HYDROLYTIC ACTIVITY OF PHYSICALLY ADSORBED LIPASE ON SBA-15

A

Thesis Submitted

In partial fulfilment of the requirements for the

Degree of

MASTER OF SCIENCE IN CHEMISTRY

Submitted by:

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As this thesis sees the light of day, I find myself at a loss of words to express my thankfulness to almighty, but for whose gracious blessings, this work would not have been conceived, much less completed.

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Life at Thapar University could not be imagined without golden friends like Adeti, Jaspreet, Nayanjot and Shilpa, with whom I have shared the best time of my life and who were always there in the hour of need.

Place: Patiala
Date: 15th July, 11

Regards,
Rinipal Kaur
(RINIPAL KAUR)

DEDICATED TO MY PARENTS.....

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Mandeep Kaur Chahal

Mandeep Kaur Chahal

Place: Patiala

Date: 14-7-2011

CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled, **"Hydrolytic activity of physically adsorbed lipase on SBA-15"**, in partial fulfilment of the requirement for the award of the degree of **Masters of Science in Chemistry in the School of Chemistry and Biochemistry, Thapar University, Patiala,** is my own work during the period of January 2011 to June 2011, under the supervision of **Dr. Amjad Ali,** Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Patiala

Date: 14-7-2011

Mandeep Kaur Chahal

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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CERTIFICATE

This is to certify that the thesis entitled **"Hydrolytic activity of physically adsorbed lipase on SBA-15"**, being submitted by Ms. Mandeep Kaur Chahal to **Thapar University, Patiala**, in partial fulfilment of the requirement for the award of the degree of Master of Science in Chemistry, is a bonafide work carried out under my supervision, and that no part of this thesis has been submitted for the award of any other degree.

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LIST OF CONTENT

ABSTRACT

In order to prepare the support for the immobilization of lipase, the large pore sized SBA-15 spherical particles were prepared using Pluronics P123 ($PEO_{20}PPO_{70}PEO_{20}$) as surfactant. Cetyltrimethyl ammonium bromide (CTAB) was used as a co-surfactant, ethanol as a co-solvent and cyclohexane as swelling agent. Further, fiber-like or rod-like mesoporous SBA-15 was synthesized by self assembly of Pluronics P123 via pre-hydrolysis of silica source (TEOS) under stirring conditions at 40 °C for 2 days. The mesoporous SBA-15 was characterized by Powder X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and Fourier Transformed Infrared (FT–IR) techniques. The *Aspergillus niger* lipase has been physically adsorbed on SBA-15 material at different pH values and maximum lipase adsorption (317.5 mg/g) was observed at pH 3. The hydrolytic activity of lipase adsorbed on SBA-15 has evaluated for the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP). Kinetic parameters viz., Michaelis-Menten constant (K_m), Maximum rate of reaction (V_{max}), Turnover number (k_{cat}) and Specificity constant (k_{cat} /K_m) for immobilized *Aspergillus niger* lipase were determined by using Michaelis-Menten and Lineweaver-Burk approach.

CHAPTER 1

INTRODUCTION AND LITERATURE SURVEY

1.1. INTRODUCTION

Technical advances in various fields, such as adsorption, separation, catalysis, drug delivery, sensors, photonics, nanodevices, bio-immobilisation, ion-exchange, optics and photovoltaics require the development of ordered porous materials with controllable structures and systematic tailoring pore architecture. Zeolites or microporous materials, whose pore sizes are less than 1.2 nm, are far away from these demands. Nowadays the research on the mesoporous molecular sieves has been one of the hot points in the study field of catalysis because these materials show a great capacity to act as versatile catalysts or catalyst supports [1]. Mesoporous materials with regular geometries have been recently paid much attention owing to their great potentials in practical applications such as catalysis, adsorption, bio-immobilization, separation, sensing, medical usage, ecology and nanotechnology [2-5].

According to the International Union of Pure and Applied Chemistry (IUPAC), the prefix meso-refers to a region 2 to 50 nm, macro- is a region > 50 nm and micro- is a region < 2 nm. The small mesopores limit the kinds of ions and molecules that can be admitted to the interior of the materials. In addition, control over the pore size offers the possibility of molecular sieving or molecular selectivity. Mesoporosity can also endow a material with a high surface area exceeding 1,000 m^2/g and pore volume greater than $1cm^3/g$. This greatly expands the potential of the materials for application to adsorption and as a support for immobilized catalytic or sensing moieties [6].

Enzymes have been used for several years to modify the structure and composition of foods but they have only recently become available for large-scale use in industry, mainly because of the high cost of enzymes. Economical usage of lipases in industry requires enzyme immobilization, which enables enzyme reuse and facilitation of the continuous process. Immobilized enzymes were defined by Katchalski-Katzir at the first Enzyme Engineering Conference, held at Henniker, NH, USA, in 1971, as the confinement or localization of enzyme physically in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously [7].

Immobilization of enzymes is an important process in biotechnological applications like separation, catalysis and sensors, which typically depend on successful immobilization of biomolecules onto or within a suitable host. Immobilization of enzymes on insoluble support is one of the most effective methods to obtain an immobilized biocatalyst [8]. Immobilization process seems to offer mainly the economical advantages. However, there are a number of advantages to attaching enzymes to a solid support and a few of the major reasons are as follows:

- Enzymes can be reused.
- Processes can be operated continuously and can be readily controlled.
- Products are easily separated.
- Effluent problems and materials handling are minimized.
- In some cases, enzyme properties (activity and stability) can be altered favorably by immobilization.
- Provides higher purity and product yields, product inhibition is less apparent.
- Greater pH and thermal stability.
- No contamination due to added enzyme.
- Continuous operation.
- Greater flexibility in reactor design [9].

Hence, silica-based porous materials, because of their high surface area and tunable pore diameter, are regarded as suitable hosts for large molecules such as proteins [10]. The ordered mesoporous silica with a well-defined pore size such as MCM-41 is not suitable, since bulkier enzymes (diameter > 40 Å) cannot access the pores. Mesoporous silica with uniform large channels and high stability, namely SBA-15, has recently been synthesized [11, 12]. The synthesis makes use of triblock copolymers as structure-directing agents for silica [13]. This allows for the accurate control of the porous structure over a wide range of pore size (50-300 Å).

The selection of an immobilization technique is based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of the lipase utilization, deactivation and regeneration characteristics, cost of immobilization procedure, toxicity of immobilization reagents,

and the desired final properties of the immobilized lipase. The various enzyme immobilization methods are shown in Figure 1.

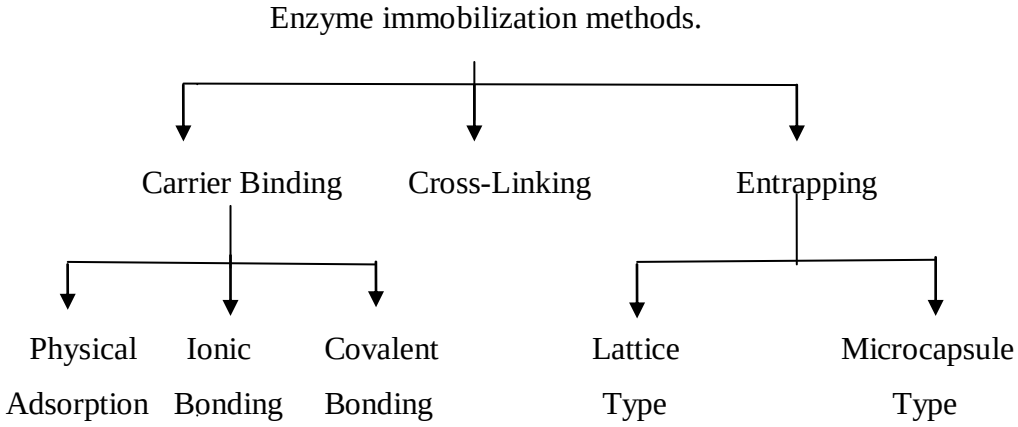


Figure 1: Various enzyme immobilization methods.

The most widely used method for immobilization is by physical adsorption. The forces binding proteins to hydrated silica surfaces include electrostatic, hydrogen bonding and weak van der Waals interactions. Electrostatic protein–surface interactions are likely to be the strongest, particularly when the adsorption is performed at a pH where the enzyme possesses a positive charge, because silica carries negative charges at pH values above 2.5 [14].

In present work, SBA-15 has been prepared as support and used for the immobilization of commercially available lipase (*Aspergillus niger*) by physical adsorption. Enzyme activity of the immobilized enzyme has been evaluated, using *p*-nitrophenyl palmitate as substrate.

