of ethyl butyrate at time t (in hours); and w_0 is the total biocatalyst mass (g). Total biocatalyst mass is the support plus the adsorbed CRL in the case of immobilized lipase and mass of lipase when CRL is used. The yield per gram of

$$\text{the biocatalysts (mmol converted ethyl but/g)} \cong \frac{(N_{FA})^0 \times (X_{ac})_t}{w_0}$$

5.8 Activity of free and immobilized lipases with respect to transesterification reaction between ethyl butyrate and n-butanol

5.8.1 Effect of various biocatalysts: Progress of alcoholysis reaction in the presence and absence of water activity

The synthesis of butyl butyrate as a function of incubation time was investigated at optimum water activity of lipases (Fig. 5.2 (a)) and those in the absence of water activity (Fig 5.2(b)). The specific activity values for the free and the immobilized lipases as well as the yield are shown in the Table 5.2.

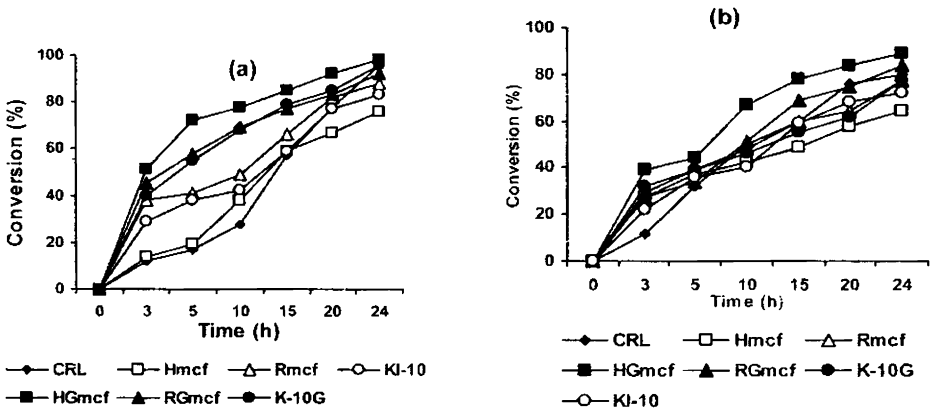


Fig 5.2 Percentage transesterification (butyl butyrate) at optimum water activity of free and immobilized lipases as a function of incubation time at 0.01M substrate, 100mg (free), 0.5g (ads), 0.2g (cv) enzyme concentration at 40°C (a) (a_w=0.33) (b) in the absence of water activity

Candida rugosa have been reported to be excellent catalysts for transesterification of short chain alcohols. Free CRL lipase gave 96% conversion after 24 h. It was reported that C.rugosa displayed the highest transesterification rates (0.483 μmol/min/mg of enzyme) at 0.33 water activity region [35] and hence this enzyme was chosen for this study. No transesterification was detected with dried enzymes. The rate of transesterification was less in the absence of water activity as the amount of bound water in the enzyme may cause a denaturation effect which does not favour the transesterification reaction. The maximum conversion obtained by C. rugosa for butyl butyrate synthesis in hexane could be due to high specificity of this lipase for C4 substrates [36].

Table 5.2 Yield and transesterification activity in the synthesis of butyl butyrate for free and immobilized lipases

Sample	Specific activity (mmol/h.g)	Yield(mmol/g)
Free CRL lipase	4	96
H_{MCF}	1.1	25.3
R_{MCF}	0.73	17.6
KI-10	0.69	16.7
HG_{MCF}	2	49
RG_{MCF}	1.8	43
K-10G	1.9	46.5

The covalently bound systems gave higher conversion than the adsorbed ones both in the presence and absence of water activity as in the case of hydrophobic supports due to the inclusion of organic moieties. They are less hydrated and hence the enzyme adsorbed on such supports compete successfully for water and showed enhanced activity compared to the adsorbed ones. Among the covalently bound systems HG_{MCF} gave the highest conversion. In the case of adsorbed systems, R_{MCF} gave higher conversion rather than H_{MCF} which may be due to the attainment of effective water activity of 0.33 in the case of enzyme

adsorbed on the external surface of R_{MCF} with high surface area. In the case of H_{MCF} most of the lipase molecules are occluded inside the pores and hence the attainment of water activity may not be uniform ad hence a lower degree of transesterification is observed. The activities on silica and clay were very low when the immobilization was performed in buffer and the lipase activity disappeared from the buffer solution. The lipase activities of the immobilized ones from heptane were higher than those of lipases immobilized from buffer. Hence heptane was chosen as the immobilization medium of lipases on all the supports for the transesterification reaction.

The transesterification activity and yield (Table 5.2) was higher for H_{MCF} samples and R_{MCF} exhibited greater activity than KI-10. The activity and yield were much higher for the covalently bound systems.

5.8.2 Effect of lipase concentration and temperature

The result of lipase quantity on transesterification of ethyl butyrate with n-butanol is shown in Fig. 5.3. There was an increase in the ester yield with the increasing lipase quantity. However, further increase in the lipase quantity of 20% did not have that much effect on ester yield.

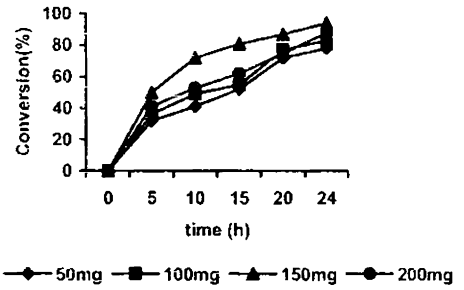


Fig 5.3 Effect of catalyst loading (CRL), ethyl butyrate,0.01mol., n-butanol, 0.01M., hexane to make total volume of 5ml, temperature, 40°C.

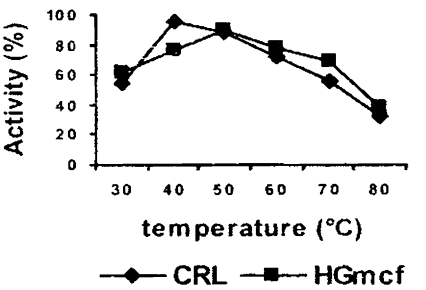


Fig 5.4 Effect of temperature on the activity of free and HG_{MCF}

This leveling-off behavior of transesterification at higher lipase quantity is typical and has also been reported by other researchers [37, 38]. The addition of

larger lipase quantity was not practical since the matrix and lipase together made the solution extremely viscous, so it will not help to further increase the ester yield. Due to the attainment of equilibrium there was no further increase in ester yield with increase in enzyme concentration. All further experiments were carried out with 100mg of the free enzyme.

The effect of temperature was studied in the range of 30 to 80°C, with 0.01 M of each reactant, free enzyme (100 mg), HG_{MCF} (200mg) and heptane (10ml) (Fig 5.4). It was found that the rate of the reaction and conversion increased with an increase in temperature from 30 to 50°C after which there was a decrease in both cases. The enthalpic contribution to the enzymatic rate enhancement suggests that there are important electrostatic and hydrogen-bonding interactions in the transition state of the enzymatic reaction, which are responsible for increased rate with temperature. The optimum temperature shifted to 50°C for HG_{MCF} while it was 40°C for the free enzyme. It seemed that hydrophobic groups on the matrix may be protecting the enzyme against denaturation at higher temperature. The conversion decreased from 60 to 80°C. This indicated that the enzyme tertiary structure might have been disrupted causing it to denature at higher temperatures [39]. Therefore, all further experiments with free and immobilized lipases were carried out at 40°C.

5.8.3 Effect of solvent on alcoholysis

To investigate the effects of solvents on the reaction, five solvents with different log P values were chosen to examine their effects on the activity. As expected, higher conversions were obtained for the reactions using the solvent with higher log P than others as reaction media. The lipase exhibits higher alcoholysis activities in a non-polar solvent (hexane) than polar solvents (Table 5.3).

n-Hexane gave the highest conversion both in the case of free and immobilized lipases. The impairment of polar solvents to enzyme by depriving the essential water binding to the active site seems to be incontrovertible. In another way,

the effects of reaction media on the activity of the immobilized lipase might be also related to the activity of the substrate molecules dissolved in these media as well as the water activity. While our results seem to suggest that the nature of the carrier might affect the lipase activity by creating different microenvironment to influence the solvation and diffusion of substrate and product in organic media [40].

Table 5.3 Effect of solvents with different log P values on the activity of free and immobilized lipases

	Solvents (Relative activity (%))				
Sample	**1,4-dioxane**	**Toluene**	**Isoctane**	**Hexane**	**heptane**
Free CRL	58	45	37	96	82
H_{MCF}	47	39	35	79	63
R_{MCF}	55	4	42	88	78
K-10	51	42	37	84	69
HG_{MCF}	66	57	51	96	77
RG_{MCF}	59	51	43	90	70
K-10G	62	54	47	94	74

The protective surface of the support may prevent the water monolayer from being stripped and maintain the three dimensional structure of its active protein conformation. In conclusion, the present results suggested that n-hexane appears to be the most suitable solvent for transesterification reaction.

Solvents with a log $P > 4$ are known to have a positive effect on enzyme activity. Solvents with a log $P < 2$ are known to reduce enzyme activity [41]. The higher enzymatic activity in the non-polar solvent may be attributed to the minimum distortion of the hydration layer around the enzyme by the solvent, thereby leaving the enzyme in an active state whereas the polar solvent, due to its high affinity for water, might remove the essential hydration layer around the enzyme, thus decreasing enzyme activity. These trends are in accordance with the data reported by Yadav et al. [42] on the transesterification of vinyl acetate (with n-decanol, 2-ethyl-1-hexanol, benzyl alcohol, cinnamyl alcohol, 1-phenylethyl alcohol and 2-phenylethyl alcohol) using an immobilized lipase in *n*-heptane.

5.8.4 Effect of water activity

The reaction rate profile of lipase from C. rugosa, and the immobilized lipases were measured at fixed initial water activity ranging from 0.12 to 0.96 (Fig. 5.5). The free and the immobilized lipases exhibited maximum transesterification activity at a water activity of 0.33 ($MgCl_2.7H_2O$). Water is known to have inhibition effect on lipase activity in the transesterification reaction. This phenomenon has also been observed by other researchers [43, 44] and it was thought to be the inhibitive effect on the lipase activity caused from the negative effect of existing water in the reaction. The transesterification rate was lower at higher water activity of 0.96 for all lipases used and similarly lower transesterification degree was observed with very low a_w salt hydrates.

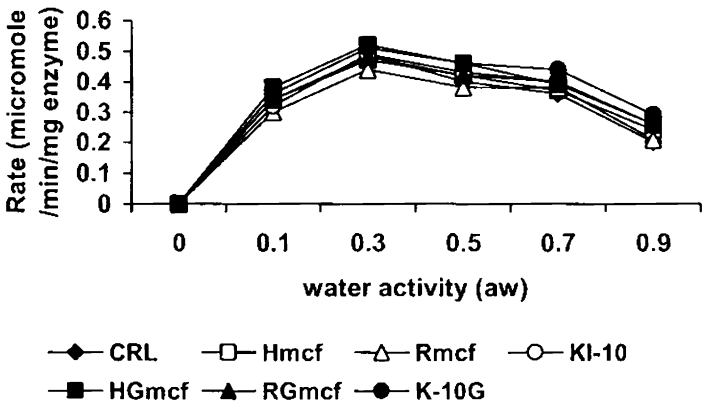


Fig 5.5 Reaction rate profile of lipases in alcoholysis as a function of water activity at 0.01 substrate, 100mg g enzyme concentration, 0.5g ads lipase, 0.2g covalently bound lipase, at 40°C. Equilibration carried out with LiCl, (a_w: 0.12); $MgCl_2$ /$7H_2O$ (a_w: 0.33); $Mg(NO_3)_2$ (a_w:0.54); NaCl (a_w: 0.75) and KNO_3 (a_w: 0.96).

At the lowest water activity of 0.33, the conversion of ethyl butyrate achieved the maximal values in the case of free and immobilized lipases. The covalently bound ones showed greater transesterification rate in all the water activity levels than the adsorbed ones. Overall, these results suggest that water activity strongly influenced the hydration level of the enzyme which in turn affected the transesterification activity. At

higher water activity (0.96) all the lipases used showed lower reaction rates and this could be due to severe diffusion limitation due to aggregation of enzyme or increased reverse reaction (hydrolysis) at higher water activities [45].

When the reactions were carried out at a higher water activity level (0.96), the yield of butyric acid from hydrolysis of ethyl butyrate increased monotonically with low butyl butyrate ester synthesis. It is known that, in alcoholysis, reaction proceeds via an acyl enzyme complex, which then reacts with the alcohol substrate. When the water activity is increased, water can also react with the acyl enzyme complex. As a result, a change in ratio of the rates of reactants takes place that favours hydrolysis. However, at low water activity level (0.33), a shift of equilibrium towards synthesis of butyl butyrate (96%) was observed with reduced hydrolysis. This suggests that the transesterification reaction occurs probably at two stages and a split water activity control as suggested by Ujang and Vaidya [46] could be beneficial for maximum activity. According to this method, during the initial stages of the reaction, higher level of water activity is required for hydrolyzing the ester (substrate). In later stages, the reaction is stepped down to lower water activity level for the formation of desired ester (product). Ibrahim and Robb [47] have shown that the activity of silica bound subtilisn was not restored by hydration after the essential water has been removed. The same results were reported by Furukawa et al. [48], who conducted the esterification of (-) menthol with butyric acid employing the C. rugosa lipase deposited on Celite. The authors suggested that the decrease of the activity of immobilized lipase is related to the higher water content induced deformation of the activated structure pertaining to the immobilized lipase at lower water activity.

5.8.5 The effect of the reactants concentration on the transesterification initial reaction rate

The effect of ethyl butyrate and n-butanol concentrations on the initial reaction rates with free CRL lipase were investigated by transesterifying various fixed initial quantities of ethyl butyrate with different concentrations of n-butanol and vice versa. Fig. 5.6 shows that when the concentration of n-butanol increased,

the initial reaction rate also increased; reaching a maximum at the acid concentration of 0.03 M. Concentrations above 0.03M did not increase the initial reaction rate. This behavior can be an indication of inhibition effect of n-butanol on the enzyme activity. Further, subsequent increase in ethyl butyrate concentration (Fig 5.7) led to an increase in the initial reaction rate in the concentration range studied. This means that ethyl butyrate concentration has no inhibition effects on the enzyme activity.

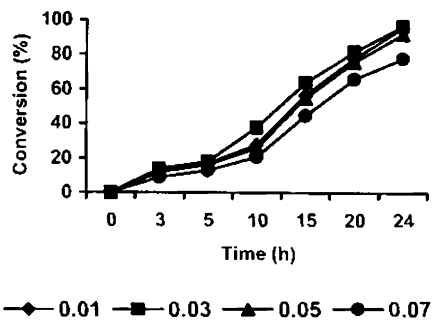


Fig 5.6 Effect of concentration of n-butanol. Ethyl butyrate, 0.01mol., hexane to make total volume of 10 ml, 100mg (CRL)., temperature, 40°C.

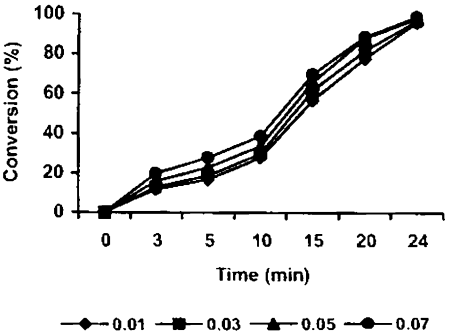


Fig 5.7 Effect of concentration of ethyl butyrate. n-butanol, 0.01mol., hexane to make total volume of 10ml, 100mg (CRL)., temperature, 40°C.

Therefore, all further reactions were carried out by using 0.01 mol of n-butanol.

Similarly, the effect of ethyl butyrate to n-butanol molar ratio in the case of HG_{MCF} is presented in Fig. 5.8 and Fig 5.9. It was found that increasing the concentration of ethyl butyrate increased the rate and conversion. Thus ethyl butyrate does not show any inhibitory effect for the concentration range considered in this study. However, the rate was found to decrease beyond 0.03 mol of n-butanol (Fig. 5.8). It could be due to a dead-end inhibition complex formation by n-butanol with the lipase, as described for transesterification of isoamyl alcohol with ethyl acetate [49].

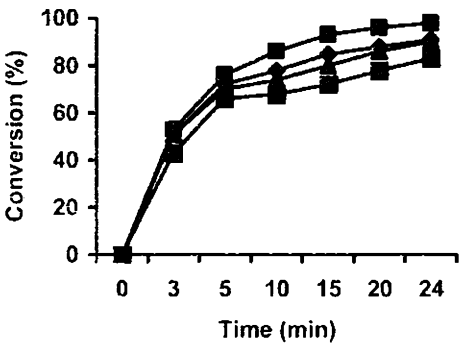


Fig 5.8 Effect of concentration of n-butanol. Ethyl butyrate, 0.01mol., hexane to make total volume of 10 ml, 200mg (HG_{MCF})., temperature, 40°C.

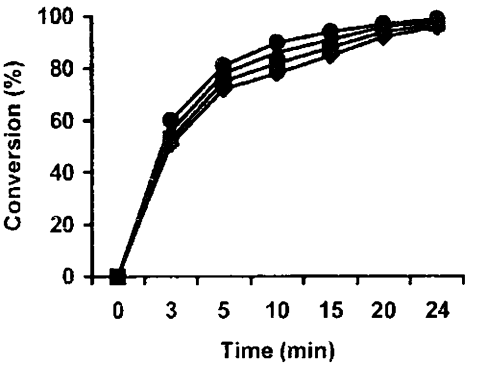


Fig 5.9 Effect of concentration of ethyl butyrate. n-butanol, 0.01mol., hexane to make total volume of 10 ml, 200mg (HG_{MCF})., temperature, 40°C.

Alcohol plays a complex role as an acyl acceptor, enzyme inhibitor and modifier of the enzyme active center [50]. The alcohol adsorbs to the immobilized enzyme and blocks the entry of substrates causing the reaction to stop [51]. The accepted mechanism of inhibition by alcohol is the formation of a non-reactive dead-end complex between enzyme and alcohol [52]. Many previous studies have also reported inhibition only by alcohol for the synthesis of octyl laurate [53] and geranyl acetate [54]. For example, Shimada et al. [55] found that immobilized Candida antarctica lipase was inactivated in a mixture containing greater than 1.5 molar equivalents of methanol in oil in a solvent-free system.

5.8.6 Thermal Stability measurement

From a commercial point of view, the thermal stability of enzymes is one of the most important features for the application of the biocatalyst. The thermal stability of both free lipase and the immobilized lipases was investigated by storing samples for 2 h at 50 and 55°C as shown in Fig. 5.10. The remaining activities were expressed as relative percentage to the original activities.

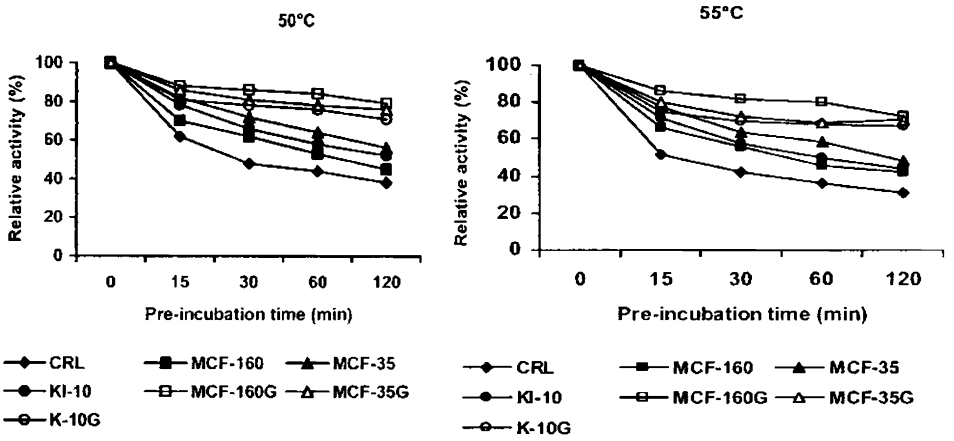


Fig 5.10 Thermal stability of the free and the immobilized lipases at 50 and 55°C

According to Fig. 5.10, all the of adsorbed lipases hold over 55% activity at 2 h, the covalently bound ones retained 75 % of their activity while the free enzyme retained only 38% of the activity after 2 h at 50°C. The free lipase retained about 38% and 30% of its initial activity at 50 and 55° C after a 120 min incubation period. R_{MCF} retained higher activity than H_{MCF}. After a 120 min heat treatment at 55°C, adsorbed ones retained about 43% its initial activity and the covalently bound ones retained more than 65% of their initial activity. Similar results have been previously reported for various covalently immobilized enzymes [56, 57]. Excessive heat will break down the tertiary structure of the enzyme, and the weaker conformation of free lipase was easier to destroy while the support matrices provided greater thermal stability for the immobilized lipase.

These results indicate that the thermal stability of immobilized lipases is much better than that of the free one owing to the formation of covalent bond between the enzyme and the supports, which prevents the conformation transition of the enzyme at high temperature. The low differences observed were not very significant but could perhaps be explained by the different average numbers of links between the enzyme and the support. The results showed that for the immobilized enzymes, the decrease of activity is much slower than that of free enzyme, which suggest that after immobilization, the enzyme molecules can refold back to its active site after thermal

treatment to some extent while for the free enzymes in solution, because of the long-range migration and aggregation, the lipase molecules cannot refold completely after being cooled down to room temperature, which resulted in irreversible denaturation and drastic decrease in enzymatic activity. The improved stability of immobilized enzymes may be related to the prevention of autolysis, thermal denaturation [58] and an increase in enzyme rigidity [59] by raising the temperature.

5.8.7 Retention of activity by immobilized lipases in continuous cycles

The operational stability of an immobilized enzyme without appreciable loss of enzyme activity is important for the economic viability of a biosynthetic process. The operational stability of the immobilized enzyme is the main characteristic that limits a potential application of immobilizates in industrial scale [60, 61]. In Figure 5.11, the relative activities of native and immobilized lipase are plotted against the number of reaction cycles. The immobilized catalyst was filtered off, washed with heptane and reused.

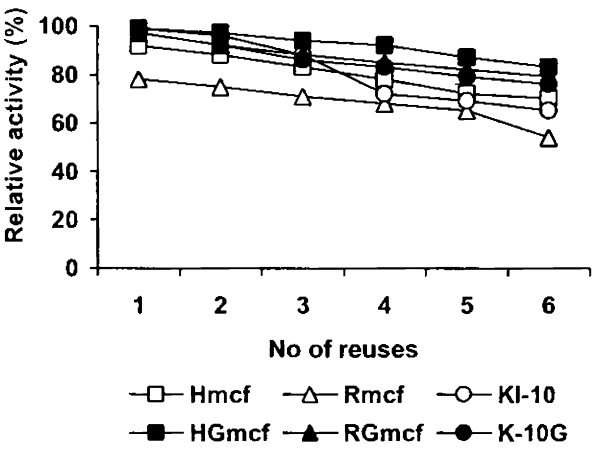


Fig 5.11 Reusability of the immobilized lipases

It was observed that, H_{MCF} demonstrated more than 70% activity after 6 runs while R_{MCF} and KI-10 retained 60% activity after the same. The difference observed is due to the difference in the structure of the supports. The interaction between lipase and H_{MCF} would be beneficial to reduce the enzyme deactivation. The biocatalyst

immobilized on pure-R_{MCF} suffered severe desorption of enzyme, which caused a decrease of about 54% after six runs. The reason of the drop in conversion after fourth cycle for may be the deactivation of lipase due to the washing with hexane, which could also be a potential inhibitor. Another reason could be the changes in the composition of the initial reaction mixture. Washing of the supports did not wash out the product (ester) and substrate completely. The presence of the product in the reaction mixture shifted the equilibrium in the backward direction (hydrolysis) and it resulted in overall loss of activity. Among the covalently bound systems, HG_{MCF} showed residual activity of 85% while RG_{MCF} and K-10G could retain over 75% activity. This may be explained by the increased stability of conformation of the enzyme upon binding to the support surface through a longer activating agent. The activity loss upon reuse could be due to weakening in the strength of binding between the matrix and enzyme and also due to inactivation of the enzyme upon immobilization such as product and/or substrate inhibition [62].

The activity loss is high when only (weak) physical bonds are involved, probably due to enzyme leaching during washings. Operational stability of immobilized enzyme with glutaraldehyde is much higher. The tight binding to the support allowed very little leaching of the enzyme. Immobilization can facilitate a decrease in enzyme consumption as the enzyme can be retrieved and reused for many repeated reaction cycles [63]. Chen and Hsieh [64] reported a loss of activity after five reuses when lipase was immobilized on ultrafine cellulose fibers prepared using an adsorption method. Ye and coworkers [65] studied lipase immobilization using the covalent bonding method and reported that the residual activity of the immobilized lipase was 67 % after 10 reuses.

5.8.8 Kinetics of reaction

In order to identify the optimal conditions for the lipase catalyzed transesterification reaction, it is essential to understand the kinetics of this reaction. Most of the earlier kinetic studies on lipase-catalyzed transesterification considered short chain

alcohols and long-chain fatty acids, observing alcohol inhibition and described by a Ping Pong kinetic model with competitive inhibition by the alcohol [66, 67].

Reactions were carried out for different initial concentrations of ethyl butyrate (0.01-0.1 M) with a constant butanol concentration. The concentration of one of the two substrates was maintained constant while the full series of concentration for the second one was investigated. All reactions were conducted for 24 h with 100mg enzyme loading at 40°C. The kinetics were determined for the industrial free Candida rugosa lipase and for HG_{MCF}. The kinetic parameters for the transesterification reaction were determined using the Ping-Pong Bi-Bi mechanism with substrate inhibition and represented by the Lineweaver and Burk plot. Many authors also demonstrated that kinetics mechanism for transesterification reaction was Ping Pong Bi Bi [68, 69].

(a) Mechanism of the reaction

The mechanism for the transesterification reaction was proposed by Varma at al [70]. The transesterification for the synthesis of butyl butyrate from ethyl butyrate is

$$\underset{\text{Butyric acid(R1)}}{CH_3CH_2CH_2COOH} + \underset{\text{Butanol(R2)}}{CH_3(CH_2)_3OH} \rightleftarrows \underset{\text{water(P1)}}{H_2O} + \underset{\text{Butyl butyrate(P2)}}{CH_3CH_2CH_2COO(CH_2)_3CH_3}$$

For the transesterification reaction, ethyl butyrate (R1) binds first with enzyme to form an ester–enzyme complex. This complex is transformed by a unimolecular isomerization reaction to give acyl–enzyme intermediate complex with release of first product ethanol (P1). The second substrate, butanol (R2), reacts with this acyl-enzyme complex to form a binary complex with enzyme. This binary complex undergoes a unimolecular isomerization to a butyl butyrate–enzyme complex, which releases another product, butyl butyrate (P2), and free enzyme.

$$E + R1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ER1 \longrightarrow E^*P1 \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} E^* + P1$$

$$E^* + R2 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftarrows}} E^*R2 \longrightarrow EP2 \underset{k_{-4}}{\overset{k_{+4}}{\rightleftarrows}} E + P2$$

The expression of reaction rate for the Ping Pong Bi Bi with alcohol inhibition as given in Eq. (1)

$$V_i = \frac{V_{max f}[C_{R1}][C_{R2}]}{[C_{R1}][C_{R2}] + K_{R2}[C_{R1}]\left(1 + \frac{[C_{R1}]}{K_{IR1}}\right) + K_{R1}[C_{R2}]\left(1 + \frac{[C_{R2}]}{K_{IR2}}\right)} \quad \text{.....(1)}$$

where V_i is the initial reaction rate, V_{max} is the maximum reaction rate, K_{R1} and K_{R2} are the Michaelis-Menten constant for ethyl butyrate and n-butabol, respectively. K_{IR2} is the inhibition constant for alcohol. $[C_{R1}]$ and $[C_{R2}]$ are the initial concentration for ethyl butyrate and n-butanol, respectively.

(b) The Burke–Lineweaver (double-reciprocal) plot

Fig. 5.12 illustrates that, when the mole number of ethyl butyrate increased from 0.01 to 0.1 M, all the plots of $1/V_i$ as a function of 1/[But,ol] converged towards the same lower curve.

On the other hand when $1/V_i$ was plotted as a function of $1/n_{ethyl\ but}$ (Fig. 5.13.), the lines are parallel to each other at different values of n-butanol, which shows inhibition of the enzyme by alcohol. This indicated competitive inhibition by n-butanol, which would imply that the latter formed a complex with the enzyme that prevented the formation of the active complex that would lead to the product. No such inhibitory effect was found for ethyl butyrate. Similar observations were observed when the inverse initial rate, plotted as a function of the inverse mole numbers of the two substrates in the case of HG_{MCF} presented in Fig. 5.14 and Fig 5.15. The mole number of the substrates ethyl butyrate and n-butanol were 0.01M, 0.02M, 0.03M, 0.05M, 0.075M, 0.1M and all possible combinations between these concentrations for the two substrates were examined.

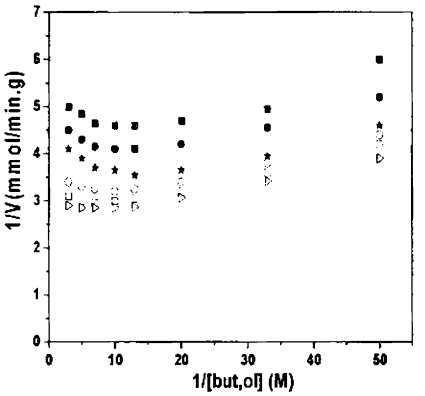


Fig5.12 Lineweaver and Burk representation of the variation of the maximum reaction rate of transesterification, *Vi*, with the initial molar concentration of ethyl butyrate, with butanol in presence of 100mg enzyme (CRL) at 40°C. Legends represent butanol concentrations (M) of 0.01 (■), 0.02 (●), 0.03(★), 0.05 (○), 0.075 (□), 0.1 (▷) temperature, 40°C.

