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An Insight on Recent Advances on Immobilization Methods for Industrial Enzymes and its Relevance to Xylanases

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ABSTRACT

Immobilization is the key for optimizing the operational performance of an enzyme in industry. Immobilization of an enzyme provides greater resistance to changes in physiological conditions such as pH or temperature and leads to the reuse of catalysts, easier reactor operation and separation of products with a wider choice of bioreactors. By virtue of this it provides a cost-effective industrial process. It allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and can be used again in a far more efficient process and so can be widely used in industries for enzyme-catalyzed reactions. Glycoside hydrolases are enzymes hydrolyzing glycosidic linkages in complex plant polysaccharides and are thus utilized in a number of industries using plant polysaccharides as the raw material. Xylanases are glycoside hydrolases diversely used in various industries including the paper and pulp industry, brewing, animal feed, starch, textiles, etc. The present review describes the state of the art of basic immobilization strategies of enzyme immobilization. We further focus on immobilization of xylanases for commercial applications in various industries.

Keywords: enzyme, glycoside hydrolase, immobilization, xylanases

CONTENTS

INTRODUCTION

Enzyme immobilization is attachment of enzyme to inert insoluble materials that increases its resistance for change in conditions like pH and temperature and allows the enzyme to be reused. It is a very efficient process and is widely used in industries for enzyme catalyzed reactions. The history of bio-immobilization can be divided into several phases: the early days (1916–1940s), the underdeveloped phase (1950s), the developing phase (1960s), the developed phase (1970s), the post-developed phase (1980s), and the rational design phase (1990s–present) (Leeuwlaan 2005a).

Different methods for the immobilization of enzymes are critically reviewed basically, divided into three main categories, viz. binding to a prefabricated support (carrier), entrapment in organic or inorganic polymer matrices, and cross-linking of enzyme molecules. Use of novel supports such as mesoporous silicas, hydrogels, and smart polymers, novel entrapment methods and cross-linked enzyme aggregates (CLEAs) have been emphasized in various works (Sheldon 2007). Commercialization of biocatalysts requires rapid and effective immobilization techniques. Selective immobilization of proteins to self-assembled monolayers presenting active site-directed capture ligands was a major development in immobilization. MacBeath and Schreiber (2000) immobilized a series of proteins on aldehyde-terminated glass slides and showed that these proteins were able to interact with other molecules in solution. First conceived by MacBeath and Schreiber a number of other research groups have further refined the concept of peptide microarray by introducing a variety of immobilization and screening methods (Zhu *et al*. 2001; Hodneland *et al.* 2002).

The first industrially used immobilized enzymes was reported by Tosa *et al.* (1967) who developed the immobilization of *Aspergillus oryzae* aminoacylase for the resolution of synthetic racemic D-L amino acids. Some major applications of immobilized enzymes are the industrial production of sugars, amino acids, and pharmaceuticals (Takamatsu *et al.* 1993).

Various methods developed for immobilizing enzymes include adsorption method, covalent binding method, cross linking method, entrapping method and entrapping by microencapsulation. All these immobilization techniques can be broadly classified as reversible and irreversible methods.

REVERSIBLE METHODS

Adsorption is the simplest method used for immobilization of cells and enzymes. It is reversible interaction between the enzyme and the support material (**Fig. 1**). Interactions such as vanderwall forces, ionic and hydrogen binding are utilized which includes no chemical activation or modification. The immobilization can be obtained on various mat-