1.2. LITERATURE SUEVEY

A procedure for producing mesoporous materials (silica) was patented in 1970 [15]. It went almost unnoticed and in 1990, Yanagisawa and co-workers described the preparation of mesoporous silicas with uniform pore size. However, the pores in these materials were generally irregularly spaced and broadly distributed in size. Mesoporous silica nanoparticles (MSNs) were independently synthesized in 1990 by researchers in Japan [16]. They were later produced also at Mobil Corporation laboratories in 1992. Quaternary ammonium cationic surfactants such as Cetyltrimethyl ammonium bromide ($C_{16}H_{33}N(CH_3)_3$-Br, CTAB) were first used as templates to prepare highly ordered M41S [17] mesoporous silicate molecular sieves under hydrothermal, basic conditions. The M41S family includes MCM-41 [18, 19] with two-dimensional hexagonal alignment of mesopore channels, MCM-48 [20] with three dimensional cubic orders, and the layered material MCM-50[21]. Thereafter, silica based mesoporous materials with different mesostructures have been synthesized usually using tetraethylorthosilicate (TEOS) as the hydrolysable silica building block, and long chain ammonium surfactants, amines or triblock copolymers as templating agents. Some of the well known materials are HMS (hexagonal mesoporous silica) [22], MSU (Michigen state university) [23], SBA (Santa Barbara amorphous) [24, 25], FSM (folded sheet materials), MTS (Micelle templated silica), PMO (Periodic mesoporous organosilica), and PMS (Periodic mesoporous silica). In addition to preparation of various mesoporous silica structures, incorporation of heteroatoms such as Cu, Zn, Al, B, Ga, Fe, Cr, Ti, V and Sn into mesoporous silica framework has been widely investigated. Furthermore, it is possible to synthesize mesoporous structures of the materials other than silica. Methodology to prepare mesoporous silica via the template synthesis is extended to preparation of a various mesoporous metal oxides, such as TiO_2, Ta_2O_5, Nb_2O_5, ZrO_2, Al_2O_3 and V_2O_5 as well as synthesis of mesoporous aluminophosphate [26]. These types of materials yield reflections in the low angle region of a powder X-ray diffraction pattern [27].

Silica based mesoporous materials with different mesostructures have been synthesized usually using tetraethylorthosilicate (TEOS) as the hydrolysable silica building block, and long chain ammonium surfactants, amines or triblock copolymers as templating agents. Some of the well known materials are MCM-41 (*p6mm*), MCM-48 (*Ia3d*), HMS and MSU (wormlike), KIT-6 *(Ia3d*), SBA-1 and SBA-6 (*Pm3n*), SBA-2 and SBA-12, SBA-15 (*p6mm*) and SBA-16 (*Im3m*). As extensively investigated, amphiphilic molecules can self-organize into a diversity of supermolecular structures including spherical micelles, hexagonal rods, lamellar liquid crystals and other assemblies, which can be used as templates to tune pore structure and size [28]. Figure 2, represents the mechanism of formation mesoporous material in which amphiphilic molecules organize into spherical micelles and leads to the formation of mesoporous silicates after the removal of template.

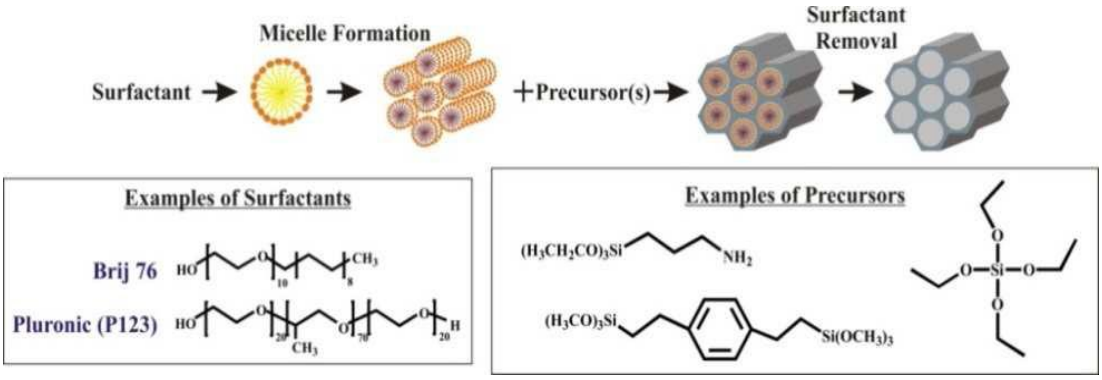


Figure 2: Illustration of the steps involved in the synthesis of surfactant templated silicate materials [29].

However, M41S materials are limited to a pore diameter of approximately 80 Å and furthermore, they have significant external surface areas. These characteristics limit use in size-selective separations of large biomolecules such as proteins and enzymes [30]. Zhao et al. [31] extended the family of highly ordered mesoporous silicates by synthesizing SANTA BARBARA AMORPOUS (SBA) type materials. These have pore size raging between 20 and 300 Å and use non ionic block copolymers as structure directing agents in highly acidic media. SBA-15 was synthesized using tri-block copolymer poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide), which is commercially available as pluronics P123 ($PEO_{20}PPO_{70}PEO_{20}$). SBA-15 is a hexagonal mesoporous material which possesses

large BET surface area (>700m^2/g) with large pore diameter and large pore wall thickness. Among ordered mesoporous nanomaterials, SBA-15 attracts more attention owing to its excellent hydrothermal stability, less condensed structure, unique micro-mesoporosity, high pore volumes and surface areas which is essential for the supports to immobilize proteins and enzymes or catalysts. This material possesses large, uniform, and ordered channels, along with a complementary net of micropores which provides connectivity between the ordered channels through the silica.

Researchers focused on the application of mesoporous silica for enzyme and protein immobilization, hybrid sol-gel films and monoliths for optical and electrochemical sensing of inorganic species [32]; mesoporous silica nanoparticles for biosensing [33]; zeolites and mesoporous silicates for electrochemical detection [34]; and sol-gels and templated mesoporous materials for fluorescence-based sensing [35].

To enable practical applications of SBA-15, synthesis strategies have been developed to form thin films [36], spheres [37, 38], fibers [39] and membranes [40]. There are few reports in the literature that describe the synthesis of spherical mesoporous particles with pore diameter >40 Å [37, 38]. Slower reaction rates increase the surface curvature, which leads to the formation of curved structures, such as spheres and gyroids. Slower reaction rates can be achieved with reduced stirring rate, low acidity and the addition of a co-solvent [37]. Ma et al. synthesized 3–6 μm spherical mesoporous particles with a maximum pore size of 105 Å [38]. However, it was reported that P123 never yielded monodisperse spheres, due to very strong hydrophobic forces that lead to a tendency to attain elongated cylindrical silicated-surfactant micelles. Parameters such as stirring rate, temperature, ionic strength, reactant composition and pH can influence the morphology of SBA-15 particles [41]. The synthesis of spherical mesoporous SBA-15 with larger pore diameter, 127 Å, with particle diameters in the range of 4–10 nm were obtained using pluronics P123 ($PEO_{20}PPO_{70}PEO_{20}$) as a surfactant coupled with Cetyltrimethyl ammonium bromide (CTAB) as a co-surfactant and ethanol as co-solvent played a very important role in the formation of silica spheres, as it delays the reaction rate of the SBA-15 synthesis [42]. Fiber-like or rod-like mesoporous SBA-15 silicas with different lengths and diameter of macrostructures and pore diameter could be synthesized via a one-step method at low temperature ranging from 25 to 40 ºC under

low concentration of P123 (0.67 wt%) without the addition of inorganic salts, where pre-hydrolyzed silica species may favor the self assembly of silica-polymer hybrid micelles [43].

Significant improvement in the pore properties of SBA-15 brought about by carboxylic acids and hydrothermal treatment. A comparative study of the pore properties of SBA-15 samples prepared under non-hydrothermal and hydrothermal conditions, in the absence and presence of carboxylic acids such as succinic, tartaric and citric acids has been carried out [44]. In the absence of carboxylic acid, flake-like and spheroid particles were generally obtained irrespective of the preparative procedures. On the other hand, stirring of the pre-mix induces a rod-like morphology in presence of carboxylic acids.

Highly ordered, extremely hydrothermal stable SBA-15/Al-SBA-15 has been synthesized using mixed surfactants (triblock copolymer, P123 and semifluorinated surfactant, FSO-100) as a template at a high aging temperature (140-180 ºC) with the assistance of NaCl [45].

