ACTIVITY OF THE IMMOBILIZED *Candida rugosa* LIPASE

A

Thesis Submitted

In partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

IN

CHEMISTRY

Submitted By:
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CERTIFICATE

This is certify that the thesis entitled "**Activity of the immobilized *Candida rugosa* lipase**" being submitted in partial fulfillment of requirements for the award of degree of **Master of Science in Chemistry**, submitted in **the School of Chemistry and Biochemistry, Thapar University, Patiala** is a bonafide work carried out under the supervision of **Dr. Amjad Ali,** Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala and that no part of this project has been submitted for the award of any other degree.

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in this thesis entitled, " **Activity of the Immobilized *Candida rugosa* lipase** " in partial fulfillment of the requirement for the award of Degree of **Master of Science in Chemistry**, submitted in **the School of Chemistry and Biochemistry, Thapar University,** Patiala, is an authentic record of my own work carried out under the supervision and guidance of **Dr. Amjad Ali**, Assistant Professor , School of chemistry and biochemistry, Thapar University, Patiala and refers other researcher's work which are duly listed in the reference section.

The matter embodied in this thesis has not formed the basis for the award of any other degree of this or any other university.

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In the end, I wish to express my deep sense of gratitude to my family, for supporting and encouraging me at every step of my work. It is power of their blessings, which has given me the courage, confidence and zeal for hard work.

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Regards,

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ABSTRACT

In the present study *Candida rugosa* lipase has been immobilized on MCM-41 support through sol – gel process. The immobilized enzyme was entrapped by the poly condensation of silica precursors namely tetramethoxyorthosilane /tetraethoxyorthosilane(TEOS) and iso-butyltrimethoxysilane (iso- BTMS). The immobilized enzyme was characterized by scanning electron microscopy, Fourier Transformed Infrared (FT–IR) and UV–Visible Spectrophotometer.

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 *Candida rugosa* lipase were determined by using Michaelis-Menten and Lineweaver-Burk approach.

CHAPTER 1

INTRODUCTION AND LITERATURE SURVEY

1.1 INTRODUCTION

Enzymes are biological catalysts with high selectivities in food industry. The application of an enzyme for a reaction is limited by the high cost of the enzyme. If an enzyme is catalysed on a rigid support such as MCM 41 or SBA 15 ,this limitation can be removed because immobilzed catalyst causes easy seperation and reusability of the enzyme .

Heterogenous catalysts are catalytic active components.They are being carried on a solid support i.e porous solid to increase the reaction rate and are widely used in the production of energy and fuels.Heterogenous catalysts are more useful than homogenous catalysts in the following ways : easy catalyst sepeartion and recovery , regeneration and use.However , homogenous catalysts are still used in food , chemical and pharmaceutical companies and these catalysts have some properties such as high selectivity and accessibilty to all catalytic active sites. But due to environmental problems , homogenous are no longer used these days due to inherent problems such as corrosion , toxicity, difficulty in catalyst handling,seperation from the reaction system,high cost and creation of the solid waste.Comparison between homogenous catalyst and heterogenous catalyst is shown in the table below:

Table 1 Homogenous catalyst versus heterogeneous catalysis [1]

	Homogenous	Heterogeneous
Activity	good	poor
Selectivity	good	Fair
Catalyst Description	fair	Poor
Catalyst Recycling	poor	Good
TON	fair	Good
Quantity of Catalyst	fair	Good

There are different methods that can be used to immobilize the enzymes. For example: adsorption, covalent attachment to solid supports and entrapment with polymers, entrapment of enzymes in an inorganic /organic hybrid polymer matrix is in high demand. An enzyme procedure uses an aqueous solution of enzymes, a general acid or base such as sodium fluoride as catalyst, and alkoxysilanes such as tetramethoxysilane (TEOS) or tetramethoxysilane (TMOS) as precursors.

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. For the economical usage of lipase in industry enzyme immobilization is required, this enables enzyme reuse and facilitation of the continuous process [2]

Immobilized Enzymes

An enzyme that is attached to an inert, insoluble material such as calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride) is said to be an immobilized enzyme. It provides increased resistance to changing conditions such as pH or temperature.

There are three different methods of immobilization. They are as follows: [2]

- **Adsorption on glass, alginate beads or matrix**

In this method enzymes are attached to the outside surface of an inert material. The active site of the immobilized enzyme may be blocked by the matrix or bead which will reduce the activity of the enzyme since this method is not a chemical reaction. This method is slow.

- **Entrapment**

The enzyme is trapped in insoluble beads or microspheres, such as calcium alginate beads. However, this insoluble substance hinders the entry of the substrate, and the exit of products.

- **Cross linking**

  Through chemical reaction enzymes are covalently bonded to a matrix .This method is more efficient than the two other techniques. The enzyme activity is only affected by immobility because during the chemical reaction the binding site does not cover the enzyme's active site.

Advantages of Attaching an Enzyme to a Solid Support

There are a number of advantages for attaching an enzyme to a solid support:

1. Reusability of enzyme is possible.
2. Processes can be operated continuously and can be readily controlled.
3. Separation of product is easy.
4. Minimized effluent problems and materials handling.
5. With the help of immobilization enzyme properties (activity and stability) can be altered favorably.
6. Provides higher purity and product yields, product inhibition is less apparent.
7. Provide greater pH and thermal stability.
8. No contamination due to added enzyme.
9. Greater flexibility in reactor design [3].

Entrapment of lipase entails capture of the lipase with a matrix of a polymer [4]. The immobilized lipase by entrapment is more stable than physically adsorbed lipase and covalent bonding method uses a simple method and the immobilized enzyme maintains its activity and stability.