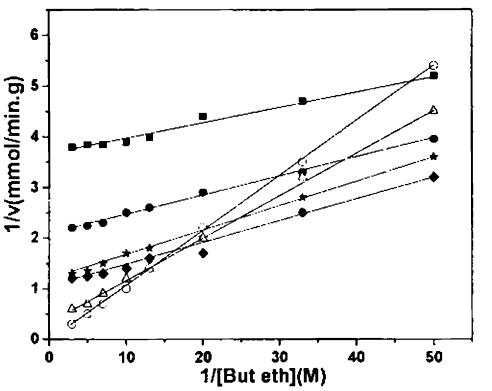
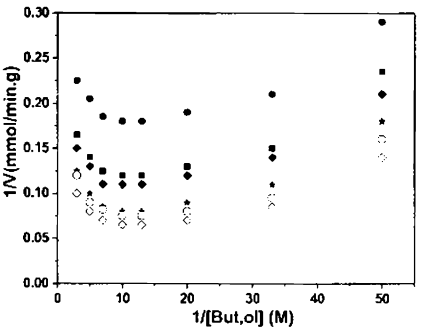


Fig 5.13 Lineweaver and Burk representation of the variation of the maximum reaction rate of transesterification, *Vi*, with the initial molar concentration of n-butanol, with ethyl butyrate in presence of 100mg enzyme (CRL) at 40°C. Legends represent ethyl butyrate concentrations (M) of 0.01 (♦), 0.02 (★), 0.03(●), 0.05 (Δ), 0.075 (■), 0.1 (○)

The Michaelis constants are given by Equation (1). A large value of these constants indicates weak substrate binding (that the ES complex is less stable) while small values of these constants indicate that the enzyme requires only a small amount of substrate to become saturated. The smaller the value, the stronger the affinity. The most important factor is the dissociation constant (K_I) for binding inhibitor to enzyme and a high value of this constant indicates that there is minimal inhibition. The values of the kinetics parameters were calculated by nonlinear regression using Graph Pad Prism software. The kinetic parameters, V_{maxf}, K_{R1}, K_{R2} ,K_{IR2}, obtained from Equation (1) are listed in Table 5.4. $K_{m.But}$ for free lipase was lower than $K_{m.But\ eth}$ which indicated higher affinity for n-butanol and hence the inhibition observed. There was no inhibition observed with ethyl butyrate due to the higher value of $K_{m.But\ eth}$.



ig 5.14 Lineweaver–Burk plot of reciprocal initial reaction rates Vs reciprocal butanol at fixed ethyl butyrate concentrations: (●) 0.01 M, (■) 0.02 M, (♦) 0.03 M, (★) 0.05 M, (○) 0.075M and (◊) 0.1 M. Reaction conditions: 0.2 g HG_{MCF}; 40°C and 10ml hexane

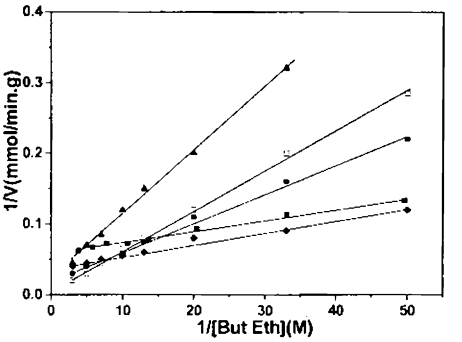


Fig 5.15 Lineweaver–Burk plot of reciprocal initial reaction rates Vs. reciprocal ethyl butyrate at fixed butanol concentrations: (♦) 0.01 M, (■) 0.02 M, (●) 0.03 M, (□) 0.05 M, and (▲) 0.1 M. Reaction conditions: 0.2 g HG_{MCF}; 40°C and 10ml hexane

Table 5.4 Kinetic parameters for the synthesis of butyl butyrate from ethyl butyrate and n-butanol using free CRL and HG_{MCF}

Catalyst	Parameter	Value
Free (CRL)	V_{max}	19.62 mmol min^{-1} g^{-1}
	K_{mBut}	0.38M
	$K_{mBut\ eth}$	0.47M
	Ki_{But}	1.29M
HG_{MCF}	V_{max}	10.74 mmol $min^{-1}g^{-1}$
	K_{mBut}	1.67M
	$K_{mBut\ eth}$	1.13M
	Ki_{But}	0.89M

It appears that the kinetics constants $K_{m,But}$ and $K_{m,But\ eth}$ were increased after immobilization except for the inhibition constant by n-butanol ($K_{i,But}$) and V_{max} which decreased. $K_{m,But}$ was also higher compared to free enzyme but lower than $K_{m,But\ eth}$. The apparent Michealis–Menten constant for CRL lipase increased after immobilization. K_m and V_{max} undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change for the substrate. These variations can be attributed to several factors such as protein conformational

changes induced by the attachment to the support, steric hindrances, variations in the microenvironment and diffusional effects [71, 72]. The apparent increase in K_m for the immobilized enzyme indicates an alteration in the affinity of the enzyme towards the substrate upon covalent immobilization on silica or lower possibility to form a substrate–enzyme complex. The reduction in V_{max} for immobilized enzymes as compared to free ones is mainly due to their partial inactivation caused by less favourable conditions of catalysis following the immobilization process.

5.9 Conclusions

The present work investigated the study of the reaction parameters governing the enzymatic synthesis of butyl butyrate via transesterification reaction with free and immobilized lipases on mesocellular silica foams and clay. The reaction conditions reported in the present study are mild and 'clean' as compared to chemical methods. The key points from the present study are:

- The influence of water activity on the synthesis of butyl butyrate by transesterification reaction (alcoholysis) using free Candida rugosa and the immobilized lipases were studied and it was found that the transesterification rate is lower in the absence of water activity. The yield and the transesterification activity were higher for the covalently bound systems.
- The free and immobilized lipase exhibited higher alcoholysis activities in non-polar solvents.
- A direct relationship between water activity (a_w) and reaction rate was observed. Lipases from C. rugosa was active at a_w 0.33. At higher water activity (a_w: 0.96) the reaction equilibrium favoured the hydrolysis of ethyl butyrate and the equilibrium was shifted to synthetic mode (butyl butyrate) when operated at low water activity (0.33).
- The decrease in transesterification rate with increasing n-butanol concentration confirmed the inhibition by n-butanol whereas no such

effect was observed with ethyl butyrate. This study showed that the kinetic mechanism Bi-Bi Ping-Pong with inhibition by n-butanol remained unchanged for the reactions catalyzed by the industrial powder of Candida rugosa or for the covalently bound HG_{MCI}.

- The immobilized lipases showed improved thermal and operational stability compared to free enzyme.
- The Michaelis constant (K_m) for n-butanol was lower than K_m for ethyl butyrate which showed the higher affinity for n-butanol. The maximum reaction velocity (V_{max}) decreased after immobilization and K_m showed an increase revealing an affinity change for the substrate or may be due to the conformational changes of the enzyme resulting in a lower possibility to form substrate–enzyme complex.
- The properties and stability of the immobilized lipase exhibited characteristics that may be suitable for industrial biotransformations.

References

[1] E. Santaniello, P. Ferraboschi, P. Grisenti, Enzyme Microb. Technol., 15 (1993) 367.

[2] T. Garcia, A. Coteron, M. Martinez, J. Aracil, Chem. Eng. Sci., 55 (2000) 1411.

[3] R. Garcia, T. Garcia, M. Martinez, J. Aracil, Biochem. Eng. J., 5 (2000) 185.

[4] M. Rizzi, P. Stylos, A. Rich, M. Reuss, Enzyme Microb. Technol., 14 (1992) 709.

[5] M. Liaquat, R. K. Owusu, Food Chem. Toxicol., 65 (2000) 295.

[6] B. Gilles, H. Yamazaki, D.W. Armstrong, Biotechnol. Lett., 9 (1987) 709.

[7] G. Langrand, C. Triantaphylides, J. Baratti, Biotechnol. Lett., 10 (1988) 549.

[8] V. M. Balcao, M. C. Vieira, F. X. Malcata, Biotechnol Prog., 12 (1996) 164.

[9] R. Fernandez-Lafuente, P. Armisen, P. Sabuquillo, G. Fernandez-Lorente, J. M. Guisan, Chem Phys Lipids., 93 (1998) 185.

[10] P. J. Halling, Biochem Soc Trans., 25 (1997) 170.

[11] A. M. Klibanov, Trends Biochem Sci., 14 (1989) 141.

[12] P. Pepin, R. Lortie. Biotechnol Bioeng., 63 (1999) 502.

[13] G. Bell, P. J. Halling, B. D. Moore, J. Partridge, D.G. Rees, 13 (1995) 468.

[14] P. J. Halling, Enzyme Microb Technol., 16 (1994)178.

[15] E. Zacharis, I. C. Omar, J. Partridge, D. A. Robb, P. J. Halling, Biotechnol Bioeng., 55 (1997) 367.

[16] G. Bell, A. E. M. Janssen, P. J. Halling, Enzyme Microb Technol., 20 (1997) 471.

[17] D. K. Oladepo, P. J. Halling, V. F. Larsen, Biocatalysis, 8, (1994) 283.

[18] P. J. Halling, R. H. Valivety, Physical, chemical nature of low systems for biocatalysis especially phase behaviour, water activity and pH, in Biocatalysis in Nonconventional Medi, Elsevier, Amsterdam (1992) pp. 13–21.

[19] Y. L. Khmelnitsky, S. H. Welsh, D. S. Clark, J. S. Dordick, J. Am. Chem. Soc., 116 (1994) 2647.

[20] A. O. Triantafyllou, E. Wehtje, P. Adlercreutz, B. Mattiasson, Biotechnol. Bioeng., 54 (1997) 67.

[21] R. Awang, M. Basri, S. Ahmad, A. B. Salleh, J Am Oil Chem Soc., 77 (2000) 609.

[22] R. H. Valevety, P. J. Halling, A. R. Macrae, Biochim Biophys Acta., 1118 (1992) 218.

[23] L. Greenspan, J. REs Nat Bureau Stds, 81a (1977) 89.

[24] British Standards Institute, Specification for laboratory humidity ovens (noninjection type) (1964) BS 3718.

[25] K. Nakamura, M. Kinoshita, A. Ohno, Tetrahedron., 50 (1994) 4681.

[26] S. Karlsson, S. Backlund, F. Eriksson, G. Hedstrom, Colloids Surf., B., 10 (1998) 137.

[27] T. Debechea, C. Marmet, L. Kiwi-Minskerb, A. Renkenb, M. A. Juillerat, Enzyme Microb. Technol., 36 (2005) 911.

[28] H. El. Rassy, A. Perrard, A. C. Pierre, Chem Bio Chem., 4 (2003) 203.

[29] J. C. Santos, H. F. de Castro, World J. Microbiol. Biotechnol., 22 (2006) 1007.

[30] P. Lozano, A. B. Perez-Marın, T. De Diego, D. Gomez, D. Paolucci-Jeanjean, M. P. Belleville, J Membr Sc., 201 (2002) 55.

[31] F. Borzeix, F. Monot, J. Vandecasteele, Enzyme MicrobTechnol., 14 (1992) 791.

[32] H. F. De Castro, R. de Lima, I. C. Roberto, Biotechnol Prog., 17 (2001) 1061.

[33] T. De Diego, P. Lozano, S. Gmouh, M. Vaultier, J. L. Iborra, Biomacromolecules, 6 (2005) 1457.

[34] P. J. Halling, Biotechnol Tech., 6 (1992) 271.

[35] G. V. Chowdary, S. G. Prapulla, Process Biochem., 38 (2002) 393.

[36] G. Langrand, N. Rondot, C. Triantaphylides, J. Baratti, Biotechnol Letts., 8 (1990) 581.

[37] J. Lu, K. Nie, F. Xie, F. Wang, T. Tan, Process Biochem., 42 (2007) 1367.

[38] A. V. L. Pizarro, E.Y. Park, Process Biochem., 38 (2003) 1077.

[39] M. D. Romero, L. Calvo, C. Alba, A. Daneshfar, H. S. Ghaziaskar, Enzyme Microb. Technol., 37 (2005) 42.

[40] C. R. Wescott, A. M. Klibanov, Biochimica et Biophysica Acta., 1206 (1994) 1.

[41] B. Yang, S. J. Kuo, P. Hariyadi, K. L. Parkin, Enzyme Microbial Technol., 16 (1994) 577.

[42] G. D. Yadav, A. H. Trivedi, Enzyme Microbial Technol., 32 (2003) 783.

[43] L. Li, W. Du, D. Liu, L. Wang, Z. Li, J Mol Catal B: Enzym., 43 (2006) 58.

[44] E. Z. Su, M. J. Zhang, J. G. Zhang, J. F. Gao, D. Z. Wei, Biochem Eng J., 36 (2007) 167.

[45] T. Yamane, Y. Kojima, T. Ichiryu, M. Nagata, S. Shimizu, Biotechnol Bioeng., 34 (1989) 838.

[46] Z. Ujang, A. M. Vaidya. Appl Microbiol Biotechnol., 50 (1998) 318.

[47] C. O. Ibrahim, D. A. Robb, Asia Pac.J. Mol. Biol. Biotehnol., 3 (1995) 198.

[48] S. Furukawa, T. Ono, H. Ijima, K. Kawakami, J. Mol. Catal. B: Enzym., 15 (2001) 65.

[49] M. Rizzi, P. Stylos, A. Riek, M. A. Reuss,. Enzyme Microb Technol., 14 (1992) 709.

[50] Y. C. Cheng, S. W. Tsai,. Enzyme Microb Technol., 32 (2003).362.

[51] J. W. Chen, W. T. Wu, J Biosci Bioeng., 95 (2003) 466.

[52] G. D. Yadav, P. S. Lathi, Biochem Eng J., 16 (2003) 245.

[53] H. E. Rassy, A. Perrard, A. C. Pierre, J. Mol. Catal B: Enzym., 30 (2004) 137.

[54] W. Chulalaksananukui, J. Condoret, D. Combes, Enzyme Microb Technol., 14 (1992) 293.

[55] Y. Shimada, Y. Watanabe, A. Sugihara, Y. J. Tominaga, J.Mol Catal B : Enzym., 17 (2002) 133.

[56] G. Jeganathan, A. Nakhla, J. Bassi, Hazard. Mater., 145 (2007) 127.

[57] M. V. Kahraman, G. Bayramoglu, N. Kayaman-Apohan, A. Gungor, Food Chem., 104 (2007) 1385.

[58] Z. He, Z. Zhang, M. He, Process Biochem., 35 (2000) 1235.

[59] M. D. Trevan, Immobilized Enzymes—an Introduction and Applications in Biotechnology, Wiley, Chichester (1980) p. 11.

[60] R. N. Silva, E. R. Asquieri, K. F. Fernandes, Process Biochem., 40 (2005) 1155.

[61] G. Czichocki, H. Dautzenberg, E. Capan, K. D. Vorlop, Biotechnol Lett., 23 (2001) 1303.

[62] O. V. Lomako, I. I. Menyailova, L. A. Nakhapetyan, Y. Nikitin, A. V. Kiselev, Enzyme Microb. Technol., 4 (1982) 89.

[63] S. D. Nirpjit, J. Kaur, J Biotechnol Appl. Biochem., 36 (2002) 7.

[64] H. Chen, Y. L. Hsieh, Biotechnol. Bioeng., 90 (2004) 405.

[65] P. Ye, Z. K. Xu, Z. G. Wang, J. Wu, H. T. Deng, P. Seta, J. Mol. Cat., 32 (2005) 115.

[66] M. P. Bousquet Dubouch, M. Graber, N. Sousa, S. Lamare, M. D. Legoy, Biochim Biophys Acta (BBA) – Protein Struct Mol Enzymol., 1550 (2001) 90.

[67] V. Dossat, D. Combes, A. Marty, Enzyme Microb Technol., 30 (2002) 90.

[68] J. Xiong, J. Wu, G. Xu, L. Yang, Chem Eng J., 138 (2008) 258.

[69] G. D. Yadav, P. S. Lathi. J. Mol. Catal. B : Enzym., 32 (2005) 107.

[70] M. N. Varma, G. Madras, J.Chem. Technol. Biotechnol., 83 (2008) 1135.

[71] M. S. Moly Eldin, M. Portaccio, N. Diano, S. Rossi, U. Bencivenga, A. Dva, P. Canciglia, F. S. Gaeta, D. G. Mita, J. Mol. Catal., 7 (1999) 254.

[72] T. Uhlich, M. Ulbricht, G. Tomaschewski, Enzyme Microb Technol., 19 (1996) 124.

Chapter 6

HYDROLYSIS OF ESTERS – IN AQUEOUS AND ORGANIC MEDIA

Part – I

Hydrolysis in Aqueous media

Contents

The steadily growing interest in lipases over the last two decades stems from their biotechnological versatility and the ability of these enzymes to catalyze a broad spectrum of bioconversion reactions with tremendous potential in various areas such as in food technology, biomedical sciences and chemical industry. Many of these applications are performed with immobilized lipases. The immobilization is an advantageous method that improves the stability of the biocatalyst and provides for its repeated use and the easy separation of the catalyst from the reaction medium. Obviously, the problem of selecting the support material and the proper technique is very important and therefore the pursuit for suitable materials has not yet been ceased. Affinity between supports and enzyme is more important for the efficient immobilization of enzyme. In the search for suitable materials, in this study mesocellular silica foams as well as clay were selected as supports for adsorption and covalent binding of Candida Rugosa lipase. The selection criteria were based on the enzyme loading onto the support, enzyme activity and immobilization efficiency. The activities of the immobilizates of commercial nonspecific Candida rugosa lipase were assessed in an aqueous medium and organic medium by the hydrolysis of long chain fatty esters as a reaction system. Hereby, the factors influencing the course of the hydrolysis with free and immobilized enzyme with the best performances were studied. Among the factors investigated were the enzyme concentration, the pH and the temperature of the reaction mixture, as well as the reusability and storage stability of the immobilizates.

6.1 Introduction

Lipases are the hydrolytic enzymes that can catalyze a wide range of reactions such as hydrolysis, alcoholysis, transesterifications, aminolysis and enantiomer resolution. Lipases (EC 3.1.1.3) have gained considerable importance as versatile biocatalysts for thc hydrolysis/synthesis of a wide range of esters and amides [1–3]. The lipases suffer a conformational change from an inactive state (closed form) to an active state (open form), exposing the active site to the reaction medium and increasing its catalytic activity, the presence of hydrophobic surfaces promotes the stabilization of this open form (the so-called interfacial activation) and improve their activity during immobilization [4, 5].

Total hydrolysis of ester bonds in triacylglycerols may be accomplished at high temperatures and pressure in the presence of steam. Fatty acids can alternatively be produced by ambient pressure, saponification or chemically catalyzed hydrolysis. However, the use of lipases for enzymatic splitting of fats in the presence of excess water is more appealing since the reaction proceeds under mild conditions of pressure and temperature with specificity and reduced waste [6]. This technology is currently employed in the production of fatty acids, diglycerides, monoglycerides, flavoring agents for dairy products and detergents for laundry and household uses. It is generally accepted that lipase catalyzed hydrolytic activity does not correlate with its esterification activity or transesterification activity, because the transfer reaction is carried out in a organic solvent. Lipase-mediated hydrolysis of vegetable oils or triglyceride in non-conventional media such as super critical fluids [7, 8], and organic solvents [9, 10] has also been attempted. A colorimetric method is convenient as an assay method for enzyme activity [11]. Hence p-nitrophenyl esters has been focusssed as a chorogenic substrate for the measurement of hydrolytic activity.

The esterolytic activity of lipases is routinely estimated by employing the para-nitrophenyl palmitate (pNPP) assay described by Winkler and Stuckmann [12].

The basis of this assay protocol is the colorimetric determination estimation of para-nitrophenol (pNP) released as a result of enzymatic hydrolysis of pNPP at 410nm.

In many cases the non-orientable immobilized lipases lost catalytic activity and efficiency to some extent. The properties and microstructure of supports have great impact on the performance of immobilized enzymes. It is, thus, important that the choice of support materials and immobilization methods for enzyme should be well justified. Generally, the efficiency of enzyme immobilization is decided by the support size (or surface area). However, this is questionable in the case of lipase, because of the explicit structure of the enzyme protein. Literature reports about the 3D structure of lipases indicate that lipases are fairly hydrophilic as well as hydrophobic proteins and their main hydrophobic area is the extremely hydrophobic region surrounding the catalytic site [13].

Lipases immobilized onto various carriers were investigated in terms of their hydrolytic [14, 15], synthetic activity [16, 17] or both [18, 19]. It was reported that the hydrophilicity or hydrophobicity of the carriers used in immobilization of lipase affected the lipase activity [20, 21]. Nanofibrous membranes were fabricated from poly(acrylonitrile-co-maleic acid) (PANCMA) by the electrospinning process and after tethering it with chitosan and gelatin were employed for the covalent immobilization of Candida rugosa lipase. In comparison with the immobilized lipase on the nascent nanofibrous membrane, there was an increase of the V_{max} value for the immobilized lipases on the chitosan and gelatin-modified nanofibrous membranes. The pH and thermal stabilities of lipases increased upon immobilization [22].

Microporous poly (styrene–divinylbenzene) copolymer constitutes excellent support for covalent immobilization of lipase from T. lanuginosu with 85% yield and could retain full activity after 30 days storage and fifteen repeated batch reactions [23]. Electrospun polysulfone nanofibrous membranes

containing PVP or PEG as additives were applied to immobilize C.rugosa lipases by physical adsorption by Zhen et al. Compared with free lipase, the optimum temperature for adsorbed lipase activity increased, pH value got lower and thermal stability increased. The V_{max} and K_m values for immobilized preparations were lower and higher those for free preparations [24]. Lipase immobilized to chitosan beads by the binary method showed a higher thermal and storage stability in soybean oil hydrolysis compared to soluble lipase in the work done by Ting. et al. [25]. High catalytic activity for hydrolysis reactions was observed when using TEOS as precursor while those prepared with methyltrimethoxysilane (MTMS) showed low activities in the hydrolysis reactions in the work done by Soares at al. [26]. Immobilized lipase using dry and wet chitosan beads retained 78% and 85% of its initial activity after 10 batch hydrolytic cycles and also good storage stability with high resistance to pH and temperature changes in the work done by Chiou et al.[27].

In this study the catalytic ability of lipase immobilized on six different supports for the hydrolysis has been evaluated. p-nitrophenyl palmitate (pNPP), a well known substrate for lipase hydrolysis was selected as the substrate in this work. The effect of temperature, pH, thermal stability, effect of metal ions and alcohols, reusability, storage stability and kinetic properties were investigated.

6.2 Substrate preparation and lipase assay

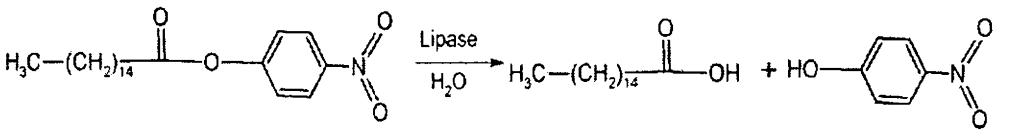
6..2.1 Activity assay of lipase in aqueous media

A modified version of the procedure by Winkler and Stuckmann [12] based on the hydrolysis of *p*-NPP was used throughout this study to measure lipase activity. The stock substrate solution was prepared as per the method of Kordel et al. [28] with slight modifications, by dissolving *p*-NPP was in 2-propanol to obtain a 16.5mM stock solution. One milliliter of the stock substrate solution was added to 9ml of 0.01M phosphate buffer, containing 0.4% (w/v) Triton X-100 and 0.1% (w/v) arabic gum, pH 7.0. Lipase activity

was assayed spectrophotometrically by measuring the rate of hydrolysis *p*-NPP at 410 nm and 37°C in a UV spectrophotometer. The change in absorbance at 410 nm and 37°C was read at 60 s intervals for a period of 20 min. The reaction mixture composed of 900µl of freshly prepared substrate solution and 100µl of the enzyme extract. Enzyme activity was expressed as unit/ml (U/ml), where one unit of activity was defined as the amount of enzyme that catalyzed the release of 1µmol of *p*-nitrophenol (*p*-NP) per min under the assay conditions. The extinction coefficient of *p*-NP at pH 8.0 was determined as 17,500M^{-1} cm^{-1} per liter.

6..2.2 Activity assay of lipase in the organic medium

The reaction rate of the free and immobilized lipase preparations in heptane was determined according to the process described by Pencreac'h and Baratti [29]. In the standard conditions, the reaction mixture was composed of 2mL of n-heptane containing 10mM *p*-NPP in an Erlenmeyer flask. The reaction was started by the addition of 10 mg free lipase preparation (or 50 mg immobilized lipase preparation). The mixture was incubated at 37°C. After 5 min of reaction, agitation was stopped, the lipase powder was allowed to settle for 30 s, and the clear supernatant was withdrawn. Fifty microlitres of supernatant was immediately mixed with 1.0mL, 10mM NaOH, directly in 1.0mL cuvette of the spectrophotometer. The *p*-NP was extracted by the aqueous alkaline phase. It displayed a yellow color because of the alkaline pH. The absorbance was read at 410 nm against a blank without enzyme and treated in parallel. Molar extinction coefficient of $17.4\times10^{3}M^{-1}$ cm^{-1} for *p*-NP was estimated using the standard solutions of *p*-NP in n-heptane.



p-nitrophenyl palmitate **palmitic acid** **p-nitrophenol**

6.3 Measurement of lipase activity

6.3.1 Effect of pH on immobilization and activity

It is well known that the procedure of enzyme immobilization on insoluble supports has a variety of effects on the state of ionization and dissociation of the enzyme and its environment [30]. Immobilization was carried out in different buffer solutions in order to determine a suitable buffer for the immobilization of lipase on silica and clay (Fig 6.1). Taking advantage of the structural characteristics of mesoporous materials, enzyme molecules can be directly immobilized through the interaction with surface silanol groups by hydrogen-bonding [31] or electrostatic forces [32]. The morphologies of mesoporous silicas also play an important role in their immobilization performance for enzymes [33].

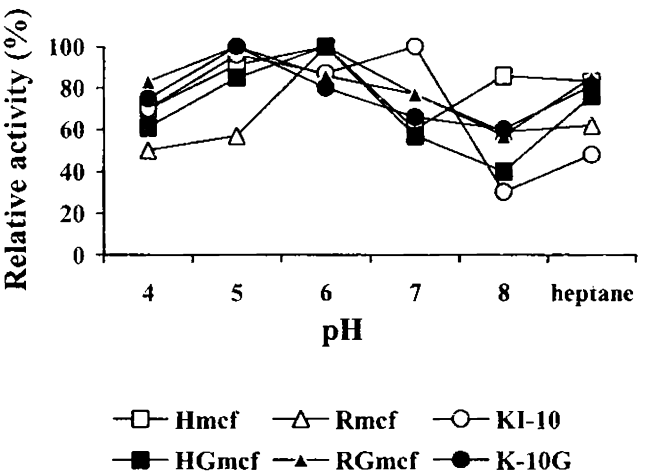


Fig 6.1 Effect of immobilization pH on the activity of the immobilized lipase

The optimum pH for the adsorbed systems H_{MCF}, R_{MCF} and KI-10 were 6 and 7 respectively. The pH at which maximum immobilization took place in the case of covalently bound systems was 5 in the case of HG_{MCF} and K-10G while it was 6 in the case of RG_{MCF}. There was decrease in the immobilization capacity at alkaline side of the pI of the lipase. Heptane was also chosen as immobilization medium for all the immobilizates but it was found that the

enzyme bound was less when compared to the immobilization in pH solutions. In the case of hydrolytic reactions, no marked differences in activity were found between the coupling media used. The pH-buffering capacity of clay mineral may protect the adsorbed enzyme from denaturation [34]. Because the isoelectric point (pI) of CRL is 5 to 6.5, the protein is kept at its most stable conformation at these pH values. Although the active and the stable confirmation and the stable conformation of a protein may not be the same, in this case, the neutral protein surface may enhance the hydrophobic interaction between the lipase and the modified silica surface.

It has been reported that the maximum amount adsorbed of enzyme occurs at a pH near the pI of the protein–substrate complex and not at the pI of the protein [35, 36]. The candidates for the dominant driving forces for the adsorption of CRL lipase include hydrophobic interactions (a kind of Van derWaals attraction), electrostatic repulsion and attraction between the amino acid residues on the surface of CRL and silanol groups on the surface of the silica walls of the mesoporous materials, the intramolecular cohesive attraction and repulsion. Hydrophobic interactions are more dominant near the pI than electrostatic interactions. These hydrophobic interactions originate from (1) the interaction between the nonpolar side chains of the amino acids residues on the surface of CRL and surface siloxane bridges or (2) from the CRL-CRL interactions between the hydrophobic side chains of neighboring CRL molecules adsorbed on the surface of MCF silica and clay [37].

It is supposed that for silicates, which are able to promote cationic exchange through silanol residues, the adsorption mechanism must be produced through the positive charges of protonated lysines of the enzymes. Lipase (from C. Rugosa) has an isoelectric point of 4.6. The charge effects of the support on the shift in pH are not observed if the ionic strength in the bulk is high, persumably because of the compression of the diffuse double layer on the carrier [38, 39].

At pH 5.0, the enzymatic polarity might be weakened, which could enhance the lipase binding onto the hydrophobic surface as observed in the case of HG_{MCF}, RG_{MCF} and K-10G due to the introduction of the spacer arm groups onto the support. The hydrophobic and electrostatic interactions between the enzyme and the support are the important contributions to the adsorption strength for lipase. When the hydrophobic interaction governs the adsorption process, the adsorbed protein amount should not be influenced significantly by the pH changes of the protein solutions for adsorption. However, if electrostatic forces are important for the adsorption, changing the pH value over the isoelectric point of the lipase (about 4.6) should have a large impact on the adsorption [40].

Lipase immobilization on the pure silica and clay occurred by various binding mechanisms, from simple adsorption by van der Waals or ionic forces. Best results were obtained when lipase adsorption was conducted near the enzyme isoeletric point (pI 4.6), due to a favourable charge distribution on the amino acid residues, which prevented a possible change of enzyme conformation. As there is no repulsion between the amino acid residues on the surface of adsorbed lipase molecules near the pI, the lipase molecules are more densely packed on the adsorbent surface. As a result, the amount of adsorption increases near the pI of lipase.

Lipase immobilization in pH range of 5.0 – 6.5 provided relatively high activity values because enzyme conformation, vital 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 [41]. Similar results were obtained by other authors [42, 43] when immobilizing Candida rugosa lipase on poly(acrylonitrile-co-maleic acid) hollow fibre and Mucorjavanicus lipase on SBA-15 mesoporous silica. The immobilized lipase from Bacillus coagulans BTS immobilized on glutaraldehyde activated Nylon-6

by covalent binding was fairly stable in the pH range 7.5-9.5 with optimum pH 7.5 [44]. The immobilized lipase of C. rugosa was stable in the pH range of 5-8 with optimum pH 9 [45].

It has been seen that there can be a good adsorption between enzyme and support at a certain pH even when the demand for opposite charges is not fulfilled (isoelectric point). This indicated that the adsorption is dominated by hydrophobic/hydrophilic interactions rather than electrostatic interactions. The same conclusions were drawn by Deere et al. [46].

pH is one of the most influential parameters altering enzymatic activity in an aqueous medium. Immobilization of enzyme is likely to result in conformational changes of enzyme resulting in a variation of optimum pH. Commonly, there is a decrease of enzyme activity after its immobilization.

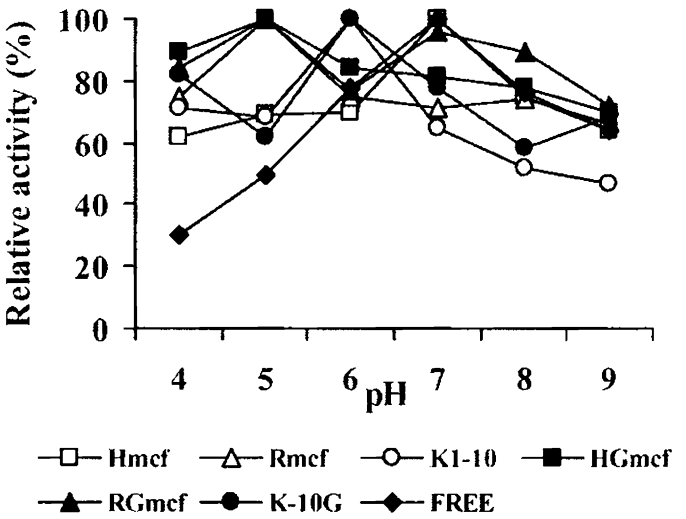


Fig 6.2 Effect of pH on the activity of the free and immobilized lipases

This is attributed to the minor modification in the three-dimensional structure of the enzyme that may lead to the distortion of amino acid residues involved in catalysis, the presence of random immobilization which causes the

analytic approach to the active site of the enzyme hindered, and the limitations imposed by the slow mass transfer of substrate or products/from the active site of the enzyme. Fig. 6.2 illustrates the effect of pH on the activity of the free and the immobilized lipases. The maximum lipase activity is exhibited at pH 7.0 by free as well as H_{MCF} indicating the absence of conformational changes in the enzyme after adsorption. The optimum pH for maximum activity in the case of R_{MCF}, HG_{MCF}, RG_{MCF} and KI-10 (K-10G) was 5 and 6. This behaviour was expected and is common in the lipase immobilization.

