Mesoporous Silica as Supports for Biocatalysts:
Introduction and Literature survey

Recently, the demand for well ordered mesoporous materials has triggered major synthetic efforts in academic and industrial laboratories due to commercial interest in their use as adsorbents, catalysts, catalyst supports and adsorption of large bio-molecules owing to their high specific surface area, large specific pore volume and pore diameter. Biocatalyst technology, as a part of a broader “chemical biotechnology,” is increasingly important as a tool for chemical synthesis. Its application is driven by consumer demand for new products and by industrial attempts at increasing profits via cost reduction, as well as government and regulatory pressures, new technologies and scientific discovery. Current applications of biocatalysts include the production of high fructose corn syrup, aspartame, semi-synthetic penicillins and award-winning cancer drugs. Thermostable α-amylases from Bacillus species are of great industrial importance in the production of corn syrup or dextrose. Despite these applications, biocatalysts cannot reach their potential without a concerted effort on the part of industry, non-profit and government funding agencies, as well as academic and national laboratories. One of the means of reducing the total cost in the enzyme industry is immobilization.
1.1 Mesoporous materials

Porous materials are classified into several kinds by their pore size. According to IUPAC notation a mesoporous material is a material containing pores with diameters between 2 and 50 nm., microporous materials have pore diameters of less than 2 nm and macroporous materials have pore diameters of greater than 50 nm; the mesoporous category thus lies in the middle[1]. Typical mesoporous materials include some kinds of silica and alumina that have similarly-sized fine mesopores. Mesoporous oxides of niobium, tantalum, titanium, zirconium, cerium and tin have also been reported [2]. According to the IUPAC notation, a mesoporous material can be disordered or ordered in a mesostructure [3]. The first mesoporous material, with a long range order, was synthesized in the early 90s, by a research group of the former Mobil Oil Company. Since then, research in this field has steadily grown. Technical advances in various fields, such as adsorption, separation, catalysis, drug delivery, sensors, photonics, and nanodevices, require the development of ordered porous materials with controllable structures and systematic tailoring pore architecture [4, 5]. The structural capabilities at the scale of a few nanometers can meet the demands of the growing applications emerging in processes involving large molecules, for example, biology and petroleum products. Zeolites or microporous materials, whose pore sizes are less than 1.2 nm, are far away from these demands. These motivations spark the proliferation of mesoporous materials [6-8].

1.2 Ordered mesoporous materials via nonionic-surfactant-templating approach

In materials science, building blocks play key roles which have controllable properties, as well as ordered, complex and integrated structures. For synthetic
chemistry, supramolecular assembly represents a powerful methodology in the creation of large, discrete, ordered structures. A large number of materials have been developed, particularly periodic mesoporous solids. They combine liquid-crystal packing with rigid frameworks. Exceptional properties of highly ordered mesostructures, i.e. their large surface areas and uniform pore sizes endow them with powerful properties with countless potential applications in adsorption, separation, catalysis, and photonics.

The synthesis of mesoporous molecular sieves is mainly concerned with “building mesopores”. In general, two classes of materials have often been integrated as components in this mesoporous family, including mesoporous molecular sieves with open framework structures, mesoporous silicate replicas constructed by nanowire arrays. Mesoporous molecular sieves, which are obtained from the organic inorganic assembly by using soft matter, that is, organic molecules or supramolecules (e.g., amphiphilic surfactants and biomacro molecules) as templates, clearly contribute the main mesoporous family members. Surfactants are mostly used as templates. The open framework and tunable porosities endow mesopores accessible to large biomolecules, metal ions and reagents. The organic inorganic self-assembly is driven by weak noncovalent bonds such as hydrogen bonds, van der Waals forces and electrovalent bonds between the surfactants and inorganic species. Cooperative assembly between organic surfactants and inorganic precursors is generally involved, forming inorganic/organic mesostructured composites. Mesoporous molecular sieves can be obtained after the removal of surfactants. Therefore, the surfactant self-assembly is particularly essential for the formation of highly ordered mesostructures. On the basis of the current
knowledge on the surfactant self-assembly, the mesoporous materials can be rationally designed and the synthesis can be controlled.

Two most common representatives of ordered silica are MCM-41 (Mobil Composition of Matter) and SBA-15 (Santa Barbara Amorphous). Both of them possess a well-order two-dimensional (2D) hexagonal (p6mm) array of mesopores. Depending on the shape of the supramolecular template, hexagonal phase MCM-41 [9], cubic phase MCM-48 [10] and lamellar phase MCM-50 [11] have been discovered. MCM-41 materials are usually synthesized in a basic medium in the presence of cationic surfactants such as cetyltrimethylammonium. Its pore diameter may be adjusted in the range from approximately 20-100 Å by changing the length of the alkyl chain of the surfactant molecule, by adding expander molecules (such as 1,2,3-trimethylbenzene) which increase the size of the hydrophobic region of the micelles, and by changing the synthesis conditions [12-14]. However, the silica walls of MCM-41 are thin (generally less than 15 Å, without additional treatment) resulting in low stability in the presence of water. Indeed, MCM-41 materials readily lose their hexagonal structure upon treatment in boiling water for short periods of time [15-16]. This lack of hydrothermal stability is a considerable drawback with respect to the use of MCM-41 kind materials in applications requiring the presence of water especially for the immobilization of enzymes.