1.2 LITERATURE SURVEY

Many research efforts have been put forward towards the development of carrier-bound immobilized enzymes. These enzymes are used in continuous processes and due to more efficient separation, recycling and reuse of costly enzymes, these carriers – bound enzymes were able to reduce the costs [2-3]. By enzyme immobilization the enzyme performance such as activity, stability and selectivity can also be achieved. [4-6]. The Immobilized lipase are much more stable by entrapment as compared to physically adsorbed lipase such as the covalent bonding method uses a relatively simple procedure. The immobilized lipase also maintains its activity and stability [6]. Various methods are being used for trapping lipases in a polymer matrix [7]. Enzyme entrapment in an inorganic polymer matrix is a most popular method that can be used for this purpose. This method [8] is based on sol–gel process. The enzymes immobilization increases reuse and reduces process cost [9].

1.2.1 Immobilization on Supports: Carrier-Bound Enzyme

The properties of both the enzyme and the carrier material govern the properties of supported enzyme preparation. Immobilized enzymes are provided with specific chemical, biochemical, mechanical and kinetic properties because of the interaction between the enzyme and the carrier material. There are various types of support (carrier):

1. Synthetic organic polymer
2. Biopolymer
3. An inorganic solid

1. Synthetic organic polymers

Acrylic resins such as Eupergit C are widely used as supports. Eupergit C is a macro porous copolymer of N, N’-methylene-bi-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide with average particle size of 170 mm and a pore diameter of 25 nm. It is highly hydrophilic and stable, both chemically and mechanically, over a pH range from 0 to 14, and does not swell or shrink even upon drastic pH changes in this range. It binds proteins via reaction of its oxirane moieties, at neutral or alkaline pH, with the free amino groups of the enzyme to form covalent bonds which have long-term stability within a pH range of pH 1 to 12 as shown in the figure below

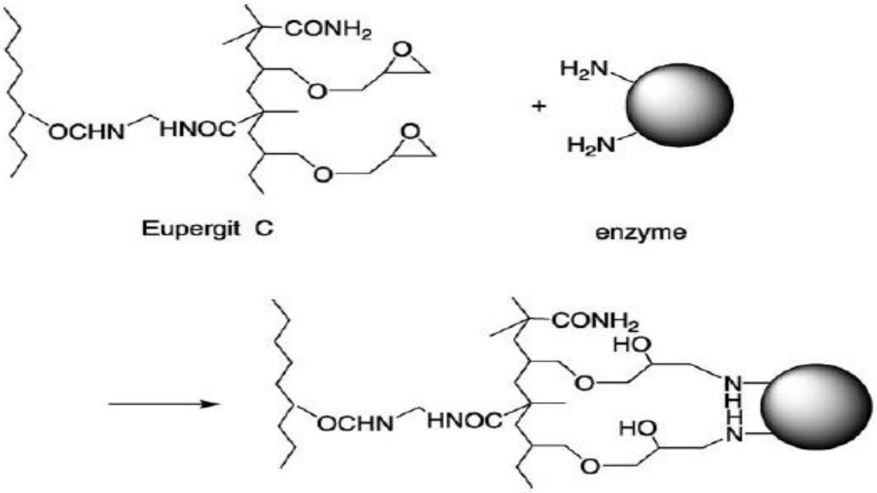
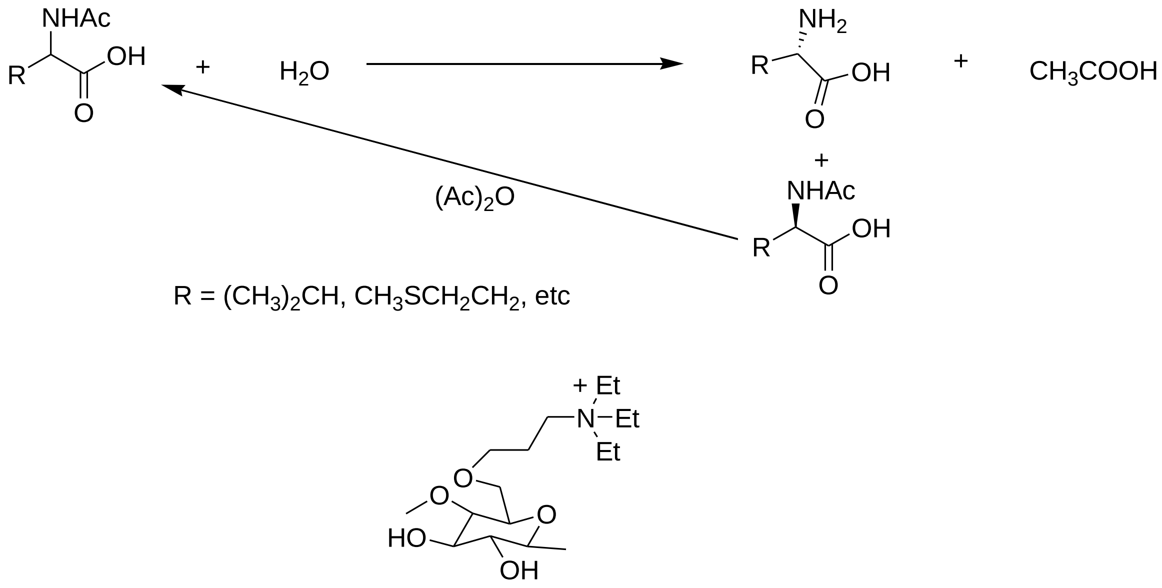


Figure 1: Immobilization of Enzymes on Eupergit C [12]

2. Biopolymers

Various biopolymers have been widely used as support for immobilized enzymes. Mainly water-insoluble polysaccharides such as cellulose, starch, agarose and Chitosan and proteins such as gelatin and albumin are being used. Tanabe process is the first industrial application of an enzyme in a biotransformation. The Enzyme was immobilized by ionic adsorption on DEAE-Sephadex which consists of cellulose modified with diethylaminoethyl functionalities and the process was performed in continuous operation in a fixed-bed reactor as shown in figure 2:



DEAE-Sephadex

Figure 2: Tanabe Aminoacylase Process Inorganic Supports [13]

3. Inorganic Supports

Number of inorganic solids can be used for the immobilization of enzymes, e.g., alumina, silica, zeolites and mesoporous silica's such as MCM-41, and SBA-15.Immobilized catalase on nanoporous silica spheres, having a surface area of 630 mg and mesoporous with a pore size of up to 40 nm and subsequently assembled a nano-composite shell coating composed of three layers of poly-dimethyldiallylammonium chloride (PDM) and 21 nm silica nanoparticles. The resulting immobilisate displayed an activity 75 times that of catalase immobilized on mesoporous silica spheres.