Furthermore, the pH profiles of the immobilized lipases are broader than that of the free enzyme, which means that the immobilization methods preserved the enzyme activity over a wider pH range. The free enzyme is inactivated at lower pH values. The covalently bound systems could retain higher activity in both acidic and alkaline pH than the free enzyme. It is believed that the enzymes are adsorbed mainly due to the physical interactions including electrostatic ones and hydrogen bond formation. This does not perturb the secondary and ternary protein structure. The adsorption of lipase is typically determined by electrostatic and hydrophobic interactions (a kind of van der Waals attraction) and the cohesive attraction and repulsion of the amino acids present in the lipase molecule. The shift depends on the method of immobilization as well as the structure and charge of the matrix. In another research, investigated by de Castro and co-workers [47], optimum pH for immobilized lipase on polyvinyl alcohol activated with glutaraldehyde was found to be 8.8. An optimum pH of 7 was reported by Kang and Rhee [48] using C. rugosa lipase immobilized by adsorption on swollen Sephadex for the hydrolysis of olive oil. Recently, Liu et al. [49] used micron-sized magnetic beads for lipase immobilization and the optimum pH of free enzyme during olive oil hydrolysis shifted from 7 to 8 after immobilization. Binding of enzymes to polycationic supports, such as chitosan would result in an acidic shift of the pH optimum [50].

6.3.2 pH stability studies

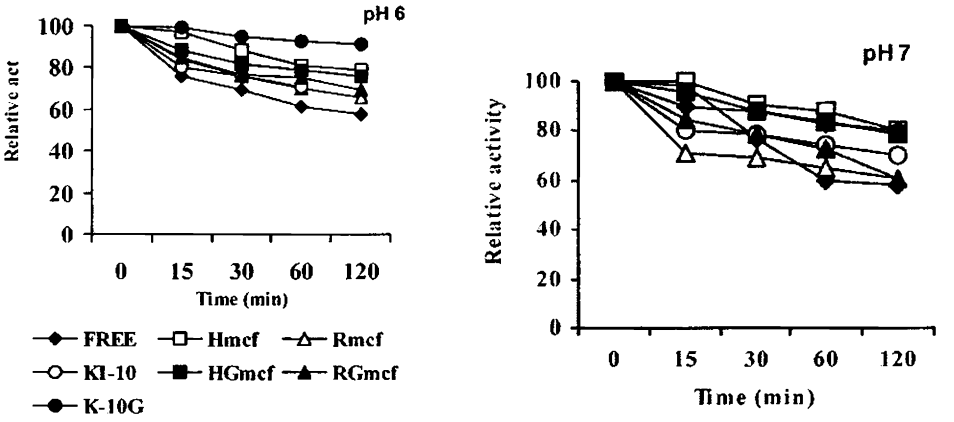


Fig 6.3 Stability of the free lipase and the immobilized systems at pH 6 and 7

Fig 6.3 shows the pH stability of the free and the immobilized lipases at various pH. Free lipase was inactivated when subjected to prolonged incubation time in a buffer. The immobilized systems exhibited higher activity at pH 6. The pH stability of lipase could be enhanced by the immobilization process. It was attributed to the formation of covalent bonds, which limited the transition of enzyme conformation, against the change of pH in the case of covalent bound systems. On the other hand, the results suggested that the immobilized lipases possessed better pH stability than the free one.

6.3.3 Effect of metal ions and chemicals on the activity of free and immobilized lipase

Understanding the role of lipase inhibitors may provide a better perceptive of their mechanism of action [51] and successful identification of potent and specific inhibitors have resulted in their application in certain treatments [52]. The influence of various metal ions on free and immobilized lipases is summarized in Table 6.1. The effects vary according to the metal ions and to their concentration. Among the metal ions used in this study, Mn (II) metal ion increase the activity of the enzyme by almost 126% respectively. While metal ions Co (II) and Ca (II)) caused a decrease of enzyme activity by

about 20% and 90% . Hg (II) completely deactivated the enzyme activity. This is likely due to the proximity of the SH group to the catalytic and interfacial binding site but spatially remote from the catalytic site, this may have induced the marked loss of activity [53, 54].

Table 6.1 Stability of the free and the immobilized lipases in the presence of salts and detergents[a]

Metal cations/Chemicals	Sample						
	Free enzyme	H_{MCF}	R_{MCF}	KI-10	HG_{MCF}	RG_{MCF}	K-10G
	Residual activity (%)[b]						
Control	100	100	100	100	100	100	100
EDTA	123	280	76	168	217	169	215
$MnCl_2$	126	172	122	132	180	118	153
$HgCl_2$	0	0	0	0	0	0	0
$CoCl_2$	84	165	72	158	182	138	134
Triton X-100	129	297	157	204	213	188	205
$MgCl_2$	109	115	102	107	122	119	114
$CaCl_2$	8	84	94	82	96	88	86

a The enzyme was incubated in the presence of various compounds at 28°C for 1h. Control, without the addition of any substance.

b Residual activity (%) is the activity obtained after incubating the enzyme with various compounds at 25°C for 30 min compared to control.

The catalytic triad of lipases has been recognized to consist of Ser, His, and Glu or Asp [55, 56], thus the bulky Hg^{2+} group might cause steric interference to the approach of the substrate to the active site. Mg^{2+} had no appreciable effect on lipase activity which was similar to the findings of Aryee et al. [57]. EDTA (1mM) and Mn^{2+}(1mM) activated the enzyme by 23% and 26%, respectively similar to the results of Lima et al. [58].

It was observed that the inhibitory effect of these ions was less pronounced with the immobilized enzyme. This may be due to the protection of

the immobilized enzyme by the carrier. This protection may result from the structural changes in the enzyme molecule introduced by the applied immobilization procedure and consequently, lower accessibility of these inhibiting ions to the active site of the enzyme. Similar results have been reported for other immobilized enzymes [59, 60, 61]. Generally, the explanation for these various effects lies in the alteration of the enzyme conformation. As widely reported in the literature [62, 63], enzymes can be modulated by interaction of metal ions with amino acid residues involved in their active sites. Such interactions can either increase (positive modulation) [60] or decrease (negative modulation) of the enzyme's catalytic activity [64]. Surfactants possess the common property of lowering the surface tension when added in small amounts to water, and this could affect enzyme catalysis. In this study, addition of 1% Triton X-100 activated lipase activity by 29%. An inhibition effect was observed with Triton X-100 in the results of Aryee at al. and Lima et al. [57, 58].

6.3.4 Effect of organic solvents on activity and stability of lipase

The stability in organic solvents is an important characteristic of lipases. It can determine whether the enzyme can be used to catalyze synthetic reactions and also to predict which solvent would be better to perform the reaction. The selection of organic solvent for this study was based on their degree of hydrophobicity (or polarity), denoted by their log *P* values [65]. The lower the hydrophobicity of the solvent, the greater its affinity to water and the higher the likelihood of the solvent stripping off the essential water molecules that surrounds the enzyme. Conversely, increasing hydrophobicity and decreasing polarity increases the formation of multiple hydrogen bonds, and exponentially increases the rate of lipase-catalyzed reactions, and resistance to denaturation [66].

The organic solvent with log $P < 2$ are unsuitable for enzymatic reactions [67]. Thus, water-miscible organic solvents are "toxic" to the enzymes and exert great

deactivation effect on the enzymes. In our study a lower concentration (10%) of the water-miscible organic solvents were employed.

Table 6.2 Stability of free and immobilized lipases in organic solvents

Solvents	Sample							
	log P[a]	Free enzyme	H_{MCF}	R_{MCF}	KI-10	HG_{MCF}	RG_{MCF}	K-10G
	Residual activity (%)[b]							
Control[c]		100	100	100	100	100	100	100
Acetone	-0.23	43	62	58	63	177	112	135
Isopropanol	-0.28	93	251	100	105	176	84	157
DMSO	-1.35	115	197	86	87	120	117	117
Methanol	-0.76	92	212	147	19	116	100	105
n-butanol	0.8	106	175	60	89	154	64	88
Ethanol	-0.24	48	61	55	58	66	62	65
n-hexane	3.5	200	274	126	142	221	133	157
n-heptane	4.0	150	265	85	111	193	90	152
Benzene	2.0	215	109	99	104	120	105	113
Toluene	2.5	195	165	132	163	147	188	224

a *log P value defined as the partition coefficient of any organic solvent between water and 1-octanol.*

b *Residual activity (%) is the activity obtained after incubating the enzyme with various organic solvent as compared to control.*

c *Control without any organic solvent, taken as 100%.*

There was almost ≥ 90 % retention in activity in the case of isopropanol, methanol and n-butanol while in acetone (log P =−0.23), there was only 43% remaining activity after 30 min incubation (Table 6.2). In early trials with acetone as precipitant, a marked loss of activity was consistently observed and this has been attributed to the ability of acetone to form stronger hydrogen bonds and its subsequent contribution to augment loss of activity. However, the immobilized enzymes showed much activation after incubation with the water immiscible organic solvents except in the case of acetone and ethanol. There was an inhibition in the case of adsorbed systems while an activation in the case of covalently bound systems. Most of the activation effects were seen in the

case of H_{MCF} and HG_{MCF}. It has been suggested that this inhibitory effect results from the direct contact of organic solvents with enzyme which causes the denaturation and inactivation of enzyme or may be due to the stripping off the essential water from enzyme surface.

In contrast, the enzyme was quite stable and active in water immiscible solvents with 215, 195, 200 and 150 % activities after 30 min of incubation in benzene, toluene, hexane and heptane respectively (Table 6.2). In the case of immobilized enzymes all the systems except H_{MCF} showed less activation in water miscible organic solvents. The H_{MCF} system could maintain 274% and 265% activity after incubation with hexane and heptane. This was similar to the findings made by Sztajer et al. [68] and Lima et al. [58]. 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, keeping a flexible conformation and therefore increasing the activity [69]. An activity enhancement was demonstrated when F. Oxysporum f. sp. vasinfectum lipase was incubated with n-hexane [70]. On the other hand, lipase from Bacillus sp was inhibited in the presence of n-hexane [71]. In studies on the use of lipase for transesterification and synthesis of esters, the reactions have previously been carried out in media containing water-immiscible organic solvents and a small amount of water, due to the fact that enzymes are less susceptible to denaturation in such systems. The effectiveness of enzyme catalysis in organic solvents improves with increasing log P value, but only up to a point [72] since the nature of the solvent, and its structure have been shown to greatly influence solvent effect in non-aqueous milieu catalysis.

6.3.5 Effect of temperature on the activity of immobilized enzyme

The effect of temperature on the activity of free and immobilized lipases for p-NPP hydrolysis at pH 7.0 in the temperature range of 25–60°C is shown in Fig. 6.4. The optimum reaction temperature of the free enzyme was at 35°C,and that of the immobilized lipases H_{MCF} and KI-10 were heightened to 50°C while that of R_{MCF} rose to 45°C. In the case of covalently bound systems, the optimum temperature was

increased to 55°C in the case of HG_{MCF} and K-10G while it was 50°C for RG_{MCF}. This is consistent with previous findings, indicating that enzymes immobilized on carriers by covalent bonding could lead to higher stability [73].

In other words, the immobilized lipases hold much more excellent heat resistance than that of the free enzyme. At 70°C, the free enzyme lost all of the activity, whereas immobilized enzyme retained 40% of the initial activity. Comparing with the free lipase, the relative activity of the immobilized lipases expressed a slow decrease, however, the relative activity of the free enzyme was decreased speedy so that there is only 20% of its relative activity at 70°C, which is probably due to its thermal denaturation. The increasing temperature would be suitable for the adjustment of the conformational integrity of the enzyme immobilized by covalent binding to the carrier materials [74], so the immobilized lipases still hold over 60% of their relative activities. In fact, upon immobilization, the interaction between the enzyme and the support can provide a more rigid external backbone for lipase molecules, the effect of higher temperatures in breaking the interactions that are responsible for the proper globular, catalytically active structure which becomes less notorious or a low restriction in the diffusion of the substrate at high temperature.

The effect of temperature in hydrophobic interaction may also be important at higher temperatures during the unfolding process, the proteins expose buried hydrophobic amino acid residues on the surface. Thus the contact area between the protein and the hydrophobic groups of the matrix should increase, resulting in an increase in the hydrophobic interaction of the proteins for the adsorbent at higher temperature.

A similar shift in optimum temperature from 37°C (free lipase) to 55°C was obtained when Candida rugosa lipase was immobilized by covalent binding on hybrid matrix of polysiloxane-polyvinyl alcohol chemically modified with activating agents as glutaaldehyde, sodium metaperiodate and cabonyldiimidazole [75]. Yang et al.

reported big shift in optimum reaction temperature from 37°C for the free lipase to 50°C for immobilized lipase [76]. The optimum temperature of free lipase in the hydrolysis of olive oil shifted from 37°C to 50°C when it was immobilized onto micron-size magnetic beads [77].

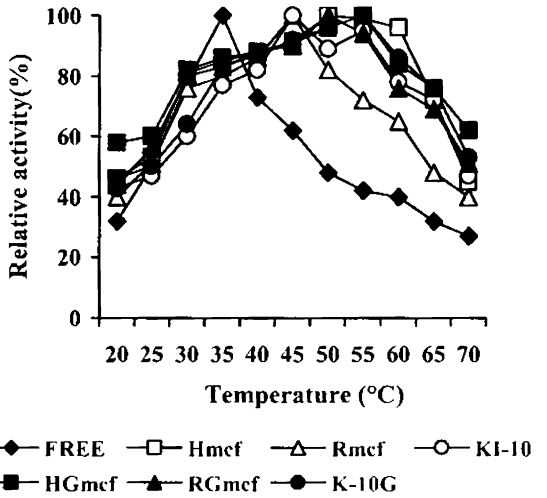


Fig 6.4 Effect of temperature on the activity of free and immobilized lipases.

A specific site, where the unfolding process begins during inactivation, characterizes an enzyme. Immobilization protects this unfolding nucleus only in one fraction of enzyme molecule, which could block the native unfolding pathway, resulting in enhanced stability. The second fraction with no protection behaved like a soluble enzyme.

6.3.6 Thermal stability

From a commercial point of view, the thermal stability of enzymes is one of the most important features for the application of the biocatalyst. Fig. 6.5 shows the thermal stabilities of the free and immobilized lipases. Free and immobilized enzymes were incubated for 2 h at 50°C and 55°C.

According to Klibanov [78], two types of enzyme instability, such as thermal, should be distinguished. One of them is a heat-induced, cooperative unfolding usually almost instantaneous and reversible, of enzyme molecules.

The other is a time-dependent, gradual, irreversible loss of enzymatic activity on exposure to high temperatures. The first kind was evaluated in the optimum temperature assays, and the second one was evaluated in the experiments of thermal stability. It is well known that enzymes in solution are not stable and their activities would also decrease gradually during use. The increase in optimum temperature was caused by the changing physical and chemical properties of the enzyme. The covalent bond formation, via amino groups or cysteine groups of the immobilized lipase might also reduce the conformational flexibility and result in higher activation energy for the molecule to reorganize the proper conformation for binding to substrate [79].

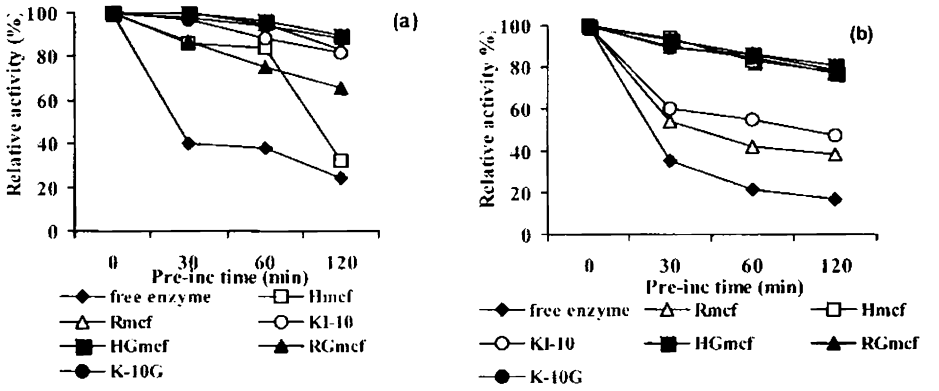


Fig 6.5 Thermal stabilities free lipase and immobilized lipases at **(a)** 50°C **(b)** 55°C

All the immobilized systems were more thermally stable than the free enzyme and the highest thermal stability was achieved with H_{MCF}. It can be seen that the free lipase losses its initial activity within around 120 min, at 50°C and 55°C while the adsorbed systems KI-10 and H_{MCF} hold over 80% their initial activities after 2 h heat treatment. R_{MCF} could retain only 30 % activity after the same thermal treatment probably because of the rapid denaturation of the enzyme molecules as they are immobilized only on the external surface rather than inside the pores in the case of H_{MCF}. On the other hand, the covalently bound systems could retain almost 90% activity after

120 min heat treatment while RG_{MCF} could retain only 66% activity. Unexpectedly higher thermal stability (residual activity in 2 h: 88%) was observed for HG_{MCF} and K-10G, which is inconsistent with other's conclusion that the enhanced hydrophobic interaction can increase the thermal stability of lipase [74]. The chemical interaction between the amino groups and aldehyde groups provide additional stability to the covalently immobilized systems. The adsorbed enzyme also exhibits a reduction in mobility but as time proceeds this reduction in mobility is eliminated due to the weak nature of the enzyme support bond and therefore the protein unfolds leading to a loss of activity. The covalently bound lipase onto HG_{MCF} and K-10G present an improved thermal stability and thus may be attractive biocatalysts for industrial purposes. The improved stability of immobilized enzymes may be related to the prevention of autolysis [80].

6. 3.7 Reuse stability of the immobilized lipase

For immobilized enzyme, one of the most important advantages is reuse stability, which can effectively reduce the cost in industry applications. Fig. 6.6 shows the effect of repeated use on the activities of these immobilized enzymes. The results may be explained from the support structure and surface property. Free lipase lost almost all it activity after six cycles. The relative activity of H_{MCF} decreased to 75% after 6 uses while that of the HG_{MCF} could retain 85% activity. This may be due to the greater stabilization of the hydrophobic bonds formed between the enzyme and the support with greater surface area and pore diameter compared to the other supports. The larger pore size could effectively protect the protein against conformation changes to less active forms as well as maintain the enzyme inside the material with lesser leaching than the other materials. Concerning the physical immobilization, the H_{MCF} material revealed the best residual activity after five reaction cycles.

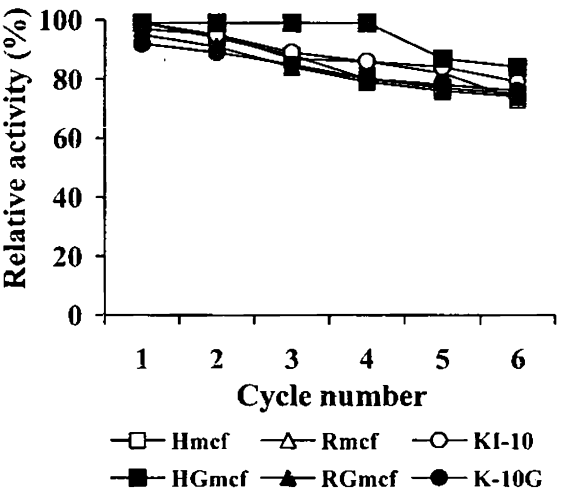


Fig 6.6 Effect of repeated use capability on the activity of free and immobilized lipase

In the case of R_{MCF} and KI-10, greater retention in activity was observed in the case of KI-10 due to the structural characteristics of clays. As the enzyme was adsorbed only on the external surface in the case of RG_{MCF} the enzyme might have been proned to more leaching effect during the consecutive cycles leading to a decrease in activity. These results were due to the inactivation of the enzyme caused by the denaturation of protein and the leakage of protein from the supports upon use. When the covalently bound counterparts are compared both the systems retain less activity compared to the adsorbed ones. These differences may be due to the denaturation of the enzyme as a result of the lesser number of bonds formed between the support and the enzyme which could not give a better stability to these supports. In comparison the activity retention of HG_{MCF} was higher than all the other supports. Less desorption or leaching of the bound enzyme in case of HG_{MCF} might be due to very tight binding of the lipase on the hydrophobic supports. The pore diameter which is about three times higher than the molecular diameter of the enzyme provided both lowered diffusion limitation and also increased the specific surface area for enzyme immobilization and hydrolysis reaction in the case of H_{MCF} and enables free access of substrates to their

active centers. After 10 reuses, the residual activity of immobilized Candida rugosa lipase is 55% for the chitosan-modified nanofiber membrane and 60% for the gelatin-modified nanofiber membrane in the work done by Ye et al. [22]. The immobilized porcine pancreatic lipase in SBA-15 retained only 40 % activity after five reuses [81] and immobilized PPL on nanoscale functionalised silica dioxide still retained 56% of their original activities after the 15 reuses [82].

6.3.8 Storage stability

To determine the change in activity of the immobilized lipase with time, free and immobilized lipase was stored at 4°C for a period of 40 days and the result is shown in Fig. 6.7. In the free form, it has a short life and therefore its use is restricted. After immobilization, the life of enzyme improved and therefore it can be kept for long term usage.

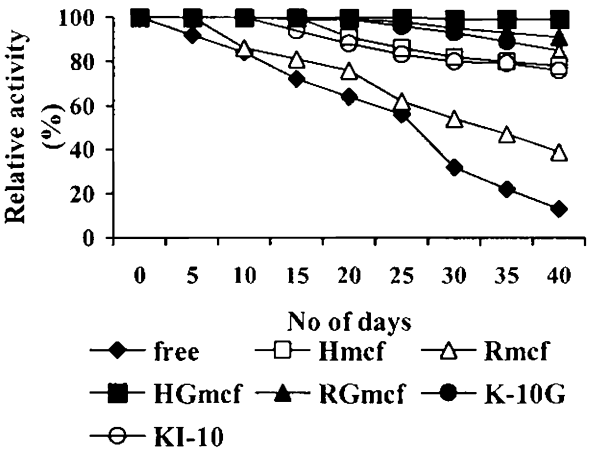


Fig 6.7 Effect of storage stability on the activity of free and immobilized lipase.

It can be seen from the results that the free enzyme lost more than 70% of its activity after 25 days storage. The decrease in activity of the free lipase might be due to its susceptible autolysis during the storage time. Under the same storage conditions, the activity of the immobilised lipase decreased more slowly than that

of the free lipase. The activity of the covalently bound systems did not change significantly even after 40 days and they could maintain more than 85% activity compared to the adsorbed ones, H_{MCF} and KI-10 which could maintain more than 75 % activity. There was gradual reduction in activity in the case of R_{MCF} after 20 days storage as the enzyme get easily denatured and also due to the leakage of enzyme in aqueous media. The extended stability of the covalently bound supports could be attributed to the prevention of structural denaturation as a result of the covalent bonding of lipase molecules onto the modified supports. The difference observed in the case of the six immobilized supports could be perhaps be explained by the different average numbers of links between the enzyme and the support [83]. These links contribute to the rigidification of the proteins three-dimensional structure and thus to the stabilisation of the enzyme. Thus, the immobilised lipase exhibits a higher stability than the free counterpart.

Candida rugosa lipase was covalently immobilized onto silica gel: via glutaraldehyde (L_{GAL}) and via hydrophobic spacer arm (1,6 diamino hexane) (L_{SA}) which preserved about 70% and 60% of their initial activities at 5°C while only 54% and 36% of their initial activities at 25°C respectively [84]. Immobilized Thermomyces lanuginosus lipase onto polyglutaraldehyde–activatedpoly(styrene–divinylbenzene) (STY–DVB), retained 80 and 75% of its initial activity in 60 and 90 days storage at 4°C [85].

Part II

Hydrolysis in Organic media

Contents

6.4 Introduction

As biocatalyst, enzymes have been always studied and used in aqueous medium. However, the discovery that enzymes possess catalytic activity in media with low water content has made it possible to run enzymatic reactions in nonconventional media such as organic solvent, supercritical fluid or gas phase [86, 87]. Lipases are surface-active enzymes because binding to emulsified triglyceride substrates markedly increases their hydrolytic activity compared with their activity toward dissolved substrates [55]. One challenge for the use of enzymes in organic media is the decreased catalytic activity, which are in general orders of magnitude lower compared to aqueous solution. Several methods to overcome this disadvantage have been investigated. More recently, the reaction by itself was investigated as a tool to study enzymatic properties. However, the activity measurements used preparative reactions which need sophisticated extraction and separation procedures such as high-performance liquid chromatography or gas liquid chromatography, which are not always available in a biochemistry laboratory. Therefore, there is need for a simpler method for the determination of lipase activity in organic media.

Another parameter which influences the activity of enzyme is the amount of water present in the organic solvent. A minimum amount of water on enzyme surface is necessary to retain its catalytic activity. The necessity of water has been explained in terms of introducing flexibility to the protein backbone and the screening of charged groups at the enzyme surface. This was confirmed by the finding that enzyme activity

in organic solvents does not depend on the total concentration of water in the reaction system, but on the number of water molecules on the enzyme surface. The use of immobilized lipases in organic media, rather than aqueous media, has several advantages, such as the favorable shift in thermodynamic equilibrium of the product over the hydrolysis reaction, increased solubility of non-polar substrates, elimination of side reactions, easy recovery of enzyme and product, and increased enzyme thermostability [88, 89, 90].

The reaction catalyzed in organic media (esterification or transesterification) was usually different from the one in aqueous media (hydrolysis), which makes these comparisons difficult because the transfer reaction is carried out in a organic solvent. In organic media, lipases were mostly used for preparative purposes in organic synthesis. The discovery that enzymes possess catalytic activity in media with low water content has made it possible to run enzymatic reactions in mixtures containing an organic solvent. Among the different systems potentially useful to run reactions in nonconventional media, the direct dispersion of a solid biocatalyst in an organic solvent is the most widely used [91]. Direct comparisons of enzyme activity in aqueous and organic media are scarce in the literature [92].

Lipase from Candida rugosa was covalently immobilized onto (PANCMA) membrane surface on which the carboxyl groups were activated with 1-ethyl-3-(dimethyl-aminopropyl) carbodiimidehydrochloride/N-hydroxyl succinimide as coupling agent. Using the hydrolysis reaction of p-nitrophenyl palmitate in aqueous and organic media, and it was found that enzyme activity in n-heptane increased due to immobilization and also that the residual activity was higher in the organic media compared to aqueous media [93]. The protein content and the rates of hydrolysis of p-nitrophenyl palmitate (pNPP) in water (soluble enzyme and emulsified substrate) and in heptane (soluble substrate and insoluble enzyme) were measured for thirty-two commercial lipase preparations in the work done by G. Pencreac'h, et al. [94]. It was found that there was no direct correlation between activities in water and in heptane as assayed with the same hydrolytic reaction. The reaction rate in n-heptane was found to be

only 0.16% of that in water in the p-NPP hydrolysis in n-heptane by a lipase preparation from P seudomonas Cepacia [29].

A simple colorimetric assay for the determination of lipase activity in organic media using hydrolysis of p-nitrophenyl fatty acids esters were adopted in this study. The main advantage of this assay is the use of the same hydrolytic reaction in both aqueous and organic media, which makes the activity comparison much easier. A relatively hydrophobic solvent (n-heptane) (i.e., with a high value of log P) was selected to maintain the maximum amount of water in the enzyme solid phase and to obtain a good solubilization of the substrate. Because both the solvent (not dried before use) and the biocatalyst contained water, no addition was initially made. The optical absorption of pNP in organic solvent is very low compared to that in alkaline solutions. Therefore, it cannot be used for an accurate and sensitive determination of reaction rates in organic media. To increase sensitivity, an extraction step was introduced in the experimental procedure to get the pNP in an alkaline aqueous phase.

The properties of the free and immobilized lipase preparations were compared in aqueous and organic media respectively. Factors affecting lipase activity were investigated, such as water content, temperature and substrate concentration.

6.4.1 Effect of various catalysts in organic media

The relative activities of all the immobilized lipases were found to be greater in aqueous media then in organic media (Table 6.3). It may come from the fact that, in organic media, the catalysis is heterogeneous (the lipase in insoluble in the solvent). Therefore, diffusional limitations may reduce the activity for highly active lipase preparations yielding to a narrow range of activity. The amount of available water, which is a substrate of the reaction, could be too low to allow good reaction rates. The structural flexibility of the enzyme molecules is lower in organic solvent than in water, which may reduce the activity.

Table 6.3 Activity of the immobilized biocatalysts in aqueous and organic media

Carrier	Relative activity (%) (In aqueous media)	Relative activity (%) (In organic media)
H_{MCF}	89	79
R_{MCF}	75	63
KI-10	78	69
HG_{MCF}	96	88
RG_{MCF}	83	70
K-10G	97	86

6.4.2 Effect of temperature

Effect of temperature on the activity of the free and immobilized lipase preparations was studied in the temperature range of 20–55°C. The results are shown in Fig. 6.8. This point is interesting, because enzyme stability is increased at a low water content which may affect the profile of activity against temperature [95].

The enzyme activity in heptane increased with the temperature increasing from 20 to 37°C and then kept almost constant from 37 to 55°C. For the immobilized lipase preparation in both medium there was no change in optimum temperature but the immobilized preparations could retain higher activity with increase in temperature from 20 to 45°C and then kept constant at higher temperature in organic medium. These results demonstrated approximately that the immobilized enzyme preparations showed their catalytic activities at higher reaction temperature in both aqueous and organic media compared to the free ones. It could be attributed to either the creation of conformation limitations on the enzyme movements as a result of covalent bond formation between the enzyme and the matrix or a low restriction for the diffusion of substrate at higher temperature. The activity of both the free and immobilized lipase preparations kept almost constant at higher temperature in the organic medium than in the aqueous medium could be due to the low water content which might affect the profile of activity against temperature.