The Santa Barbara group has contributed largely to the development of the meso ordered materials formed with non ionic Pluronics as structure-directing agents in acid medium [17]. The two-dimensional
hexagonal form SBA-15 (p6mm) and the cubic (Im_33m) form called SBA-16, as well as several other structures, were first synthesized by this group. There has been a great interest in these structures and the potential for controlling parameters such as the structure, the pore size and the wall thickness is naturally very appealing. This new class of materials offers the unique opportunity to tailor the pore size of the mesoporous host to accommodate small and large biomolecules by changing the synthesis conditions. SBA-15 materials have been synthesized in an acidic medium with the use of commercially available, low-cost bio degradable, nonionic supramolecular templates [17, 18]. Amphiphilic Pluronic surfactants, commonly used in the synthesis of SBA-15, are formed from hydrophilic poly(ethylene oxide, EO) and hydrophobic poly(propylene oxide, PO) blocks and have the following structure \( \text{EO}_m\text{PO}_n\text{EO}_m \) in which \( n \) and \( m \) can be varied. SBA-15 materials prepared using \( \text{EO}_{20}\text{PO}_{70}\text{EO}_{20} \) (Pluronic P123) exhibit surface areas of 690–920 m\(^2\)/g, pore volumes between 0.56 and 1.23 mL/g, pore sizes between 47 and 89 Å, and unusually thick walls between 31 and 64 Å [19]. Because of thicker silica wall compared to that of MCM-41, SBA-15 materials show a much higher thermal and hydrothermal stability, being stable for at least 48 h in boiling water [17, 18]. Another characteristic feature of SBA-15 materials is its unique dual pore system formed by hexagonally arranged cylindrical mesopores with micropores within the walls, which provide connectivity between large pores [20-24]. The textural characteristics of SBA-15 materials can be easily changed using the same experimental approaches as the aforementioned for MCM-41 (change in surfactant structure, addition of swelling agents and variation of the synthesis conditions) [25-27]. In this
context, it was found that heat treatment during synthesis or ageing stages of SBA-15 preparation is more effective for increasing the mesopore diameter and pore volume rather than the addition of 1,3,5-trimethylbenzene [25,26]. Variation of experimental conditions used for the SBA-15 synthesis represents an easy and efficient method for controlling the textural properties that can be beneficial for certain applications, for example, a good accessibility of the pores for the reagents can be reached, providing a great enhancement in the activity and selectivity of the catalyst. The optimization of the synthesis of the SBA-15 has received a lot of attention in the past few years. However, the problem dealing with how to control good pore size of SBA-15 materials has not been solved yet. SBA-15 silica displayed significantly higher stability under various conditions (steaming, high temperature) compared with MCM-41 silica. Therefore, it is frequently used for synthesis of various advanced materials.

1.3 Mechanism of formation of SBA-15

The general idea of using block copolymers as templates is based on the fact that amphiphilic block copolymers self assemble in certain solvents to give robust, very regular superstructures that feature structural motifs on the nanometer scale (lyotropic liquid-crystalline phases). Typically, the constituting entities are spherical or cylindrical micelles or lamellar sheets with a characteristic cross-section of 5-100 nm.

The block copolymer self-assembly technique is probably the best examined mode of self-organization and is governed by the microphase separation dictated by the mutual incompatibility of the different blocks, one
being soluble and the other being insoluble in the solvent to be used ("amphiphilic" polymers). In principle, block copolymers self-assemble or microphase-separate in a variety of solvents, in particular water, but also alcohols or THF, which enables a certain width of chemistry for inorganic framework generation.

The mechanism is most likely via an $\text{S}^0\text{H}^+\text{X}^-\text{I}^+$ double layer hydrogen bonding interaction. Here $\text{I}^+$ are inorganic silicate precursor cations, and $\text{X}^-$ are counter-anions. It is the cooperative interaction between inorganic and organic species at molecular scale that leads to assembly to 3D or 2D ordered arrangements. Silicate polyanions can interact with cationic surfactant molecules through coulombic forces. The polymerization of silicate species at the interface changes the charge density of inorganic layers, and in turn, the arrangement of surfactants. The matching of charge density at the surfactant/inorganic interface governs the assembly process [28].