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Invited Mini-Review

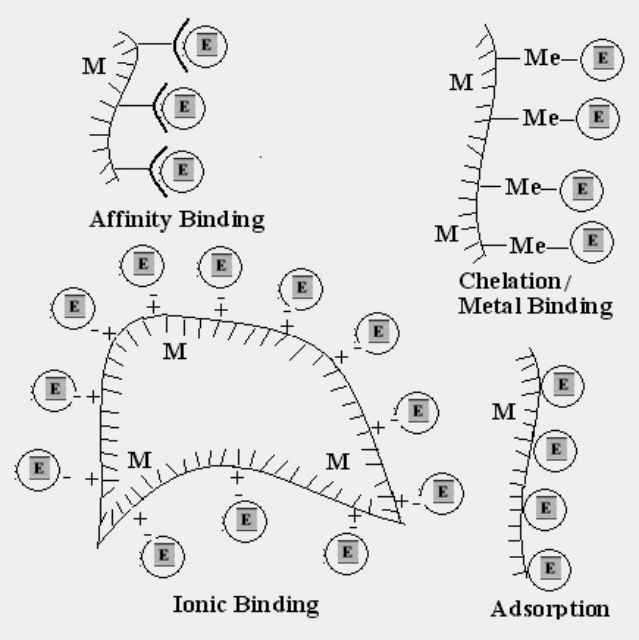

Fig. 1 Reversible immobilization methods.

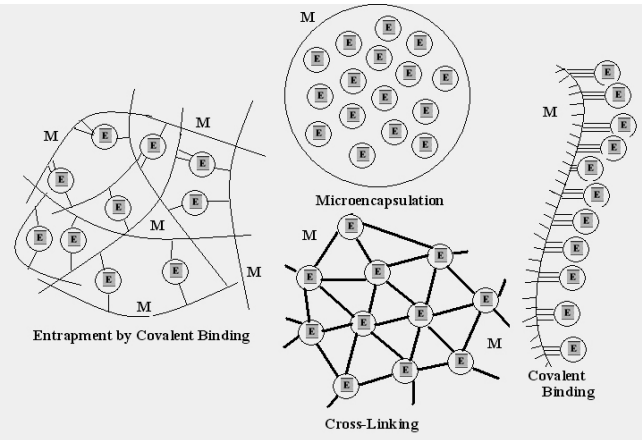

Fig. 2 Irreversible immobilization methods.

rices such as charcoal, clay alumina, silica, DEAE-cellulose (diethylaminoethyl-celluose), CM-cellulose (carboxymethyl-cellulose), various ion exchange matrices, porous carbon, clays, hydrous metal oxides, glasses, and polymeric aromatic resins (Cantarella *et al.* 1997a). Adsorption can be beneficially used industrially as it is simple, cheap and quick process for immobilization and causes little or no damage to enzyme or support material. Some limitations of the process include non specific binding, steric hindrance to the support and leakage of enzyme from the support.

IRREVERSIBLE METHODS

Immobilization by covalent bonding is the retention of enzyme on the solid support by the covalent bond formation (**Fig. 2**). The bond is formed between the functional groups present on the surface of support material and the functional groups belonging to amino acid residue on the surface of the enzyme (Cantarella *et al.* 1997b). Functional groups most often involved are the amino group (NH_2) of lysine and arginine, the carboxyl group of aspartic acid and glutamic acid, the hydroxyl group (OH) of serine and threonine and sulfydryl group (SH) of cysteine.

Another approach of irreversible immobilization is cross-linking (**Fig. 2**). Here the enzymes are joined to each other to form a large, three dimensional complex structure and can be achieved by physical or chemical method. Cross linking is rarely used because of poor mechanical properties and poor stabilities. It is often used to prevent the leakage of enzyme from polymerized gel or to trap the enzyme around preformed polymer molecules. The cross-linked enzyme aggregates formed do not have mass-transfer limitations (Sheldon *et al.* 2000).

Entrapment of an enzyme in polymeric network is a physical enclosure of the enzyme in a small space (**Fig. 2**). Entrapment of enzymes refers to process by which enzymes are embedded in a matrix formed by chemical or physical means (Leeuwlaan 2005b). It differs from adsorption and covalent binding in that enzymes molecules are free in solution, but restricted in movement by the lattice structure of the gel. It is of two type matrix entrapment, and membrane entrapment. Matrix entrapment uses various matrices for immobilization. Matrices such as Ca-alginate, agar, κ-carrageenin, polyacrylamide and collagen are generally used. Membrane entrapment is generally done using membrane of nylon, cellulose, polysulfone and polyacrylate, etc.