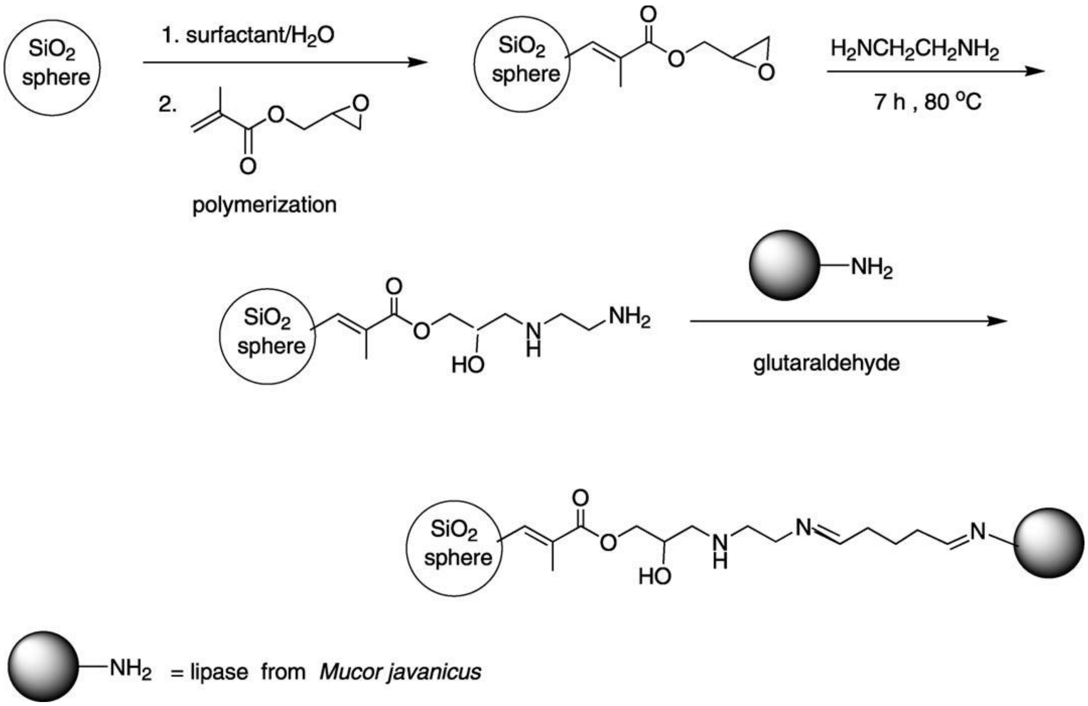


Figure 3: Immobilization of a lipase on silica nanoparticles [12]

It has been achieved to synthesize spherical particles of mesoporous particles (SBA15) with particle diameter of 4-10 micron. The spherical SBA-15 particles with pore diameter of 127 have a very high capacity of 700 mg/g for lysozyme at pH 7.

Due to increase in temperature the pore size of the SBA15 materials decreases the fraction of the microporosity present in the pores. The increase in temperature increases the hydrophobic volume of the surfactant aggregates and thus results in a large pore size. Swelling agent (TMB) effects the properties of SBA- 15. Pore size increases due to the addition of swelling agent. As the amount of swelling agent is increased, the pore diameter and pore volume increase. Incorporation of swelling agent has no effect on the surface area of the materials. Table show the effect of swelling agent on spherical particle properties.

Table 2: Effect of Swelling Agent on Spherical Particle Properties

Materials	**TMB:P 123 (g/g)**	**BET (m^2/g)**	**Pore Volume (cm^3/g)**	**BJH Pore Diameter A**
S-4	0	756	1.18	64.4
S-3	0.30	743	1.64	105
S-5	0.50	768	1.63	127.4

Ethanol decreases the fraction of microporosity present in the pores and thus plays a very important role in determining the characteristics of the SBA-15 particles. The increase in temperature increases the hydrophobic volume of the surfactant aggregates and thus results in a larger pore size. Effect of ethanol on characteristics of the SBA -15 is sown with the help of a table.

Table 3: Effect of Ethanol on Adsorbent Properties [14]

Materials	**TMB:P 123 (g/g)**	**BET (m^2/g)**	**Pore Volume (cm^3/g)**	**BJH Pore Diameter A**
E-1	20	756	1.18	64.4
E-2	30	741	1.12	63.4
E-3	40	733	1.07	62.9

Similarly, other than ethanol and swelling agent i.e. TMB (trimethylbenzene) CMC (critical micelle concentration) is an important characteristic of a surfactant. Surfactants (surface active agents) are amphiphilic molecules, i.e. they are composed of a hydrophilic (water-loving), and a hydrophobic (water-hating) part. When the concentration of surfactants is low in an aqueous solution, the surfactants are located as separate molecules in the air/water interface. This reduces the surface tension since it is larger for water than for the hydrocarbons. Increasing the surfactant concentration in the solution further reduces the pore size of the enzyme and by decreasing the surfactant concentration, the pore size of the enzyme increases.

Number of inorganic solids can be used for the immobilization of enzymes, e.g., alumina, silica, zeolites and mesoporous silica’s such as MCM-41, and SBA-15.