A large number of studies have been carried out to investigate the formation and assembly of mesostructures on the basis of surfactant self-assembly. Two main pathways, that is, cooperative self-assembly and "true" liquid-crystal templating processes, seem to be effective in the synthesis of ordered mesostructures. The most popular mechanism for the formation of mesoporous solids is known as the cooperative formation mechanism which was first proposed by Stucky and coworkers.
This cooperative formation mechanism in a nonionic surfactant system was investigated by in situ techniques. Goldfarb and co-workers investigated the formation mechanism of mesoporous silica SBA-15, which are templated by triblock copolymer P123 (EO\textsubscript{20}PO\textsubscript{70}EO\textsubscript{20}) by using direct imaging and freeze-fracture replication cryo-TEM techniques, in situ electron paramagnetic resonance (EPR) spectroscopy, and electron spin-echo envelope modulation (ESEEM) experiment [29,30]. They found a continuous transformation from spheroidal micelles into threadlike micelles. Bundles were then formed with dimensions that are similar to those found in the final materials. The elongation of micelles is a consequence of the reduction of polarity and water content within the micelles due to the adsorption and polymerization of silicate species. Before the hydrothermal treatment, the majority of PEO chains insert into silicate frameworks, which generate micropores after the removal of templates. Moreover, they found that the extent of the PEO chains located within the silica micropores depended on both the hydrothermal ageing temperature and the Si/P123 molar ratio. The formation
dynamics of SBA-15 was studied by Flodstrom et al. on the basis of time-resolved \textit{in situ} $^1$H NMR and TEM investigations [31]. They observed four stages during the cooperative assembly, which are the adsorption of silicates on globular micelles, the association of globular micelles into floes, the precipitation of floes, and the micelle-micelle coalescence. Khodakov et al. proposed a structure with a hydrophobic PPO core and a PEO-watersilicate corona in the first stage [32]. Then the cylindrical micelles pack into large domains.

The initial liquid-crystal template mechanism first proposed by Mobil's scientists is essentially always "true", because the pathways basically include almost all possibilities [9, 10]. In this pathway, true or semi-liquid-crystal mesophases are involved in the surfactant templating assembly to synthesize ordered mesoporous solids. The condensation of inorganic precursors is improved owing to the confined growth around the surfactants and thus ceramic-like frameworks are formed. After the condensation, the organic templates can be removed by calcination, extraction, etc. The inorganic materials "cast" the mesostructures, pore sizes, and symmetries from the liquid-crystal scaffolds.

1.4 Synthesis of Mesoporous Silicate Molecular Sieves

1.4.1 Hydrothermal Method

Mesoporous silicates are generally prepared under "hydrothermal" conditions. The typical sol-gel process is involved in the "hydrothermal" synthesis. A general procedure includes several steps. First, a homogeneous solution is obtained by dissolving the surfactant(s) in a solvent. Water is the most common solvent and medium. Silicate precursors are then added into the solution where they undergo hydrolysis catalyzed by acid or base and transform to a sol of silicate oligomers. As a result of the interaction between
oligomers and surfactant micelles, cooperative assembly and aggregation give precipitation from a gel. During this step, microphase separation and continuous condensation of silicate oligomers occur. The formation of mesoporous silicates is rapid, only 3-5 min in cationic surfactant solutions, which is reflected by the precipitation and 30 min for non ionic surfactants. Hydrothermal treatment is one of the most efficient methods to improve mesoscopic regularity of products [33]. After the solution reaction, the mesostructures undergo reorganization, growth, and crystallization during hydrothermal treatment. The treating temperature is relatively low, between 80 and 150 °C, in which the range of 95-100 °C is mostly used.

1.5 Fundamental principles that govern the design and synthesis of mesoporous silica

- Amphiphilic block copolymers self assemble as lyotropic liquid crystalline phases.

- Alkoxy silane species are hydrolysed.

\[ \text{Si(OEt)}_4 + n\text{H}_3\text{O}^+ \rightarrow \text{Si(OEt)}_{4-n}\text(OH}_2^+ + n\text{EtOH} \]

- EO moieties of the surfactant in strong acid media associating with hydronium ions.

\[ \text{REO}_m + y\text{HX} \rightarrow \text{REO}_{m-y}[(\text{EO})\text{H}_3\text{O}^+]y.....\text{YX}^- \]

Organic inorganic self-assembly is driven by weak non covalent bonds such as H bonds, van der Waals forces and electrovalent bonds between the surfactants and inorganic species.

\[ \text{REO}_{m-y}[(\text{EO})\text{H}_3\text{O}^+]y...\text{YX}^-...\text{I}^+ \equiv \text{S}^0\text{H}^+\text{XT}^+ \]
1.6 Enzymes as catalysts

Biocatalysis has a large impact in the chemical world. Uses are as divergent as chiral enzymatic transformations within an organic synthesis for a drug or for microbial desulfurization of diesel fuels. It suggests the potential and challenge of applying biocatalysis to all feedstreams. “Chemical biotechnology” is the rapidly growing application of biotechnology to chemical production [34-36]. It often goes hand-in-hand with green chemistry and the use of renewable feedstocks. Other applications of biotechnology lead to new products, new manufacturing methods and improved deleterious impacts on the environment.