In recent years, most researches have been focussed on the preparation of functionalized mesoporous silica thin films. Many routes exist for designing hybrid inorganic-organic mesoporous silicates [46]. The surface modified mesoporous silica with organic functionalities enhance the interactions of guest molecules with support host materials by chemical bonding with the help of cross-linker in addition to physical forces (i.e. van der Waals, hydrogen bonding, hydrophobic and electrostatic interactions). The synthesis of functionalized mesoporous silicas was initially reported by Mann and co-workers in 1996 [47]. General process of organic functionalization is played by two approaches with direct synthesis and post synthesis methods. The direct synthesis involves co-condensation of tetraalkoxysilanes and organo-silanes. Another method for the preparation of functionalized mesoporous materials is post synthesis method by grafting process. This method treats calcined mesoporous silicas with the functional organo-silanes. Generally speaking, organic functional groups are covalently attached to the pore surface by the reaction of the organo-silane using appropriate solvent under reflux condition such as toluene [48]. Many functional groups including aliphatic hydrocarbon, phenyl and amine are used for the functionalization, have been studied. Various organo-silanes were used for functionalization such as 3-aminopropyltrimethoxysilane (APTMS), 3-mercaptopropyltrimethoxysilane

(MPTMS), hexamethyldisilazane (HMDS), phenyltrimethoxysilane (PTMS), methacryloxy-methyltrimethoxysilane (MAMTMS), 2-(3,4-epoxycyclohexyl) ethyltrimethoxysilane (ECETMS) and *N*(aminoethyl)-aminopropylmethyldimethoxysilane (AEAPMDMS). PTMS, MAMTMS, ECETMS and AEAPMDMS have a specific organic functional group such like hydrophobic phenyl ring, vinyl group, epoxide group, and amine group, respectively. These functional groups (vinyl, epoxide, and amine group) could be useful for various applications such as a linker of functional organic materials or active metal for heterogeneous catalysts. Rhodium immobilized on the aminated SBA-15 catalyst, SBA-15/AEAPMDMS/Rh, was investigated as a 1-octene hydroformylation [49].

Crack free amino-functionalized ordered mesoporous organic-inorganic hybrid silica thin films with large pore size have been directly synthesized by sol-gel dip coating [50] on glass substrate using tetraethoxysilane (TEOS), 3-aminopropyltriehoxysilane (APTES) and triblock co-polymer ($(H(OCH_2CH_2)_{20}(OCH(CH_3)CH_2)_{70}(OCH_2CH_2)_{20})OH$, Pluronics P123) as silica source, amino-precursor and structure-directing agent, respectively for immobilization of cytochrome c [51].

Immobilization and encapsulation of enzymes on solid inorganic materials has been the focus of intense studies due to great potential applications of the resulting hybrid materials in biocatalysis [52] and as biosensors [53]. Inorganic supports with surfaces favorable for the immobilization of enzymes, which results in high enzyme activity, have been extensively studied in recent years [54]. Compared with other inorganic supports, recently developed ordered mesoporous silicas [55, 56], namely, MCM-41, SBA-15, meso-cellular foams (MCF), and MCM-48, are supposed to be more suitable for the immobilization of enzyme [57] because of their structural characteristics such as large surface area, high pore volume, well-ordered pore structure, and abundant surface silanol groups [58]. The immobilization performance, as well as the assayed activity and stability of the different enzymes immobilized on different mesostructure materials, have been well-investigated [59, 60].

The immobilization on SBA-15 of different protein and enzymes, such as *porcine pancreatic* lipase (PPL) [61], β-glucosidase [62], cytochrome c [63], *Mucor javanicus* [64], *porcine pepsin* (PEP) [65], *Pseudomonas* sp. (PSL) [66], horseradish peroxidase (HRP) [67], *Candida Antactica* lipase [68],

haemoglobin (Hb) [69], subtilisin, trypsin, α-amylase, chloroperoxidase and lysozyme has been recently investigated.

Among various enzymes, lipases are of great interest owing to their properties and applications. Lipases are ubiquitous enzymes that in nature catalyze triacylglycerol hydrolysis. They are the most used enzymes for biotechnological applications both for large-scale processes and for the fine chemical synthesis. Moreover, lipases show high chemo-, regio- and stereo-selectivity and can be used in organic media. Immobilized enzymes, and especially lipases, can be used both in aqueous and in nonaqueous media. Only ordered mesoporous materials can be used as support to prepare immobilized bio-catalyst. Some of ordered mesoporous materials are shown in Table 1.

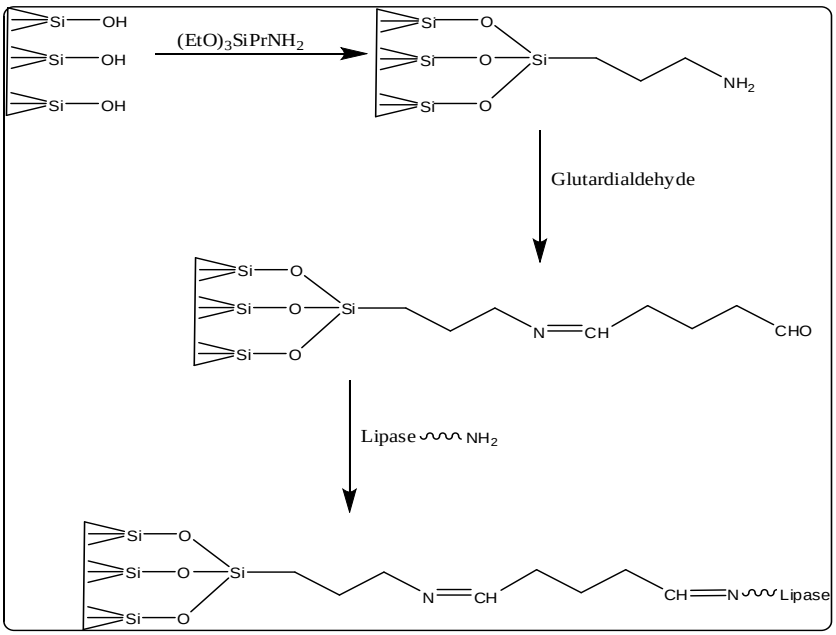
Table 1: Ordered mesoporous solids able to take up biomolecules

Mesoporo-us solid	Space group	Estimated pore diameter/nm	Comments	Refer-ences
MCM-41	p6mm	2–5	Hexagonal array of 1-D channels prepared under alkaline conditions with cationic surfactant.	70
SBA-15	p6mm	5–10	Hexagonal array of 1-D channels prepared under acidic conditions with non-ionic block co-polymer P123 surfactant	70
FDU-5	Ia3d	5–8	Bicontinuous gyroidal structure prepared under acidic conditions with non-ionic block co-polymer P123 surfactant and additives	71
SBA-16	Im3m	min.1–6;max. 4–9	Body centred arrangement of cages prepared under acidic conditions with non-ionic block co-polymer F127 surfactant	72
FDU-12	Fm3m	min.4-9;max.10–12	Face centred cubic arrangement of cages prepared under acidic conditions with non-ionic block co-polymer F127 surfactant and additives	73

Types of immobilization

Basically, three traditional methods of enzyme immobilization can be distinguished, binding to a support (carrier), entrapment (encapsulation) and cross-linking.

(1). Binding to a support can be physical (such as hydrophobic and van der Waals interactions), ionic or covalent in nature. However, physical bonding is generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength. Ionic binding is generally stronger and covalent binding of the enzyme to the support even more so, which has the advantage that the enzyme cannot be leached from the surface. In organic media, a strong enzyme/support interaction is not required, because of enzyme insolubility. Thus, physical adsorption is an appropriate immobilization method. On the contrary, as observed, enzyme leaching in water media, can be an important phenomenon. To avoid such undesired phenomenon, a stronger enzyme/ support interaction, such as chemical adsorption, should be used. Therefore, the SBA-15 support was chemically modified as reported in Scheme 1. In the first step, the reaction between the -OH groups of the SBA-15 and the 3-aminopropyltriethoxysilane was performed; then the product of this reaction was treated with glutardialdehyde. In the last step, the superficial amino groups of the lipase were made to react with the functionalized support to produce the chemically adsorbed SBA-15/lipase biocatalyst.



Scheme 1: Lipase immobilization on SBA-15 by Chemical Adsorption[74]

However, this also has a disadvantage: if the enzyme is irreversibly deactivated both the enzyme and the (often costly) support are rendered unusable. The support can be a synthetic resin, a biopolymer or an inorganic polymer such as (mesoporous) silica or a zeolite.

(2). Entrapment via inclusion of an enzyme in a polymer network (gel lattice) such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule as shown in Figure 3. The difference between entrapment and support binding is often not clear. Entrapment requires the synthesis of the polymeric network in the presence of the enzyme. For example, when an enzyme is immobilized in a prefabricated mesoporous silica the enzyme may be situated largely in the mesopores but this would not be entrapment. On the other hand, when the enzyme is present during the synthesis of a silica sol-gel the enzyme is entrapped.