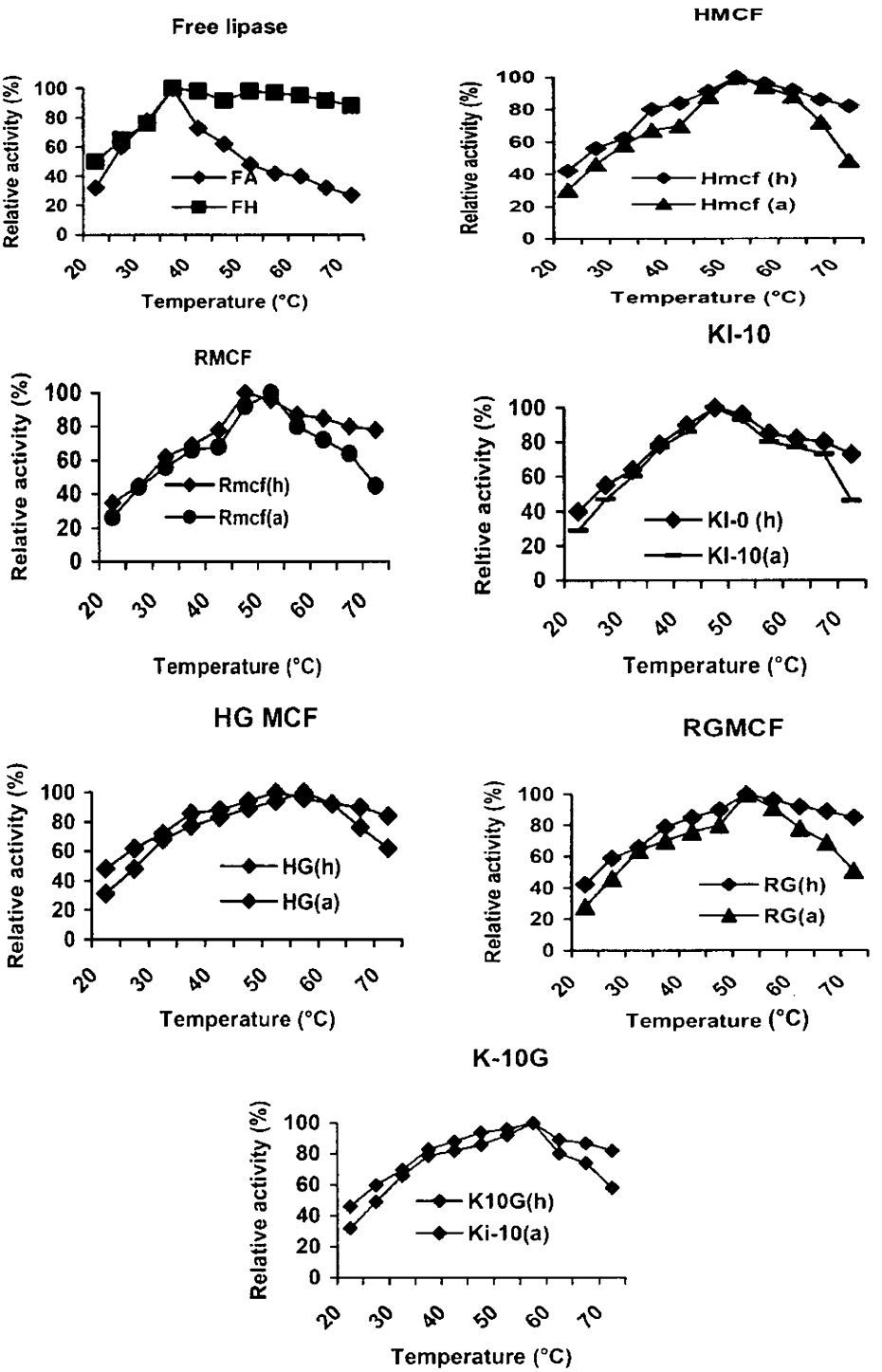


Fig 6.8 Effect of temperature on the activity of the free and immobilized lipases in organic medium.a-aqueous medium, h-heptane

6.4.3 Effect of water addition

Water plays a major role of "molecular lubricant" in enzyme resulting in conformational flexibility of enzyme, which leads to enhanced activity in non-aqueous media. A small amount of water is absolutely essential to obtain a sufficient enzyme conformational flexibility for enzyme activity by forming multiple hydrogen bonds with enzyme molecule in organic media. At higher water content, aggregation of solid particles induces diffusional limitations of substrate and a reduction in reaction rate is usually observed.

Effect of the amount of water added on the lipase activity is shown in Fig. 6.9. With no addition of water, a significant hydrolysis rate was observed but it was lower when compared to other reports. Thus, the amount of water in the system was enough for high enzyme activity. The relatively low increase observed in this study may have been related to the high amount of water already present in the lipase powder. Commercial lipase from Candida rugosa was used without treatment.

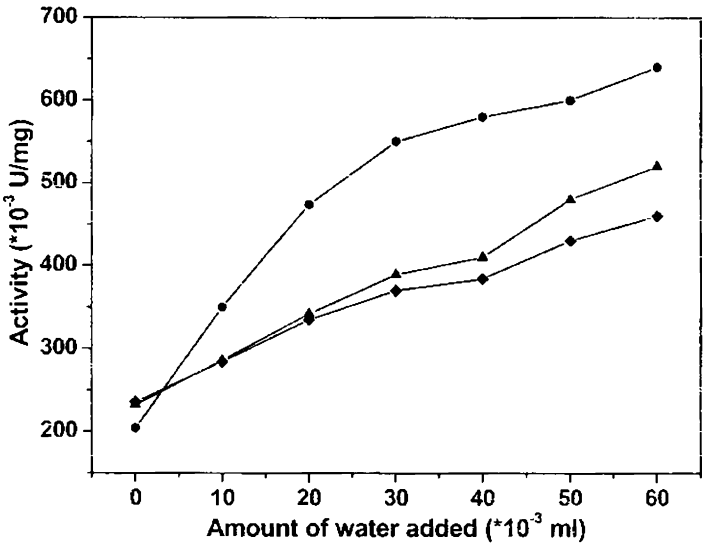


Fig 6.9 Effect of amount of water added in heptane on the activity of the free and immobilized lipases. (▲) H_{MCF} (●) free lipase (♦) HG_{MCF}

With no addition of water, the rate of *p*-NPP hydrolysis was 0.143μmol/min mg for the free lipase preparation, 0.168 μmol/min mg and 0.182μmol/min mg for the H_{MCF} and HG_{MCF} preparations. From 25μl, two liquid phases were clearly seen, with the enzyme being solubilized in the water phase. Thus, the solid-liquid system became a biphasic liquid-liquid system. The increase of the hydrolysis rate in this region could be attributed to this physical change. Similar observations have been reported by others [96].

In addition to this physical effect, water may affect the reaction rate by its direct participation as a reactant in the hydrolysis reaction. The increment of the amount of added water from 0.01 to 0.05ml led to an increase in the activity of both the free and immobilized lipase preparations. This activity increase could be attributed to the change of lipase conformation, which was more flexible and easy to access substrate for reaction.

It was observed that the increase of activity was not at the same rate for the free and immobilized lipase preparations. The increase of activity was faster for the free lipase preparation than that for the immobilized lipase preparation with the increment of the amount of water added. This could be explained by the creation of conformation limitation on the enzyme moments as a result of covalent bond formation between the enzyme and the matrix.

6.4.4 Reusability studies

The reusability of immobilized enzymes is vital for cost-effective use of an enzyme. To investigate the operational stability, the immobilized lipases were washed with n-heptane after any run and reintroduced into a fresh organic media, this being repeated up to 10 cycles. Table 6.4 shows the activity retained after 10 reuses in aqueous and organic media. It was found that the immobilized enzyme retained higher activity in organic media compared to the aqueous media. After 10 reuses HG_{MCF} could retain the highest activity (80%) compared to the other adsorbed and covalently bound systems. This may be due to the

hydrophobic activation of the lipase molecule in solvents with low polarity (n-heptane) compared to water. 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 6.4 Reusability characteristics of free and immobilized lipases

Carrier	Relative activity (%) (In aqueous media) after 10 reuses	Relative activity (%) (In organic media) after 10 reuses
H_{MCF}	48	55
R_{MCF}	30	38
KI-10	37	42
HG_{MCF}	75	80
RG_{MCF}	56	63
K-10G	66	71

The open conformation of the immobilized lipases could be retained in the organic media with low water content which may be the reason for the higher retention of activity in the organic medium compared to the aqueous medium. The lower activity of the immobilized CRL in R_{MCF} is due to the stripping of enzyme after separation and washing steps employed during the recycling reaction because it is only weakly attached to the silica surface. The immobilized lipases from Candia rugosa on PANCMA could retain 62% activity in aqueous media and 67% in organic media after 10 reuses in the work done by Ye et al. [41].

6.4.5 Storage stability

The effect of storage conditions on the activity of the immobilized enzyme is an important aspect to ensure that a long shelf life is possible. The free (0.1M, pH 7.0 phosphate buffer) and immobilized lipase were stored at 4°C and the activity measurements were carried out in organic medium after a period of 40 days (Table 6.5). Enzymes are not stable during storage in solutions and their activities decreased gradually by the time. After 40 days, there was a significant

decrease in the activity of the immobilized and free enzyme in organic medium compared to the stability retained in aqueous medium. But immobilized enzyme provided a distinctive advantage in stability over free enzyme at longer durations. The free enzyme lost all of its initial activity within 40 days in organic media while the adsorbed systems (H_{MCF} and KI-10) could retain more than 60% activity while only 30 % activity in the case of R_{MCF}. The activity retained after 40 days storage in aqueous medium was more than 75% in the case of H_{MCF} and KI-10. The lower stability observed in the organic medium is due to the low amount of water present which was not sufficient enough to maintain the flexibility and also due to the inactivation of some of the lipase molecules. The covalently bound systems could retain almost 90% activity in aqueous medium and 80% activity in organic medium. The experimental results indicate that the immobilization definitely holds the enzyme in a stable position in comparison to the free counterpart.

Table 6.5 Storage stability characteristics of free and immobilized carriers

Catalyst	Storage stability (4°C) for 40 days Relative activity (%)
Free CRL	8
H_{MCF}	66
R_{MCF}	32
KI-10	63
HG_{MCF}	100
RG_{MCF}	86
K-10G	78

As previously reported, the immobilization of enzymes via glutaraldehyde coupling on the supports resulted in a significant storage stability compared to the free counterpart [97]. The enhanced storage condition of covalently bound systems in both media can be attributed to higher

conformational stability of enzyme due to binding with the hydrophobic groups on the surface of clay and silica.

6.4.6 Kinetic parameters of free and immobilized lipase in aqueous and organic media

Kinetic constants for the hydrolytic activity of the free and immobilized lipase preparations were assayed at substrate concentration from 0.2 to 1.5mM in the aqueous medium, and from 1 to 50mM in heptane. The kinetic constants K_m and V_{max} were calculated from double reciprocal plots. The lineweaver plots of free and immobilized lipases in aqueous and organic media are shown in Fig 6.10 and Fig 6.11. The kinetic parameters obtained in both the media are compared in Table 6.6. Comparison of the K_m value for a given free and immobilized enzyme could provide information about interaction between enzyme and its support [98].

Table 6.6 Kinetic parameters of the free and the immobilized lipases

Sample	Medium	V_{max} (U/mg)	K_m (mM)
Free lipase	Aqueous	44.4	0.174
	Organic	8.68	0.272
H_{MCF}	Aqueous	36.2	0.181
	Organic	3.50	0.289
R_{MCF}	Aqueous	26.2	0.301
	Organic	4.33	0.279
KI-10	Aqueous	35.2	0.259
	Organic	0.259	0.284
HG_{MCF}	Aqueous	27	0.245
	Organic	3.27	0.332
RG_{MCF}	Aqueous	20.8	0.375
	Organic	3.6	0.309
K-10G	Aqueous	25.3	0.273
	Organic	3.93	0.293

The K_m value for the immobilized lipases was higher than that for the free lipase. An increase in K_m when an enzyme had been immobilized, indicated that the immobilized enzyme had an apparent lower affinity for its substrate than that of free enzyme does, which might be caused by the steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding, or diffusional resistance to solute transport near the particles of the support. The result showed that the affinity of immobilized lipase for pNPP was smaller than that of the free lipase, namely, the activity of immobilized lipase was lower than that of the free lipase. The V_{max} value of the immobilized lipases was smaller than that of the free enzyme.

From Table 6.6, it can be seen that the K_m values of H_{MCF} is lower than that of HG_{MCF}. This result is largely attributed to the presence of the large specific surface area of the mesocellular silica foams, which can create a more favorable interface for the mass transfer of substrate or product to or from the active site of the enzyme. Similarly the K_m value of the HG_{MCF} is lower than that of RG_{MCF} and K-10G. This is due to the easier conformational transition to form the substrate-enzyme complex.

The greater hydrophobicity benefit the adsorption of hydrophobic substrate molecules on the enzyme surface close to the active site of the enzyme and hence influence the kinetic parameter, K_m. K_m value for the immobilized lipase by covalent binding is higher than that by adsorption which shows the easiness with which the enzyme-substrate complex is formed in the case of adsorbed systems. V_{max} values of the immobilized lipases by adsorption are higher than that by covalent binding.

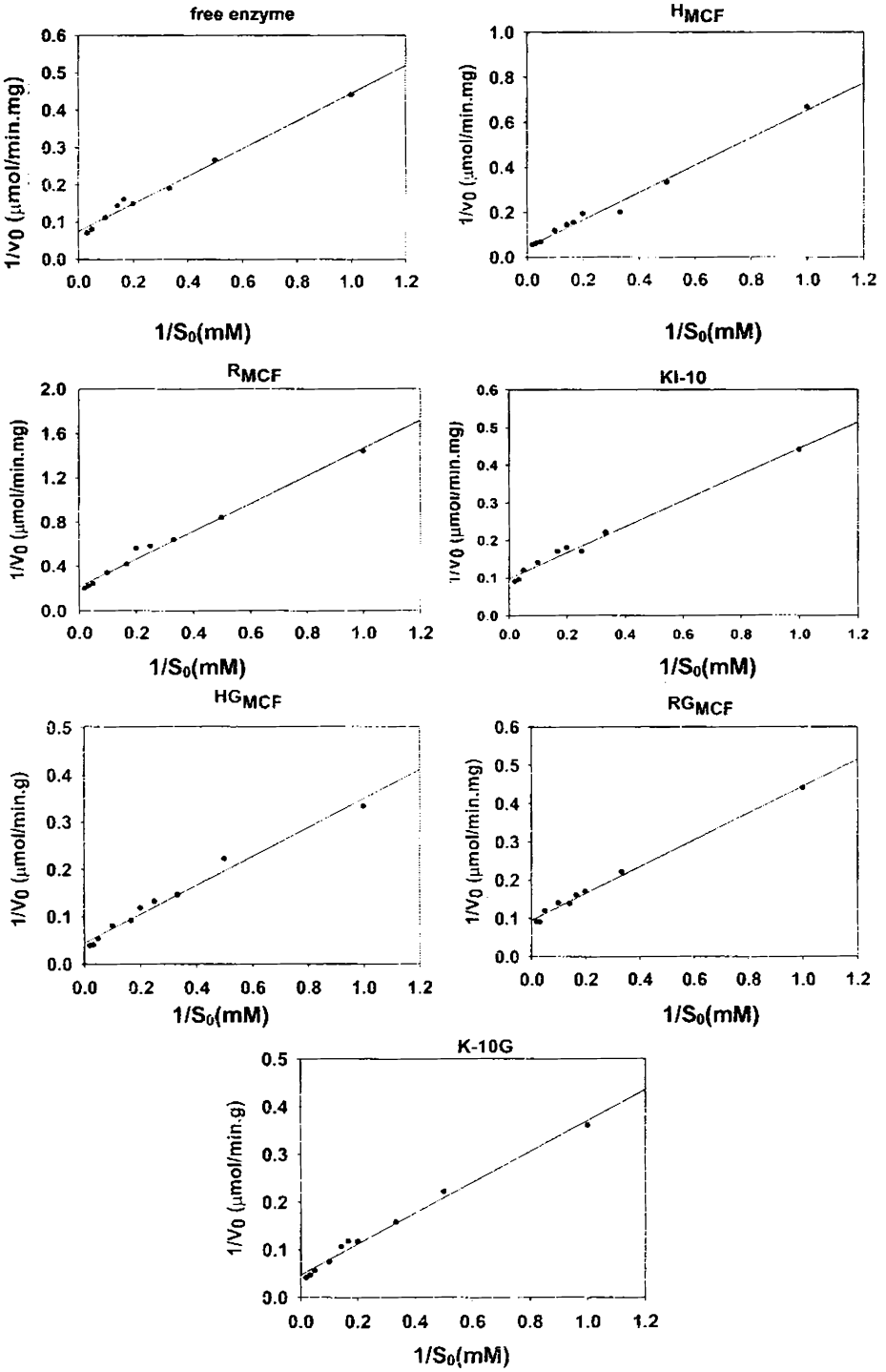


Fig 6.10 Lineweaver–Burk plots of the free and immobilized lipases in organic medium

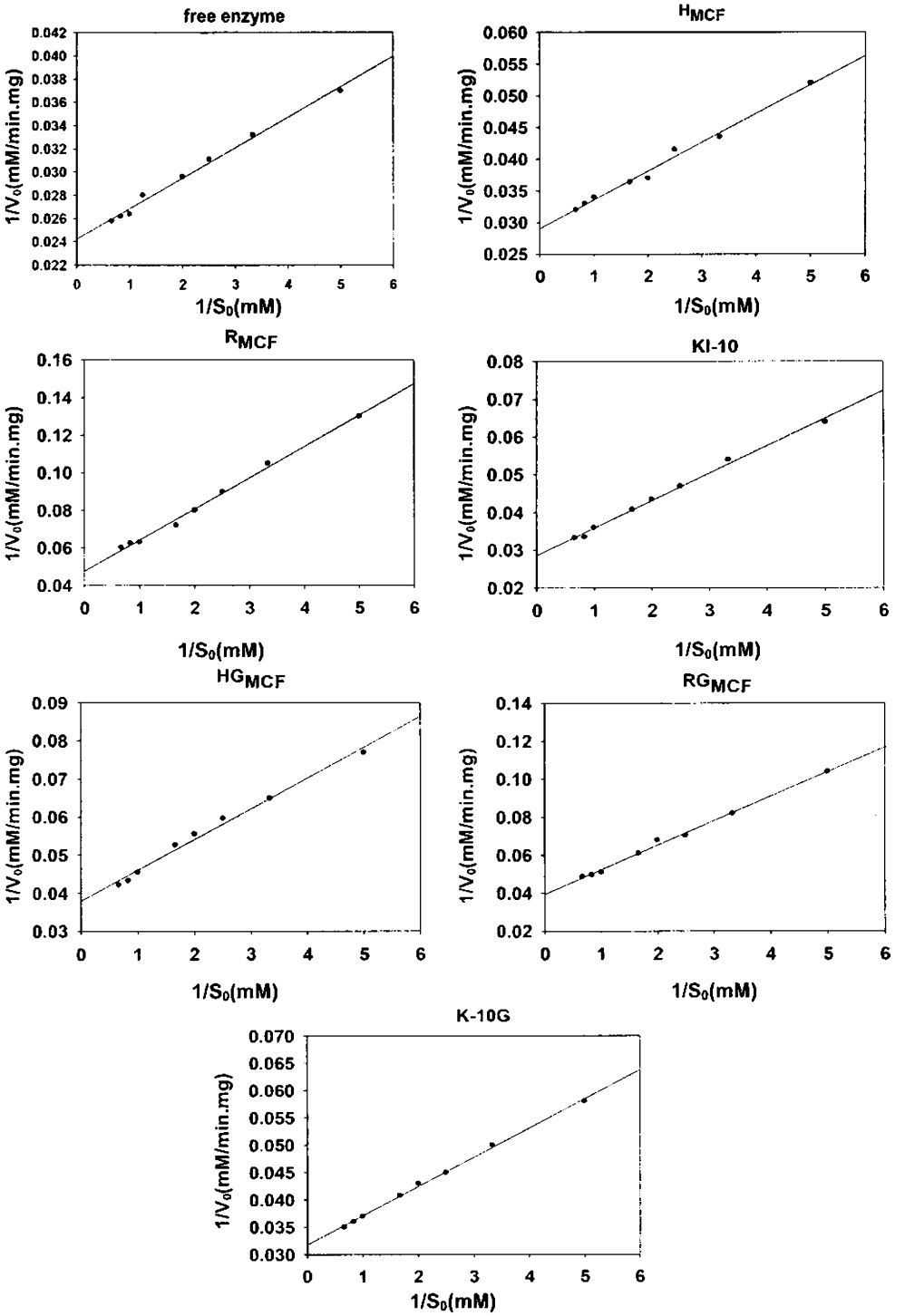


Fig 6.11 Lineweaver–Burk plots of the free and immobilized lipases in aqueous medium

The K_m value of free lipase preparation was 0.172mM in the aqueous medium and 8.68mM in heptane, respectively. A similar observation in aqueous and organic media was observed in the case of Candida rugosa lipase immobilized on PACNMA membrane [41]. The K_m value for the free lipase was nearly 50 folds higher in organic media. The V_{max} value of free lipase preparation was 44.4 U/mg in the aqueous medium and 0.272 U/mg in heptane.

The decrease in V_{max} value was about 163 fold. Therefore, it was found that the activity of the free lipase preparation in heptane (0.213 U/mg) was 0.51% of that in the aqueous medium (41.6 U/mg). There were several hypotheses to explain the relatively low activity in the organic medium. Firstly, most of the enzyme might be inactivated by the organic medium, only partial enzyme was active. Secondly, the diffusion limitation of substrate might considerably increase and lead to the decrease of hydrolysis rate.

6.4.7 Comparison of activities in aqueous and organic media

Table 6.7 shows the activities of the free and immobilized enzymes under optimum reaction conditions. It can be seen that the activity yield of lipase by chemical bonding is higher than that by adsorption. The free enzyme was 195 fold more active in water compared to that in organic solvent. The specific activity of covalently immobilized lipases is lower than their adsorbed counterparts as chemical bonding is a rigorous method which causes substantial decrease of enzyme activity. However, the amount of immobilized protein by adsorption is lower than that by chemical bonding. Due to the high surface area of H_{MCF} the diffusional resistance was markedly reduced by the reduction of the geometric size of the enzyme support and the catalytic efficiency was effectively improved compared to the covalently bound systems.

Table 6.7 Activity parameters for the free and immobilized lipases

Sample	Medium	Bound protein (mg/g)support)	Specific activity (U/mg)	Immobilization Yield(%)	Catalytic effieciency ($M^{-1}s^{-1}$) Vmax/Km ($*10^3$)	Effectiveness factor (η)	Activity yield (%)
Free lipase	Aqueous	-	41.6	-	258.13	-	100
	Organic	-	0.213	-	3.13	-	-
H_{MCF}	Aqueous	237.4	33.9	94.6	200.2	0.81	90.9
	Organic		0.239		8.25		95.4
R_{MCF}	Aqueous	92.37	24.6	78.6	86.9	0.59	78.6
	Organic		0.224		6.44		84.8
KI-10	Aqueous	50.3	29.2	87	135.59	0.79	83.7
	Organic		0.235		7.15		87.3
HG_{MCF}	Aqueous	252.8	28.9	87.9	109.9	0.60	95.9
	Organic		0.286		10.15		109.5
RG_{MCF}	Aqueous	123.5	21.4	75.3	55.33	0.47	84.7
	Organic		0.257		8.65		103.8
K-10G	Aqueous	89.6	25.7	83.8	92.4	0.56	86.5
	Organic		0.266		7.45		105.2

The maximum amount of bound protein is obtained with HG_{MCF} which also has the highest activity yield but with lowest immobilization yield compared with H_{MCF}. Due to the high surface area of R_{MCF} the amount of bound protein is greater than that of KI-10 but the activity yield and immobilization yield is less compared to KI-10. This may be due to the denaturation of the enzyme due to overcrowding on the external surface. The activity yield for all the covalently bound systems is greater than the adsorbed ones while the immobilization yield shows the reverse trend. Catalytic efficiency is also lower for the free enzyme in organic medium when compared to the immobilized counterparts. The value is greater for the covalently bound systems than the adsorbed ones probably due to the interfacial activation of the lipase in organic solvents. Activity yield of all the immobilized preparations is higher in aqueous media than that in aqueous medium

The effectiveness factor is lower than 1 for the immobilized systems in aqueous medium which depicts that immobilization has some effect on substrate and product diffusion. Activities in organic media were found to be lower than those in aqueous media, which is attributed to the low amount of water available, the lower structural flexibility of the enzymes in organic media [99] and the diffusional effects since it is a heterogeneous catalysis system yielding to a narrow range of activity. This effect is especially important with lipases for which the opening of the lid covering the active site is necessary for good activity [100]. A similar assay has been recently reported for subtilisin in organic media using amino acids nitrophenyl esters as substrate [101]. The lipase molecules that were located at the surface of the biocatalyst particles would be active, the others were inactivated by the organic solvent.

Pancreac'h and Barati [94] proposed a method to compare the activity of lipases towards PNPP in aqueous medium and in n-heptane, using the $R_{O/A}$, which is the ratio of the activity in organic solvent to the activity in aqueous medium.

It was found that the activity for the free lipase preparation in heptane increased from 0.213 to 0.239 for H_{MCF}, and the activity yield was 106%. Similar increase is

observed in the case of all the systems. The increase in activity is much higher in the case of covalently bound systems. The increase of enzyme activity could be explained by several hypotheses. Firstly, free lipase aggregated because it was insoluble in the organic medium, while immobilized lipase scattered on a large surface area could easily contact with substrates [102, 4]. Secondly, the formation of covalent bonds between the enzyme and the support surface increased the stability of the enzyme conformation against the organic medium.

Thirdly, some properties of the modified supports could benefit the activity of the immobilized lipase. This hydrophobic interaction can stabilize the "open state" conformation of lipase and favor the active site accessibility to substrates and the substrate partitioning may affect activity [104].

6.5 Conclusions

Lipase from Candida rugosa has been immobilized on mesocellular silica foams and montmorillonte K-10 via adsorption as well as covalent binding via glutaraldehyde on aminated supports. Using the hydrolysis reaction of *p*-nitrophenyl palmitate in aqueous and organic media, the properties of the immobilized lipase were assayed and compared with those of the free ones.

Montmorillonte K-10 is environmental and solvent-friendly support with large number of silanol groups and permanent charged sites which facilitate adsorption of enzymes. MCF sample featured with a well-defined 3D mesoporosity with ultralarge mesopores are more effective, as demonstrated by their higher activities observed for this sample. The ultralarge and uniform pore size of MCF would facilitate the mass transport of substrates, and allow bulky enzymes to be immobilized within the pores without steric effects. A very promising result of this work was the observation that the activity of the immobilized enzyme becomes less sensitive to reaction conditions than that of the free counterpart. The key points form this study is as follows:

- The maximum activity of CRL lipase has been achieved near the pI of CRL. The optimum pH for the adsorbed systems H_{MCF}, R_{MCF} and KI-10 were 6 and 7. The pH were maximum immobilization took place in the case of covalently bound systems was 5 in the case of HG_{MCF} and K-10G while it was 6 in the case of RG_{MCF}.
- The immobilized CRL holds much more excellent adaptability compared with free enzyme in the range pH 6.0–9.0.
- .The employment of immobilization appeared to markedly improve the thermal tolerance and stability of the CRL lipase.
- The free and the immobilized lipases exhibited marked activation in water immiscible organic solvents with a log P>4 while a deactivation was observed in the case of water-miscible organic solvents.
- EDTA (1 mM) and Mn^{2+} (1mM) activated the free as well as the immobilized lipases whereas complete deactivation was observed in the case of Hg (II).
- The immobilizates distinguished itself not only with acceptable operational stability but also with good storage stability. The high operational stability, obtained with the immobilized lipases, indicated that the immobilized lipase could successfully be used in a continuous system for the hydrolysis of lipids.
- In both medium there was no change in optimum temperature but the immobilized preparations could retain higher activity with increase in temperature from 20°C to 45°C and then kept constant at higher temperature in organic medium.
- The increase in the rate of hydrolysis is comparatively lower for the free enzyme due the high amount of water already present in the lipase powder.
- In the reusability studies the immobilized lipases retained higher activity in organic media compared to aqueous media.

- Free enzyme was completely deactivated in organic medium. The immobilized lipases retained lower activity in organic media compared to aqueous media.
- Activity yield of lipase by chemical bonding of lipase is greater than that by adsorption while the immobilization yield shows the reverse trend.
- Amount of immobilized protein by adsorption is lower than that by covalent binding.
- K_m value for the immobilized lipase by covalent bonding is higher than that by adsorption. V_{max} values for the immobilized lipase by adsorption is higher than that after functionalization which is corresponding with the activity yield.
- Catalytic efficiency is higher for the adsorbed systems than the covalently bound ones in the hydrolysis of p-NPP in aqueous medium. Effectiveness factor is lower than 1 in all cases depicts that immobilization has some effect on substrate and product diffusion.
- The activity of the free lipase in heptane (0.213 U/mg) was 0.51% of that in the aqueous medium (41.6 U/mg).
- The K_m value for the free lipase was nearly 50 folds higher in organic media. The decrease in V_{max} in the organic medium was about 163 folds which may be due to the inactivation of the enzyme in organic medium and the increased diffusional limitation of the substrate leading to a decrease in the hydrolysis rate.
- The enzyme was 195 fold more active in water compared to that in organic solvent.
- Activity yield of all the immobilized preparations in organic media is higher than that in aqueous medium.

- Catalytic efficiency is greater for the covalently bound systems than the adsorbed ones probably due to the interfacial activation of the lipase in organic solvents.
- The properties of the supports and stability of immobilized lipases exhibited interesting characteristics that may be suitable for industrial biotransformations.

References

[1] K. Hill, Pure Appl. Chem., 72 (2000) 1255.

[2] N. O. V. Sonntag, J. Am. Oil Chem. Soc., 61 (1984) 229.

[3] M. Habulin, Z. Knez, Eur. J. Lipid Sci. Technol., 104 (2002) 381.

[4] R .Fernandez-Lafuente, P. Armisen, P. Sabuquillo, G .Fernandez-Lorente, J. M. Guisan, Chem. Phys. Lipids., 93 (1998)185.

[5] V. Grazu,. O. Abian, C. Mateo, F. Batista-Vicra, R. Fernandez-Lafuente, J. M. Guisan, Biotechnol. Bioeng., 90 (2005) 597.

[6] F. X. Malcata, H. R. Reyes, H. S. Garcia, C. G. Hill, C. H. Amundson, J. Am. Oil Chem. Soc., 67 (1990) 890.

[7] H. Sovova, M. Zarevucka, Chem. Eng. Sci., 58 (2003) 2339.

[8] J. W. Hampson, T. A. Foglia, J. Am. Oil Chem. Soc., 76 (1999) 777.

[9] S. Y. Huang, C.F. Chen, J. Chin, Inst. Chem. Eng., 32 (2001) 205.

[10] Z. M. He, J. C. Wu, C.Y. Yao, K. T. Yu, Biotechnol. Lett., 23 (2001) 1257.

[11] J. P. Fay, .D. Jakober, K. J. Cheng, Can. J. Microbiol., 36 (1990) 585.

[12] U. K. Winkler, M. Stuckmann, J. Bacteriol., 138 (1979) 663.

[13] P. Grochulski, Y.G. Li, J. D. Schrag, F, Bouthillier, P. Smith, D. Harrison, B. Rubin, M.Cygler, J Biol Chem., 268 (1993)12843.

[14] G. Bayramoglu, B. Kaya, M. Y. Arıca, Food Chem., 92 (2005) 261.

[15] M. Matsumoto, K. Ohashi, Biochem. Eng. J., 14 (2003) 75.

[16] K. Lohith, S. Divakar, J. Biotechnol., 117 (2005) 49.

[17] S. Al-Zuhair, K. V. Jayaraman, S. Krishnan,W. H. Chan, Biochem. Eng. J., 30 (2006) 212.

[18] M. Petkar, A. Lali, P. Caimi, M. Daminati, J. Mol. Catal. B: Enzym., 39 (2006) 83.

[19] H. W. Yu, H. Chen, Y. Y. Yang, C. B. Ching, J. Mol. Catal. B: Enzym., 35 (2005) 28.

[20] L. Vaysse, A. Ly, G. Moulin, E. Dubreucq, Enzyme Microb. Technol., 31 (2002) 648.

[21] A. Zaidi, J. L. Gainer, G. Carta, A. Mrani, T. Kadiri, Y. Belarbi, A. Mir, J. Biotechnol., 93 (2002) 209.

[22] P. Ye, Z. K. Xu, J. Wuc, C. Innocent, P. Seta, Biomater., 27 (2006) 4169.

[23] N. Dizge, B. Keskinler, A. Tanriseven, Colloids Surf.:B., 66.(2008) 34.

[24] Z. Wang, J. Wang, Z. Xua, J. Mol. Cata:B Enzym., 42 (2006) 45.

[25] W. J. Ting, K. Y. Tung, R. Giridhar, W. T. Wu, J. Mol. Catal:B Enzym., 42 (2006) 32.

[26] C. M. F. Soares , O. A. A. dos Santos, H. F. de Castro, J. E. Itako, F. F. de Moraes, G. M. Zanin, J. Non-Cryst. Solids., 352 (2006) 3469.

[27] S. H. Chiou, W. T. Wu, Biomateri., 25 (2004) 197.

[28] M. Kordel, B. Hofmann, D. Schomburg, R. D. Schmid, J.Bacteriol., 173 (1991) 4836.

[29] G. Pencreac'h, J. C. Baratti, Enzyme Microb. Technol., 18 (1996) 417.

[30] E. Akertek, L. Tarhan, Appl. Biochem. Biotechnol., 50 (1995) 291.