The chemical industries are beginning to realize that enzymes are not only effective for catalyzing reactions of “natural” compounds within living systems, but that they can be used to catalyze reactions of “unnatural” compounds. Enzyme biocatalysts are being applied in the production of fine chemicals, pharmaceuticals and agricultural chemicals. Their attractiveness comes from high selectivity, ability for use under ambient conditions, and ease of disposal. The enzyme nitrile hydratase from a R. rhodococcus strain has been developed for the hydrolysis of acrylonitrile to acrylamide for use in plastics [37]. The enzyme is immobilized in whole cells and can produce acrylamide concentrations greater than 600 g/L. The biocatalytic approach has reached a production level of 100,000 tons/yr. The DSM-Toyo Soda process uses the enzymatic protease thermolysin for manufacture of aspartame, and is illustrative of two types of biocatalyst selectivity: chemical and stereoselectivity [37]. High-fructose corn syrup produced in large quantities is an enzyme-based product. The process includes three enzymatic
steps: the α-amylase catalyzed liquefaction of corn syrup, further hydrolysis of sugar oligomers by glucoamylase, and the isomerization of glucose to the glucose-fructose mixture. The hydrolysis of penicillin G or V to 6-aminopenicillanic acid (6f-APA) using penicillin acylase is an early success story for the use of enzymes in chemical manufacture. DuPont and Genencor have filed patents for processes and microorganisms to make 1,3 propanediol (1,3-PD) by fermentation in one step from various carbohydrate sources. The 1,3-PD is used in the production of the polyester polytrimethylene terephthalate. Cargill-Dow Polymers is developing a large-scale fermentation process alongside their other corn processing systems followed by chemical processing (a type of biorefinery) to generate polylactic acid for a multitude of applications including biodegradable sutures, biocompatible fibers, packaging, and functional replacements for commodity plastics such as styrene [38]. Enzyme recovery from a homogeneous catalytic process can significantly increase production costs. One method of lowering these processing requirements is to use a heterogeneous catalyst—an immobilized enzyme.

1.7 Immobilized enzymes

Immobilization means associating the biocatalysts with an insoluble matrix, so that it can be retained in proper reactor geometry for its economic reuse under stabilized conditions [39]. Since the second half of the last century, numerous efforts have been devoted to the development of insoluble immobilized enzymes for a variety of applications. The immobilized enzymes are more useful than the soluble counterparts: for instance as reusable heterogeneous biocatalysts, as stable and reusable devices for
analytical and medical applications as selective adsorbents for purification of proteins and enzymes as fundamental tools for solid-phase protein chemistry and as effective micro devices for controlled release of protein drugs [39-43].

The advantages of immobilization are,

- The enzyme can be easily removed from the product mixture
- The enzyme can be packed into columns and used over a long period
- Speedy separation of products reduce inhibition
- Thermal stability is increased allowing higher temperatures to be used.
- Higher operating temperatures increase rate of reaction

Although in 1916, Nelson and Griffin discovered that artificial carrier-bound invertase on Al (OH)₃ and charcoal was still catalytically active, the potential of bioimmobilization as a method of obtaining useful and reusable immobilized biocatalysts was unfortunately not recognized in the succeeding 40 years [44]. This simple fortuitous discovery has, however, been widely recognized as the cornerstone of the various enzyme-immobilization techniques currently available, because in the last half century it actually stimulated much interest and effort in exploration of insolubilized active enzymes for various studies and industrial applications that can be better met with immobilized rather than free enzymes. A systematic approach to enzyme immobilization starts in the late 1940s. From 1950s onwards much work has been devoted to the search for polymer supported materials that strongly binds the protein. Organic polymers like Eupergit C, oxirane acrylic beads, polysaccharides, carbon, polystyrenes, polyacrylates, maleic anhydride based
copolymers, polypeptides, vinyl and allyl polymers and polyamides have been commercially used as enzyme carriers [45]. Up to now, more than 5000 publications and patents have been published on enzyme immobilization techniques. Several hundred enzymes have been immobilized in different forms and approximately a dozen immobilized enzymes, for example penicillin G acylase, lipases, proteases, invertase, etc. have been used as catalysts in various large scale processes. Till 1990s organic polymeric carriers are the most widely studied supports because of the presence of rich functional groups, which provide essential interactions with the enzymes [45]. But the organic supports suffer a number of problems such as,

- Poor stability towards microbial attacks
- Poor stability to organic solvents
- Disposal issues and toxicity
- Deactivation due to protein unfolding

So the better alternatives for organic supports are the inorganic materials. Adsorption of protein over sol-gels and controlled porous glass (CPG) has been extensively studied for possible applications as biosensors and reviewed by Weetall and Avnir [46-48]. However, sol-gels are found to be unsuitable for the immobilization of proteins due to their broad pore size distribution. On the other hand, the major disadvantage of CPG materials for adsorption studies are their high cost and more importantly their surface area, which rapidly decreases with increasing pore size (30-200 nm). A large number of techniques and supports are now available for the immobilization of enzymes or cells on a variety of natural and synthetic supports. The choice
of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its ultimate application. Therefore, it will not be possible to suggest any universal means of immobilization. The most important requirements for a support material are that it must be insoluble in water, have a high capacity to bind enzyme, be chemically inert and be mechanically stable. The enzyme binding capacity is determined by the available surface area, both internal and external or the ease with which the support can be activated and the resultant density of enzyme binding sites [49]. The activity of the immobilized enzyme will also depend upon the bulk mass transfer and local diffusion properties of the system.