Several studies have reported enzymatic alcoholysis of vegetable oils in solvent and solvent-free media using both primary and secondary alcohols [14]. The conversion of grease and tallow to simple esters using several immobilized lipases also has been reported [15]. Recently phyllosilicate sol gel immobilization technique was introduced. This technique produces a highly stable biocatalyst this catalyst is able to convert greases to simple alkyl esters. Through immobilization optimum pH can be improved and also the temperature range can be widened. As compared to free enzymes immobilization of enzyme enhances the thermal stability of enzyme activity. Several lipolytic enzymes were immobilized in the pores of MCM-41 and Al-MCM-41 and used as catalysts in the gas-phase esterification of acetic acid with ethanol [14].

1.2.2 SOL GEL PROCESS

The most famous technique of lipase encapsulation is sol-gel process. This process shows the introduction of hydrophobic functionalities on the inorganic network of silica magnified the esterification activity of the enzyme relative to the traditional use of lipase powders in organic solvents. Enzymes are hydrophilic in nature because of which they show very low activity in organic media. Their immobilization in a controlled hydrophilic/ hydrophobic environment inside a solid allows greatly enhanced activity. This makes the entrapment of biocatalysts very useful in organic chemistry, and is at the origin of the commercially available immobilized lipases.

The sol-gel method does not have control over the pore size of the resulting solid which play an important role in enzyme accessibility, in substrates and in products diffusivity and accordingly in its activity. In order to achieve this lipases have been immobilized by adsorption on different types of MCM-41 materials having various hydrophilic/hydrophobic surfaces and by encapsulation in recently reported Sponge Mesoporous Silica (SMS), which is prepared using a mixture of lecithin and dodecylamine, acingt as templating agents. MCM-41 supports have been chosen for enzyme adsorption in spite of ordered mesoporous silica (SBA-15, MCM-48, MCF), because they are readily available till date to offer a wide range of pore size from 2 to 15 nm for a similar surface area of 1000 mg. The catalytic activity of ester hydrolysis of immobilized enzymes was compared with that of immobilized lipases which is obtained by the well known method of entrapment in hydrophobic silica sol-gel. Lipases are used in esterification reactions to produce esters such as sugar esters, wax esters, flavor esters, etc [17 -18].

Lipases can be categorized as immobilized or non-immobilized such as Mucor, Candida, Penicillium, Rhizopus, etc. Most of the carriers for immobilization are granular particles including celite or exchange resins [4, 7, 8, 14], this is one of the reason for the high costs. Granular immobilized enzymes can be reused by filtration, but are easily broken down by agitation in two-phase systems.

Sol-gel chemistry is used in the design of silicates [17]. Liquid silicon alkoxide precursors ($Si(OR)_4$) are hydrolyzed and condensed to form siloxane bridges, a process that is often described as inorganic polymerization and is represented below:

Hydrolysis: $Si(OR)_4 + nH2O \rightarrow HOn\text{-}Si(OR)_4\text{-}n + nROH$

Condensation: $(RO)_3\ Si\text{-}OH + HO\text{-}Si(OR)_3 \rightarrow (RO)_3Si\text{-}O\text{-}Si(OR)_3 + H_2O$

and $(RO)_3Si\text{-}OR + HO\text{-}Si(OR)_3 \rightarrow (RO)_3Si\text{-}O\text{-}Si(OR)_3 + ROH$

The most common precursors that can be used are TEOS, tetramethoxysilane and TMOS, tetramethoxysilane. Colloidal sol of condensed silicate species can eventually interconnect as an immobile three dimensional network encompassing the space of its reaction container (gel, Figure 4). A gel which when dried under ambient conditions or with heat will typically cause shrinkage as solvent leaves the micro pores of the silicate network is known as xerogel. In order to remove solvent, supercritical drying can be applied yielding a product that is more similar to the size and shape of the original gel. Such aerogels may have low solid volume fractions near 1% and, therefore, very high pore volumes. The use of basic pH and an excess of water can result in particulate precipitation. Thin films or membranes can be generated by the deposition of the gel. The isoelectric point of silica is in the pH range 1-3. This value determines the surface charge of a condensing silicate or material in solution due to protonation and deprotonationation of silanol groups (Si-OH)