[31] M. Arica, V. Hasirrci, J. Chem. Technol. Biotechnol., 58 (1993) 287.

[32] D. Tanyolac, B. Yuruksoy, A. Ozdural, J. Biochem. Eng., 2 (1998) 179.

[33] D. He, Y. Cai, L. Nie, S. Lao, Biochem. Eng. J., 6 (2000) 7.

[34] J. M. Sarkar, A. Leonowicz, J. M. Bollag, Soil Biol. Biochem., 21(1989) 223.

[35] A. Vinu, V. Murugesan, O. Tangermann, M. Hartmann, Chem. Mater., 16 (2004) 3056.

[36] A. Vinu, V. Murugesan, M. Hartmann, J. Phys. Chem. B., 108 (2004) 7323.

[37] A. Salis, D. Meloni, S. Ligas, M. F. Casula, M. Monduzzi, V. Solinas, E. Dumitriu, Langmuir, 21 (2005) 5511.

[38] A. D. McLaren, P. Lacker, Adv. Enzymol., 33 (1970) 245.

[39] E. Katchalski, I. Silman, R. Goldman, Adv. Enzymol. Relat. Areas Mol. Biol., 34 (1971) 445.

[40] T. Gitlesen, M. Bauer, P. Adlercreutz, Biochim Biophys Acta., 1345 (1997) 188.

[41] P. Ye, Z. K. Xu, Z. G. Wang, H. Wu, H. T. Deng, P. Seta, J. Mol. Catal. B: Enzym., 32 (2005) 115.

[42] P. Sabuquillo, J. Reina, G. Fernandez-Lor ente, J. M. Guisan, R. Fernandez-Lafuente, Biochim. Biophys. Acta., 1388 (1998) 337.

[43] J. M. S. Rocha, M. H. Gil, F. Garcia, J. Biotechnol., 66 (1998) 61.

[44] S. Pahujani, S. S. Kanwar, G. Chauhan, R. Gupta, Biores.Technol., 99 (2007) 2566.

[45] T. C. Hung, R. Giridhar, S. H. Chiou, W. T. Wu, J. Mol. Catal. B: Enzym., 26 (2003) 69.

[46] P. Chen, D. Chu, Y. Sun, J. Chem. Technol. Biotechnol., 69 (1997) 421.

[47] C. Santos, A. V. Paula, G. F. M. Nunes, H. F. de Castro, J. Mol. Catal. B: Enz., 52 (2008) 49.

[48] S. T. Kang, J. S. Rhee, Biotechnol. Bioeng., 33 (1989) 1469.

[49] X. Liu, Y. Guan, R. Shen, H. Liu, J. Chromatogr. B., 822 (2005) 91.

[50] F. Vaillant, A. Millan, M. Dornier, Process Biochem., 35 (2000) 989.

[51] F. Marguet, C. Cudrey, R. Verger, G. Buono, Biochim. Biophys. Acta., 1210 (1994) 157.

[52] S. Kotsovolou, A. Chiou, R. Verger, G. Kokotos, J. Org. Chem., 66 (2001) 962.

[53] B. A. Raso, H. O. Hultin, Comp Biochem. Physiol., 89B (1988) 671.

[54] R. Verger, L. Srada, P. Desnuelle, Biochim.Biophys Acta., 242 (1971) 580.

[55] R. Verger, Lipases. In: Borgstrom B, Brockman HL, editors. Pancreatic lipases. New York, NY: Elsevier (1984) 83–150.

[56] A. A. Guidoni, F. Bendouka, J. D De Caro, M. Rovery, Biochim Biophys Acta., 660 (1981) 148.

[57] A. N. A. Aryee, B. K. Simpson, R. Villalonga, Enzym.Microb. Technol., 40 (2007) 394.

[58] V. M. G Lima, N. Krieger, D. A. Mitchell, J. D. Fontana, Biochem Eng J., 18 (2004) 65.

[59] T. Kimura, M. Yoshida, M. Ogara, T. Nakakuki, Agri.Biol.Chem., 53 (1999) 1843.

[60] B. Krajeweska, J. Chem.Technol. Biotechnol., 52 (1991) 151.

[61] M. A. Abdel-Naby, A. A. Sherif, A. B. Al-Tanash, A. T. Mankarios, J. Appl. Microbiol., 87 (1999) 108.

[62] M. Bataillon, A. P. N. Cardinali, N. Castillon, F. Duchiron, Enzym.Microb. Technol., 26 (2000) 187.

[63] N. B. Ghanem, H. H. Yusef, H. K. Mahrouse, Bioresour. Technol., 73 (2000) 113.

[64] J. D. Breccia, F. Sineriz, M. D. Baigori, G. R. Castro, R. Hatti-Kaul, Enzym. Microb. Technol., 22 (1998) 42.

[65] D. R. Gjellesvik, J. B. Lorens, R. Male, Eur J Biochem., 226 (1994) 603.

[66] M. Persson, D. Costes, E. Wehtje, P. Adlercreutz, Enzyme Microb Technol., 30 (2002) 916.

[67] M. Pogorevc, H. Stecher, K. Faber, Biotechnol Lett., 24 (2002) 857.

[68] H. Sztajer, H. Lunsdorf, H. Erdmann, U. Menge, R. Schmid, Biochim Biophys Acta., 1124 (1992) 253.

[69] P. Grochulski, L. Yunge, J. D. Schrag, F. Bouthillier, P. Smith, D. Harrison, B. Rubin, M. Cygler, J Mol Chem., 268 (1993) 12843.

[70] P. Rapp, Enz. Microbiol. Technol., 17 (1995) 832.

[71] A. Sugihara, T. Tani, Y. Tominaga, J. Biochem., 109 (1991) 211.

[72] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol Bioeng., 30 (1987) 81.

[73] Y. X. Bai,Y. F. Li, Y. Yang, L. X. Yi, J. Biotechnol., 125 (2006) 574.

[74] L. H. Demirciog, H. Beyenal, E. A. Tanyolac, N. Hasirci, Polymer Int., 35 (1994) 321.

[75] J. C. Santos, P. D. Mijone, G. F. M. Nunes, V. H. Perez, H. F. de Castro, Colloids Surf., B, 61 (2008) 229.

[76] Yang, Dongsoo, Rhee, J. Shick, Biotechnol Bioeng., 40 (1992) 748.

[77] X. Liu, Y. Guan, R. Shen, H. Liu, J. Chromatogr. B., 822 (2005) 91.

[78] A. M. Klibanov, Nat. Insight., 409 (2001) 241

[79] S. Phadtare, V. P. Vinod, P. P. Wadgaonkar, M. Rao, M. Sastry, Langmuir, 20 (2004) 3717.

[80] S. Sharma, P. Kaur, A. Jain, M. R. Rajeswari, M. N. Gupta, Biomacromolecules, 4 (2003) 330.

[81] Y. Li, G. Zhou, W. Qiao, Y. Wang, Mater. Sci. Eng., B., 162 (2009) 120.

[82] Y. Bai, Y. Li, Y. Yang, L. Yi, Process Biochem., 41(2005) 770.

[83] B. Ratner, T. Horbett, R. Rapoca, Macromol., 26 (1993) 3247.

[84] Gul Ozyilmaz, J. Mol. Catal. B: Enzym., 56 (2008) 231.

[85] N. Dizgea, B. Keskinlera, A. Tanriseven, Colloids Surf., B., 66 (2008) 34.

[86] M. N. Gupta, Eur. J. Biochem., 203 (1992) 25.

[87] A. Marty, W. Chulalaksananukul, P. M. Willemot, J. S. Condoret, Biotechnol. Bioeng., 39 (1992) 273.

[88] V. M. Balcao, A. L. Paiva, F. X. Malcata, Enzyme Microb. Technol., 18 (1996) 392.

[89] G. Kunkova, J. Szilva, J. Hetflejs, S. Sabata, J. Sol–Gel Sci. Technol., 26 (2003) 1183.

[90] Gill, E. Paster, A. Ballesteros, J. Am. Chem. Soc., 121 (1999) 9487.

[91] Y. L. Khmelnitsky, A. V .Levashov, N. L Klyachko, K. Martinek, Enzyme Microb. Technol., 10 (1988) 710.

[92] A. Zaks, A. M. Klibanov, J. Biol. Chem., 263 (1988) 8017.

[93] P. Y. Z. Xu, Z. G. Wang, J. Wu, H. T. Deng, P. Seta, J.Mol. Catal B: Enzym., 32 (2005) 115.

[94] G. Pencreac'h, J. C. Baratti, Enzyme Microb. Technol., 28 (2001) 473.

[95] A. Zaks, A. M. Klibanov, Science, 224 (1984) 1249.

[96] F. Monot, F. Borzeix, M. Bardin, J. P. Vandecasteele, Appl. Microb. Biotechnol., 35 (1991) 759.

[97] R. Fernandez-Lafuente, J. M. Guisan, Enzyme Microb. Technol., 39 (2006) 877.

[98] M. Y. Chang, R. S. Juang, Enzyme Microb Technol., 36 (2005) 75.

[99] J. Partridge, P. R. Dennison, B. D. Moore, P. J. Halling, Biochim Biophys Acta., 1386 (1998) 79.

[100] M. Norin, F. Haeffner, K. Hult, O. Edholm, Biophys J., 67 (1994) 548.

[101] A. Reimann, D. A. Robb, P. J. Halling, Biotechnol. Bioeng., 43 (1994) 1081.

[102] G. Pencreac'h, J. C. Baratti, Appl Microbiol Biotechnol., 47 (1997) 630.

[103] P. J. Halling, Enzyme Microb Technol., 16(1994) 178.

Chapter 7

CROSSLINKED β- GLUCOSIDASE IN MESOCELLULAR SILICA FOAMS: CHARACTERIZATION AND CATALYTIC ACTIVITY STUDIES

Contents

β-glucosidase can be used by the food industry to increase the bioavailability of the isoflavones in the human intestine, and by the beverage industry to improve the aromatic composition of juices and wines. It is well known that glycosidic compounds are useful products in pharmaceutical, food, cosmetic and fine chemical industries. In addition, since both the immobilization procedure and solid support add to the cost of the insoluble enzyme, there is a great interest in cheap substrates on which β-glucosidase can be immobilized by a simple and economic process. To overcome the shortcomings as a result of adsorption and covalent binding of enzymes on solid supports, a simple and highly effective method of crosslinking enzymes via multipoint attachment was adopted in this context which involves simple adsorption of enzyme followed by enzyme crosslinking using glutaraldehyde. The pure supports as well as the crosslinked supports were characterized by Nitrogen adsorption studies, FTIR, TG/DTG and NMR. All the studies confirmed the incorporation of enzyme as well as aldehyde moieties into the support. The optimal immobilization conditions (enzyme loading, immobilization time and amount of glutaraldehye) were examined. Also, the operation stabilities of immobilized enzymes when used repeatedly (temperature, pH, storage, etc.) and the kinetic properties were studied and compared with those of free enzyme. This is of practical importance for further applications. The results are promising for a future technological application of the immobilized enzyme in wine-making.

7.1 Introduction

Flavor compound synthesis by biotechnological processes plays nowadays an increasing role in the food industry. This is the result of scientific advances in biological processes, making use of microorganisms or enzymes as an alternative to chemical synthesis, combined with recent developments in analytical techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), infrared (IR) or mass spectrometry (MS). Research in this area for new products and bioprocesses is also enhanced by a growing market and increasing public concern for the wholesomeness and chemical safety of food ingredients.

β-glucosidase

Glycoside hydrolases (also called **glycosidases**) catalyze the hydrolysis of the glycosidic linkage to generate two smaller sugars. They are extremely common enzymes with roles in nature including degradation of biomass such as cellulose and hemicellulose. Glycoside hydrolases are classified into EC 3.2.1 as enzymes catalyzing the hydrolysis of O- or S-glycosides.

β-Glucosidase (βG, EC 3.2.1.21) is one of the most interesting glycosidases, especially for hydrolysis of glycoconjugated precursors, in musts and wines, and the release of active aromatic compounds. β-Glucosidase (E.C.3.2.1.21) comes from many sources including bacteria, animals, and plants, which exhibits wide substrate specificity and is capable of cleaving β–glucosidic linkages of conjugated glucosides and disaccharides [1]. It can hydrolyze cellooligosaccharides and cellobiose into glucose and is capable of hydrolyzing anthocyanins that are the main coloring agents found in the foods of vegetable origin [2]. The main interest in this enzyme is related to its potential applications in food processing industry (for example, the production of wines and fruit juices) for improving organoleptic product properties [3, 4].

The principal reaction catalyzed by this class of enzymes is the hydrolytic cleavage of β-glucosdic linkages of low molecular glycosides. Recently this enzyme was also studied for its potential to liberate aroma rich terpenes. Such aroma precursor compounds are found in different fruits (mango, passion fruit, grapes) [5, 6] bonded to glucosides and the β-glucosidases were more specific and effective than the acid hydrolysis process for liberating terpenols from terpenylglucosides.

Mechanism

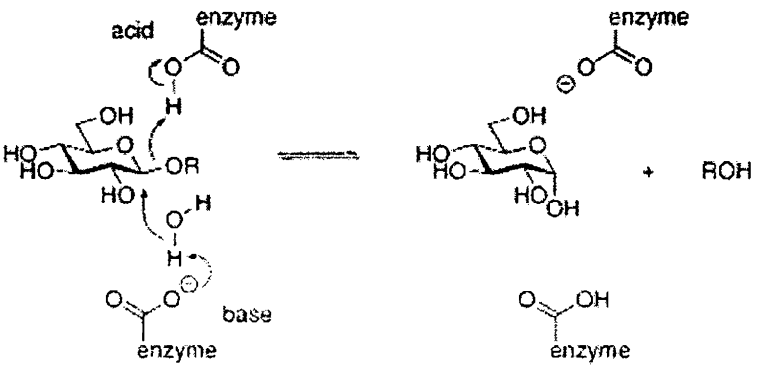


Fig 7.1 Mechanism for the hydrolysis of glucosides by β-glucosidases

β-glucosidases utilize two enzymic residues, typically carboxylate residues, that act as acid and base respectively as shown in Fig 7.1.

7.2 Immobilization of β- glucosidase

The limited half-life of the immobilized enzyme through adsorption, which is a consequence of it being progressively released in the solution, is a drawback to its possible practical applications. In order to stabilize enzyme bonding to the support, β-glucosidase was immobilized by covalent bonding and/or cross-linking. Utilizing this latter procedure, a considerable enzymic load can be achieved on the support, as well as an optimum half-life. Electrostatic bonds between the OH~ groups of silica and the NH_2 groups of the enzyme together with hydrogen bonds and Vander Waals forces are formed during the phase of simple adsorption. In addition to these bonds, bonds between GA and the enzyme (Schiff bases) are

also formed during crosslinking. These latter bonds set up a network which prevents the enzyme from being released.

Immobilization methodologies, of β-GL together with other glycosidases, have been studied to increase the organoleptic characteristics in food. Immobilization of β-glucosidase from different sources had been frequently described using different supports and methods to bind enzymes and matrices [7- 9]. Equal weights of clay and chitosan were mixed to prepare the composite beads, in wet (without freeze-drying) or dried (with freeze-drying) form, which were used as immobilization supports for β-glucosidase after cross-linking with glutaraldehyde in the work done by Chang et al. The immobilized enzymes exhibited good storage stability and exhibited broader temperature and pH ranges after immobilization of β-glucosidase [10, 11]. High immobilization yields and residual activity levels was obtained when β-glucosidase was immobilized on amine agarose gel via oxidation of its carbohydrate chains [12].

Glucosidase activity was strongly reduced in the presence of clay minerals montmorillonte and Al-montmorillonite [13]. This was explained by the strong adsorption of the enzyme on the clay minerals causing an unfolding of the enzyme on the clay mineral surface [14]. The stability of β-glucosidase immobilized on chitosan after crosslinking with glutaraldehyde, showed good stability in wine and acid buffer in the work done by Martino et al. [15]. In the study carried out by Mathijs et al. β-glucosidase was immobilized on silica via two different procedures and it was found that the enzyme coupled with dextran dialdehyde and subsequent attachment with aminopropyl silica showed enhanced thermostability than the other counterpart which may be due to the different number of links formed between the enzyme and the carrier [16].

Inorganic and organic matrices have been used, the inorganic matrices were α and γ alumina, and bentonite; while the organic matrices were: Eupergit-C®, Xad 7®, cellulose as such and functionalized, amine agarose and chitosan gels. Sandy alumina showed an excellent immobilization capacity for β-glucosidase in

the work done by Fadda et al. [17]. An increase in thermal stability and a shift towards alkaline pH was observed when β-glucosidase was immobilized onto siliochrome which was modified with APTES and glutaraldehyde [18]. Several papers have described the immobilization of β-glucosidase including adsorption or covalent bonding on silica, alumina, chitin and chitosan [19-22].

7.3 Cross-Linked Enzyme Aggregates (CLEAs)

The development of robust immobilized biocatalysts that are stable over a broad range of pH and temperature and are tolerant to organic solvents is a major challenge in industrial biocatalysis [23]. A major breakthrough in this field was the development of cross-linked enzyme crystals (CLECs), biocatalysts which combine the features of essentially pure protein with high specific activity [24, 25] and high tolerance to organic solvents [26, 27]. Cross-linked enzyme aggregates (CLEA) are strictly speaking not immobilised enzymes. Their straightforward preparation combines protein precipitation followed by cross-linking with a suitable dialdehyde, usually with glutaraldehyde, without the need for expensive protein purification [28, 24].

Lipase (LP) was immobilized on the surface of the supporting materials carboxymethylcellulose (CMC) and diethylaminoethyl cellulose (DEAE-C) by using cross-linked enzyme aggregation (CLEA) by glutaraldehyde. The activity of enzyme aggregates coated on DEAE was approximately 2 times higher than that of enzyme aggregates coated on CMC. This is explained by the fact that enzyme aggregates with amine residues are more efficient than those with carboxyl residues [29].

Recently, inorganic mesoporous silica materials have attracted much attention as a host of enzymes because of their controlled porosity and high surface area [30-33]. α-Chymotrypsin (CT) and lipase (LP) were immobilized in SBA-15 mesoporous silica via CLEA approach and it was found that the immobilized aggregratres showed improved stability as well as negligible activity decrease under harsh shaking condition for one week while the adsorption and covalent

attachment resulted in more than 50–90% enzyme inactivation under the same condition [34]. Lipase PS from Burkholderia cepacia was successfully immobilized in sol–gels under low methanol conditions using lyophilization in order to dry the gel. The enzyme was also cross-linked with glutaraldehyde to CLEAs without any additive and were employed for the highly enantioselective acylations of 1-phenylethanol (**1**), 1-(2-furyl)ethanol (**2**) and *N*-acylated 1-amino-2-phenylethanol (**3**) with vinyl acetate in organic solvents. The CLEA of lipase PS showed a significant increase in activity upon immobilization (174%) and the lipase PS-CLEA is highly enantioselective in acylation reactions than the sol-gel preparation which showed better reusability characteristics compared to CLEA's [35]. The CLEA approach was employed to adsorb α-chumotrypsin onto Hexagonal mesoporous silica (HMMS) followed by enzyme crosslinking with glutaraldehyde. The resulting CLEAs containing α-chymotrypsin (CLEA–CT) did not show any activity decrease under rigorous shaking for one month, which demonstrates a huge success of this approach [36]. Nanometer-scale CLEAs of a-chymotrypsin (CT) and lipase (LP) was prepared in HMMS using GA as the crosslinking agent which demonstrated increased stability under harsh shaking conditions for 2 weeks with negligible loss of activity and also high loading capacity. This was due to the ship-in-a-bottle approach of CLEAs in HMMS [37]. By crosslinking reactive amine residues of the enzyme with glutaraldehyde (GA), crosslinked enzymes (CLEs) [38] and crosslinked enzyme crystals (CLECs) [39] were developed to increase the operational stability of enzymes and to facilitate their recovery and recycling.

As a possible application of mesocellular silica foams (MCF), we attempted to immobilize enzyme β-glucosidase in MCF. An extremely effective method as so-called Cross-Linked Enzyme Aggregates (CLEAs) was employed to immobilize β-glucosidase onto mesocellular silica foams which employs the adsorption of enzymes followed by enzyme crosslinking using glutaraldehyde. The schematic representation of CLEA-GL in MCF is shown in Fig 7.2.

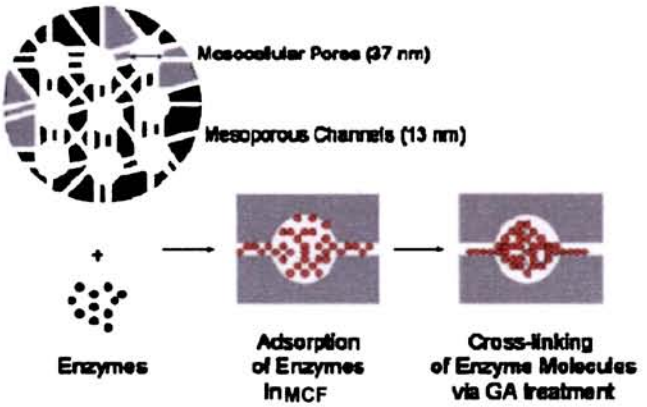


Fig 7.2 Schematic representation for CLEAs in MCF (crosslinked enzyme aggregates in mesocellular silica foams).

CLEAs in MCFs exhibit high activity retention and stability and can be readily recovered and recycled without any loss of activity. The approach of crosslinked enzyme aggregrates (CLEAs) in mesoporous silica is a simple and effective method for enzyme stabilization [40]. Along with the higher specific activity than that of adsorbed enzymes, this CLEA approach is much simpler than that of covalent attachment by obviating the tedious processes for silica functionalization and enzyme attachment. The multipoint attachment of enzyme molecules is well-known to stabilize activity by preventing enzyme denaturation [41]. This CLEA approach has resulted in highly-loaded, stable, and active enzyme immobilization due to a ship-in-a-bottle effect that takes advantage of the bottle-neck pore structure.

This simple but powerful approach of CLEAs in mesoporous media opens up a new possibility for enzyme stabilization and will contribute to a variety of enzyme applications in biosensors, bioremediation, and bioconversion.

7.4 Immobilization and activity measurements

7.4.1 Synthesis of MCF carriers

The purely siliceous mesocellular silica foams (MCF) were prepared according to the direct hydrothermal method described in [42] using a Pluronic P123 triblock copolymer surfactant with 1, 3, 5-trimethylbenzene (TMB) as the organic swelling agent with TMB/P123 = 1 (w/w).

7.4.2 Immobilization of enzymes on MCFs

CLEAs of β-GL (CLEA-β-GL) were prepared according to the procedure reported by Kim et.al [37]. CLEAs of β-GL (C LEA-GL) were prepared as follows.

MCF (10 mg) was mixed with 2 ml of free β-GL (glucosidase) in a buffer solution (2mg/ml in sodium acetate buffer, pH 4.8) sonicated for 3 s, and incubated at room temperature under shaking condition. After 5hrs of incubation for β-GL adsorption, the samples were briefly washed by an aqueous buffer (100mM sodium acetate buffer, pH 4.8), and then incubated in the same buffer containing 0.1% glutaraldehyde (GA). After GA treatment for 30 min, the samples were excessively washed by a phosphate buffer (100mM sodium phosphate, pH 8.0). The samples were washed four times by phosphate buffer (10 mM sodium acetate buffer pH 4.8) and stored at 4°C. The protein amount leached from the silica was done following Lowry's method [43]. The final enzyme loading in the silica was calculated from the difference between the initial β-GL amount and the leached amount of β-GL into washing solutions. The optimal GA concentration was found to be 0.1%. At lower concentrations (0.05 %) enzymic activity was lower due to the release of the enzyme into the saline washing solution. Optimal performance was obtained with 0.1% GA and a crosslinking time of 4hrs.

Adsorbed GL samples were prepared in the same procedure as that of CLEA-GL, but no GA was added during the GA treatment step. For covalent attachment of GL, aminated MCF (10 mg) was incubated in 1.5 ml of 0.1% GA solution (100mM sodium phosphate, pH 8.0) for 1h, and washed five times with the same buffer containing no GA. Then, the aldehyde-functionalized MCF was incubated in GL solution (2mg/ml, sodium acetate buffer, pH 4.8) and stirred for 4h at 4°C.

7.4.3 Measurement of β-glucosidase activity

The activity of free β-glucosidase in the solution was determined by adding 0.1ml enzyme to 0.9mL of 0.1M acetate buffer (pH 4.8), which contains 5mM of substrate *p*-nitrophenyl β-D-glucopyranoside substrate [15]. The reaction mixture

was incubated with stirring at 25°C for 1 min and stopped by adding 2mL of $1MNa_2CO_3$. The absorbance of the final product *p*-nitrophenol was measured at 400 nm using an UV/visible spectrophotometer and the activity was calculated based on a molar extinction coefficient of 18,300 $dm^3/(mol\ cm)$. One activity unit of β-glucosidase is defined to be the amount of this enzyme required for hydrolyzing 1μmol substrate/min. The activity of β-glucosidase immobilized on the silica was similarly measured, except that 0.1ml of the solution was replaced by 0.1ml deionized water and a given amount of immobilized enzyme.

7.5 Results and Discussion

Physicochemical characterization studies

7.5.1 N_2 adsorption measurements

Fig 8.3 shows the N_2 adsorption and desorption isotherms of mesocellular silica foams and CLEA-GL. All the samples prepared show type IV isotherms for nitrogen adsorption at 77K, which is typical [44] of mesoporous solids with H1 type hysteresis loops at high relative pressures. In the case of MCF, before immobilization, sharp inflection step is observed. An important loss of adsorbed volume is observed as well as a shift towards low p/p_0 values for the mesoporous uptake curve is seen after cross linking indicating decrease in the mesoporosity of the material. The surface area decreases from 595 m^2/g to 352 m^2/g after crosslinking and the pore diameter from 161 Å to 47 Å which can be mainly attributed to the binding of CRL and aldehyde molecules into the mesopores (Table 7.1).

Table 7.1 Textural property of MCF and CLEA-GL

Immobilizate	Surface area (m^2/g)	Pore diameter(Å)	Pore volume (cm^3/g)
MCF	595	161	2.49
CLEA-GL	352	47	0.82

The pore size distribution curve shifted to a smaller size after crosslinking. All values of pore size, surface area, and pore volume were reduced by forming CLEA-GL in the pores of MCF's suggesting that more GL could be loaded in MCFs.

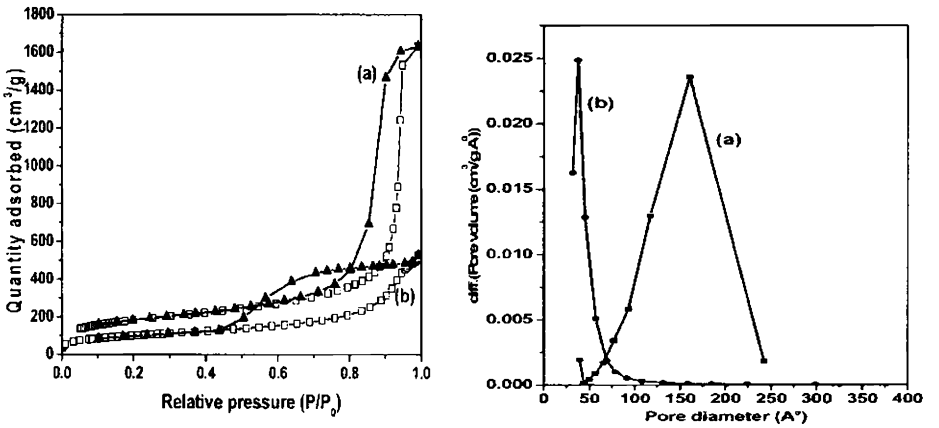


Fig 8.3 N_2 adsorption/desorption isotherms and pore size distributions of **(a)** MCF160 **(b)** CLEA-GL

7.5.2 Thermogravimetry

Fig 7.4 shows the TG/DTG profiles obtained for immobilized β-glucosidase on silica and that after crosslinking with glutaraldehyde. The typical TG and DTG curves of M-GL demonstrate the significant weight loss between 200-400^{0}C, which is attributed to the enzyme decomposition.

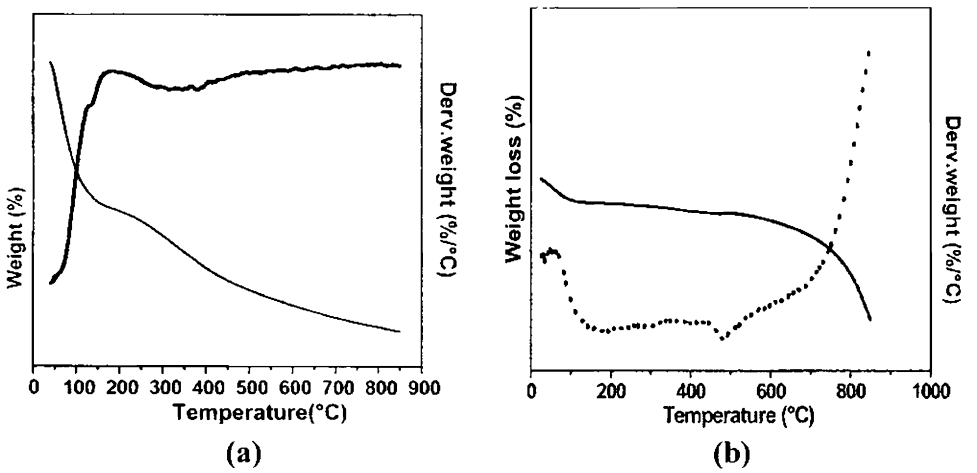


Fig 7.4 TG/DTG curves of **(a)** MAI **(b)** CLEA-GL

After crosslinking (CLEA-GL) there is only a single weight loss extending from 100-650°C which is attributed to the decomposition of the organic groups of glutaraldehyde as well as the enzyme moieties which showed that the thermal

stability of the cross linked β-glucosidase has increased considerably after binding with glutaraldehyde.

7.5.3 FTIR studies

The FTIR spectra of adsorbed β-glucosidase and that of CLEA-GL are shown in Fig 7.5.

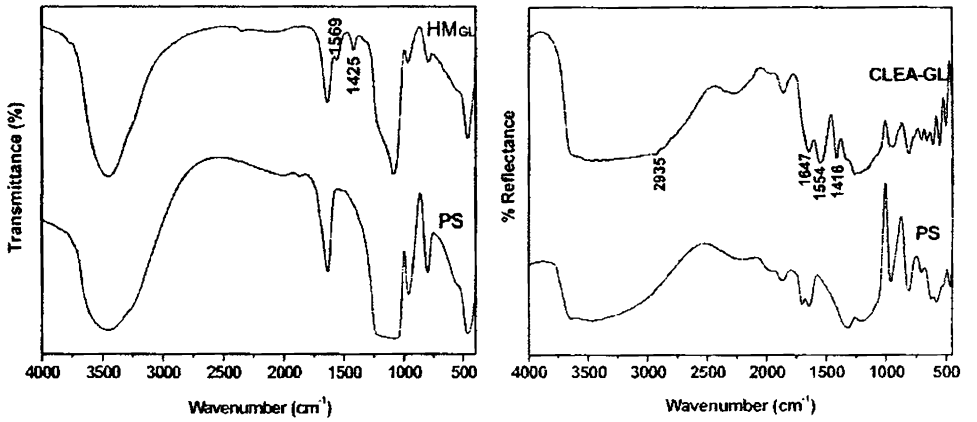


Fig 7.5 IR spectra of the (A) PS (pure silica) and enzyme adsorbed (HM_{GL}) and (B) CLEA-GL

In addition to the characteristic peaks of MCF silica at 1630 cm^{-1} assigned to the –OH vibration of physisorbed H_2O and the Si–O–Si bands at around 1000–1250, 800, and 475 cm^{-1}, the amide I (1630cm^{-1}) and amide II (1540 cm^{-1}) of β-GL was observed after immobilization which confirmed the retention of native secondary structure of protein and biological activity. The C-H simple bending vibration occurs at 1425cm^{-1} for free β-Glucosidase (GL) and with increased intensity at 1416cm^{-1} in the case of CLEA-GL which confirmed the presence of aldehydic group after crosslinking. C-H stretching vibration frequency is seen at 2935 cm^{-1} with contributions from glutaraldehyde. The amine-glutaraldehyde reaction produces an imine N=C bond, Schiff-base, seen at 1647 cm^{-1} while an ethylenic C=C bond formed by resonance stabilization of the imine appears at 1554cm^{-1} with increased intensity which confirmed the crosslinking of glutaraldehyde with enzyme molecule.