Template assisted synthesis of mesoporous materials with designed structure and function will provide the most suitable surface for enzyme immobilization which is to be discussed in detail later [49].

1.8 Methods of Immobilization

When immobilizing an enzyme to a surface, it is most important to choose a method of attachment that will prevent loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme. The commonly used methods for immobilization are discussed below. Choice is governed by a number of factors some of which will not be apparent until the procedure is tried.

1.8.1 Carrier-binding

The carrier-binding method is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. In general,
an increase in the ratio of hydrophilic groups and in the concentration of bound enzymes, results in a higher activity of the immobilized enzymes. Some of the most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel. According to the binding mode of the enzyme, the carrier-binding method can be further sub-classified into: a. Physical adsorption b. Ionic binding and c. Covalent binding [50-52].

a. **Physical adsorption**

Physical adsorption of an enzyme onto a solid is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid. A major advantage of adsorption as a general method of insolubilizing enzymes is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap, easily carried out, and tends to be less disruptive to the enzymic protein than chemical means of attachment, the binding being mainly by hydrogen bonds, multiple salt linkages, and Vander Waal’s forces. In this respect, the method bears the greatest similarity to the situation found in biological membranes in vivo and has been used to model such systems. A disadvantage is the weakness of the adsorptive binding forces; adsorbed enzymes are easily desorbed by temperature fluctuations and even more readily by changes in substrate concentration and ionic strength [53].

b. **Ionic binding**

The physical adsorption can be turned as ionic binding as the material for adsorption and proteins acquire charge when immersed in suitable
solvents. Depending on the pH of the medium, the biocatalyst protein can be positively or negatively charged. Such charged species can easily be coupled to ionic supports. Although ionic binding is stronger than physical adsorption; the biocatalyst is subject to leaching due to ionic strength and pH changes in the medium. It is extremely simple and the coupling is rather mild in nature [54].

c. Covalent binding

The most intensely studied insolubilization technique is the formation of covalent bonds between the enzyme and the support matrix. The functional groups of proteins suitable for covalent binding under mild conditions include (i) the alpha amino groups of the chain end and the epsilon amino groups of lysine and arginine, (ii) the alpha carboxyl group of the chain end and the beta and gamma carboxyl groups of aspartic and glutamic acids, (iii) the phenol ring of tyrosine, (iv) the thiol group of cysteine, (v) the hydroxyl groups of serine and threonine, (vi) the imidazole group of histidine, and (vii) the indole group of tryptophan. Covalent bonding should provide stable, insolubilized enzyme derivatives that do not leach enzyme into the surrounding solution [55].

1.8.2 Cross-linking

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Many efforts were devoted to the development of cross-linked enzyme crystals (CLEC) suitable for biotransformations in non-aqueous media or in organic–water mixtures, because of the greater stability of the enzymes under hostile conditions.
Remarkably, it has been noticed that the performance of the CLEC obtained is highly dependent on the predetermined conformation of the enzyme molecules in the crystal lattice. Thus, selection of a highly active enzyme conformation by varying the crystallization conditions becomes crucial for the creation of highly active, stable and selective CLEC [56].

In other process of cross-linking, the individual biocatalytic units are joined to one another with the help of bi-or multi-functional reagents. In this way, very high molecular, typically insoluble aggregates are obtained. Cross-linking is a relatively simple process. The most commonly employed bifunctional reagent is glutaraldehyde [57]. In addition, di-isocyanates, (hexamethylene di-isocyanates) are often used as linking agents [58]. The disadvantages of cross-linking are that it is not suitable for packed bed operation and access to innermost catalytic sites is limited by the unfavorable conditions of diffusion. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity.

1.8.3 Entrapping

The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. It is done in such a way as to retain protein while allowing penetration of substrate and products. This method is particularly common for immobilization of whole cells. Suitable matrix materials are polymers, alginate, carrageenan, pectin, agar and gelatin. Entrapment of cells in alginate gel is popular because of the
requirement for mild conditions and the simplicity of the used procedure. Several reports are available employing alginate gel [59].