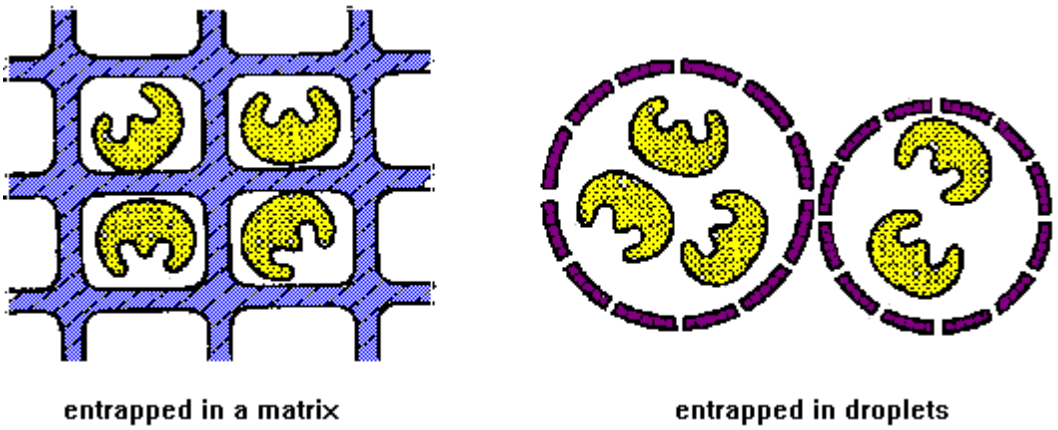


Figure 3: Schematic illustration of entrapment methods (http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

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(3). Cross-linking of enzyme aggregates or crystals, using a bifunctional reagent, to prepare carrierless macroparticles as shown in Figure 4. Moreover, immobilization of an enzyme on a carrier often leads to the loss of more than 50% native activity, especially at high enzyme loadings [75]. Consequently, there is an increasing interest in carrier-free immobilized enzymes, such as cross linked enzyme crystals (CLECs) [76], and cross-linked enzyme aggregates (CLEAs). This approach offers clear advantages: highly concentrated enzyme activity in the catalyst, high stability and low production costs owing to the exclusion of an additional (expensive) carrier.

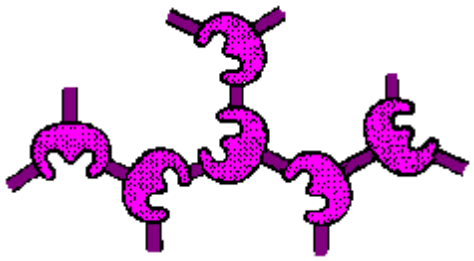


Figure 4: Schematic illustration of cross-linking method (http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

It should be pointed out that, from the literally thousands of papers on enzyme immobilization, it is difficult to make comparisons of the different methodologies as most authors compare the performance of the immobilized enzyme, prepared using a particular technique, with that of the free enzyme but do not compare different methods of immobilization [9].

CHAPTER 2

OBJECTIVES

1. Preparation of large pore SBA-15 mesoporous materials for the immobilization of enzyme.

2. To study the physical adsorption of *Aspergillus niger* lipase on SBA-15 at different pHs (3, 4, 5, 6, 7 and 8).

3. To study the activity assay of immobilized enzyme by hydrolysis of *p*-nitrophenyl palmitate and determination of kinetic parameters viz., Michaelis-Menton constant (K_m), maximum rate of reaction (V_{max}), turnover number (k_{cat}) and specificity constant (k_{cat} / K_m).

CHAPTER 3

MATERIALS AND METHODS

3.1. CHEMICALS AND INSTRUMENTS

Pluronic acid ($PEO_{20}PPO_{70}PEO_{20,}$ P123), Cetyltrimethyl ammonium bromide (CTAB), *p*-nitrophenyl palmitate (*p*-NPP) and *Aspergillius niger* lipase were purchased from Sigma-Aldrich. Tetraethyl orthosilicate (TEOS), cyclohexane (99.5%), ethanol (99.9%), hydrochloric acid (35-38% pure) and acetic acid (99.5%) were purchased from LOBA chemie INDIA. Salts $NaH_2PO_4.H_2O$, Na_2HPO_4, CH_3COONa and CH_3COOH used for the preparation of buffer were also purchased from LOBA chemie, INDIA and used without further purification.

Powder X-ray diffraction (XRD) data was collected on Panalytical's X'Pert Pro with Cu Kα radiation. The samples were scanned in the range of $2\theta = 0\text{-}12°$ at the scanning speed of 2°/min. XRD was used to determine the particle size and the type of crystal planes found in the catalyst. Field emission scanning electron microscopy (FESEM) was performed on FESEM JEOL JSM 6510LV JAPAN to collect the SEM images of the SBA-15. Fourier transformed infrared (FT-IR) spectras were obtained at ambient temperature by using Thermo Scientific (NICOLET iS10) FT-IR instrument. Absorption spectra to determine the kinetic parameters were recorded on Perkin Elmer (Lambda-35) UV-Visible spectrophotometer. Insoluble material from the lipase solution was removed by using REMI RESEARCH CENTRIFUGE. Software Origin Pro 8.0 was used to draw the curves and was fitted using the protocols available with the software.

3.2. PREPARATION OF THE BUFFER SOLUTIONS

The buffer solution was prepared by using the Henderson-Hasselbalch equation:

pH= pKa + log{[salt]/[acid]}

(a) Preparation of 50 mM phosphate buffer:

50 mM Phosphate buffer at pH = 6, 7, 8 was prepared by using sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$) and sodium phosphate dibasic (Na_2HPO_4) in de-ionised water.

For pH =6; 595.60 mg of $NaH_2PO_4.H_2O$ and 97.13 mg of Na_2HPO_4 were dissolved in 100 mL of de-ionised water.

For pH =7; 266 mg of $NaH_2PO_4.H_2O$ and 435 mg of Na_2HPO_4 were dissolved in 100 mL of de-ionised water.

For pH =8; 40.95 mg of $NaH_2PO_4.H_2O$ and 667.85 mg of Na_2HPO_4 were dissolved in 100 mL of de-ionised water.

Preparation of 50 mM acetate buffer:

Solutions of 0.02 M CH_3COOH and 0.03 M CH_3COONa were prepared in distilled water.

To prepare 100 mL of acetate buffer of pH 3; 98.95 mL of 0.02 M acetic acid and 1.05 mL of 0.03 M sodium acetate were mixed.

To prepare 100 mL of acetate buffer of pH 4; 90.0 mL of 0.02 M acetic acid and 10.0 mL of 0.03 M sodium acetate were mixed.

To prepare 100 mL of acetate buffer of pH 5; 48.63 mL of 0.02 M acetic acid and 51.37 mL of 0.03 M sodium acetate were mixed.

The pH of the buffer was measured by pH meter and adjusted, if required, by adding HCl or NaOH. The buffer solutions used immediately after cooling at room temperature and stored, if required, at low temperature.

CHAPTER 4

SYNTHESIS OF SBA-15, LIPASE IMMOBILIZATION AND ACTIVITY OF IMMOBILIZED ENZYME

4.1. SYNTHESIS OF MESOPOROUS SBA-15

4.1.1. SBA-15 synthesis using swelling agent

Mesoporous SBA-15 was prepared by following the literature reported sol-gel method with slight modification [42]. Triblock copolymer Pluronic acid (P123, $PEO_{20}PPO_{70}PEO_{20}$) was used as a structure directing agent. Initially, 3 g of P123 was dissolved in 60 ml (1.5 M) HCl. CTAB (0.6 g) and cyclohexane (1.9 mL) was mixed with 25 mL deionized water separately, and then mixed with P123 solution and later ethanol (20 mL) was added to resulted reaction mixture. TEOS (10 mL) was added drop by drop and the resulted mixture was initially vigorously stirred for 45 min at 35-45 °C followed by refluxing for 60 h at 90-120 °C. The solid product thus obtained was filtered and washed with deionised water repeatedly and dried at 130 °C in electric oven for 12 h. The solid thus obtained was calcined at two different temperatures viz., 650 and 750 °C for 5 h to remove the organic template. The SBA-15 thus obtained was characterized by FT-IR, powder XRD and SEM techniques.