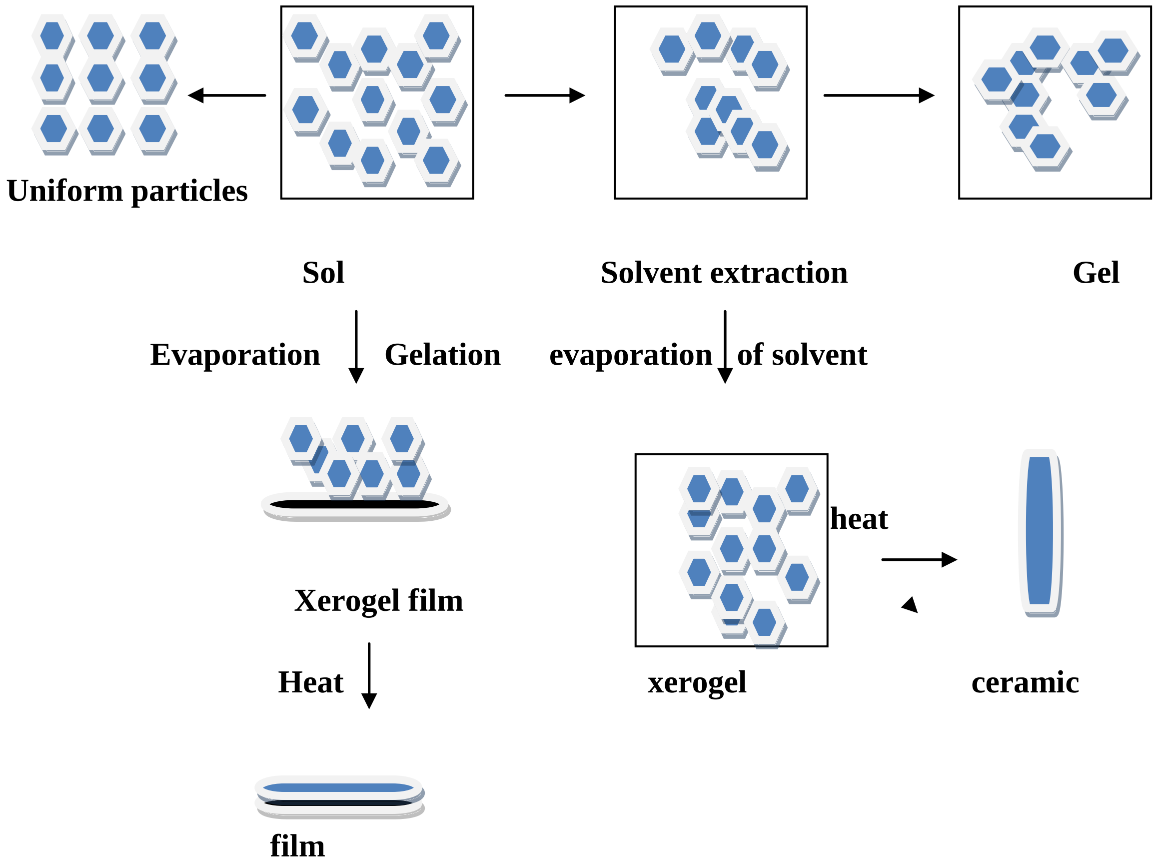


Figure 4: Overview of the Sol-gel Process illustrating the differences between Xerogels and Aerogels [21]

Surfactants are often removed by calcination, or burning, to produce molecular sieves with narrow pore size distributions and highly ordered mesostructures. These types of materials yield reflections in the low angle region of a powder X-ray diffraction pattern. The M41S family includes MCM-41 with two-dimensional hexagonal alignment of mesopore channels, MCM-48 with three dimensional cubic orders, and the layered material MCM-50. FSM-16 is a mesoporous silicate with hexagonal order and is formed by structural rearrangement of layered clay by intercalating surfactant micelles as in the figure 5. Since the first reports of M41S, many different surfactants, precursors and combinations of the two have been studied. Alkyl amines have been used to prepare the HMS group of materials [23], the wormlike mesopore systems were templated with poly (ethylene oxide) surfactants [24], and block copolymers have generated the SBA family of materials [25]. The product designated SBA-15 has hexagonal order like MCM-41 and often features micropores that allow interconnectivity between pore channels.

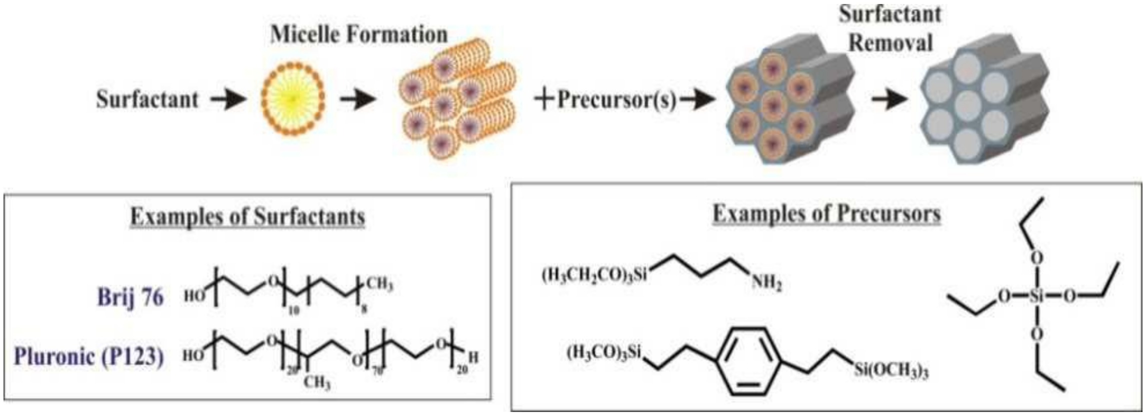


Figure 5: Illustration of the Steps Involved in the Synthesis of Surfactant Templated silicate Materials. [26]

1.2.3 Entrapment

Entrapment is one of the immobilization techniques that can be defined as physical restriction of enzyme within a confined space or network. Gelation of polyanionic or polycationic polymers by the addition of multi-valent counter-ions is a simple and common method of enzyme entrapment. Alginates are one of the most frequently used polymers due to their mild gelling properties and non toxicity. Alginate is an anionic linear copolymer composed of 1, 40-linked β-D-mannuronic acid and α-L-guluronic acid in different proportions and sequential arrangements. Enzymes are entrapped by drop-wise addition of an aqueous solution of sodium alginate and the biocatalyst to a hardening solution of a Ca^{2+} salt. The cation acts a cross linking agent towards the alginate biopolymer and the droplets precipitate as beads with the biocatalysts entrapped within the network. Even though various enzymes have been immobilized in alginate gel beads, effects of immobilization conditions on loading efficiency and immobilization yield have not been fully examined [25-27].

The entrapment of lipases in sol–gel material has been undertaken to improve the enzyme stability. The sol–gel method is allowed to make the support material and to entrap the enzyme in a single step, starting from a solution containing both the matrix precursors and the enzyme, so that when the gelation occurs, the enzyme remains entrapped and uniformly dispersed in the gel. Moreover, due to high hydrolysis and polycondenzation rates offered by silica precursors, a neutral pH could be adopted, avoiding any possible configurational modification of the enzyme induced by acid or basic environments. So far, silica has been used as a support for enzyme immobilization only in the form of a silica composite [28, 29], obtained by casting on an electrode a solution containing silica and an enzyme. Lipases, chosen as model enzyme, are used in a growing number of industrial applications, both in aqueous and in non aqueous media. In particular, immobilized lipases have been successfully used in organic synthesis [30-32]. Also, lipases have been recently used in biosensors for the determination of lipids in biosensing [33, 34]. So far, immobilization of lipases by the sol–gel technique has been carried out only using silica-based materials [35-37] Lipase being a versatile enzyme has been selected in the present work for the immobilization on MCM 41 support.