7.5.4 ^{29}Si and ^{13}C Nuclear magnetic resonance spectroscopy

The ^{29}Si MAS spectrum of the parent MCF sample (Fig 7.6) exhibited two broad resonances at -112 ppm for a Q^4 environment and at -102 ppm for a Q^3 environment together with a shoulder at -93 ppm ascribable to Q^2 species. After adsorption of the enzyme and further crosslinking with glutaraldehyde the Q^2 peak almost disappeared reflecting that geminal silanols allowed a better functionalization than isolated ones. There was a pronounced decrease in the intensity of Q^3 peak compared to Q^4. There was a shift in the peak position by 1ppm due to the strain imparted on the matrix due to the crosslinking step.

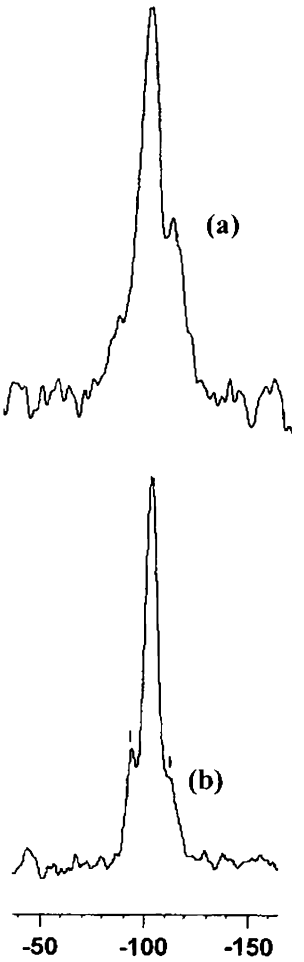


Fig 7.6 ^{29}**Si** NMR of **(a)** MCF160 **(b)** CLEA-GL

The ^{13}C NMR of CLEA-GL (Fig 7.7) exhibited peaks due to the enzyme groups at 10, 23, 71 and 101 ppm with decreased intensity. The effective binding of aldehyde was evident from the peak at 184 ppm.

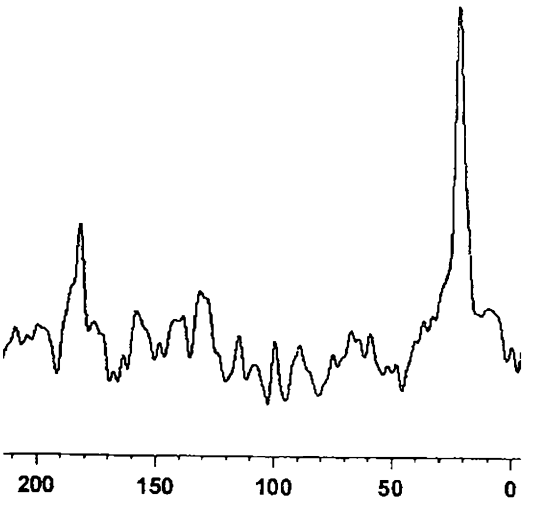


Fig 7.7 ^{13}C NMR of CLEA-GL

7.6 Biochemical characterization of free and CLEA-GL

7.6.1 Stability and activity of various immobilizates

The mesopores of MCF supports were used as a template for the preparation of CLEAs, which can enhance the enzyme loading by utilizing all the pore volume, pore diameter and surface area and possibly prevent the enzyme leaching. It is anticipated that the above characteristics would place a good resistance against leaching β-GL from MCF's.

Figure 7.8 shows the stability of free GL (β-glucosidase), adsorbed GL and CLEA–GL in aqueous buffer (10mM sodium acetate, pH 4.8) at room temperature. At each time point, the residual activity of GL was measured by the hydrolysis of p-ntrophenyl β-D glucopyranoside in an aqueous buffer (10mM sodium acetate, pH 4.8), and the relative activity was calculated from the ratio of residual activity to the initial activity. The enzyme stability of various immobilizates was investigated by incubating each sample at room temperature and under harsh shaking condition.

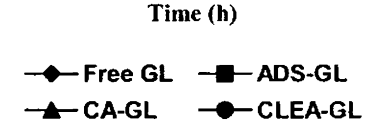


Fig 7.8 Stability of CLEA-GL, Adsorbed GL, covalently bound-GL and free GL in a shaking condition at 30°C. CLEA-GLs were prepared with 2mg/ml and 0.1% glutaraldehyde concentration.

In harsh-shaking condition, the activity of free GL rapidly dropped, and no measurable activity remained after 24 h incubation. ADS-GL in MCF and CA-GL marginally enhanced the enzyme stability, but the relative activities after 24h incubation were 50% and 68%, respectively. CA-GL showed a fairly-good stabilization of enzyme activity by retaining 65% of initial enzyme activity after 48h incubation. However, CLEA-GL in MCF showed negligible activity decrease after 48h incubation in the same condition. CLEA-GL also showed an impressive stabilization of enzyme activity when compared to other immobilization approaches. This impressive stability under rigorous conditions demonstrates that the mesocellular pore structure with interconnected windows was successful in preventing enzyme aggregates in the main mesocellular pores (16 nm) from being leached out. Most of the β-glucosidase in the mesoporous channels are crosslinked with CLEAs in mesocellular pores and do not leach out from MCF's. The successful enzyme stabilization via the CLEA approach can be explained by the effective prevention of enzyme leaching as well as the improvement of intrinsic enzyme stability via multipoint covalent linkages between enzyme molecules.

Table 7.2 Enzyme loadings and activities of various immobilizates

Samples	Initialy loaded enzyme (μg in 1mg silica)	Finally-loaded enzyme (μg in 1mg silica)	Specific activity (μmol/min/ mg enzyme)	Activity yield (%)	Immobilization yield (%)
Free GL	-	-	12.3		
ADS-GL	150	87	5.2	72.4	87.3
CA-GL	150	132	8.5	77.4	82.7
CLEA-GL	150	139	7.8	88.9	75.3

Table 7.2 shows the results of enzyme loadings via various immobilization methods: adsorption (ADS), covalent attachment (CA), and CLEA approaches. The activities of ADS, CA, and CLEA samples are also summarized. The specific activity of CLEA-GL in MCF was 7.8 μmol/min per mg enzyme, which was higher than that of ADS-GL. These higher specific activities of CLEA samples imply that GA crosslinking prevents the enzyme denaturation and inactivation, leading to a better substrate transfer by allowing mesopores to be less populated with denatured and inactivated enzymes and also due to the inhibition of auotolysis. The specific activity of CLEA sample was lower than that of CA sample. The CA samples of GL showed higher specific activities presumably due to the fact that the enzymes are immobilized mainly at the entrance part of pores, facilitating rapid enzyme-substrate reaction.

All the adsorbed samples showed a significant reduction of enzyme loading while the CLEA samples retained most of initially adsorbed enzymes. The final enzyme loading amount of ADS-GL and CLEA-GL in MCF were 87 and 139μg/mg silica, respectively. This result revealed the importance of GA treatment in preventing the leaching of initially-adsorbed enzymes from MCF. Adsorbed GL places more serious mass-transfer limitations on the substrate than CLEA–GL, even though the internal porosity of MCF's with

adsorbed GL contains smaller amounts of GL molecules than that with CLEA–GL. This was due to the denaturation and/or autolysis of adsorbed GL molecules, which leads to an increase of their occupied volume and more serious mass-transfer limitation for substrates in MCF's. The combination of CA approach and subsequent binding of the enzyme with a high aspect ratio resulted in steric hindrance by the covalently-attached enzyme molecules at the inlet part of pores against the penetration of other enzyme molecules into the pores. The CLEA approach does not place this steric hindrance since it starts with the enzyme adsorption, leading to higher enzyme loading.

The activity yield is higher in the case of CLEA-GL compared to adsorbed and covalently bound samples. The immobilization yield was lower for the CLEA-GL than the adsorbed and the covalently bound samples as the enzyme might have undergone a transition to a less active state upon covalent immobilization followed by glutaraldehyde treatment. This CLEA approach, simply crosslinking fully-adsorbed enzymes molecules has resulted in highly-loaded, stable, and active enzyme immobilization.

7.6.2 Optimisation of glutaraldehyde concentration for crosslinking

Figure 7.9 shows the stability of CLEAs-GL treated with various concentrations of GA after adsorption of GL (2mg/ml) into MCF's. Treatments with relatively low GA concentrations (0.01 and 0.05% GA) resulted in a marginal improvement of enzyme stability and there was still GL leaching observed with the samples, which was confirmed by the measurement of the leached GL in the supernatant. Upon increasing the GA concentrations to 0.1 or 1%, the GL stability greatly improved. With these samples, there was no measurable decrease in GL activity detected for 48h incubation period under shaking. The impressive stability is due to the mesocellular pore structure of MCF's and the inhibition of autolysis by glutaraldehyde crosslinking.

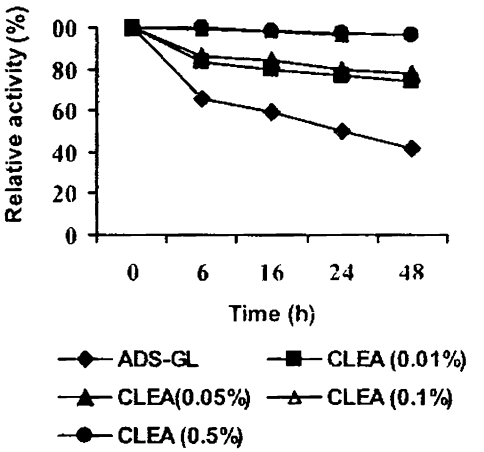


Fig 7.9 Stability of CLEAs-GL-MCF prepared with 2mg/ml GL and various glutaraldehyde concentration at 30°C.

To prevent the leaching problem, adsorbed GL was treated with various glutaaldehyde concentrations (0.0, 0.05, 0.1 and 0.5%w/w). With this treatment, the loading capacity of the resulting GL improved upto GA concentration of 0.1%.

Table 7.3 Loading capacity and activity of CLEA-GL in MCF prepared with various GA concentrations

SAMPLES	FREE GL	ADS-GL	CLEA-GL (0.01%)	CLEA-GL (0.05%)	CLEA-GL (0.1%)	CLEA-GL (0.5%)
GL loading amount in MCF (μg in 1mg silica)	-	87	118.7	125.2	139	130
Specific activity (μmol/min per mg GL)	12.37	5.23	8.35	8.26	7.83	4.31

The specific activity also showed a gradual increase with increase in aldehyde concentration but with 0.5% glutaraldehyde, the activity decreased probably due to the denaturation of enzyme. This may be due to the reticulation among enzyme molecules favored by the use of a bifunctional molecule and therefore affecting the activity. Another reason for the low activity may be due to a partial denaturation of enzyme as a result of "rigidification" of the enzyme molecule or steric hindrance which prevents the

substrate from reaching the active site. Without GA crosslinking (GA 0%), adsorbed GL in MCF's continuously leached out from MCF's which is evident from the lower enzyme loading per mg silica and also the lower specific activity compared to CLEA-GL. Thus β-glucosidase activity was stabilized when the samples were treated with 0.1% glutaraldehyde.

7.6.3 Activity as a function of pH

The pH activity profiles of crosslinked and soluble β-glucosidase are shown in Fig. 7.10. The optimum pH at which βGL binds to MCF while maintaining maximum activity was determined by performing the immobilization process at various pH values. Usually, carboxyl and amino groups create a unique ionic atmosphere, which may be altered by the interaction with the solid support surface changing the microenvironment at the active site [45].

The relative activity of free enzyme increases and reaches its optimum at pH 6 whereas the optimal pH of the immobilized enzyme exhibited a shift of 0.5 pH units towards the alkaline zone as compared to the free enzyme (from 6.0 to 6.5). The immobilized enzyme was more stable in lower and higher pH in comparison to free enzyme. Free β-GL deteriorates when pH is increased above 6 and also below pH 5. The relative activity of CLEA is over 75% in the pH range 4.5–6.0.

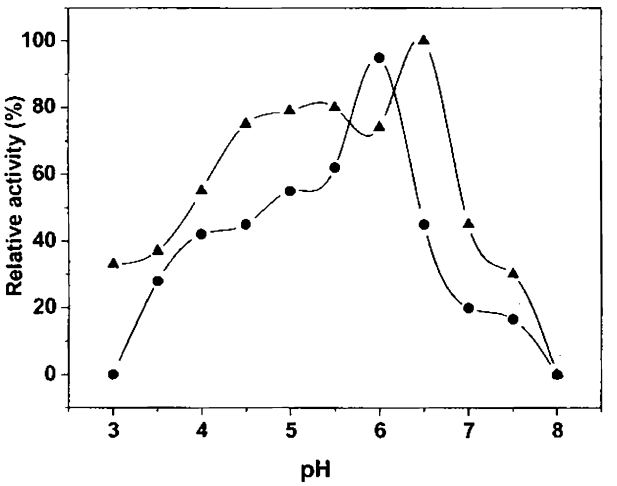


Fig 7.10 Effect of pH on the relative activity of (●)free and (▲) CLEA-GL at 25°C.

The CLEA's exhibited a broader pH range with higher activity than the free βGL. The shift observed in the optimum pH of soluble and immobilized enzyme was probably caused by the presence of negative charges dominating on the matrix surface. At this pH, the surface of the SiO_2 framework of MCF has an overall negative charge since the isolectric point of SiO_2 is pH ~2. Almond β-glucosidase has isoelectric points of 4.9 and 6.5 [46].

The almond glucosidase isozymes reported so far in literature include: isozyme A (135 kDa, p*I* 6.9, two subunits)] ,isozyme B (190–200 kDa, p*I* 4.8, two subunits, isozymeAB (155 kDa, p*I* 5.6, two subunits), isozyme C (58 kDa, p*I*4.55, one subunit), isozyme D (52.5 kDa, p*I* 4.4, one subunit), isozyme E (52.5 kDa, p*I* 6.4, one subunit),and isozyme F (90 kDa, p*I* 4.8, one subunit). The main component of the commercially supplied almond β-glucosidase (Sigma Co.) was found to be the isozyme A. The overall net charge of the protein is slightly positive. Therefore, an electrostatic interaction between the two is expected. When the pH of the immobilization is increased above pH 6.5 less βGL is immobilized. At pH 7.5, i.e. above the isoelectric point of βGL, βGL is not immobilized by MCF, which is likely a result of the net negative charge of both the silicate framework and βGL. The forces involved in the enzyme-matrix interaction are mostly of an electrostatic nature. Thus from these results, it is observed that the isoelectric point of SiO_2 and the overall framework charge play important roles in immobilizing enzymes. The strength of the electrostatic interaction between the enzyme and the silicate surface is very important in maintaining the overall activity of the enzyme. Leaching of the enzyme from the CLEA-GL material was tested with water by repeatedly washing the immobilized enzymes and measuring the activity of the washed solution. The activity obtained from the washed solutions was minimal.

The dried composite beads prepared from chitosan and activated clay exhibited higher activity in the pH range 5.0-8.0 than the wet composite and also

there was a shift in the optimum pH for both the composites to the acidic region compared to the free β-GL [11].

7.6.4 Effect of temperature on the enzyme activity

The effect of temperature on the activity of free and immobilized β-glucosidase is shown in Fig. 7.11. The optimal temperature was shifted from 50°C for free β-glycosidase to 60°C for CLEA-GL. As the case of pH effect, the immobilized enzymes have a broader tolerance range to heat than free enzymes. In general, the immobilization support has a protecting effect at high temperatures when enzyme deactivation occurs. The conformational flexibility of the enzyme is affected by immobilization. The immobilization step causes an increase in enzyme rigidity, commonly reflected by an increase in stability towards denaturation by raising the temperature [47]. Above a certain temperature an enzyme will tend to lose the compact three-dimensional structure which is required for catalytic activity.

Yan and Lin [48] also indicated that the activity of free β-glucosidase reaches maximum at 55°C but only 70% of the 55°C activity is obtained in the temperature range 50–60°C. This likely indicates a higher thermal stability for the immobilized enzymes.

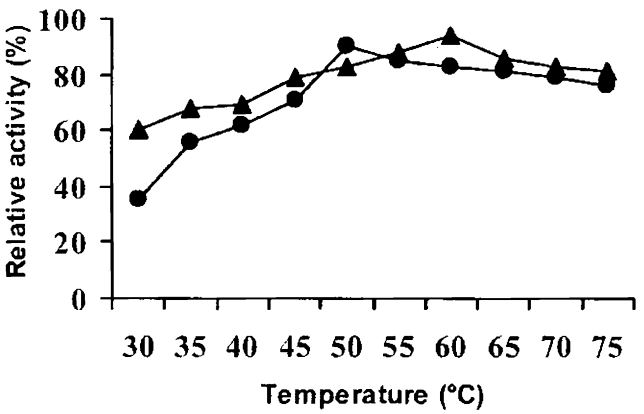


Fig 7.11 Effect of temperature on the relative activity of (●) free and (▲) CLEA-GL at pH 4.8

Crichton et al. noted a large difference in thermoresistance between immobilized β-GL dextran conjugates and native enzymes attached onto glutaraldehyde-activated porous silica. They explained the enhanced thermoresistance by the rigidity of the enzymes which occurs when the enzyme is linked to a suitable polymer [49]. In contrast, the temperature-activity profile of the soluble and immobilized β-glucosidase from A. niger remained almost unaltered. In some cases, both the optimal temperature and the temperature-activity profile change [50, 51] upon immobilization, whereas in other cases they do not [52].

7.6.5 Thermal stability studies

Fig 7.12 shows the long-term stability profiles for free β-glucosidase and CLEA-GL activities at 55°C and 60°C. If the heat stability of an enzyme is enhanced by immobilisation, the potential utilisation of such enzymes would be extensive. It is often found that immobilized enzyme has a higher thermal stability than free enzyme because of the reduction of conformational flexibility in immobilized enzyme [53]. Free enzyme was rapidly inactivated at higher temperatures after prolonged heat incubation, whereas at these temperatures the CLEA-GL was relatively stable. Because immobilization and cross linking provided more rigid external backbone for enzyme molecules, the effect of higher temperatures in breaking the interactions that were responsible for the proper globular, catalytic active structure, became less prominent, thus increasing the thermal stability of the immobilized glucosidase.

The thermal stability of the crosslinked enzyme preparations enhanced considerably, as compared with that, of the native enzyme. Free β-glucosidase retained less than 30% of its initial activity after treatment at 60°C for 3h while CLEA-GL retained more than 50% of its initial activity. CLEA-GL retained more than 60% activity at 65°C while the free enzyme retained only 30 % activity after 3h heat treatment.

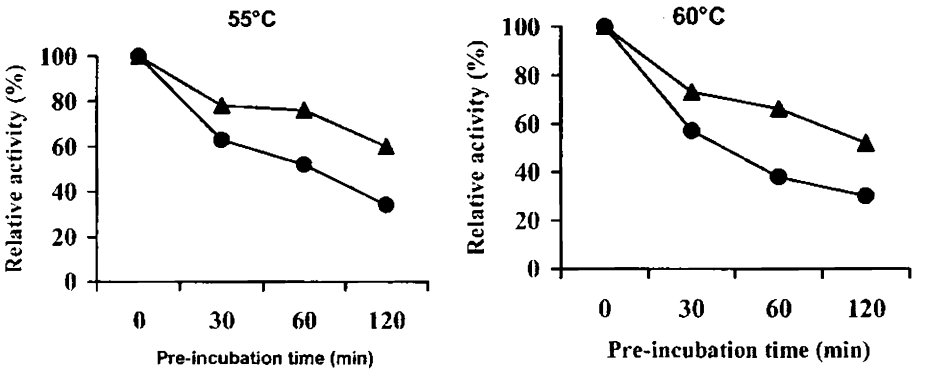


Fig 7.12 Effect of temperature on enzyme stability (•) free β-glucosidase (▲) CLEA-GL

The immobilized β-glucosidase on chitosan–clay composite composites could retain more than 60% activity at higher temperature when compared to free enzyme which retained only 30% activity [11]. On the other hand, the high activity of immobilized enzymes at low temperatures is probably a result of favored adsorption of enzymes on activated clay [54]. An important increase in thermal stability was observed when less stable β-glucosidases (from T. reesei, N. crassa and almonds) were immobilized [55]. The major factor in this contrasting result was probably related to the different microenvironment for the enzyme provided by the different supports.

7.6.6 pH stability of free and immobilized enzymes

The stability profiles for free enzyme and the crosslinked enzyme aggregrates are shown in Figure 7.13. The crosslinked β-glucosidase retained more than 50% of its original activity at pH values 5.5 and 6 after incubation for 5h, and it maintained more than 70 % of its activity after 1h compared to the free enzyme which retain only 40% activity after 5h incubation in the respective pH solutions. CLEA-GL was less sensitive to pH changes than the native enzyme. Free enzyme demonstrated maximum activity at pH 6 and hence its optimum value. The optimum pH of CLEA-GL is 6.5. Extremes of pH will however cause a time-dependent, essentially irreversible denaturation in the case of free enzyme.

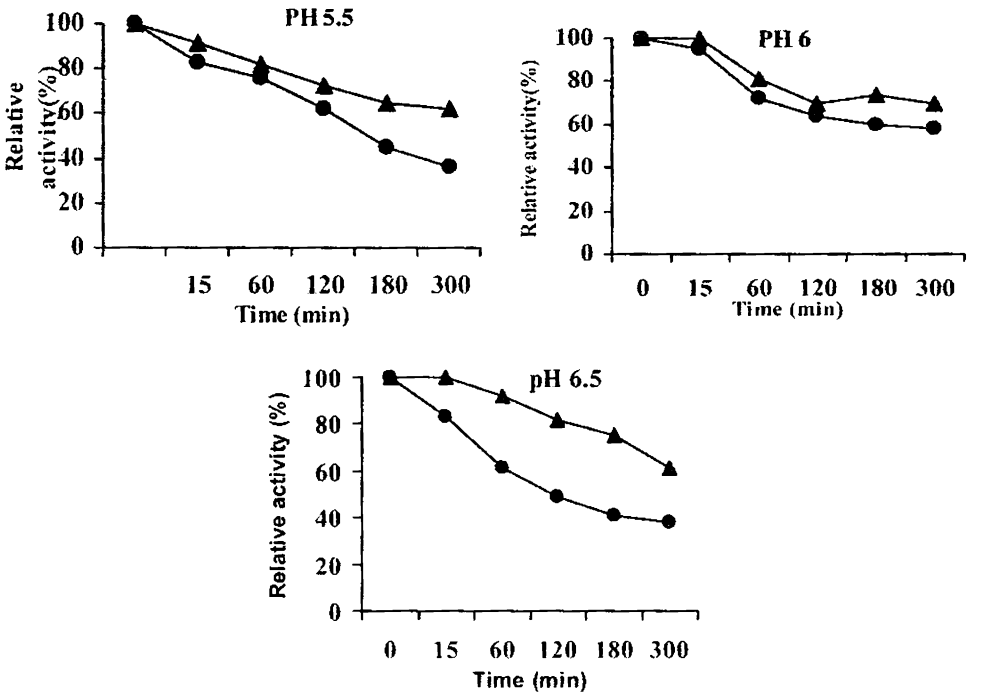


Fig 7.13 pH stability of (•) free and (▲) CLEA-GL at different pH at 25°C

The stability of the enzyme against higher pH was significantly improved by immobilization. This is more evident when the enzyme is immobilized since the support itself may change the pH value around the catalytic site. This effect is known as the partitioning effect, directly related to the chemical nature of the supporting material that induces electrostatic or hydrophobic interaction between the matrix and the molecular species dissolved in the solutions. The resulting CLEA's hold excellent adaptability in a wider and higher pH compared with free enzyme. It is known that polyionic matrices cause the partitioning of protons between the bulk phase and enzyme microenvironment causing a shift in the optimum pH value [56]. The adaptability should depend on the method of immobilization, as well as the structure and charge of the carrier [57, 58], furthermore, the screen function of the structure would play an important role in enhancement the adaptability.

In studying the remaining activities of free and immobilized laccase on magnetic chitosan microspheres, Jiang et al. [59] have found that the immobilized laccase is stable in the pH range 5.0–6.0 while free laccase is stable in the pH range 7.0–9.0. This indicates that the immobilization appreciably improves the stability of laccase in the acidic region.

7.6.7 Effect of various cations and chemicals

To inspect whether metal ions are essential to glucosidases some univalent and bivalent ions were selected to prepare the reaction buffer. Table 7.4 shows the effects of metal ions on the relative activity towards PNPG.

PNPG hydrolysis was increased by 10% in the presence of Co^{2+}, Mn^{2}+, Mg^{2+}. However, Ca^{2+}, Cd^{2+}, Zn2+ and Fe^{2+} had no effect on the stability of the enzyme. The enzyme was indeed greatly inhibited by Ag^{-}, Cu^{2+} and Hg^{2+}. This may indicate that thiol groups are involved in the active catalytic site. However, Mn^{2+} did significantly stimulate enzyme activity. Cu showed an even more pronounced inhibitory effect. $CaCl_2$, $ZnCl_2$, $FeCl_2$ and EDTA had little effect on the activity.

Table 7.4 Effect of various metal cations and chemicals on the activity of β-glucosidase and CLEA-GL

Cation/chemical	Sample (Relative activity (%))			
	1mM		50mM	
	Free	CLEA-GL	Free	CLEA-GL
Control	100	100	100	100
$MnCl_2$	109.5	98	101.3	84
$CaCl_2$	91	85	87	80
$CdCl_2$	98.3	62	78	48
$CuCl_2$	48	43	22	17
$CoCl_2$	109.9	96.5	103.8	72
$HgCl_2$	4.8	3.1	0	0
EDTA	97.9	81	94.7	78
$MgCl_2$	111.5	92	98	83
$ZnCl_2$	89	83	77	74
$AgNO_3$	3.1	1	0	0
$FeCl_2$	72	62	66	57

The enzyme activity depends on the protonation of the amino acids at the reactive site and that enzyme activity is inhibited by complexation of the reactive sites by the heavy metal cations. Activation by Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, may be explained by stabilization of the enzyme structure [60]. Divalent cations such as Ca^{2+} and Mg^{2+} at 1mM concentrations in the reaction media almost showed not much influenced on activity of the immobilized β-glucosidase while at 50 mM, there was a significant decrease when compared to free enzyme (50mM) which may be due to the destabilization effect of metal salts on enzyme structure after immobilization. There was not much effect on free enzyme with higher concentration of salts used except in the case of $CuCl_2$, $AgNO_3$ and $HgCl_2$ where there was complete inactivation.

The mechanisms of the effect of these metal ions on the enzyme activity are not known. α-glucosidases and β–glucosidase could catalyze the synthesis of glucosides in lower yields without metal ion which indicated that metal ions are not essential for enzymatic synthesis of glucosides in the study carried out by Wang et al.[61]. KCl and $MgSO_4$ had little effect on the two β-glucosidases from almond and apple seed at 5mM concentration while $CaCl_2$, $MnCl_2$, $CoCl_2$ and $ZnSO_4$ inhibited the enzymes to some extent while heavy metal salts, such as $CuSO_4$ and $AgNO_3$, severely inhibited the activity [62]. The effects of various cations and reagents on β-glucosidase from Aspergillus Niger activity were investigated by Riou et al. and it was found that there significant inactivation with Ag^{+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} and slight stimulation by Mn^{2+} while Co^{2+} and Ca^{2+} had no activity dependence [63].

7.6.8 Activation by alcohols

The effect of alcohols on the activity of beta glucosidase activity is shown in Table 7.5. Methanol and ethanol increased the activity of beta glucosidase by 1.1 fold and 1.3 fold while propanol and butanol increased the activity by 1.6 and 2 fold (0.5M). However ethanol, propanol and butanol inhibited the activity of the enzyme at above 1-2 M.

Table 7.5 Effect of various alcohols on the activity of free β-glucosidase and CLEA-GL

Alcohol	Conc (1 M) (Relative initial rate of hydrolysis (%)		Conc (0.5M) (Relative initial rate of hydrolysis (%)	
	Free enzyme	CLEA-GL	Free enzyme	CLEA-GL
Control	100	100	100	100
Methanol	93	35	111.3	98
Ethanol	115.2	58	127.5	103.3
Propanol	98	66	160.3	130.7
Butanol	102.7	78	200	170.8
Pentanol	109.4	80	204.9	185.4
Hexanol	132.8	89	218.1	189.3

A 2 fold increase was observed in the case of pentanol and hexanol at lower concentrations of alcohol. Activation by alcohols has been observed for β-glucosidases from Candida entomophil [64] and fusarium oxysporum [65]. Such a phenomenon as described by Pamberton et al. [66] is the result of glycosyl transferase activity. At higher concentrations of alcohols the enzymes were inhibited probably because of protein denaturation. In the case of CLEA-GL though activation was observed at low alcohol concentration the increase was much less when compared to the free enzyme.

As the chain length of the alcohol was increased, the rate of release of p-nitrophenol also increased. Maximal activity was induced by the presence of hexanol. Enzyme activation is a function of the length of the alkyl chain. At 70°C (pH 3.5), an approximately 225% increase in relative activity was observed in the presence of hexanol when compared to the activity in the absence of alcohol and around 170% activation at 30°C (pH 7.5) was observed. in the study carried out by Goyal et al. [67].

7.6.9 Kinetics of enzymatic reactions

The time changes of product concentration (*p*-nitrophenol) at different substrate concentrations, S_0 (*p*-nitrophenyl β-D glucopyranoside, PNPG) for free β-glucosidase and CLEA-GL are shown in Fig. 7.14 and Fig 7.15. The slope of the straight line is the initial rate of reaction V (mM/min). The relation between the rate of reaction and substrate concentration can be described by the Michaelis–Menten equation [55, 68].

$$V = \frac{V_{max} S}{K_M + S}$$

where V_{max} is the maximum rate of reaction and K_M is the Michaelis constant (mM). Figure 8.16 shows Lineweaver-Burk plots of data obtained in experiments where the rates of the reactions catalysed by native and crosslinked β-glucosidases were monitored at varying pNPG concentrations.

$$\frac{1}{V} = \frac{1}{V_{max}} + \left(\frac{K_M}{V_{max}}\right)\frac{1}{S}$$

The values of V_{max} and K_M by free and immobilized β-glucosidase at 25°C are listed in Table 7.6. The reaction follows Michaelis-Menten kinetics over the range of substrate concentrations studied. The K_m values of the crosslinked enzyme (8.2mM) obtained were higher than those of the free enzyme (5.9mM), suggesting that the crosslinked network limited the permeation rate of substrate and product. This means that internal diffusional effects are mainly responsible for the increase of Michaelis constants. The reduction in affinity of the enzyme may be due to the conformational changes on immobilization.