4.1.2. SBA-15 synthesis without swelling agent

Rod like or fiber like SBA-15 mesoporous material was prepared at low temperature without swelling agent. During synthesis, Pluronic acid (2 g) was dissolved in HCl (1.6 M, 300 g) and kept at 25 °C. TEOS (4.25 g) was pre-hydrolyzed in HCl (0.05 M, 1.5 g) under vigorous stirring at room temp for 2 h until a clear solution was obtained. The clear solution was mixed with P123 solution and stirred for 30 mins at 25 °C. Then, mixture was kept under stirring at 40 °C for 2 days. The solid product was collected by filtration, washed with water and dried at 25 °C for 12 h. The solid thus obtained was calcined at 650 °C to remove organic template. The SBA-15 thus obtained was characterized by FT-IR, powder XRD and SEM techniques.

4.2. PREPARATION OF ENZYME SOLUTION

The solution of *Aspergillus niger* lipase of concentration 3.4 mg/ml was prepared by shaking 68 mg of enzyme in 20 mL buffer solution (50 mM) of pH =3, 4, 5, 6, 7 and 8 at 25 °C. The acetate buffer was used for making the buffer solution of pH 3, 4, 5 and phosphate buffer was used for making the buffer solution of pH 6, 7, 8. The clear *Aspergillus niger* solutions were collected and stored in refrigerator for further use.

4.3. Immobilization of *Aspergillus niger* lipase on SBA-15

The *Aspergillus niger* lipase has been immobilized on SBA-15 support (prepared by using swelling agent) by physical adsorption. SBA-15 (80 mg) was added to *Aspergillus niger* lipase solution (20 mL) having 3.4 mg/mL concentration. In order to study the effect of pH on lipase adsorption on support, experiments have been performed at various pHs (3, 4,5, 6, 7 and 8). The mixture was incubated using orbital shaker at 28 °C at 170 rpm. The supernatant was separated from solid support by centrifugation at 6000 rpm. The lipase concentration of supernatant was measured by recording the absorption spectra at 277 nm at regular time intervals. The amount of lipase immobilized was calculated by taking the difference of initial lipase concentration from concentration of lipase in supernatant after the adsorption experiments.

The amount of lipase immobilized on SBA-15 was calculated by following the Bradford method [77]:

$$P= (C_0-C_1)V/W$$

Where, P is amount of *Aspergillus niger* adsorbed on SBA-15 (mg/g);

C_0 and C_1 are initial and final lipase concentration in reaction medium (mg/mL);

V is volume of reaction medium (mL);

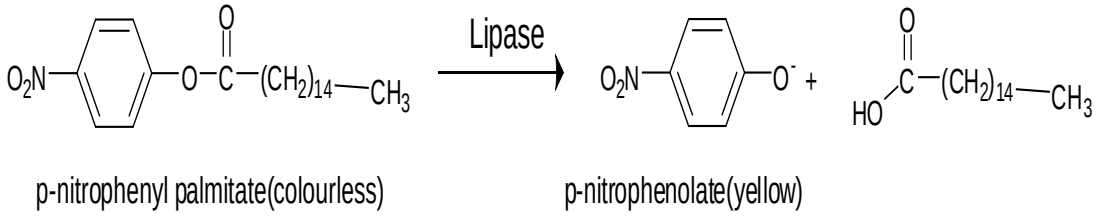
W is weight of support (g).

When maximum immobilization took place, the solid support containing immobilized lipase was separated from supernatant by centrifugation. The biocatalyst so obtained was dried at room temperature and stored at 4 °C temperature in refrigerator.

4.4. LIPASE ACTIVITY ASSAY

4.4.1. Preparation of *p*-nitrophenyl palmitate (*p*-NPP) solution

The stock solution of the substrate, *p*-nitrophenyl palmitate (*p*-NPP), of concentration 1.2 mM has been prepared in pure ethanol and stored in refrigerator. The same solution has been used for making the *p*-NPP solution of appropriate concentrations. *p*-nitrophenyl palmitate has been taken as model compound for esters and upon hydrolysis, it yielded chromogenic *p*-nitrophenol as shown in Scheme 2, and could be easily followed by UV-Visible spectroscopy.



Scheme 2: Hydrolysis of *p*-nitrophenyl palmitate to *p*-nitrophenolate

4.4.2. Hydrolysis of *p*-nitrophenyl palmitate using immobilized *Aspergillus niger* lipase

In a typical hydrolysis experiment, 30 mL phosphate buffer (50 mM, pH 7), 30 mg of immobilised enzyme and then 2 mL *p*-nitrophenyl palmitate of varying concentrations (0.8 mM, 1.0 mM and 1.2 mM) were mixed together in 50 mL round bottom flask. The reaction mixture was stirred till the completion of the reaction and after regular time intervals (10 mins), 2 mL of reaction mixture was withdrawn and centrifused to remove clear supernatant from solid biocatalyst. The supernatant thus obtained was recorded in the range of 360 to 450 nm against a blank having all the components of reaction mixture except the substrate. *p*-nitrophenol (λ_{max} = 403 nm) formed during the hydrolysis reaction has been quantified by recording the UV-Visible spectra of supernatant in the range of 360 to 450 nm.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. CHARACTERIZATION OF MESOPOROUS SBA-15

5.1.1. X-ray diffraction (XRD)

Small angle XRD patterns of SBA-15 are shown in Figure 5. The XRD pattern of SBA-15 (prepared by using swelling agent) showed four well resolved peaks at very low angles (2θ = 0.7850°, 0.9743°, 1.3118° and 1.6372°).

These peaks have been indexed as (100), (110), (200) and (210) reflections respectively. These four peaks are in agreement with the presence of 2-D hexagonal structure as reported in literature [78, 79].

The SBA-15 (prepared without swelling agent) showed less resolved XRD pattern as compared to SBA-15 (prepared using swelling agent). The first intense peak at 1.2370° showed d_{100} of 71.36 Å. SBA-15 (prepared without swelling agent) had only one intense (100) peak, indicating imperfect 2-D hexagonal symmetry [43].

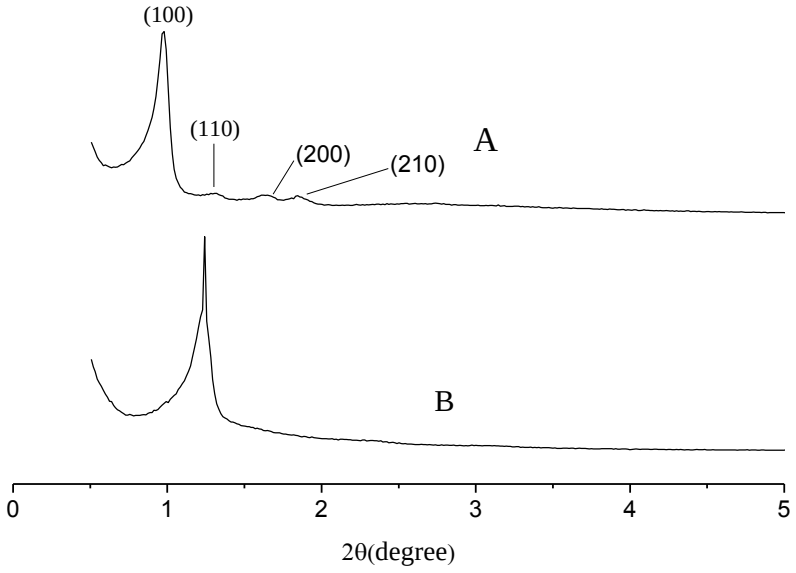


Figure 5: Small angle XRD patterns of (A) SBA-15 (prepared using swelling agent); and (B) SBA-15 (prepared without swelling agent).

5.1.2. FT-1R spectra

The FT-IR spectras of prepared SBA-15 are shown in Figure 6. The spectra reveals that SBA-15 prepared either using swelling agent or without swelling agent show, two peaks viz., at 1080 cm^{-1} due to Si-O-Si asymmetric vibration and at 803 cm^{-1} due to symmetric stretching of Si-O-Si.