This method differs from the covalent binding and cross linking in that the enzyme itself does not bind to the gel matrix or membrane. Thus the conditions necessary for continuous use of the enzyme pool are accomplished. Membrane confinement can be achieved by three methods; micro-encapsulation, liposome technique and membrane reactors. All three methods result in retention of the biocatalyst within a defined space by a semi-permeable membrane, which can be crossed by the substrate(s) and product(s) but is impermeable to the biocatalyst(s) [59].

1.9 Mesoporous materials as supports for immobilization

Silica-based materials have been widely used in chemical industries for many decades, as catalysts in petrochemical refining, synthesis of fine chemicals and pharmaceutical products and as sorbents in chromatographic and environmental applications [60,61]. However, the majority of these applications use natural or synthetic zeolites with structural repeats on the 1-2 nm scale, and pore sizes less than 1 nm. Consequently, these are not suitable hosts for bio-macromolecules such as proteins (typical molecular size of 3-30 nm). The use of zeolites in biotechnology is therefore rather limited.

Mesoporous materials have a clear advantage over microporous zeolites and zeotype molecular sieves for the transformation of large organic molecules. For example, they fulfill many of the requirements for enzyme carriers such as high specific surface areas (up to ca. 1500 m²/g), high specific pore volumes (up to ca. 1.5 cm³/g), well-ordered pore structures with
uniform mesopores adjustable in diameter from about 1.5 to 30 nm, sufficient functional groups for enzyme attachment hydrophilic/hydrophobic character, water insolubility, chemical and thermal stability, mechanical strength, suitable particle form, regenerability, and toxicological safety [49].

The discovery of ordered high surface area silicas with pore sizes of 5nm and above opened the way to the study of well-defined biomolecule–mesoporous silica hybrids. In particular, it has been possible to immobilize a range of small to medium size enzymes, such as proteases, lipases and peroxidases, via physisorption, encapsulation and tethering on the internal surfaces of the solids. Use has also been made of silicas functionalized for this purpose. In many cases the immobilized enzymes are both active and reusable. Here we review the studies on enzymes immobilized on ordered mesoporous solids and assess the need for careful studies in real applications. In 1996 Diaz and Balkus first attempted to immobilize enzymes onto mesoporous MCM-41[62]. Since then, many research groups have established that many factors have a strong influence on the enzyme loading and on the activity of the resultant biocatalyst, including the relative sizes of the mesopore and the enzyme, surface area, pore size distribution, mesopore volume, particle size, ionic strength, isoelectric point, and surface characteristics of both the enzyme and the support.

The observation that some enzymes retain their functionality upon immobilization on ordered mesoporous supports triggered significant research activity in encapsulating enzymes as well as other bioactive components. Examples of a variety of biological molecules adsorbed onto ordered mesoporous silica and carbon materials were summarized by
Hartmann [49]. Immobilized enzymes in mesoporous materials have found applications in peptide synthesis [63], pulp bio-bleaching [64], biocatalysis [65-67] and biosensors [68-69]. Most studies have been carried out using the hexagonal MCM-41-type/SBA-15-type material because of its easy availability and good reproducibility in synthesis and modification. To create more suitable biocatalysts, biosensors, or to separate proteins by using Mesopores silicas (MPSs), it is of great importance to understand the factors that influence the immobilizing behaviour of proteins within MPSs. It has been found that two factors may greatly influence the immobilization properties of MPSs. The first is the size of the mesopore, or more specifically the mesopore size relative to the protein molecule size. Diaz and Balkus found that the amount of protein loading into mesoporous silica MCM-41 in a limited contact time decreased with increasing protein molecular weight [62]. This is expected if the pore size of the mesochannels is sufficiently large for 'comfortable' entrapment of biomolecules [66, 70]. Kisler et al. have demonstrated that the rate of adsorption in MCM-41 materials depends strongly on the size of the adsorbing molecule relative to the pore size for a range of biomolecules [71]. The second factor that influences the immobilizing behaviour is the surface characteristics of the MPS (mesoporous silica) and proteins. The surface charges of MPS and the proteins must be complementary, because it is generally accepted that the electrostatic interaction between protein and MPS is one of the most important factors that influence adsorption and desorption [66, 67, 72]. Some researchers studied the factors that may influence the surface properties of proteins, for example, the pH [62,66] and ionic strength [65] of the protein solution. Takahashi et al. [73] believe that MCM-41 and SBA-15 prepared with cationic and non-ionic
surfactants, respectively, have different surface characteristics, and therefore different properties of adsorption. Lei et al. reported that suitable organically functionalized mesoporous hosts provide higher affinity for charged protein molecules and the more favoured microenvironment results in exceptional immobilizing efficiency [72]. Wright and coworkers have also investigated the adsorption and desorption property of SBA-15 functionalized by thiol, chloride, amine, and carboxyl groups and it has been found that the interactions of the enzyme-support depended strongly on the nature of the functional groups attached to the surface. Fan reported that the amount and size of entrance in mesoporous materials may greatly influence the bioimmobilization behaviours of MPS [74-75] and it has been revealed that for SBA-15 type MPSs, the morphology plays an important role in the immobilization ability [75]. On the other hand, some protein adsorption behaviour has not been well understood. Deere et al. suggested that the hydrophobic interactions dominate rather than electrostatic interactions in the desorption process of cytochrome c from commercial Kieselgel silica [76]. Lie et al. believed that there are electrostatic, hydrogen bond, and hydrophilic interactions between protein and MPSs functionalized by amine and carboxyl groups [72]. In the investigations of bioimmobilization within the same MPSs (e.g. SBA-15), their immobilization behaviour of similar proteins vary significantly according to different researchers [66,77]. Therefore, there should exists other factors that might have been ignored during former studies. Considering the large pore volume (~1.0 cm³/g) of MPS, the previously reported specific immobilization capacity of MPS is still relatively low (<200 mg/g) Furthermore, it often takes several hours, even four days for the immobilization of proteins to reach equilibrium [65,71].
The stability of adsorbed enzymes should be discussed within two different concepts of enzyme stability: one is the intrinsic enzyme stability and the other is the operational enzyme stability. By definition, the intrinsic stability represents the stability of enzyme molecules themselves while the operational stability means the persistence of the enzyme activity during a process, i.e., under conditions of use.