Table 7.6 The maximum rate of reaction (V_{max}) and the Michaeli's constant (K_m) using free and immobilized β-glucosidase (S_0=0.25-2.5mM PNPG)

Sample	V_{max} (mM/min)	K_m (mM)	R^2
Free enzyme	0.0135	5.9	0.9989
CLEA	0.0120	8.2	0.9970

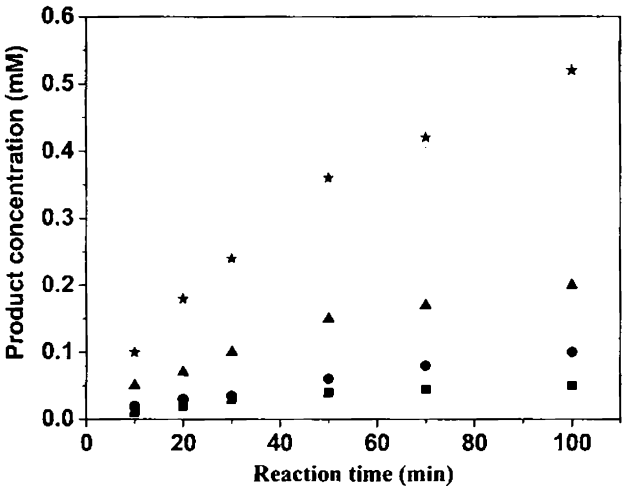


Fig 7.14 Time profiles of catalytic reaction of free β-glucosidase under different substrate concentrations at 25°C and pH 4.8 (acetate buffer).S_0(mM)- (■)-0.25, (●)-0.5, (▲)-1, (★)-2.5

On the other hand, the V_{max} of the immobilised enzyme (0.0120 mM/min) was smaller than those of the native enzyme (0.0135mM/min). The decrease in specific activity and increase in K_m value due to mass transfer and diffusional limitations has been reported by several authors [69, 70, 71].

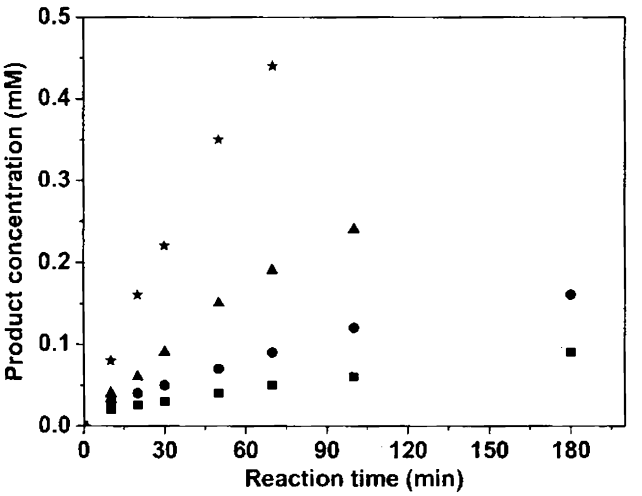


Fig 7.15 Time profiles of catalytic reaction of CLEA-GL under different substrate concentrations at 25°C and pH 4.8 (acetate buffer). S_0(mM)- (■)-0.25, (●)-0.5, (▲)-1, (★)-2.5

Probably, the change in affinity of the enzyme to its substrate is caused by structural changes in the enzyme introduced by the immobilization or by lower accessibility of the substrate to the active sites of the immobilized enzyme. The reduced V_{mac} value can be explained by the reduced flexibility and deformation of β-glucosidase enzyme molecules after being crosslinked. The increased K_m, value can be attributed to the increased mass-transfer limitation for the substrate in MCF silica containing CLEA–GL.

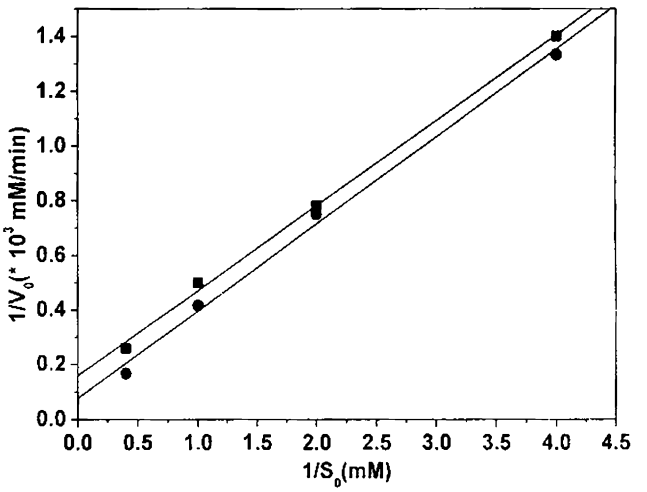


Fig 7.16 The Lineweaver–Burk plot of catalytic reaction using (■) β-glucosidase and (●) CLEA-GL .

The K_m value found for vanilla β-glucosidase was 3.3 mM for PNPG and a V_{max} =11.5 IU mg^{-1} protein) in the kinetics study carried out by Dignum et al. towards p-nitrophenol [72]. Lineweaver-Burk plots of free and immobilized beta-glucosidase on chitosan gave K_m values of 2×10^{-4} and 55×10^{-4}M with PNPG [73]. The K_m value remained the same (0.248×10^{-3}M) and V_{max} value was found to be slightly lower in the case of beta glucosidase immobilized on sandy alumina in the work done by Fadda et al. [74]. The immobilization of beta-glucosidase on polyacrylamide gel had no effect no effect on K_m values (4-15mM) while the K_m value increased by a factor of 1.20 when immobilized in alginate beads. The V_{max} values were 51.9% and 35.4% smaller for the immobilized supports compared to the free enzyme [75].

7.6.10 Operational stability of immobilized enzymes

Fig. 7.17 shows the activity of free β-and CLEA-GL after repeated use. A good operational stability is one of the most important criteria for industrial utilization of immobilized biocatalyst. To evaluate the reuse stability, the crosslinked systems were washed with sodium acetate buffer (pH 4.8) after any run and reintroduced into a fresh solution, this being repeated up to 10 cycles. The crosslinked enzyme aggregrates retained more than 85% activity even after the 10^{th} cycle. Besides that, no activity was found in the buffer solution containing the crosslinked aggregrtaes due to the highly stable and active preparation, which meant that there was no enzyme released from the support. The higher stability of the crosslinked aggregrates might be due to the crosslinking of the enzyme with glutaraldehye which creates a more stable preparation than the adsorbed couterpart.

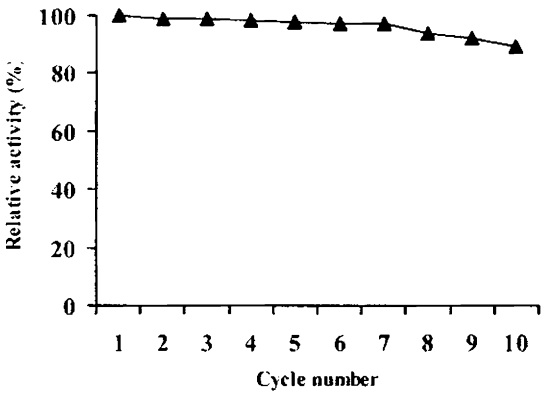


Fig 7.17 Effect of repeated use capability on the activity of (▲) CLEA-GL

The activity of the enzyme immobilized on dried composite is 1.83 and 1.65 times higher than those on wet chitosan and wet composite after 50-times reuse, and maintains 74% of the activity at the second operation [11]. Bickerstaff [76] reported that immobilization restricts the movement of the backbone and side chains of the enzyme molecule thereby preventing intermolecular interaction and unfolding of the polypeptide chain. This advantage increases the stability of the enzyme and retains its activity after repeated use. The loss in activity after some cycles is due to the natural inactivation of enzyme as a result of time dependent denaturation of the enzyme protein.

7.6.11 Storage stability of free and immobilized enzymes

Fig. 7.18 shows the storage stability of free and immobilized β-glucosidase at 4°C for 60 days. More than 85% of the original activity is lost for free enzyme after 10 days when stored in the optimum buffer solution. In the free form, it has a short life and therefore its use is restricted. After immobilization, the life of enzyme improves and therefore it can be kept for long- term usage. CLEA-GL could retain almost 100% activity after 60 days storage. The immobilization procedure stabilized the enzyme substantially.

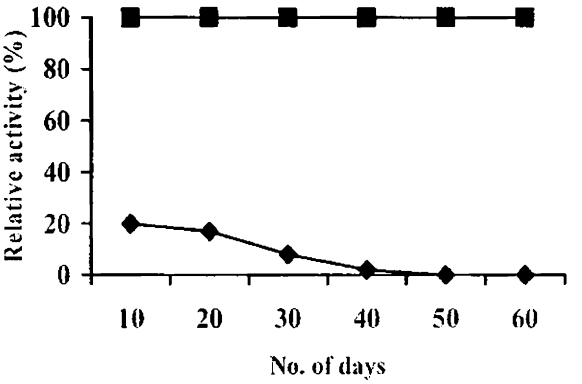


Fig 7.18 Storage stability of (●) free β-glucosidase and (▲) CLEA-GL in pH 4.8 at 4°C for 60 days

The activities of wet and dried-composite immobilized beta glucosidases are 4.9 and 10.4 times higher than that of free enzyme, respectively. However, the wet and dried composite beads swell and break apart after being stored for 10 days at 4°C [11]. In the work of Cetinus and Oztop [77], catalase stored at 5°C was more stable than at 25°C. Free enzyme retained 50% of its activity after 18 days, the immobilized catalase stored wet (in buffer) retained about 50% of its activity level for 25 days and when stored dry (at room temperature) retrained about 50% of its activity level for 5 days. In the word done by Roy et al., immobilized beta-glucosidase on polyacrylamide and cyanogens bromide activated sepharose gel beads [78], the soluble enzyme lost all its activity after 6 days storage at room temperature while the immobilized enzymes were more stable for a long period of time.

7.7 Conclusions

Mesocellular silica foams was used as the immobilization supports for β-glucosidase which was then crosslinked with glutaraldehyde to obtain Crosslinked enzyme aggregrates (CLEA's) of β-GL in MCF's.

The main conclusions from the present study on immobilization of β-glucosidase are as follows:

- Mesocellular silica foams was synthesized by microemulsion templating technique and were used for the successful development of Crosslinked enzyme aggregrates (CLEA's) of beta glucosidase.
- The decrease in specific surface are, pore diameter and pore volume suggests the incorporation of enzyme and aldehyde molecules into the mesopores. The incorporation was confirmed from TG, FTIR and NMR studies.
- Immobilization broadened the pH optimum and the temperature dependant activity profile of the enzyme. The results confirmed that immobilization of β-GL on MCF imparts greater resistance to environmental factors such as pH, temperature etc without appreciable loss of activity.
- CLEA-GL in MCF showed negligible activity decrease after 48h incubation in harsh shaking condition due to the prevention of enzyme leaching as well as the enzyme stability attained by multipoint covalent linkages between enzyme molecules.
- The K_m values of the crosslinked enzyme were higher than those of the free enzyme, suggesting that the there is a diffusion imparted to the substrates and products by the crosslinked network.
- CLEA-GL exhibited superior operational stability and good storage stability.
- Our approach using glutaraldehyde for the purpose of crosslinking captures very high enzyme loadings and prevents leaching, providing a more stable

and active immobilized enzyme system than those obtained by simple adsorption and covalent binding.

- The immobilized β-glucosidase was found to be very stable and this system described is an example of the use of a biocatalyst for the flavor enrichment of beverages that could be employed in industry.
- Siliceous mesostructured cellular foams show great potentials as carrier materials for enzymes to give very active biocatalysts, and in that they well exceed the most conventional silica gels and perhaps also popular polymeric carriers which opens up a new possibility for enzyme stabilization and will contribute to a variety of enzyme applications in biosensors, bioremediation, and bioconversion.

References

[1] M. P. Dale, W. P. Kopfler, I. Chait, L. D. Byers, Biochem. J., 25 (1986) 2522.

[2] H. T. Hang, Agric. Food Chem., 3 (1995) 141.

[3] Z. Gunata,, S. Bitteur, R. Baumes, J. C Sapis, C. Bayonove, Rev. Fr. OenoL., 122 (1990) 37.

[4] O. Shosheyov, B. A. Bravdo, R. Ikan, J. Agric. Food Chem., 38 (1988) 1387.

[5] Y. Z. Guanata, C. L. Baynove, R. E. Cordonnier, A. Arnaud, P. Galzy, J. Sci. Food. Agri., 30 (1990) 499.

[6] Y. Vasserot, A. Arnaud, P. Galzy, Bioresour Technol., 43 (1993) 269.

[7] F. Biset, Sternberdg, Appl. Environ. Microbiol., 35 (1978) 750.

[8] Venardovs, E. Klei, W.Sundstromd, Enzyme Microbiol. Technol., 2 (1980) 112.

[9] M. Rao, Deshpandev, C. H Misha, Biotechnol. Lett., 5 (1983) 75.

[10] M. Y Chang, H. C. Kao, R. S. Juang, Int. J. Biol. Macromol., 43 (2008) 48.

[11] M. Y. Chang, R.S. Juang, Biochem. Eng.J., 35 (2007) 93.

[12] G. Spagna, R. N. Barbagallo, P. G. Pifferi, R. M. Blanco, J. M. Guisan, J. Mol. Catal B: Enzym., 11 (2000) 63.

[13] G. Gieger, H. Brandl, G. Furrer, Soil Biol.Biochem., 30 (1998) 1537.

[14] H. Quiquampax, J. Abadie, M. H. Baron, Mechanisms and consequences of protein adsorption on mineral surfaces Pn:Proteins at interface II: Fundamentals and Applications. (eds:T.A.Horbertt et al) 321-333., American Chemical Society, Symposium series No.602.

[15] A. Martino, R G. Pifferi , G. Spagna, Process Biochem., 31 (3) (1996) 287.

[16] G. Matthijs, E. Schacht, Enzym. Microb. Technol., 19 (1996) 601.

[17] M. B. Fadda, M. R. Dessi, A. Rinaldi, Biotechnol. Bioeng., 33 (1989) 777.

[18] O. V. Lomako, 1. I. Menyailoval, A. Nakhapetiakl, I. Kozlovskayka, Rodioxova, Acta biotechnol., 2 (1982) 179.

[19] J .Woodward, J. Biotechnol., 11 (1989) 299.

[20] C. David, M. A. Neish, D. Baeyans-Volant, Nouv. J.Chim., 8 (1984) 701.

[21] D. W. Sundstrom, H. E Klei, R. W. Coughlin, G. J. Biederman, Biotechnol. Bioeng., 23 (1981) 473.

[22] M. Rao, V. Deshpande, C. Mishra, Biotechnol. Lett., 5 (1983) 75.

[23] C. H. Wong, G. M. Whitesides, Enzymes in Synthetic Organic Chemistry; Pergamon: Tarryton, New York (1994).

[24] L. Cao, F. van Rantwijk, R.A Sheldonm, Org Lett., 2 (2000)1361.

[25] R. Schoevaart, M. W. Wolbers, M. Golubovic, M. Ottens, R. A. Sheldon, Biotechnol Bioeng., 87 (2000) 754.

[26] N. St. Clair, M. Navia, J. Am. Chem. Soc., 114 (1992) 7314.

[27] J. Lalonde, Chemtech., 15 (1997) 38.

[28] A. L. Margolin, Trends Biotechnol., 14 (1996) 223.

[29] Lee, Gowoun, H. Jool, J. Kim, J. H. Lee, J. Microbiol. Biotechnol., 4 (2007) 17.

[30] M. E. Davis, Nature, 417 (2002) 813.

[31] J. Lee, K. Sohn, T .Hyeon, J .Am. Chem. Soc., 123 (2001) 5146.

[32] C. Lei, Y. Shin, J. Liu, E. J. Ackerman, J Am Chem Soc.,124 (2002) 11242.

[33] P. Schmidt-Winkel, W. W. Lukens Jr., D. Zhao, P.Yang , B. F. Chmelka, G. D.Stucky, J Am Chem Soc., 121 (1999) 254.

[34] Moon Il Kim, J. Kim, J. Lee, S. Shin, H. N. Chang, Microporous Mesoporous Mater., 111 (2007) 18.

[35] P. Hara, U. Hanefeld, L. T. Kanerva, J. Mol. Catal B: Enzym., 50 (2008) 80.

[36] J. Lee, J. Kim, J. Kim, H. Jia, M.Il Kim, J. H. Kwak, S. Jin, H. N Alice, P. Chang, J.Porous Mater., 1(7) (2005) 744.

[37] Moon Il Kim, J. Kim, J. Lee, H. Jia, H. Bin Na, J. K. Youn, H. G. Park, Biotechnol. Bioeng., 96 (2007) 210.

[38] M. S. Doscher, F. M. Richards, J. Biol. Chem., 238 (1963) 2399.

[39] D. Haring, P. Schreier, Curr.Opin. Biotechnol., 3 (1999) 35.

[40] J. Lee, J. Kim, J. Kim, H. Jia, M. I. Kim, J. H. Kwak, S. Jin, A. Dohnalkova, H. G. Park, H. N. Chang, Small, 1 (2005) 744.

[41] V. V. Mozhaev, N. S. Melik-Nubarov, M. V. Sergeeva, V. Siksnis, K. Martinek, Biocatal., 3 (1990) 179.

[42] P. S. Winkel, W. Wayne, P. Yang, I. D. Margolese, J. S. Lettow, J. Y. Ying, G. D. Stucky, Chem. Mater., 12 (2000) 686.

[43] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. J. Randall, J.Biol.Chem., 193 (1951) 265.

[44] S. J. Gregg, K. S. W. Sing, In Adsorption, Surface Area and Porosity; Academic Press: New York (1982).

[45] A. Garcia, S .Oh, C. R. Engler, Biotechnol. Bioeng., 33 (1989) 321.

[46] Y. Hsuanyu, K. J. Laaidler, Canadian. J. Biochem. Cell Biol., 63 (1991) 167.

[47] M. A. Abdel-Naby, Appl. Biochem. Biotechnol., 38 (1993) 69.

[48] T. R. Yan, C. L. Lin, Biosci. Biotechnol.Biochem., 61 (1997) 965.

[49] J. P. Lenders, P. Germain, R. R. Crichton, Biotechnol. Bioeng., 27 (1985) 572.

[50] T. Mori, T. Sato, T. Tosa, I. Chibata, Enzymol., 43 (1972) 213.

[51] M. S. Carrasco, J. C. Rad, S. Gonzilez-Carcedo, Bioresour. Technol., 51 (1995) 175.

[52] Y. Yabushita, Biotechnol. Appl.Biochem., 10 (1988) 294.

[53] S. A. Cetinus, H. N. Oztop, Enzyme Microbial Technol., 32 (2003) 889.

[54] M. Y. Chang, R. S. Juang, Enzyme.Microb. Technol., 36 (2005) 75.

[55] R. Woodwarjd, Radford, Rodriguelz, A. Guadoj, D. Romerom, Acta Biotechnol., 12 (5) (1992) 357.

[56] M. Y. Arica, Y. Handan, S. Patir, J. Mol Catal B: Enzymol., 11 (2000) 127.

[57] M. Y. Arica, H. A. Oktem, Z. Oktem, A. Tuncel, Polym Int., 48 (1999) 879.

[58] T. Uhlich, M. Ulbricht, G. Tomaschewski, Enzyme Microb. Technol., 19 (1996) 124.

[59] D. S. Jiang, S. Y. Long, J. Huang, H. Y. Xiao, J. Y. Zhou, Biochem. Eng. J., 25 (2005) 15.

[60] A. Karnchanatat, A. Petsom, P. Sangvanich, J. Piaphukiew, S. Anthony Whalley, C. D. Reynolds, FEMS Microbiol Lett., 270 (2007) 162.

[61] R. Wang, X. Yanga, R. Gao, Y. Yanga, Xi. Wanga, S. Cao, J. Mol. Catal B: Enzym., 56 (2009) 131.

[62] H. L. Yu, J. H. Xu, W. Y. Lu, G. Q. Lin, Enzyme Microb.Technol., 40 (2007) 354.

[63] C. Riou, M. Salmon, M. J. Vallier, Z. Günata, P. Barre, Appl. Environ. Microbiol., 64 (1998) 3607.

[64] Y. Gueguen, P. Chemardin, P. Galzy, A. Arnaud, Enzyme.Microb.Technol., 17 (1995) 90.

[65] P. Christakopoulos, P. W. Goodenough, B. J. Macris, K. Bhat, Eur. J. Bio. Chem., 224 (1995) 379.

[66] M. S. Pamberton, R. D. Brown, G. H. Emert, Can. J. Chem. Eng., 58 (1980) 723.

[67] K. Goyal, P. Selvakumar, K. Hayashi, J. Mol. Catal. B: Enzym., 15 (2001) 45.

[68] G. Delanoy, Q. Li, J. Yu, Int. J. Biol. Macromol., 5 (2005) 89.

[69] J. Woodward, R. Krashaik, D. Smith, G. S. Zachry, Biotechnol. Bioeng. Symp., 11 (1981) 619.

[70] R. A. Muzzarelli, A. G. Barontini, R. Roccheti, Biotechnol. Bioeng., 18 (1976) 1445.

[71] M. E. Young, P. A. Carroaad, Biotechnol.Bioeng., 11 (1980) 947.

[72] M. J. W. Dignuma, R. van der Heijdenb, J. Kerlerc, C. Winkelc, R. Verpoortea, Food Chem., 85 (2004) 199.

[73] J. D. Desai, R. Ray, A. Desai, Ferment.Technol., 64 (3) (1986) 255.

[74] M. B. Fadda, M. R. Dessi, A. Rinaldi, G. Satta, Biotechnol.Bioeng., 33 (1989) 777.

[75] N. Ortega, M. D. Busto, M. P. Mateos, Bioresour. Technol., 64 (1998) 105.

[76] G. Bickerstaff, J. Biochem., 11 (1980) 163.

[77] S. A. Cetinus, H. N. Oztop, Enzyme Microbial Technol., 26 (2000) 497.

[78] S. K. Roy, S. K. Raha, S. L. Chakarabarty, Enzyme.Microb.Technol., 11 (1989) 43.

Chapter 8

ADSORPTION BEHAVIOUR OF LIPASES ON DIFFERENT SUPPORTS: A COMPARISON STUDY

Contents

Carriers with different physico-chemical properties were employed in order to obtain physical adsorption of Candida Rugosa lipase. The present study compares the results of lipase immobilization on the pure and functionalized samples of mesocellular silica foams with respect to their enzyme loading, activities, binding capacity and coupling. The stability of these systems in terms of leaching studies was evaluated. The results were compared with the conventional supports like silica gel and alumina and also the reported values from literature. It seems that the binding affinity for lipase is much pronounced for adsorbed systems rather than covalently bound ones. The results showed that MCF 160 has a high loading capacity and strong binding ability for lipase. Thanks to the low mass transfer resistance, the mesocellular siliceous silica has greatly enhanced the rate of immobilization. The amount of lipase activity adsorbed on these supports was related to the pore size of the silicates. As a comparison, the immobilized lipase activity was much higher than that of many frequently used porous materials like alumina and silica gel. The search for an inexpensive support has motivated our group to undertake this work dealing with the selection of potential inorganic matrices which can be recognized by lipases, at molecular level, as solid surfaces. The main goal of this study is to investigate the potential of mesoporous silica's as hosts for lipase immobilization. We have explored the effect of pore structure on enzyme immobilization and catalytic activity.

8.1 Screening of supports for enzyme immobilization

For enzyme immobilization, it is very important to choose a proper support since its interaction with enzyme may have a great influence on the stability and kinetics of the enzyme. There are various supports available for enzyme immobilization, which can be classified into three general types: inorganic particles, synthetic polymers and natural macromolecules. The natural macromolecules, such as chitosan, gelatin, cellulose, agarose and carrageenan, are biocompatible and non-toxic for enzyme immobilization. Nevertheless, the weak mechanical stabilities of these natural materials have limited their applications. On the contrary, the inorganic particles and synthetic polymers, for example, porous glass, magnetic iron oxide, polypropylene, polyacrylonitrile and nylon normally possess good mechanical stability, but are relatively non-biocompatible. Therefore, it is still needed to fabricate perfect supports for enzyme immobilization.

The success and efficiency of physical adsorption on the enzyme on a solid support depends on several parameters: the size of the protein to be adsorbed, the specific area of the carrier and the nature of its surface, in terms of functional groups, porosity, and pore size are crucial. According to some authors, a convenient pore size is around 100 nm for lipase immobilization [1, 2]. More suitable pores may be in the diameter range of 50~100 nm because of their excellent comprehensive behavior in enzyme loading and internal diffusion coefficient [3]. Catalytic efficiency depends on the equilibrium of these three parameters: monolayer enzyme arrangement, pore size (assuming homogeneous distribution) and surface area.

The amount of adsorbed enzyme per amount of support increases with the enzyme concentration, reaching a plateau at the saturation of the carrier: different adsorption isotherms are obtained for different enzyme–carrier systems. Many supports have been studied including polymers and resins [4], silica and silica-alumina composites [5], and carbonaceous materials [6]. These systems generally have a low mechanical strength and often exhibit severe diffusion limitations,

leading to a relatively low enzymatic activity [7, 8]. To minimize the internal diffusion limitation, porous supports are mostly used in particulate form [9]. Silica and other inorganic materials with pore diameter of around 8 nm, allow only the immobilization of small enzymes within the pores. The larger ones can only be placed on the external surface of the support particles [11]. The use of meso- or macroporous supports decreases these unwanted effects and the derivatives usually retain higher activities and have higher enzyme loading [11]. Mesostructured foam-like silica was more favorable primarily because of its low diffusion limitations. Featuring with an open porosity and ultra large pore size, mesocellular silica foams (MCF) have attracted great recent attention as new promising adsorption and catalytic materials owing to their highly accessible and open porosity by providing more favorable conditions for mass transfer compared to their conventional mesoporous counterparts [10, 11].

On account of the relatively high surface hydrophobicity of lipases, simple adsorption of lipases on suitably hydrophobic supports has been the more popular strategy over covalent conjugation methods [12, 13]. This method [14] has proven very useful to achieve hyperactivation of most lipases: the hydrophobic surface of the matrix induces the conformational change on lipases necessary to enable free access of substrates to their active centers.

By choosing different carriers, it could be possible to obtain different effects on the "open-lid" conformation of the immobilized lipase; discovering the connection between structure of the carrier and achieved effect is essential to understand the lipase activation mechanism. Our work was focused on the physical adsorption of Candida rugosa lipase (CRL), a fungal lipase, on pure mesocellular silica foams and clay as well as on glutaraldehyde modified supports. On these basis, we propose the use of a rational approach to obtain immobilized lipase on mesocellular silica foams and clay and to compare the binding capacity with the conventional supports like silica gel and alumina. The present study reveals the

superior properties of mesocellular silica foams in protein adsorption and enzymatic catalysis and paves the way to the technological applications.

8.2 Experimental

8.2.1 Immobilization of lipase by adsorption

Lipase solutions (2-20 μg/ml) were prepared by adding appropriate amounts of lipase powder to phosphate buffer solution (0.1 M, pH 7.0) which were then added to 100mg of the supports. After this, the supports with the lipase solution are shaken gently in a water bath at 30°C for 24 h. Finally, the supports were taken out 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 [15]. 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.

8.2.2 The leaching at different time intervals

In a typical adsorption experiment, 40mg of silica and clay supports was dispersed to 20ml lipase buffer solution ($2mgml^{-1}$) in a vessel covered to prevent evaporation. The mixture was stirred at 30°C for 24 h. The enzyme-leaching test was performed by subjecting the immobilized enzymes suspended in 0.1M phosphate buffer at pH 7 in a vessel (covered to prevent evaporation) to harsh shaking condition [16]. Then, the leaching of enzyme from the mesoporous supports was measured. The leaching of the immobilized lipase can be determined by the lipase content of the supernatant at different time intervals.

8.3 Lipase adsorption isotherms

To assess the performance of the mesocellular silica foam, some typical commercial inorganic supports like silica gel and alumina were compared. The lipase was adsorbed on each of these supports under the same condition. The

microenvironment created by amino functionalities activated with glutaraldehyde was very positive and significant in the preferential binding of lipase.

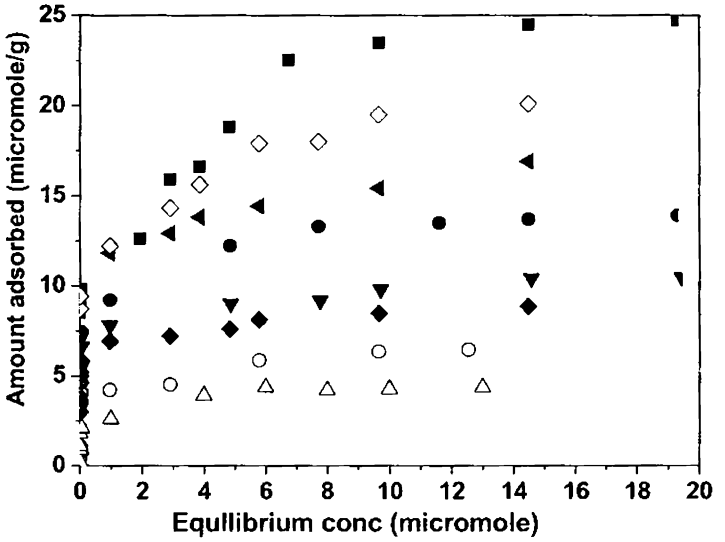


Fig 8.1 Adsorption isotherms of lipase onto various supports
■- MCF160, ◊-MCF160G, ◄-MCF35G, •-KI-10, ▼-alumina, ♦-MTI, ○-K-10G, Δ-silica gel

It was mainly owing to the hydrophobic surface property in the covalently bound systems. The study of Bastida et al. [12] indicated that hydrophobic supports have a purifying function in lipase immobilization through selective adsorption. The more suitable pore structure also contributes to the enhancement of the immobilized-lipase activity. For each support, an increment of protein concentration enhances the driving force for the adsorption and increases the adsorbed protein amount correspondingly. Furthermore, when the protein concentration is higher than 20 μmol, the increase slows down gradually with increasing protein concentration.

The absorption capacity of lipase for the various supports is in the order H_{MCF}>HG_{MCF}> RG_{MCF} > KI-10> alumina> MTI> K-10G> silica gel. The grade of affinity of the enzyme molecules for each carrier was evaluated from the adsorption isotherms of lipase on clay, mesocellular silica foams and conventional supports (silica gel and alumina).

The adsorption efficiency of proteins on the pure and the functionalized supports were studied. The adsorption isotherms of lipases on various supports are shown in Fig. 8.1. The maximum amounts of lipase adsorbed on MCF 160, MCF 35, KI-10, MCF160G, MCF35 G and K-10G were 24, 6.4, 14, 20, 9 and 17μmol/g support. The amount of protein bound onto silica gel and alumina are 4.3μmol/g and 11μmol/g. Silica gel exhibits a very low loading of the protein. In the case of alumina, due to its surface charge it exhibited higher adsorption capacity than MCF35. Maximum adsorption was achieved with MCF160 after 24 h (24 μmol lipase/g). In agreement with the kinetic studies, in which MCF160 displays a higher affinity for lipase (24μmol/g), the isotherm exhibits a sharp initial rise and finally reaches a plateau (L-type). Slightly less lipase was adsorbed onto MCF160G (20μmol/g). As MCF has a pore diameter of 161 Å compared to 98 Å for HG_{MCF}, the relative binding is not likely to be limited by the access to the pores but more related to the available total surface area of mesoporous material.