Chapter2

Objectives

1. Immobilization of *Candida rugosa* lipase on mesoporous MCM- 41 by entrapment technique.
2. To study the activity of the immobilized enzyme towards the hydrolysis of *p*-nitrophenyllaurate and study the kinetics of the reaction.
3. Application of the immobilized lipase for the transesterification of used cotton seed oil.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Instruments

Candida rugosa lipase, Tetraethoxysilane (TEOS, 95%), Iso-butyltrimethoxysilane (iso-BTMS, 97%) and *p*-notrophenyllaurate were procured from Sigma-Aldrich, USA.
Cotton seed oil was purchased from the local shops located at Patiala. Methanol, ethanol, Hexane (GC grade), and sodium fluoride (NaF) were purchased from Loba chemicals, India.

The SEM images were recorded on emission scanning electron microscope (FESEM), JEOL JSM 6510LV JAPAN. Fourier transformed infrared (FT-IR) spectra 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:

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

Preparation of 50 mM phosphate buffer

Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$) and sodium phosphate dibasic (Na_2HPO_4) are used in de-ionised water for the prepration of 50 mM Phosphate buffer at pH = 6, 7, 8.

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.

Table 4: Preparation of 50 mM Phosphate buffer at different pH

50mM phosphate at different pH	**Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$)**	**Sodium phosphate dibasic (Na_2HPO_4)**	**De-Ionised Water**
pH=6	595.60mg	97.13mg	100ml
pH=7	266mg	435mg	100ml
pH=8	40.95mg	667.85mg	100ml

Chapter 4

Experimental Work

4.1 Preparation of MCM - 41

A magnetic stirrer equipped with a 250 mL beaker was charged with a solution of 0.6g of CTAB, 2ml ammonia and 50ml of deionised water. The resulted mixture was stirred continuously for 1 h to obtain a homogenous and clear solution. To this 2.5ml of TEOS was added and resulted suspension was stirred at 40℃ for 20 h. After the stipulated time the resulted suspension was filtered to obtain a white solid. The solid thus obtained was washed with deionised water and ethanol and finally calcined at 550℃ for 5h.

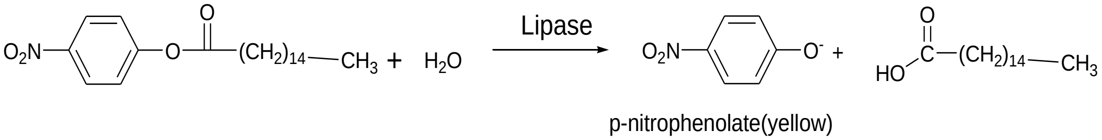
4.2 Entrapment of immobilized *Candida rugosa* lipase

A magnetic stirrer equipped with round bottom flask was charged with a solution of 60 mg of pure *Candida rugosa* (CR), 5ml water, 0.5ml sodium fluoride and silica precursors (0.8ml TEOS and 4.5 ml *iso*-BTMS) .The resulted mixture was stirred continuously for 1h and was kept at room temperature for 24h.After the stipulated time the mixture was shifted to 250ml beaker and was incubated in water bath at 33℃ . The resulted mixture was dried at 35℃ to form a ceramic powder which was later broken up and grounded in a mortar to form a ceramic powder. The resulted powder so obtained was washed with 100 ml of distilled water for 1 h at a mixing speed of 500 rpm. The wet paste was dried again at 33℃ for 24 h. CRL – MCM 41 was crushed to yield a powder and stored at 4℃.

4.3 Lipase Activity Assay

Preparation of *p*-nitrophenyl laurate solution

The stock solution of the substrate, *p*-nitrophenyl laurate (*p*-NPL), of concentration 1.0 mM has been prepared in pure ethanol and stored in refrigerator. The same solution has been used for making the *p*-NPL solution of appropriate concentrations. *p*-nitrophenyl laurate has been taken as model compound for esters and upon hydrolysis, it yielded chromogenic *p*-nitrophenol as shown in the figure below, and could be easily followed by UV-Visible spectroscopy.



Hydrolysis of *p*-nitrophenyl laurate to *p*-nitrophenolate

Hydrolysis of *p*-nitrophenyl laurate using immobilized *Candida rugosa* lipase:

In a typical hydrolysis experiment, 5 mL phosphate buffer (50 mM, pH 8), 15 mg of immobilized enzyme and then 2 mL *p*-nitrophenyl laurate of varying concentrations (0.5 mM, 0.3 mM and 0.7 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 centrifuged to remove clear supernatant from solid biocatalyst. The absorption of supernatant was subjected to the absorption in the range of 360 to 450 nm against a blank having all the components of reaction mixture except the substrate. *p*-nitrophenol formed during the hydrolysis reaction has been quantified by the absorption band appearing at λ_{max} = 403 nm.

Chapter 5

Results and Discussions

Characterization of immobilized lipase (CRL-MCM-41)

5.1 Scanning Electron Microscopy:

The FESEM image of immobilized enzyme *Candida rugosa* lipase MCM41 (CRL-MCM41) was taken. SEM image reveals that (CRL-MCM-41) was found exist in spherical shaped particle of 2μm.