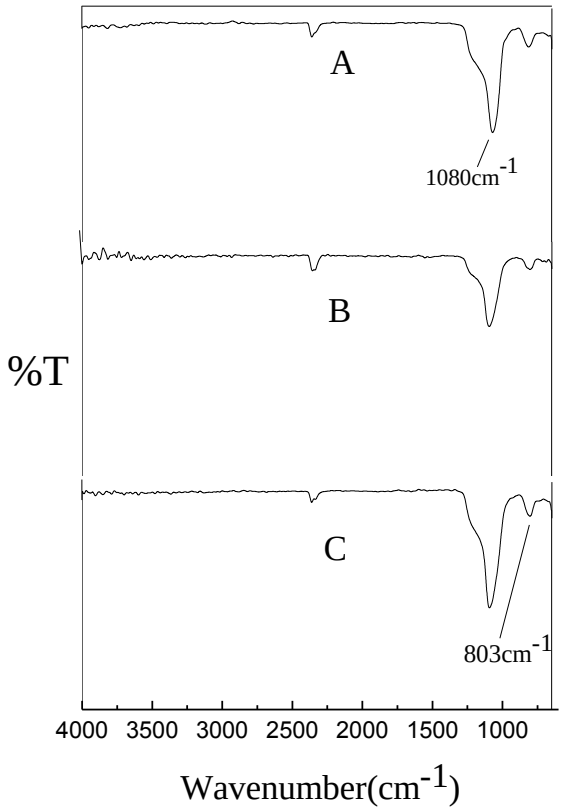


Figure 6: FT-IR spectra of SBA-15 (prepared using swelling agent) calcined (A) at 650 °C; at (C) 750 °C; SBA-15 (prepared without swelling agent) and calcined (B) at 650 °C.

5.1.3. Scanning Electron Microscopy (SEM)

The FESEM images of SBA-15 prepared using swelling agent and SBA-15 without swelling agent are shown in Figure 7, and Figure 8, respectively. The same study reveals that SBA-15 prepared using swelling agent were found to exist in spherical shaped particle of 5 µm, while the SBA-15 prepared without swelling agent were found to exist in the cluster of rods of 10 µm length.

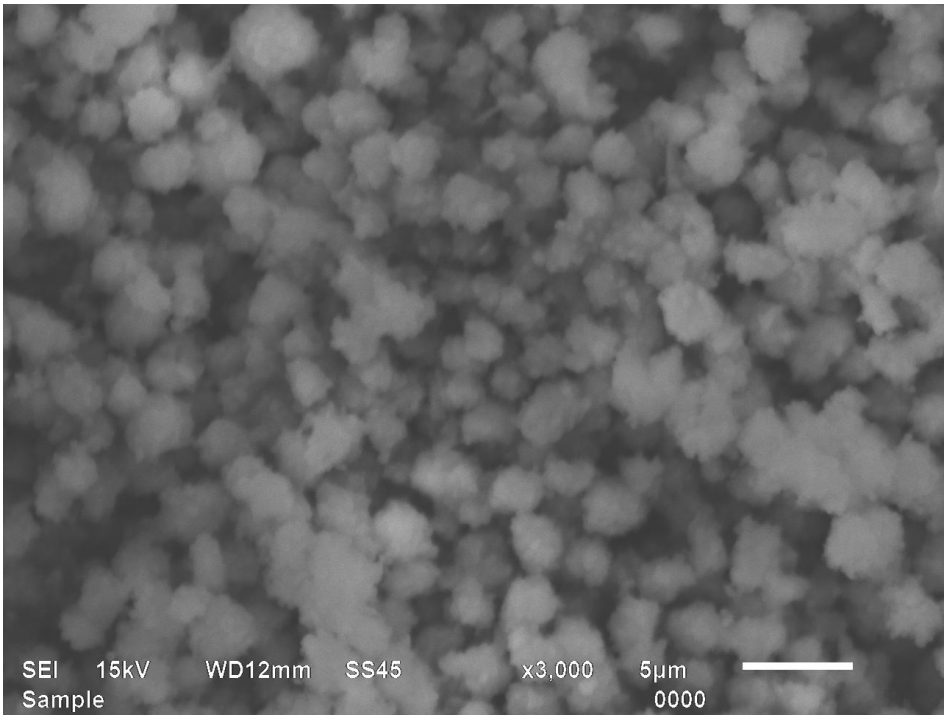


Figure 7: SEM image of spherical SBA-15 (using swelling agent).

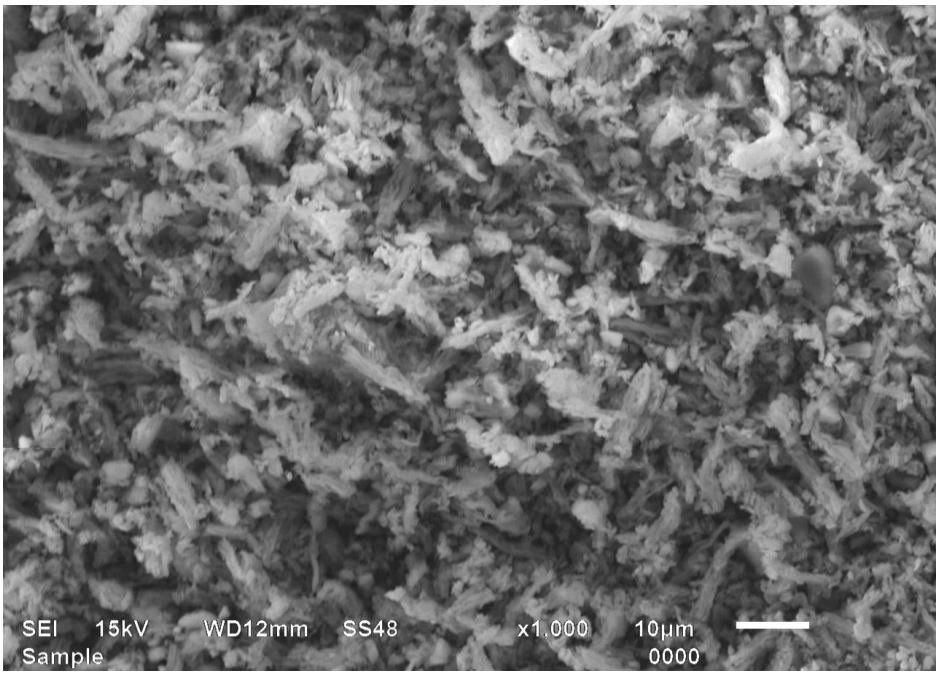


Figure 8: SEM image of fiber like or rod like SBA-15 (without swelling agent).

5.2. CHARACTERIZATION OF SBA-15 WITH IMMOBILIZED LIPASE

FT-IR spectra of SBA-15 after adsorption of lipase have been shown in Figure 9. In the FT-IR spectra of lipase immobilized SBA-15, appearance of two new bands at 1700 cm^{-1} and 1530 cm^{-1} corresponding to C-O stretching and N-H deformation, respectively support the immobilization of lipase on SBA-15.

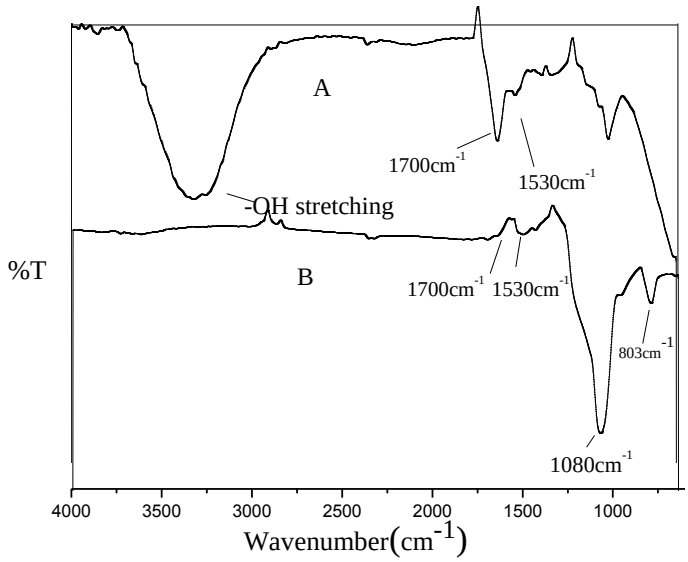


Figure 9: FT-IR spectra of (A) pure *Aspergillus niger*; and (B) SBA-15 with immobilized *Aspergillus niger* at pH = 7.

5.3. EFFECT OF pH ON LIPASE IMMOBILIZATION ON SBA-15

The lipase has been immobilized on SBA-15 via physical adsorption at different pHs viz., 3, 4, 5, 6, 7 and 8. The enzyme immobilization on SBA-15 was calculated by recording the absorption spectra of supernatant in the range of 240-370 nm. The decrease in the intensity of absorption band at 277 nm, support the adsorption of lipase on SBA-15 as shown in Figure 10.