MCF 160 has a much reduced surface area of 595 m^2/g compared to that of MCF35 with surface area of (915 m^2/g). It has been recently reported that the surface of the pores with smaller pore diameter cannot be utilized in adsorption and the fractional coverage of the small pore surface may depend on the length of the diffusion path. In the adsorbent with smaller pores, R_{MCF}, pore blocking may occur due to aggregation of two or more lipase molecules. Consequently, the long diffusion path in the small mesopores will result in a greater probability for pore blocking to occur, and, thus, a smaller loading is obtained in R_{MCF}. Though the surface area and pore diameter of montmorillonite K-10 is less compared to R_{MCF} it has greater affinity for lipase and hence had higher adsorption capacity. This may be due to the different types of interaction which binds the lipase into clay much more strongly than that of R_{MCF}. Each isotherm is characterized by a sharp initial rise, suggesting a high affinity between the lipase molecule and the adsorbent surface. However, in the case of H_{MCF}, the large pore diameter facilitates the diffusion of the lipase molecules from the mesopore entrance to the interior part of

the silica, and pore blocking might not occur. Hence, it is tentatively assumed that the higher lipase adsorption capacity of H_{MCF} silica is due to the larger pore diameter, which allows full access of the mesopores by the lipase molecules. R_{MCF} and silica gel showed less affinity for enzymes. Compared with lipase dimensions (40Å), it can be observed that the enzyme was immobilized on the external area of the support in the case of MCF 35 and clay.

Fig 8.1 shows that all functionalized samples possess a higher enzyme loading and a faster adsorption rate than pure-silica supports and clay. The oxides differ widely with respect to surface character. Silica is an oxide with a weak Bronsted activity (pKa of Bronsted's sites of 7) and with a point of zero charge of 3. Alumina is also a material with a weak Bronsted activity (pKa of Bronsted sites of 8.5) and its point of zero charge is 8. Thus, under the conditions used for the experiments (pH 7) silica will carry a strong negative charge and alumina will have a small positive charge. Since the enzyme has a negative net charge under these conditions (the pKa of the lipase is 3.5), it is reasonable to assume that it will give an attractive interaction with alumina and a repulsive interaction with the silica. We, therefore, postulate that the difference in degree of binding that is seen between alumina on the one hand and silica on the other hand is related to the type of interaction that the lipase has with the pore walls. When the walls consist of negatively charged silica, the negatively charged enzyme will keep away from the walls while for the slightly positively charged alumina it will at least partly be adsorbed at the wall. Silica gel presented a wider pore size distribution with a lower pore volume due to its amorphous nature in contrast to MCF materials. So these biocatalysts gave poorer enzymatic load, catalytic efficiency as well as residual activity when compared to alumina and other supports.

8.4 Leaching studies

Enzyme leaching is a major concern for immobilized enzymes. In this study, the amount of enzyme leached out from the supports was also tested using the method reported by Serri et al. [17]. They found that the shear forces

created by the movement of liquid could significantly accelerate the enzyme leaching from the support. Therefore, the stability of the immobilization could be rapidly assessed. In this regards, after a certain period of vigorous shaking, the supernatant was collected and tested to determine the amount of enzyme that had leached out from the support. Fig 8.2 shows the profile of enzyme leaching with time for both mesoporous supports. It is noted that 23% of the enzyme leached out from R_{MCF} after 120 min reaction, suggesting that the immobilized lipase leached out off the external surface of R_{MCF} while it was only 6% for H_{MCF}. It is obvious that the leaching of the immobilized lipase increases with prolonging of incubation time. The leaching of lipase is possibly due to the weak interactions between lipase molecules and the silanol groups of R_{MCF}.

Fig 8.2 also suggests that the leaching of enzyme mostly occurred in the first 20 min and gradually stabilize after that. It is noted that the amount of immobilized enzyme leached out from the covalently bound (HG_{MCF}, RG_{MCF}, K-10G) was always lower than that leached out from the pure samples. Evidently, the functionalization of MCF resulted in the improvement in the physical stability of lipase immobilization. Lipase that has been immobilized on a solid support might leach out from the system because of desorption or detachment from the surface and the subsequent diffusion out of the support material [18]. The detachment is governed by the strength of the interactions with the surface while the diffusion process is dependent mainly upon the geometry of the pores. In the case of HG_{MCF}, less leaching of the bound enzyme was attributed to stronger multipoints interactions between lipase and the functional group on the hydrophobic support [19]. Lipase is known to have better affinity towards hydrophobic supports [17, 20]. Therefore, the presence of hydrophobic functional groups should be favorable to the immobilization.

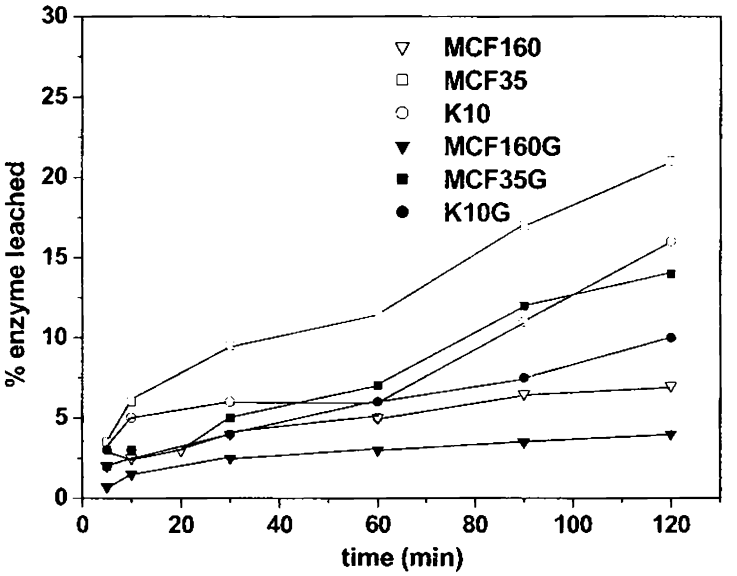


Fig 8.2 Enzyme leaching from the support materials at various shaking durations.

In the case of modified materials, the possible effect of additional hydrophobic interactions can also be detected. K-10G sample was less prone to leaching when compared to RG_{MCF}. In the case of K-10 there was a substantial leaching after 120 min which stabilized after a certain time. HG_{MCF} showed the highest stability without much loss in the enzyme after 120 min of harsh shaking condition. When the lipase is occluded in the porous network as in the case of H_{MCF}, it can probably be orientated in an advantageous way, allowing a better interaction with the silica wall that makes the process more sensitive to all these factors, thus providing certain irreversibility. In the case of all the functionalized samples, the difference with respect to its siliceous counterpart can only be explained by the presence of the organic moieties. Although they are not much hydrophobic enough to improve the immobilization, they can force the anchoring of entrapped lipase molecules in certain areas with high density of functional groups, which together with the bad connectivity of the plane group limits the leaching. In general, the presence of hydrophobic groups and pore size greater than 40A° do play a role in the leaching in aqueous media.

8.5 Comparison with other supports

Table 8.1 summarizes the activity parameters of the synthesized supports and clay with silica gel and alumina. The activity and the immobilization yields obtained are also compared with the literature values. It can be seen that the bound protein is much higher for H_{MCF} due to the larger pore diameter which is higher compared to adsorption of lipase on MCF [21], SBA-15, SBA-16, FDU-12 [22], MCM-22 and MCM-36 [23]. The amount of bound protein is higher in the case of K-10 in our reports than the previously reported ones with almost similar activity yield [24, 25]. The activity yield and immobilization yield is higher for alumina compared to silica gel. Among the support materials, MCF 160 was far superior to the other conventional supports.

Table 8.1 Comparison of the activity parameters with conventional silica gel

Sample	Bound protein (mg/g support)	Specific activity (U/mg)	Activity yield (%)	Immobilization Yield(%)	References
MCF160	237.4	33.9	89	94.6	
MCF35	92.37	24.6	80.2	78.6	
KI-10	50.3	29.3	83.8	91	
Silica gel (surface area - 500m^2/g, pore diameter-67Å)	18.6	12.1	62.4	53	
Alumina (Surface area-241m^2/g, pore diameter-52Å)	33.7	21.9	86.7	96	
REPORTED VALUES					
Candida cylindracea on Montmorilonte	22.7		87		[24]
CRL on bentonite	-	-	77	-	[25]
Rhizopus Oryzae lipase on MCF (Surface area-475m^2/g) Pore diameter -220Å Pore volume-0.63cm^3/g)	84.3	-	-	91.6	[21]
CALB lipase on SBA-15, SBA-16, FDU-12	44mg/g enzyme, 5mg/g, 28mg/g	-	--	-	[22]
CALB lipase on MCM-22, MCM-36	20mg enz/g, 4mg/g				[23]

8.6 Conclusions

The aim of this work was to determine the activities resulting from the adsorption of Candida rugosa lipase onto mesocellular siliceous foams and clay and comparing the resultant activities with those obtained through the immobilisation onto silica gel and alumina.

The main inferences from the present comparison study are:

- Of the supports examined, siliceous MCF160 was shown to be a superior support to conventional silica gel.
- Alumina showed higher binding capacity than silica gel though it has a surface area and pore diameter lower than silica gel. This effect is attributed to the positively charged alumina, but not the negatively charged silica, adsorbing the lipase, which carries a net negative charge.
- It has been observed that the lipase adsorption capacities of the adsorbents depend on the mesopore volume and the pore diameter of the adsorbent. H_{MCF} showed the highest affinity for lipase. The affinity shown by R_{MCF} is less compared to KI-10. N_2 adsorption and XRD data after lipase adsorption reveals that the β-glucosidase molecules are tightly packed inside the mesopores of MCF160.
- Negligible amount of leaching was observed in the case of HG_{MCF} as compared to H_{MCF} due to the hydrophobic interaction between lipase and silica that has increased their rigidity, thus avoiding the distortion of lipase protein and preventing enzyme from being leached away in vigorous shaking conditions.
- R_{MCF} was prone to greater leaching with incubation time than all other supports. The lipase loading in our study was higher in the case of H_{MCF} compared to other literature reports.
- The activity of the lipase immobilized on the silica foam MCF160 was higher than that on some other porous supports. The results may be

explained from the support structure and surface property: the unique three dimensional mesostructure which lowered the diffusion limitation and increased the specific surface area for enzyme immobilization and hydrolysis reaction. These results would not be possibly achieved using amorphous materials, and reveal the relevance of the internal structure of the supports for enzyme immobilization. These encouraging findings open novel and interesting prospects for mesoporous materials, and a new scenario in enzyme immobilization. The stability of immobilized lipase HG_{MCF} provided interesting solution to its possible industrial application.

References

[1] J. A. Bosley, J. C. Clayton, Biotechnol. Bioeng., 43 (1994) 934.

[2] B. Al-Duri, E. Robinson, S. McNerlan, P. Bailie, J. Am. Oil Chem. Soc., 72 (1995) 1351.

[3] L. Q. Cao. Carrier-bound Immobilized Enzyme. Weiheim: Wiley-VCH Verlag GmbH & Co. KGaA (2005) 178-179.

[4] N. N. Gandhi, V. Vijayalakshmi, S. B. Sawant, J. B. Joshi, Chem. Eng. J. 61 (1996) 149.

[5] M. Di. Serio, C. Maturo, E. De Alteriis, P. Parascandola, R. Tesser, E. Santacesaria, Catal. Today., 79 (2003) 333.

[6] G. A. Kovalenko, O. V. Komova, A. V. Simakov, V. V. Khomov, N. A. Rudina, J. Mol.Catal. A: Chem., 182 (2002) 73.

[7] G. D. Yadav, P. S. Lathi, J. J. Mol. Catal. B: Enzym., 27 (2004) 113.

[8] F. Yagiz, D. Kazan, A. N. Akin, Chem. Eng. J., 134 (2007) 262.

[9] X. S. Zhao, X. Y. Bao, W. P. Guo, F. Y. Lee, Mat. Today., 9 (2006) 32.

[10] D. T. On, S. Kaliaguine, J. Am. Chem. Soc., 125 (2003) 618.

[11] Y. J. Han, J. T.Watson, G. D. Stucky, A. Butler, J. Mol. Catal., B 17 (2002) 1.

[12] A. Bastida, P. Sabuquillo, P. Armisen, R. Ferná ndez-Lafuente, J. Huguet, J. M. Guisán, Biotechnol. Bioeng., 58 (1998) 486.

[13] R. Fernandez-Lafuente, P. Armisén, P. Sabuquillo, G. Fernández-Lorente, J. M. Guisán, Chem. Phys. Lipids., 93 (1998) 185.

[14] M. Petkar, A. Lali, P. Caimi, M. Daminati, J. Mol. Catal. B: Enzym., 39 (2006) 83.

[15] O. H. Lowry, N. J. Rosebrough, A. L. Faar, R. J. J. Randall, J. Biol.Chem., 193 (1951) 265.

[16] M. I. Kim, J. Kim, J. Lee, S. Shin, H. B. Na, T. Hyeon, H. G. Park, H. N. Chang, Microporous Mesoporous Mater., 111 (2008) 18.

[17] N. A. Serri, A. H. Kamaruddin, W. S. Long, Bioprocess. Biosyst. Eng., 29 (4) (2006) 253.

[18] D. Jeison, G. Ruiz, F. Avecedo, A. Illanes, Process Biochem., 39(2003) 393.

[19] N. Neerupma, R. Singh, J. Kaur, J. Biotechnol., 9 (5) (2006) 559.

[20] A. S. M. Chong, X. S. Zhao, Appl. Surf. Sci., 237 (2004) 398.

[21] M. R. Shakeri, K. Kawakami, Microporous Mesoporous Mater., 118 (2009) 115.

[22] E. Serra, A. I. Mayoral, Y. Sakamoto, Microporous and Mesoporous Mater., 4 (2008) 201.

[23] E. Dumitri, F. Secundo, J. Patarin, I. Fechete. J. Mol. Catal B: Enzym., 22 (2003) 119.

[24] I. E. Fuentes, C. A. Viseras, D. Ubiali, M. Terreni, J. Mol. Catal B: Enzymatic., 11 (2001) 657.

[25] M. Ghiaci, H. Aghaei, S. Soleimanian, M. E. Sedaghat, Applied Clay Sci., 43 (2009) 289.

Chapter 9

SUMMARY AND CONCLUSIONS

Contents

The discovery of mesoporous molecular sieves has expanded the available pore sizes of zeolites and zeotypes into the mesopore range, thus opening new possibilities in the adsorption of large molecules such as enzymes. The size of the protein to be adsorbed as well as the specific surface area of the carrier material and the nature of its surface (type of porosity, pore size, hydrophobic/hydrophilic nature of surface) are crucial parameters for successful immobilization. Among the most used enzymes for industrial applications lipases are one of the most suitable and studied for immobilization. The present work focused on the physical adsorption and covalent binding of Candida rugosa lipase on montmorillonite K-10 clay and Meso cellular foams (MCF) supports and to compare the activities of immobilized enzymes in a batch reactor. In the second part, the supports were well characterized by various physicochemical characterization techniques. Third part of this work focused on the performance of the immobilized systems which were evaluated and compared with the free enzyme using three test reactions: hydrolysis of esters in aqueous and organic medium and synthetic reactions involving esterification and transesterification in non-aqueous medium. In final part of this venture, as a possible application of MCF, we have developed an extremely effective method for immobilizing β-glucosidase onto mesocellular silica foams as so-called Cross-Linked Enzyme Aggregates (CLEA's) which exhibit high activity retention and stability and can be readily recovered and recycled without any loss of activity. This chapter highlights the key conclusions of the entire work and also the relevance and expected benefits.

9.1 Introduction

The last ten years have witnessed a remarkable proliferation of developments in what may be termed 'solid-phase biochemistry'. The increasing awareness of the need to create green and sustainable production processes in all fields of chemistry has stimulated materials scientists to search for innovative catalysts supports. Immobilized enzymes have, over the past 20 years, moved from the laboratory to industry as a method for the commercial production of food stuffs, pharmaceuticals and fine chemicals. The use of inorganic supports still offers the researcher many advantages over most organic materials because of the physical characteristics. Immobilization methods range from binding to prefabricated carrier materials to packaging in enzyme crystals or powders. A recent use of immobilized enzymes is in synthetic organic chemistry, an area closely related to enzyme technology.

The discovery of mesocellular foam (MCF) materials in 1999 allows a much wider choice of enzymes to be studied in the area of immobilization. Recently developed ordered mesoporous silica namely meso-cellular foams (MCF) have large surface area (500–1200m^2/g), uniform sized pores and thermal stability up to 823K and would be interesting solids for studying enzyme immobilization. Due to their characteristics (pore diameters, pore volume, specific surface area and ease of functionalisation), the mesoporous silica materials offer a great opportunity for immobilized biocatalytical processes. The supports were functionalized using silane and the coupled with glutaraldehyde which then binds to the amino group of the enzyme. The various functionalized materials were used as supports for immobilization of enzyme Candida rugosa lipase via covalent binding. Adsorption on matrices such as clays was selected due to its chemical inertness, reactive hydroxylated surfaces, low price, hydrophilic/hydrophobic properties, permanent charged sites resulting from cationic substitutions and the possibility of enhancing reaction rates by providing a better distribution of the catalyst. Most of the works have been carried out with commercially available immobilized enzymes, hence

use of inorganic supports have to be evaluated for immobilization of lipase and its subsequent utilization for ester synthesis. Due to the overwhelming interest in natural products, biotechnology should be attractive to produce flavors of natural aroma from natural precursors.

These considerations prompted us to propose to synthesize enzyme catalysts supported in ordered mesoporous host. In the present study the effect of key reaction parameters on the lipase catalysed synthesis of esters were studied. Considering the industrial importance of the esters, a better knowledge of significant factors influencing the esterification process should be relevant. The hydrolytic activities of the supports were compared with the conventional ones like silica gel and alumina. Our efforts in this area have focused on the development of suitable support materials for biotransformation in non-conventional media.

9.2 Summary

The thesis gives an account of the synthesis of mesocellular foams via oil in water microemulsion templating method and hybrid organic-inorganic silica based mesoporous materials which have been extended for the immobilization of biomolecules. To obtain a better understanding of the structure-property relationship, the activity of the immobilized enzymes is correlated with the pore sizes and functionalities of different matrices. The present work also illustrates the potential catalytic application of these systems in accomplishing synthetic organic reactions and their superiority to conventional silica gel supports.

Chapter1 presents a general introduction and elucidates the research background of the immobilized lipases. A brief review is given on the mesoporous materials and its application as the host matrix for the biomolecule immobilization. It also gives an account of the different properties of the supports used in this study and also application of these biosystems in catalytic applications. The general mechanism for the synthesis of mesoporous silica as well as their functionalisation

with organic moieties has also been outlined. Based on literature survey, the objectives of the present work have been summarized at the end of the chapter.

Chapter 2 focuses on the various experimental procedures adopted for the synthesis of catalyst supports. The detailed procedures adopted for immobilization as well as catalytic activity studies have been surmised. Various physicochemical techniques employed for the characterization of the supports are discussed.

Chapter 3 describes the results from the various physicochemical characterization techniques employed. The immobilization and functionalisation of the supports were confirmed by various techniques such as nitrogen adsorption measurements, NMR, thermogravimetry, TEM, SEM, FT-IR, CHN analysis and contact angle measurements. Conclusions are also included at the end of this chapter, based on the observations from these characterization techniques.

Chapter 4 highlights the application of the immobilized systems for lipase catalyzed esterification reaction in non-aqueous media for the synthesis of butyl isobutyrate. Various factors controlling the reaction rate and a detailed study of the kinetics were performed.

Chapter 5 reveals the discussion of the activity studies of the free and the immobilized lipases with respect to the synthesis of butyl butyrate via transesterification reaction and the dependence of water activity. The kinetic parameters have been deduced form Ping Pong bi-bi mechanism with inhibition by n-butanol.

Chapter 6 presents the biochemical characterization of the free and the immobilized lipases in the hydrolysis of esters (aqueous and organic medium).

Chapter 7 outlines the adsorption behaviour of lipase on different supports and also their comparative study with the conventional silica gel and alumina.

Chapter 8 describes the characterization and catalytic activity studies of crosslinked β-glucosidase in mesocellular silica foams.

Chapter 9 summarizes the results obtained based on the observed catalytic behaviour and the main conclusions of the study entailed in this thesis. The expected relevance and benefits of this work are also highlighted.

9.3 Conclusions

The major conclusions of the present work are outlined below:

- Mesocellular silica foams (MCF) were synthesized by microemusion templating method via two different routes (hydrothermal and room temperature). and were functionalized with silane and glutaraldehyde. Candida rugosa lipase was adsorbed onto MCF silica and clay using heptane as the coupling medium for reactions in non-aqueous media.
- From XRD results, a slight broadening and lowering of d spacing values after immobilization and modification was observed in the case of MCF160 and MCF35 but there was no change in the d-spacing in the case of K-10 which showed that the enzymes are adsorbed only on the external surface. This was further confirmed from the nitrogen adsorption measurements.
- ^{29}Si and ^{13}C CP MAS NMR studies revealed the incorporation of the organic groups onto the supports which was further confirmed from CHN results. There was an increase in contact angle with surface modification with silane and glutaraldehyde in all the supports which can be correlated with the increased activity observed for the covalently bound systems.
- Synthesis of butyl isobutyrate was conducted by employing the free and the immobilized lipases, among which MCF160G was found to be the most active catalyst.
- The influence of water activity on the synthesis of butyl butyrate by transesterification reaction (alcoholysis) using free and immobilized lipases was studied and a direct relationship between water activity (a_w)

and reaction rate was observed. MCF160G was found to be more effective in catalyzing this reaction at a water activity of 0.33.

- Using the hydrolysis reaction of p-nitrophenyl palmitate in aqueous and organic medium, it was found that the pH and thermal stability of the catalysts were greater than the free enzyme. The immobilized lipases showed good reusability and storage stability characteristics.
- The activities and kinetic parameters were evaluated in both medium and it was found that activities in organic media were found to be lower than those in aqueous media. The K_m value for the free lipase was nearly 50 fold higher in organic media. The decrease in V_{max} in the organic medium was about 163 fold.
- Among the inorganic supports screened for the immobilization of lipases it was found that MCF160 silica retained higher activity and protein than the conventional silica gel.
- The CLEA approach of crosslinking β-glucosidase using glutaraldehyde captured very high enzyme loadings and prevented leaching, providing a more stable and active immobilized enzyme system than those obtained by simple adsorption.
- MCF is a good support for the immobilization of lipase due to its high pore volume, three-dimensional and ultra-large pore structures. The interconnected nature of the large uniform pores makes these new mesostructured silicas promising candidates for supports for catalysts and in separations involving large molecules and they may be of interest in low-dielectric applications.

9.4 Relevance and Expected Benefits

Rapid advances in microbiological research have brought about a greater understanding of biological molecule function and much progress in the area of protein engineering. Often enzymes are relatively expensive compared to their substrates and are lost in the homogenous reaction process. One method of cost

reduction is to recycle and reuse the enzyme from the product stream. This would significantly improve the economics, by optimizing enzyme use, only if the separation can be done efficiently and cost-effectively. Separation can be greatly facilitated by immobilizing the enzyme on a solid matrix. Potential customers for the product vary from research labs, to academia and large pharmaceutical companies. The market place currently contains a variety of options that can be used for the immobilization of biological molecules. The engineered, silica based material would provide some property improvements and could be ideal for large-scale processes. Such materials can find use in heterogeneous catalysis, affinity chromatography, membrane reactors, bio-sensors and drug delivery. The use of immobilized enzymes for pure and applied studies is still in its infancy. By the turn of the century, it is likely that solid-phase technology will be used in many processes and analytical applications. Medicine will have find applications to improve the quality of life for patients suffering from what are now intractable diseases. Finally, the merging of 'solid phase techniques' as applied to the immobilization of enzymes, with a general understanding of enzyme behavior, will allow us to prepare artificial biocatalysts that, in many aspects, will simulate Nature's own enzymes.

The incremental costs associated with the immobilized lipase continuous process are more than 20 times smaller than the ones associated with the traditional one, arising primarily from the cost of the relatively large amount of free lipase. The enantioselective and regioselective nature of lipases have been utilized for the resolution of chiral drugs, fat modification, synthesis of cocoa butter substituents, biofuels, and for synthesis of personal care products and flavour enhancers. Therefore, there is a lot of scope to search for newer lipases with desired selectivity and substrate tolerance. Fixed bed reactors are very promising for future developments of lipase-catalyzed lipid modifications. Combinations of lipase-catalyzed reactions with genetic engineering and/or chemical-catalysis may be required to produce nutritionally optimized products at a reasonable price on a commercial basis.

The unique MCF shell have been potentially useful in applications like encapsulation, delivery, controlled release and separation technology. The powerful approach of CLEAs in mesoporous media opens up a new possibility for enzyme stabilization and will contribute to a variety of enzyme applications in biosensors, bioremediation and bioconversion.

There remains relatively little research so far on the applications of these enzyme–nanomaterials in real catalytic reactions, and demonstration of their utility remains a pressing concern. In particular, if enzyme–nanomaterial composites can be prepared that retain their enantioselectivity, their applications in pharmaceutical and fine chemical industries can be exploited. There are also many emerging biotechnologies that can benefit from composites of mesoporous solids with biological molecules. On a practical level, the large, tunable surface areas of the solids offer a vehicle for the delivery of drugs, which can include peptide-based pharmaceuticals. The high surface area of this material with hierarchically ordered porous structure could be a useful support material for enzyme catalysis and bio-molecular sieving. Functionalized mesoporous materials with high surface area, controllable pore size, narrow pore size distribution, thermal and mechanical stability and featuring high functionality, provide high affinity with the enzyme molecules leading to high enzyme loading. A considerable effort has been devoted to develop large mesopores and macroporous materials as supports for the immobilization of enzyme, functionalized large mesopores materials with a high density of amino groups and well-defined mesochannels have resulted in a revolution of the use of biomolecule for applications in separation, catalysis and sensors, which typically depends on the successful immobilization of biomolecule onto suitable host.

CURRICULUM VITAE

RESHMI.R
Research Scholar
Department of Applied Chemistry
Cochin University of Science and Technology
Cochin-22
Kerala.

K.P.SADANAM
POTTACHERRY,
THRICKODITHANAM.P.O
CHANGANACHERRY
Kerala-686105

E-mail: reshmi_mythri@yahoo.com, reshmimythri@gmail.com

ACADEMICS

Pursuing **Ph.D (Chemistry)** under the guidance of Dr. S. Sugunan, Department of Applied Chemistry, Cochin University of Science and Technology, Kerala, India [From 2005-present]. Research Topic: Physicochemical and Biochemical Characterization of Enzymes Immobilized on Inorganic Matrices.

M. Phil (Master of Philosophy:- Physical Chemistry, Catalysis and Kinetics)

Department of Applied Chemistry, Cochin University of Science and Technology, Kerala, 70.7%, (2004).

M. Sc (Master of Science:- Pure Chemistry)

St. Berchmans College, Changanacherry, M.G. University, 70.7% (2002).

B.Ed (Bachelor of Education in Physical Sciences)

N. S. S Training College, Changanacherry, M.G. University, 71.8% (2003).

B. Sc (Bachelor of Science in Chemistry

Assumption College, Changanacherry, M. G. University, 91.8% (2000).

Pre-degree (Chemistry, Physics and Biology with mathematics as optional subject)

Sacred Heart College, Thevara, M.G. University, 78.8% (1997).

PROJECTS

M. Phil Project

"Catalytic activity of α-amylase immobilized on metal oxide carriers": Physical Chemistry Laboratory, Department of Applied Chemistry, ***Cochin University of Science and Technology***, Kerala, India. [Guidance- Dr. S. Sugunan].

AWARDS

Endowment for the highest mark in final B.Sc (Chemistry) in the year 2000, Assumption College, Changanacherry

Research Experience: 5 years

EXPERIENCES / TRAINING

- Preparation and characterization of solid catalysts for immobilization of enzymes (in industrial applications) and in designed organic transformations.
- Experiences in handling instruments like Gas Chromatograph (GC), Gas Chromatograph-Mass Spectra (GC-MS), HPLC, Fourier Transform-Infrared Spectroscopy (FT-IR), Thermogravimetric analyzer and Micromeritics BET surface area analyzer.
- Working knowledge in computers.
- Capable of collaborative research.

LIST OF JOURNAL PUBLICATIONS

- R Reshmi, Sanjay. G, S. Sugunan, Catal Commun., 8, 3 (2007) 393-399.
- R. Reshmi, Sanjay. G, S. Sugunan, Catal. Commun., 7 (2006) 460.
- P. Murukesan, R. Reshmi, S. Sugunan, 'High capacity immobilization of enzymes based on mesoporous silica from sodium silicate: an efficient biocatalyst', Enzyme Microbial Technology (Communicated).
- P. Murukesan, R. Reshmi, S. Sugunan, 'Stability and activity studies of α-amylase immobilized onto mesoporous silicates prepared from cheap sodium silicate', Food Chemistry (Communicated).
- Reshmi. R, S. Sugunan, 'Crosslinked β-glucosidase onto mesocellular siliceous silica foams', Enzyme microbial Technology (Communicated).

Contributions to National/International Symposia/Conferences

- "Biochemical characteristics of crosslinked β-glucosidase on nanoporous silica foams", Reshmi. R, S. Sugunan, Current adavances in Chemical Science, November 2008, Department of Chemistry, Sacred Heart College, Thevara, Cochin, Kerala.
- "Comparison of hydrolytic activities of Candida Rugosa lipase immobilized on mesostructured cellular foams and clays", Reshmi. R, S. Sugunan, Indian Analytical Science Congress, November 2008, Munnar, Kerala.
- "Covalent attachment of lipase onto mesocellular silica foams: activity and stability studies, Reshmi. R, S. Sugunan, Current trends in Inorganic

Chemistry (CTRIC), Januray 2008, Department of Applied Chemistry, Cochin University of Science and Technology, Cochin-22, Kerala.

- Immobilization and Characteristics of Candida Rugosa Lipase onto Siliceous Mesoporous Molecular Sieves and Montmorillonite K-10 for Synthesis of flavour esters, Reshmi. R, S. Sugunan, International Conference on Advanced Materials and Composites, ICAMC, October 2007, NIST, Trivandrum, Kerala India.
- "Stabilization of enzymes using siliceous mesoporous molecular sieves", P. Murukesan, R. Reshmi and S. Sugunan, 18th National Symposium on Catalysis, Indian Institute of Petroleum, April 2007, Dehradun, India.
- "Synthesis and characterisation of lipase immobilized onto siliceous mesostructured cellular foams for synthesis of esters", Reshmi. R, S. Sugunan, International Conference on Materials for the Millenium, March, 2007, Department of Applied Chemistry, Cochin University of Science and Technology, Cochin-22, Kerala, India.
- "Stabilization of enzymes using siliceous mesoporous molecular sieves", P. Murukesan, R. Reshmi and S. Sugunan, 18th National Symposium on Catalysis, Indian Institute of Petroleum, April 2007, Dehradun, India.
- "Stabilization of α-amylase via immobilization on silica prepared from sodium silicate and its application for starch hydrolysis", P. Murukesan, R. Reshmi and S. Sugunan, National Conference on "Smart Electroceramics", March 2007, Centre for Materials for Electronics Technology, Thrissur, India.
- Enhanced reusability of α-amylase immobilized on sol-gel derived silica, P. Murukesan, R. Reshmi, S. Sugunan, National Conference in Chemistry, September 2006, Bangalore University, Central College, Bangalore.
- α-amylase immobilized on zirconia: a heterogeneous biocatalyst for starch hydrolysis", R. Reshmi, G. Sanjay and S. Sugunan, National Conference on the role of Analytical Chemistry in Materials Science and Technology, pp 26, May 2006, Munnar, Kerala.
- Synthesis and Characterisation of Mesocellular Silica Foams with Unprecedented Uniform Large Mesopores and High Surface Areas, Reshmi. R and S. Sugunan, Frontiers in Chemistry, February 2006, Department of Applied Chemistry, Cochin University of Science and Technology, Cochin, Kerala.
- Activity of α-amylase immobilized on metal oxide carriers, Reshmi. R, Sanjay. G, S. Sugunan, National Conference in Catalysis, December 2005, Goa University, Goa.
- Activity and stability of α-amylase on metal oxide carriers, Reshmi. R, Sanjay. G and S. Sugunan, Emerging Trends in Materials Chemistry, November 2005, Calicut University, Calicut.

.....ooo.....