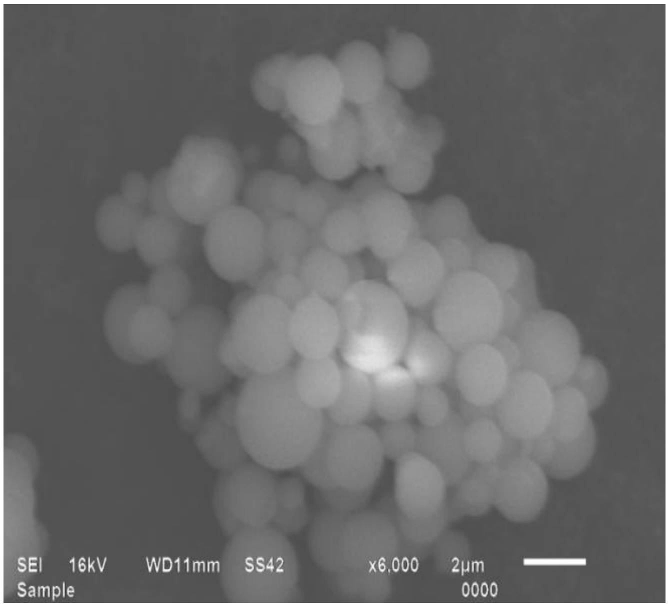


Figure 1: SEM image of CRL-MCM-41

5.2 Fourier Transform Infrared Spectroscopy

The FTIR spectra's of immobilized enzyme *Candida rugosa* lipase (CRL-MCM-41) and MCM-41is shown in the figure 2.The spectra reveals that peak of MCM-41 is due to the symmetric vibrations of Si-O-Si whereas in case of (CRL-MCM-41) is due to carbonyl stretching which comes out to be at 1634 cm^{-1}.

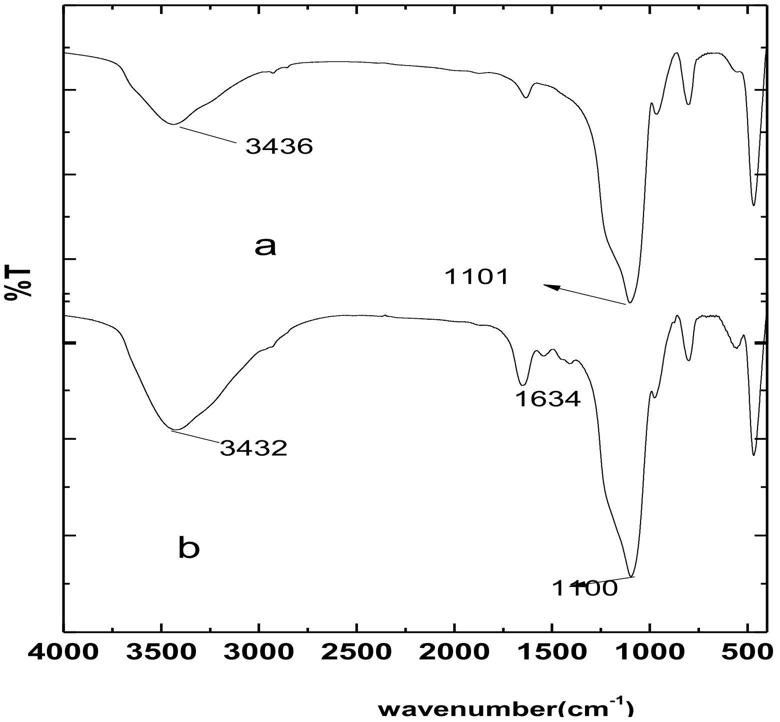


Figure 2: FTIR spectra of (a) MCM-41 (b) Immobilized *Candida rugosa* lipase (CRL-MCM41)

5.3 Hydrolysis of *p*-nitrophenyl laurate by CRL-MCM41

In order to test the hydrolytic activity of immobilized lipase, *p*-nitrophenyl laurate has been selected as substrate. The hydrolysis of the substrate was performed in the presence of pure and immobilized enzyme. The progress of the reaction was monitored by recording the absorbance at 403 nm due to the formation of *p*-nitrophenol ($\varepsilon = 14080\ M^{-1}cm^{-1}$), and shown in fig.3

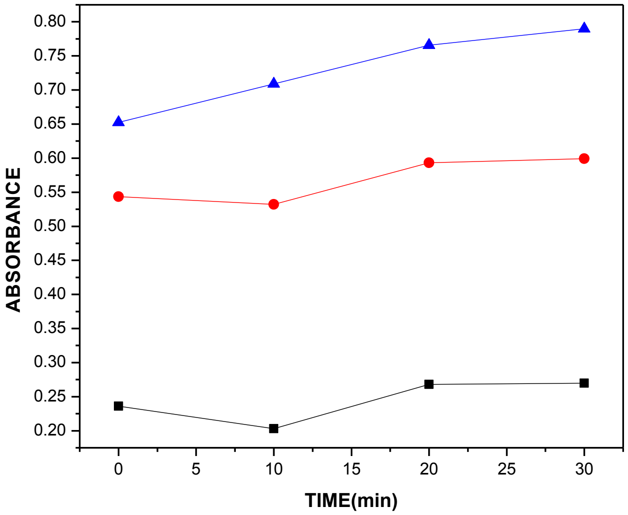


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Figure 3: Absorbance at 403 nm, showing the hydrolysis of *p*-NPL by immobilized lipase (CRL-MCM-41); (■ 0.3 mM; ● 0.5 mM; ▲ 0.7 mM).

5.4 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 (14080 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*-NPL by immobilized enzyme. It shows the increase in concentration of *p*-nitrophenol with time. The concentrations of *p*-nitrophenol have been determined for hydrolysis of *p*-nitrophenyl laurate (0.3 mM, 0.5 mM and 0.7 mM) and then graph was plotted between concentration of *p*-nitrophenol (μmol) and time to determine the initial rate as shown in Figure 4.