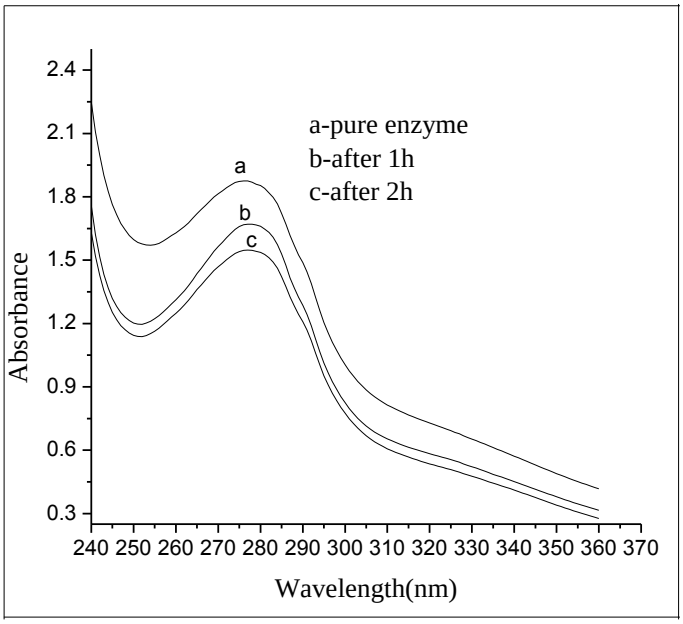


Figure 10: Absorbance of enzyme solution at 277 nm at pH 7 on SBA-15

The maximum enzyme adsorption of SBA-15 was observed at pH 3 after 2 h of contact time and maximum enzyme loading was 317.5 mg/g. The enzyme loading is function of pH of solution. Figure 11, shows clear dependence of the enzyme loading on pH.

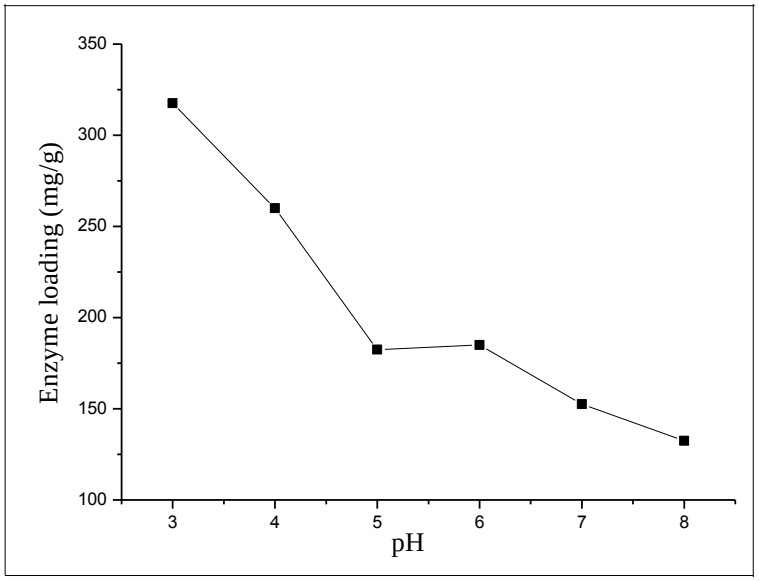


Figure 11: Effect of pH on physical adsorption of lipase on SBA-15

The forces involved during lipase adsorption of SBA-15 could be hydrogen bonding, electrostatic interaction, weak van der Waals interactions and hydrophobic interactions [80]. The isoelectric point (pI) of mesoporous silicate as SBA-15 is about 2.5 and isoelectric point (pI) of *Aspergillus niger* lipase is about 4.5. Therefore, maximum immobilization of *Aspergillus niger* lipase on SBA-15 at pH 3 is due to electrostatic interactions because at pH 3, mesporous SBA-15 is expected to possess negative charge and lipase is expected to possess positive charge.

5.4. Hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) by immobilized *Aspergillus niger* lipase

In order to test the hydrolytic activity of SBA-15 immobilised lipase, *p*-nitrophenyl palmitate has been selected as substrate. The hydrolysis reaction of *p*-nitrophenyl palmitate (ε = 14080 $M^{-1}cm^{-1}$) has been performed at pH 7. *p*-nitrophenol produced during hydrolysis reaction, show maximum absorbance (λ_{max}) at 403 nm. The progress of reaction has been monitored by recording the absorption spectra in the range of 330-460 nm as shown in Figure 12.

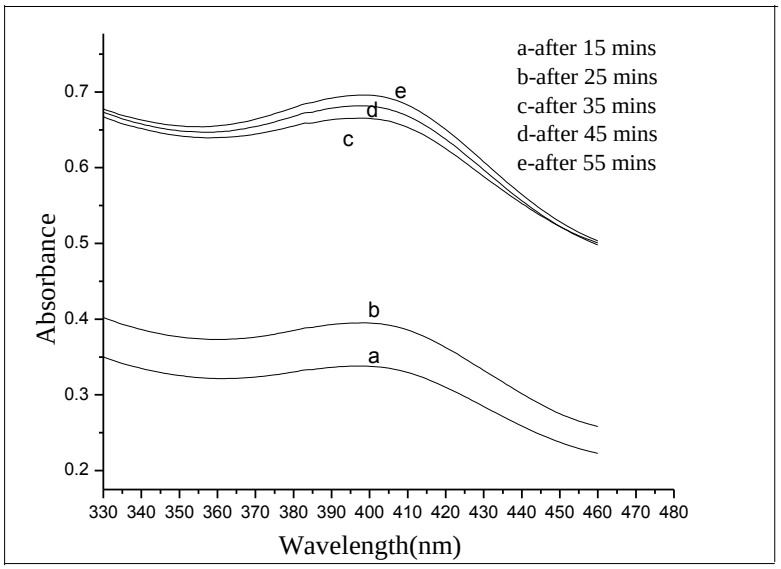


Figure 12: Absorbance spectra for hydrolysis of 1.2 mM *p*-NPP at pH 7

The similar experiments have been performed using three different substrate concentrations viz., 0.8 mM, 1.0 mM and 1.2 mM, but keeping the immobilized lipase concentration constant (30 mg/30 mL phosphate buffer), as shown in Figure 13.

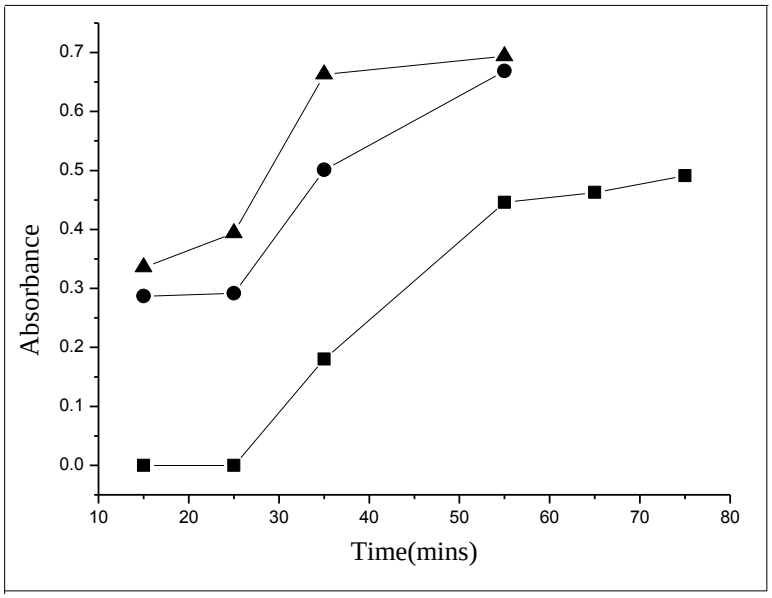


Figure 13: Absorbance at 403 nm, showing the hydrolysis of *p*-NPP by immobilized lipase; (■ 0.8 mM; ● 1.0 mM; ▲ 1.2 mM).

5.5. CALCULATIONS

The concentration of product (*p*-nitrophenol) was calculated by recording the absorbance of the reaction mixture and converting the absorbance into concentration by following the Beer-Lamberts law for absorbance as given in equation (i).

$$A = \varepsilon c l \qquad \text{(i)}$$

Where, A = absorbance at λ_{max}

ε = molar extinction of coefficient of *p*-nitrophenol taken as $14080 M^{-1}cm^{-1}$

l = path length of light traveled is 1cm i.e. width of glass cuvette.