The enzyme leaching from host materials can seriously affect the operational stability. In most studies with adsorbed enzymes, the operational enzyme stability was discussed rather than the intrinsic enzyme stability, due to the difficulties in dissecting the intrinsic enzyme stability from the results of operational enzyme stabilities. However, several recent papers discussed the improvement of intrinsic enzyme stability by confining enzymes in mesoporous materials [78, 79]. This confinement can restrain enzyme unfolding or denaturation when it is located in a pore of similar dimensions or crowded by a high concentration of enzyme molecules in the same pore. The stabilization mechanism with adsorbed enzymes in mesoporous materials has been a topic of many studies, not only for practical applications, but also for scientific interests due to the resemblance of highly concentrated enzymes in mesoporous materials to the cellular environments containing high concentration of biomolecules. Many factors are known to affect the stability of adsorbed enzymes in mesoporous materials. First, the pore size of mesoporous materials affects the adsorption behaviour and enzyme leaching [61, 62, 73, 77,]. Enzymes larger than the mesopores cannot be adsorbed within the pores. Size matching between pore and the molecular diameter of enzyme plays a key role in achieving high enzymatic stability [73, 77]. On the contrary, mesoporous materials with large pore size usually end up with poor enzyme
loading as well as poor enzyme stability due to quick leaching from the mesopores. The pore volume is also proven to determine the final amount of enzyme adsorption [62,77,80]. Secondly, the charge interaction is significant in determining the enzyme stability in mesoporous materials [66, 72, 80, 81]. If the net surface charge of enzymes is opposite to the charge of the mesopores, it will not only hasten the enzyme adsorption, but may also lead to a stable enzyme system due to the attractive interaction between two opposite charges, on the other hand, when enzymes and mesopores have the same charge, both enzyme adsorption and stability are poor due to the repulsion between the enzymes and the internal surface of mesopores. The charge status of enzymes and mesopores can be controlled by changing the pH of buffer solution [80,81] and functionalizing mesoporous materials with amino or carboxyl groups [72]. Finally, a hydrophobic modification is also known to affect the enzyme stability. Recent studies have shown that numerous functional groups, including amines, chlorides, thiols, carboxylic acids and phenyl may be attached successfully to the surface of mesoporous molecular sieves via tethering alkyl chains. These groups subsequently provide different interactions between the surfaces of the support and the enzyme molecule [82-85]. Various nanostructures for enzyme stabilization were reviewed by Kim [86].

1.10 Significance of enzyme chosen

α-amylase (EC.3.2.1.1;1,4-glucan-glucanohydrolase; endo amylase) catalyse the hydrolysis of glycosidic linkages in starch and other related oligo- and polysaccharides in an endo fashion.. These enzymes are widespread among the higher plants, animals, and microorganisms. Some of these, particularly those of bacterial and fungal origin, are commonly used in various industries
such as starch processing, paper manufacture, and pharmacology. Amylases see a great deal of use in food and fermentation industries. Thermo stable α-amylases from Bacillus species are of great industrial importance in the production of corn syrup or dextrose. Polylactic acid synthesized from corn syrup is in turn used for a multitude of applications including biodegradable sutures, biocompatible fibers, packaging, and functional replacements for commodity plastics such as styrene [87].

1.11 Structure of α-amylase

The enzyme α-amylase catalyse the cleavage of α-1, 4-glycosidic linkages of starch components, glycogen, and various oligosaccharides. This consists of a single polypeptide chain with approximately 26 % α-helix and 22 % β-sheet and has dimensions of approximately 35×40×70 (Å)[88].
Like α-amylases extracted from other sources, the polypeptide chain folds into three distinct domains. The first domain (domain A), consisting of 291 residues (from residue 3 to 103 and 207 to 396), forms a (beta/alpha) 8-barrel structure. Important active site residues can be identified as Asp231, Glu261, and Asp328, which are all located at the C-terminal end of the central (beta/alpha) 8-barrel. Domain B overlaying the active site from one side and domain C consisting of a β-structure with a Greek-key motif.