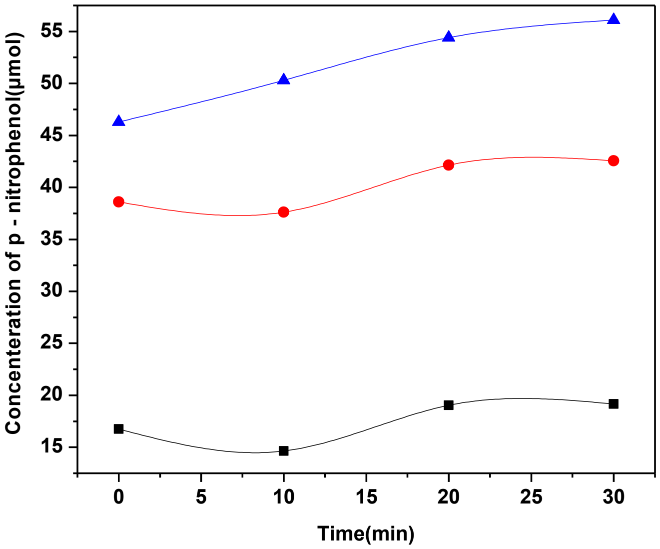

Figure 4: Concentration of *p*-nitrophenol produced during the hydrolysis of *p*-npl (■ 0.3 mM; ● 0.5 mM; ▲ 0.7 mM)

The initial rate of the reactions at various substrate concentrations were plotted as shown in Figure 5. In order to determine the various kinetic parameters of the reaction The Lineweaver-Burk mothod was employed.

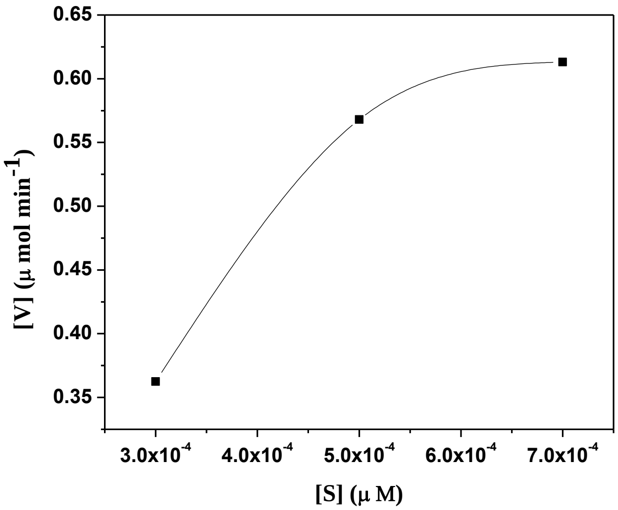

Figure 5: Michaelis-Menten Plot

5.5 DETERMINATION OF KINETIC PARAMETERS

Michaelis-Menten constant (K_m) and maximum rate of reaction (V_{max}) for immobilized *Candida rugosa* (CRL-MCM-41) have been determined by using Michaelis-Menten approach. Ii this method the Michaelis-Menten equation has been rearranged as:

$$1/V = K_m/([S]V_{max}) + 1/V_{max}$$

Where, K_m = Michaelis constant, S = substrate concentration, V = initial rate of reaction, and V_{max} = maximum rate of reaction.

The Lineweaver-Burk plot was plotted between the inverse of initial rate of reaction (1/V) and inverse of respective substrate concentrations (1/S) as shown in Figure 6. 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 axes were represented as $1/K_m$ and $1/V_{max}$ respectively. From the graph the value of Km and Vmax were found to be 3.8×10^{-3} and 2.0×10^{-6} respectively.

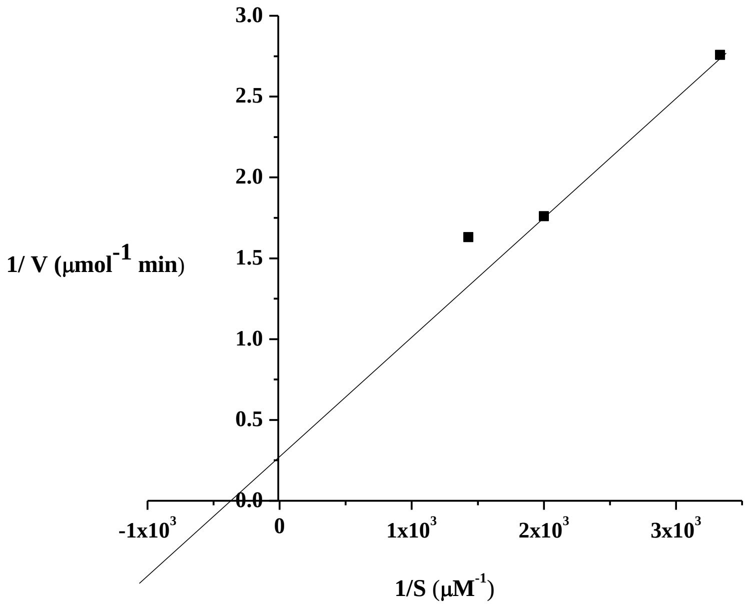

Figure 6 Lineweaver-Burk plot

5.5.1 **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}. The value of V_{max} has no absolute value but varies with the amount of enzyme used. A valuable constant in addition to K_m and V_{max} is turnover number (k_{cat}), and same could be defined as:

$k_{cat} = V_{max} / [E]$ **,**E = enzyme concentration.

The value obtained for k_{cat} is 7.1 x 10^{-2} min^{-1}

5.5.2 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 18.6 $(\mu M)^{-1}$ min^{-1} for CRL-MCM-41.

Table: 1 Observed values of the various Kinetic parameters obtained for immobilized lipase (CRL-MCM 41)

Kinetic Parameters	**Immobilized Enzyme(CRL-MCM-41)**
K_m (μM)	3.8 x 10^{-3}
V_{max} (mol min^{-1})	2.0 x10^{-6}
k_{cat} (min^{-1})	7.1 x 10^{-2}
Specific Constant ($(\mu M)^{-1}$ min^{-1})	18.6

Future studies: In present work immobilized enzyme was employed as a catalyst for the transesterification of the *p*-nitrophenyl laurate. Same could be employed as solid catalyst for the transesterification of the triglycerides in order to produce the fatty acid methyl esters (biodiesel).

Chapter 6

Conclusion

In the present work, *Candida rugosa* lipase was immobilized by sol gel technique in MCM-41. The immobilized enzyme was characterized by SEM, and FTIR studies. In order to test the catalytic activity of the immobilized enzyme, it was employed for the hydrolysis of the p-nitrophenyl laurate. The 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 lipase were determined by following Lineweaver Burk method.

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