With the progress of reaction, the concentration of *p*-nitrophenol has been increased till the complete hydrolysis of *p*-NPP by immobilized enzyme. Figure 14, shows the increase in concentration of *p*-nitrophenol with time

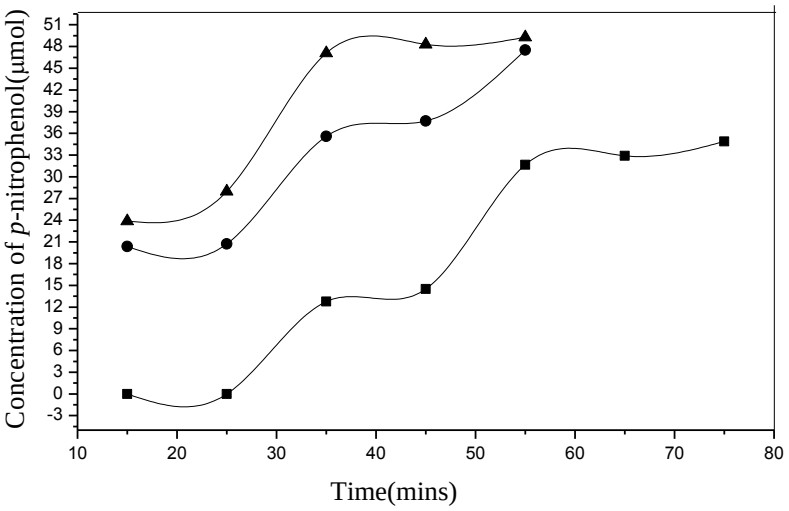


Figure 14: Concentration of *p*-nitrophenol produced during the hydrolysis of *p*-NPP (■ 0.8 mM; ● 1.0 mM; ▲ 1.2 mM).

The concentrations of *p*-nitrophenol have been determined for hydrolysis of *p*-nitrophenyl palmitate (0.8 mM, 1.0 mM and 1.2 mM) and then graph was plotted between concentration of *p*-nitrophenol (μmol) and time to determine the initial rate of reaction by using Origin pro 8.0 software using linear fitting function . The rate has been given by slope of linearly fitted line.

The rates were plotted against their respective substrate concentration to draw the Michaelis-Menten plot. The Lineweaver-Burk plot was plotted between the inverse of initial rate of reaction and inverse of respective substrate concentrations. The K_m and V_{max} were calculated from Lineweaver-Burk plot by extrapolating the line till negative X-axis and the points where this line cuts on X and Y axis were represented as $-1/K_m$ and $1/V_{max}$ respectively.

The lipase enzyme follows Michaelis-Menten kinetics, which can be indicated from the sigmoid shape of the curves drawn between initial rate of reaction (*V*) and substrate concentration (S) as shown in Figure 15.

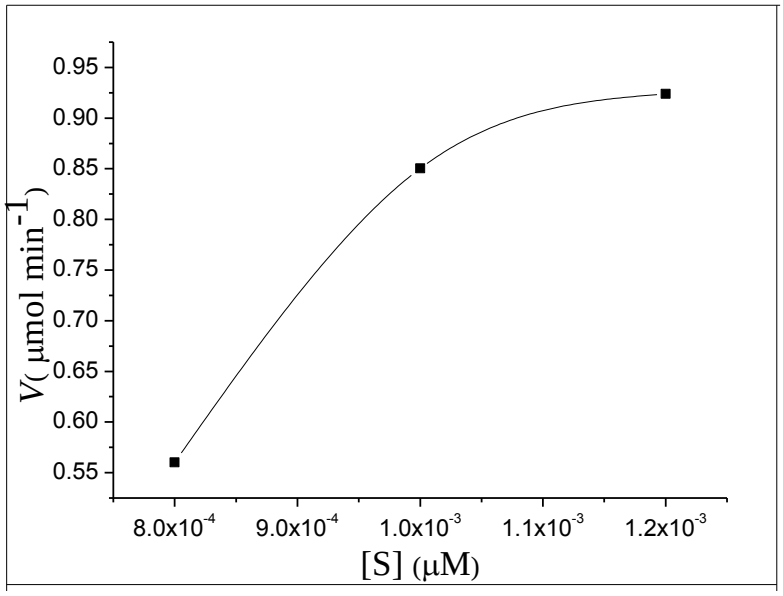


Figure 15: Michaelis-Menten curve of immobilized *Aspergillus niger* lipase for the *p*-NPP hydrolysis.

5.6. DETERMINATION OF KINETIC PARAMETERS

Michaelis-Menten constant (K_m) and maximum rate of reaction (V_{max}) for immobilized *Aspergillus niger* have been determined by using Michaelis-Menten approach. The substrate (*p*-NPP) concentration was varied in the range of 0.8 mM to 1.2 mM with the fixed immobilized enzyme concentration of 30 mg in 30 ml phosphate buffer solution at pH 7. The Lineweaver-Burk plot was plotted between the inverse of initial rate of reaction and inverse of respective substrate concentration as shown in Figure 16, and the values of K_m and V_{max} for immobilized *Aspergillus niger* lipase from this plot were found to be 2 x 10^{-3} μM and 2.5 x 10^{-6} mol min^{-1} respectively.

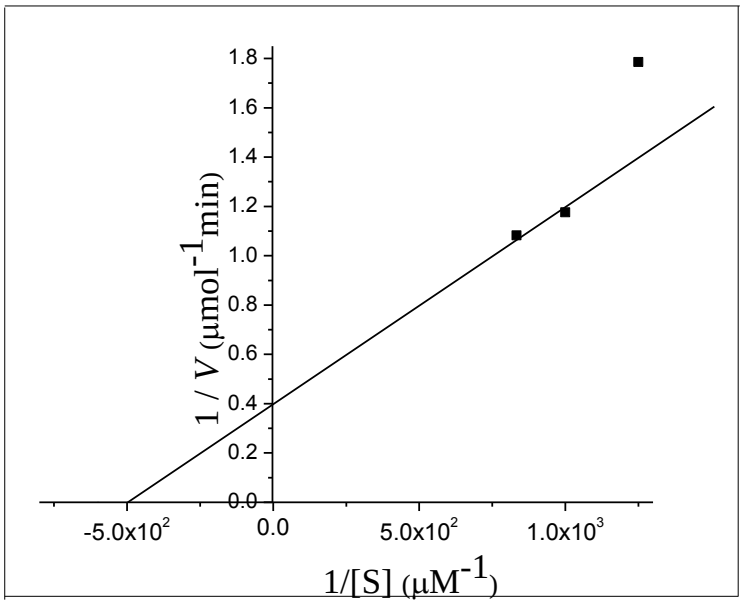


Figure 16: Linweaver-Burk plot for the hydrolysis of *p*-NPP catalyzed by immobilized lipase.

5.6.1. **TURNOVER NUMBER (k_{cat})**

K_m is characteristic of enzyme for its substrate and is independent of the amount of enzyme for its experimental determination, this is not true for V_{max}. V_{max} has no absolute value but varies with amount of enzyme used. A valuable constant in addition to K_m and V_{max} is turnover number (k_{cat}), defined as:

$k_{cat} = V_{max} / [E_t]$

$V_{max} = 78.12 \times 10^{-6}$ mol min^{-1} dm^{-3}

$[E_t] = 3.4$ mmol dm^{-3}

The value of Turnover number was found to be $k_{cat} = 1.4$ hr^{-1}

5.6.2. SPECIFICITY CONSTANT (k_{cat}/K_m)

Specificity constant is a measure of how efficiently an enzyme converts a substrate into product. It is also the apparent 2nd order rate constant at low substrate concentration. The value of specificity constant has been come out to be 11.5(μM)$^{-1}$ min^{-1}.

The observed values for the various kinetic parameters have been summarized in Table 2.

Table 2: Observed values of the various Kinetic parameters obtained for immobilized lipase

Kinetic parameter	Value
V_{max} (maximum rate of reaction)	2.5×10^{-6} mol min^{-1}
K_m (Michaelis-Menten constant)	2.0×10^{-3} µM
k_{cat} (turnover number)	1.4 hr^{-1}
k_{cat}/K_m(Specificity constant)	11.5 $(\mu M)^{-1} min^{-1}$

CHAPTER 6

CONCLUSIONS

In present work, the SBA-15 has been prepared under two different conditions viz., (i) using swelling agent and (ii) without swelling agent and characterized by powder XRD, SEM and FT-IR studies. The SBA-15 material prepared using swelling agent has been used as support for the immobilization of *Aspergillus niger* lipase enzyme. The immobilization of enzyme on SBA support has been supported by the FT-IR studies. The maximum lipase immobilization on SBA-15 (317.5 mg/g) was obtained at pH 3 and immobilized enzyme has been used to test the hydrolytic activity of the enzyme at pH 7, using *p*-nitrophenyl palmitate as a substrate. Kinetic parameters viz., Michaelis-Menten constant (K_m), maximum rate of reaction (V_{max}), turnover number (k_{cat}) and Specificity constant (k_{cat}/K_m) for immobilized *Aspergillus niger* lipase were determined by following Michaelis-Menten and Lineweaver Burk plot.

FUTURE STUDIES

Due to the time constrain, the enzymatic activity of the pure lipase enzyme has not been performed. However it is necessary and could be taken up in future so that the activity of the immobilized enzyme could be compared with that of pure one.

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