Microencapsulation is entrapment of a compound or system inside a dispersed material for its immobilization, protection, control release, structuration and functionalization. Nylon, cyclodextrin and alginate microcapsules are the best example of microencapsulation. Enzyme immobilization by encapsulation may also involve formation of liposomes (Poncelet 2006). Non conductive polymer like polyethyleneimeine (PEI) has been used to microencapsulate laccase (Hebert and Rochefort 2008).

XYLANASES

Xylanases are glycosidase which catalyze the hydrolysis of 1,4-β-D-xylosidic linkages in xylan. They are produced by plethora of organisms and are involved in the production of xylose, a primary carbon source for cell metabolism (Prade 1995). They were first reported in 1955 (Whistler and Masek 1955) and were originally termed pentosanases. After recognition by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 they were assigned the enzyme code EC 3.2.1.8. Officially termed as endo-1,4-β-xylanase, the commonly used synonymous terms include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase. Xylanases have been reported from number of bacteria and fungi like *Bacillus pumilis*, *Bacillus circulans*, *Streptomyces* sp., *Aspergillus niger* sp., *Thermomyces lanuginous*, *Trichoderma viridae*, and *Trichoderma ressei*. A locally isolated *Thermomyces lanuginous* strain SSBP produced one of the highest level of xylanase. Its thermostability (up to 80°C) and broad pH range (5.5-10) (Subramaniyan and Prema 2002).

Xylanases find varied applications in waste treatment, fuel, chemical production, food processing, paper and pulp industries, textile and starch industries, fruit and vegetable processing, brewing, wine production, baking, etc. (Sharma 1987; Wong and Saddler 1993; Prade 1995; Galante *et al.* 1998; Kulkarni *et al.* 1999; Bhat 2000, Beg *et al.* 2001; Mielenz 2001; Saha 2003; Battab *et al*. 2007; Ziaie-Shirkolaee *et al*. 2008; Jiang *et al*. 2010). They are widely used in bread making, clarification of beer and juices, waste treatment, as a biological bleacher in the pulp and paper industry (Beg *et al.* 2001; Damaso *et al.* 2003; Polizeli *et al.* 2005; Jiang *et al*. 2010). Xylanases are also extensively used in animal feed in an attempt to improve feed digestion and the performance of poultry and pigs (Bedford and Classen 1992; Thacker and Baas 1996; Barrera *et al.* 2004; Wu *et al.* 2004). Less well documented putative applications of xylanases includes applications in detergents (Kumar *et al.* 2004), in the protoplastation of plant cells (Kulkarni *et al.* 1999); in the production of pharmacologically active polysaccharides for use as antimicrobial agents (Christakopoulos *et al.* 2003) or antioxidants (Katapodis *et al.* 2003), in the production of alkyl glycosides for use as surfactants (Matsumura *et al.* 1999); and in the washing of precision devices and semiconductors (Imanaka and Sakurai 1992). The xylanases are frequently utilized alone, but are more

commonly used in conjunction with other enzymes and in particular with other hydrolases. In some instances they are also used with proteases, oxidases, isomerases etc. As resistance to thermal inactivation of enzyme is a desirable property for many industrial applications, last decade have focused an increase in research on enzymes produced by various microorganisms isolated from extreme habitats, in particular, those of hyperthermophiles and thermophiles (Vieille *et al.* 1996).

IMMOBILIZATION OF XYLANASES

Almost all methods of immobilization have been applied on xylanases for their beneficial utilization in different industrial processes. One of the early reports on xylanase immobilization by Allenza and co-workers studied the immobilization of xylanase from *Aureobasidium pullulans* onto a macroporous ceramic carrier. Despite a low coupling efficiency it was possible to run the reactor under a wide range of conditions with no detectable leaching of enzyme. The size distribution of products and rate of xylan hydrolysis were very similar for the immobilized and soluble enzymes (Allenza *et al.* 1987). In the subsequent years many novel methods have been introduced.

Xylanase from *A. niger* was non covalently immobilized on a reversibly soluble-insoluble enteric polymer Eudragit TM L-100 by Sardar and co-workers. It was found in the study that the immobilized xylanase retained 60% of its activity (Sardar *et al.* 2000).