1.12 Immobilized α-amylase

In the literature there are number of reports about α-amylase immobilization on various supports using different processes. Some commonly employed organic polymers supports are polymethylmethacrylate and gamma irradiation soluble carriers (eudragit and polyethylene imine) [89,90], carbodiimide coupling paramagnetic polyacrolein beads[91] and porous polyethylene hollow fibers activated by ethanol amine or phenol [92].

Several methods have been developed for the preparation of immobilized α-amylase using inorganic materials, each having its own advantages and disadvantages. A thermo stable α-amylase was immobilized on controlled pore glass beads. The authors concluded that by the choice of a suitable pore size of the support and of a pH where the activity of the enzyme is high, the temperature need not be elevated in order to obtain a high catalytic activity [93]. α-amylase was immobilized onto the zirconium membrane by means of covalent coupling to the glutaraldehyde pretreated colloids [94]. However, the membrane could be used at a temperature below 40 °C only to maintain its enzyme activity. Pillared clays, which possess mesopore sizes, have been extensively investigated since
the 1980s [4]. Immobilization of \( \alpha \)-amylase on montmorillonite clay was extensively studied by Sanjay & Sugunan but the enzyme was not completely entrapped within the pore. They have proposed that when the huge enzyme, \( \alpha \)-amylase was immobilized on acid activated montmorillonite K-10 the polypeptide backbone did not enter the interlayer space but was situated at the periphery of the clay. Intercalation occurred through the side chains of the amino acid residues [95]. Moreover in clays, the pore sizes are widely distributed, and the arrangement of pores is disordered. \( \alpha \)-amylase was immobilized on zirconia and alumina via adsorption. From XRD, IR and N\(_2\) adsorption studies it was confirmed that the enzyme was adsorbed on the external surface of the support [96,97].

1.13 Origin of the problem

Thermo stable \( \alpha \)-amylases from Bacillus species are of great industrial importance in the production of corn syrup or dextrose. According to the reports of Sugunan et al., complete entrapment of \( \alpha \)-amylase onto inorganic porous clays was not possible due to inappropriate pore sizes [95]. Pandya et al. investigated adsorption on functionalized SBA-15 [98]. However, the material was of low quality as the synthesis was done at room temperature. To study the effect of pore size on protein adsorption and to compare the activity of enzymes entrapped in various pore sizes, perfectly ordered mesoporous materials with similar framework is the primary requirement. Most of the authors have compared the protein adsorption on different pore sizes of MCM-41 and SBA-15 with entirely different frame works. There are only very few reports with SBA-15 of various pore sizes. We have attempted to design the nanoporous
molecular sieve conserving the mesostucture, for the complete entrapment of the enzyme \( \alpha \)-amylase of dimension 35\(\times\)40\(\times\)70 (Å).

1.14 Objectives of the present work

The present work aims at synthesizing proper materials for the immobilization of enzymes so that the stability and activity of enzymes are maintained. Cheap support rice husk silica was tried for first time as support for enzyme immobilization. As the next stage of the research the most emerging and promising support mesoporous silica were tuned to entrap the enzyme \( \alpha \)-amylase within the pore. The materials were modified with functional groups, to enhance the stability. The major objectives can be outlined as,

- to extract amorphous silica from rice husk and to test whether it is compatible for immobilization of enzymes.
- to prepare mesoporous silca SBA-15 using amphiphilic surfactant following hydrothermal route.
- to study the influence of time and temperature of hydrothermal treatment on the pore characteristics of SBA-15.
- to characterize the materials via physico chemical techniques like SAXRD, Nitrogen adsorption isotherm, thermal analysis, FTIR spectroscopy, NMR spectroscopy, SEM and HRTEM.
- to functionalize mesoporous silica with APTES and characterize the material using FTIR, SAXRD,\(^{13}\)C NMR, \(^{29}\)Si NMR and Nitrogen adsorption studies.
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- to optimize pH, time, temperature, buffer concentration for immobilization.
- to characterize the immobilized enzymes, adsorbed and covalently bound via various physico chemical techniques like SAXRD, nitrogen adsorption isotherm, thermal analysis, FTIR spectroscopy, NMR spectroscopy.
- to estimate the efficiency of immobilized enzymes in comparison to free enzymes.
- to study the influence of pH, temperature, concentration on the activity of immobilized enzymes.
- evaluate the kinetics of enzyme catalysed reactions.
- understand the reusability of immobilized preparations in two reactor types.
- examine the storage stability of these systems.
- to study the enzyme leaching at different loadings.

References

### References