Gauda and Naby covalently immobilized xylanase from *Aspergillus tamarii* on Duolite A147 pretreated with glutaraldehyde. Compared to the free enzyme, the immobilized enzyme exhibited lower optimum pH, higher optimum reaction temperature, lower energy of activation, higher *Km* and lower *V*max, greater half life, higher thermal stability as compared to free enzyme. The immobilized enzyme retained about 73% of the initial catalytic activity even after being used 8 cycles (Gauda and Naby 2002).

Hung *et al.* (2008) gave a simple and effective method for immobilization of xylanases. They used the artificial oil bodies to immobilize xylanase from *Neocallimastix patriciarum* and could successfully recover the immobilized enzyme from mixture using brief centrifugation. Upon optimization they found that the immobilized enzyme could be reused 8 times retaining 60% of its actual activity.

Gaur *et al.* (2005) non-covalently immobilized xylanase on Eudragil L-100. Xylanase obtained from *Scytalidium thermophilum* was immobilized on Eudragit L-100, a pH sensitive copolymer of methacrylic acid and methyl methacrylate. The immobilized enzyme had broader pH optima as compared to the free enzyme and the process also increased the half life of enzyme with a stabilization factor of 2. The temperature stability of free enzyme was higher than that of the immobilized enzyme.

Eudragit S-100 was also used by Ai and co-workers to immobilize xylanase from *Streptomyces olivaceoviridis* E-86 in the same year 2005 and an immobilization efficiency of 90% was obtained. The immobilized enzyme had higher optimum pH, optimum temperature, higher thermal stability and retained 81% of its initial hydrolysis activity after being recycled four times (Ai *et al.* 2005).

A new enzyme/support system to immobilize enzymes through a covalent bond on polysulfone membranes was reported by Cano and co-workers. The enzyme *endo*-1,4-β-xylanase (E.C.3.2.1.8) was attached to polysulfone previously derivatized by introducing an acrylate group (Cano *et al.* 2006). Compared with the soluble form of the protein, enzymatic membranes were more resistant to deactivation caused by pH changes or high temperatures. Therefore, the covalent enzyme immobilization process lead to an insoluble and stable form of the enzyme that retained its catalytic properties, and worked at a wide range of aqueous pH and temperature solutions than for the free form.

Sarbu along with the fellow beings found a new grafting method of acrylamide on cellulose acetate powder to make flat membranes activated with glutaraldehyde in order to form support for the enzyme immobilization. They immobilized xylanase on the acrylamide grafted cellulose acetate membranes and found that the immobilized xylanase was more resistant to inactivation by heat than the soluble enzyme (Sarbu *et al.* 2006).

Immobilization of xylanase by using barium alginate entrapment method was followed by Pal *et al.* (2006). They immobilized the partially purified xylanase obtained from *Aspergillus terreus* by solid state fermentation using wheat bran. There were very rare noticeable benefits of the immobilized enzyme, of which the major was that it showed enhanced activity in presence of Mg^{2+}. Pal and coworkers were not successful in stabilizing the immobilized beads at optimum temperature of enzyme activity.

Kapoor and co-workers immobilized xylanase from *Bacillus pumilus* strain MK 001 on different matrix using various immobilization methods. Entrapment using gelatin (GE), physical adsorption on chitin (CH), ionic binding with Q-sepharose (Q-S) and covalent binding with HP-20 beads showed the maximum xylanase immobilization efficiency. Immobilized enzyme showed higher pH optima and increased pH stability in alkaline range as compared to free enzyme. Immobilization also increased the temperature optima and stability of the enzyme at 80°C and could retain 70% of its activity after 7th cycle of the enzyme reaction (Kapoor *et al.* 2007).

Pal and Khanum carried covalent immobilization of xylanase on glutaraldehyde activated alginate beads and found that the K_m (Michaelis constant), V_{max} (maximal reaction rate), optimum pH and optimum temperature increased upon immobilization. Immobilization also led to increase in thermostability and half life of the enzyme and the enzyme could be reused 5 times while retaining greater than 85% of its original activity (Pal and Khanum 2011). The method of immobilization can overcome the problem of reduced permeability of xylan, a high molecular weight substrate, to its enzyme which is conventionally entrapped within the alginate beads.

Akdemir and coworker immobilized xylanase to amine active poly(ethylene glycol) monoacrylate (PEGMA). They activated the hydroxyl group of PEGMA by 1,1′-carbonyldiimidazole (CDI) and then immobilized the enzyme. UV-curable polymeric support formulation prepared by mixing the xylanase bonded PEGMA, aliphatic polyester, 2-hydroxyethyl methacrylate (HEMA), poly(ethylene glycol) diacrylate (PEGDA) and photoinitiator showed that immbobilization improved the temperature resistance, pH optimum of the enzyme and the immobilized enzyme retained 75% of its activity after 33 runs (Akdemir *et al.* 2011).

Production of bioethanol from lignocellulosics is of much interest in recent days due to its low cost and great potential availability. Chang and co-workers worked on the enzymes degrading these polysaccharides and showed that enzyme (xylanase, laccase and cellulase) entrapment is an important technique for the efficient use and reuse of enzymes in industrial applications (Chang *et al.* 2011).

Immobilization of xylanase from the hyperthermophilic *Thermotoga maritima* on nickel-chelate IDA (iminodiacetic acid disodium salt monohydrate)-Eupergit C 250L (Ni-E, nickel-chelate Eupergit C 250L) resulted in high immobilization yield, catalytic efficiency and bond protein. The optimum pH and temperature range of the immobilized xylanase was higher than that of the free xylanase. Immobilization increased both pH stability and thermostability. The immobilization procedure developed provides a promising solution for application of xylanases in continuous hydrolysis of ALC (auto-hydrolysis liquor of corncob). This work of immobilization of the recombinant xylanase from *Thermotoga maritima* was accomplished by Li *et al.* (2007).

Lin and coworkers immobilized Endo-xylanase secreted by the alkaliphilic *Bacillus halodurans* and found that for converting substrate xylan to soluble XOS with immobilized xylanase was determined to be 80.9%, which was lower than the use of free xylanase (99.8%). The immobi-

lized enzyme used shorter xylan chain as substrate and produced more xylobiose and xylotriose at the initial phase of reaction (Lin *et al.* 2011). In addition to this, there is one interesting report where xylanases were immobilized on various types of support matrices (Sánchez *et al.* 2011).

CONCLUSION

The present review concludes that xylanases can be immobilized using various methods described above and among all the processes physical adsorption has been found to be the most economical method of immobilization of xylanases. This method can be optimized using different matrices. It was noted earlier from previous studies that modified glutaraldehyde activated alginate beads proved to be better immobilizing material as compared to barium alginate so this fact seems equally well established (Pal *et al*. 2006; Sarbu *et al.* 2006; Pal and Khanum 2011). It was seen that xylanases can be immobilized by simple methods like on artificial oil bodies to complex UV-curable polymeric support which offers better values than other costly immobilization methods. Some of the major obstacles to the use of immobilized enzymes are included in their cost effectiveness, problem solving steps, bioreactor design and operational costs (Akdemir *et al.* 2011). Each immobilization technique is unique and has lot of beneficial effects for xylanases used in various industries. Since the cost of using enzymes in industrial process in increasing and demand for cost effective xylanases are highly recommended there is strong need of thermotolerant, thermostable and alkali-tolerant xylanases and developing their suitable immobilization technique for better application (Ghatora *et al*. 2006; Gupta and Kar 2009). These factors must be considered over increased cost associated with use of immobilized enzymes. Moreover, applications of xylanases in pulp and paper industries are to be optimized and the cost of such enzyme in increasing so there is strong need of reusing such enzyme with wider activity and potential. Most of the immobilized xylanases have shown higher temperature and pH optima and have accounted for greater thermal tolerance so immobilized xylanases can be an option and it can be more beneficial for industrial use as compared to their free counterparts. Hence, immobilized enzymes offer great prospective for future improvement in enzyme technology sector.

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