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The Art of Immobilization using Biopolymers, Biomaterials and Nanobiotechnology

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1. Introduction

The present chapter has been chosen to be the introductory chapter in the book of biopolymer due to its diversity. The core of this chapter is based on the exceptional review article by Elnashar, 2010a after some modifications.

1.1 Some important definitions

a. Definition of biotechnology

The European Federation of Biotechnology defined biotechnology as "the integration of natural sciences and engineering in order to achieve the application of organisms, cells, parts thereof and molecular analogues for products and services" (Buyukgungor and Gure, 2009). In other words, Biotech applications can be divided into 5 key sectors: biomedicine, bioagriculture, industrial biotechnology, bioenergy, and bioenvironment.

b. Definition of immobilization

An immobilized molecule is one whose movement in space has been restricted either completely or to a small limited region by attachment to a solid structure. In general the term immobilization refers to the act of the limiting movement or making incapable of movement i.e., retard the movement (Yu-Qung et al., 2004).

1.2 History of immobilization

Immobilization is a natural phenomenon existing in the universe. Microorganisms in nature are irregularly distributed and often exist in Biofilms. Biofilms are surface-attached microbial communities consisting of multiple layers of cells embedded in hydrated matrices (Kierek-Pearson and Karatan, 2005). Biofilms were first extensively studied during the 1940s but it was not until the 1970s that it was appreciated that their formation occurs in almost all natural environments. A rock immersed in a stream, an implant in the human body, a tooth, a water pipe or conduit, etc. are all sites where Biofilms develop (Carpentier and Cerf, 1993). This natural phenomenon encouraged humans to utilize it for his services.

1.3 What can we immobilize?

Many molecules have been immobilized and the majority of them are biomolecules due to their biological and biomedical applications. The following are examples of some of these molecules:

- **Proteins:**
  - Enzymes, antibodies, antigens, cell adhesion molecules and "Blocking" proteins
- **Peptides:**
  - Substances composed of amino acids
- **Drugs:**
  - Anticancer agents, antithrombogenic agents, antibiotics, contraceptives, drug antagonists and peptide/protein drugs
- **Saccharides:**
  - Sugars, oligosaccharides and polysaccharides
- **Lipids:**
  - Fatty acids, phospholipids, glycolipids and any fat-like substances.
- **Ligands:**
  - Hormone receptors, cell surface receptors, avidin and biotin
  - In immunology, small molecules that are bound to another chemical group or molecule
- **Nucleic acids and nucleotides:**
  - DNA, RNA
  - High MW substances formed of sugars, phosphoric acid, and nitrogen bases (purines and pyrimidines).
- **Others:**
  - Conjugates or mixtures of any of the above

1.4 Methods of immobilization

The methods of immobilization of the different molecules are almost the same. However, according to Cao, L. 2005 there is no general universally applicable method of certain molecule immobilization. As enzyme molecules alone or in combination with drugs, antibodies and antigens, are the most used in industries, we will be focusing on the immobilization techniques used for enzymes as a model of other immobilized molecules. The enzyme market in 2005 was around 2.65 billion dollars, with an expected annual growth of more than 9% (Ayala and Torres, 2004). On the industrial level, 75% of the enzymes were used, which is around 2 billion dollars.

However, expensive enzymes are not favored to be used in industries in the Free State as they are difficult to be separated from the products (Fig. 1a) and consequently are lost after the first use. They were alternatively immobilized on solid supports (Fig. 1b) so that they can be easily separated from the products by simple filtration or using a fluidized magnetized bed reactor system (Danial et al., 2010, Elnashar, 2010a-c; Elnashar et al., 2008, 2009a, 2009b; Elnashar and Yassin 2009a, 2009b; Mansour et al., 2007).

The main advantage for enzyme immobilization is the easy separation of the enzyme from the reaction mixture (substrates and products) and its reusability for tens of time, which reduces the enzyme and the enzymatic products cost tremendously. Beside this splendid advantage, the immobilization process imparts many other advantages to the enzyme such as:

- The ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or vice versa)
- Product is not contaminated with the enzyme
- Easy separation of enzyme from the product (especially useful in food and pharmaceutical industries)
- Enhancement of enzyme stability against pH, temperature, solvents, contaminants, and impurities.

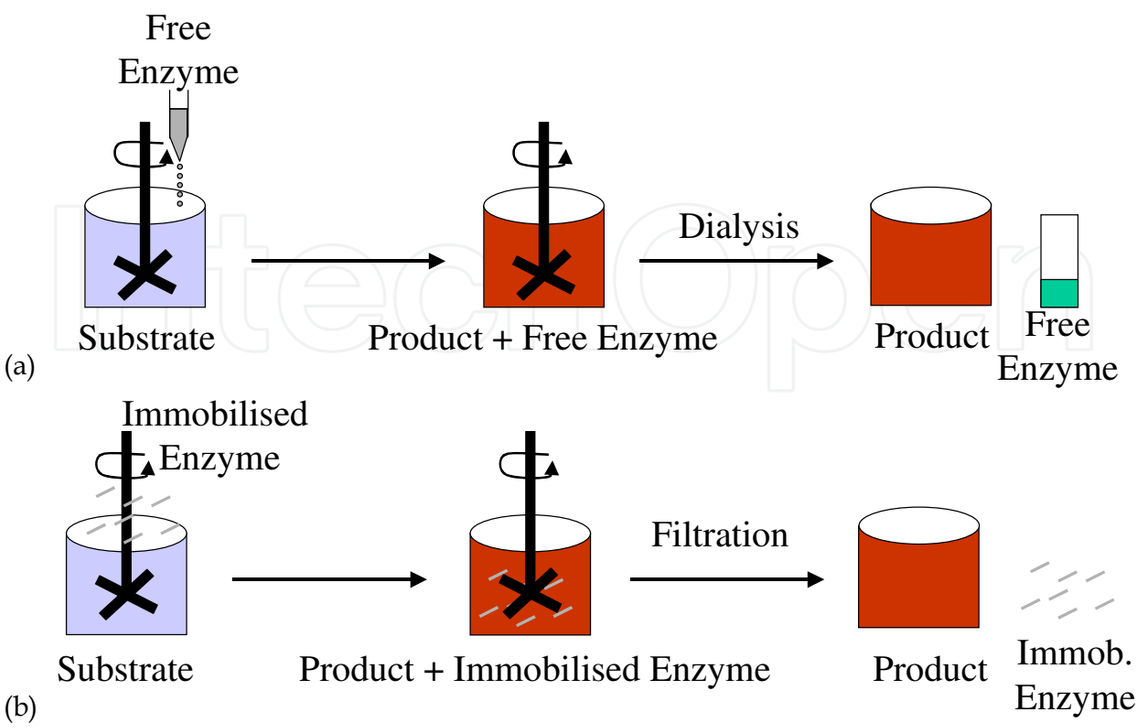


Fig. 1. Schematic diagram of free and immobilized enzyme reactions. (a), Reaction of free enzyme with substrate and formation of product, which has to be separated via dialysis; (b), Reaction of immobilized enzyme with substrate and formation of product, which can be separated via filtration or using a fluidized magnetized bed reactor system.

Immobilization provides a physical support for enzymes, cells and other molecules. Immobilization of enzymes is one of the main methods used to stabilize free enzymes (Elnashar, 2010a; Danial et al., 2010). The support material and the main methods of immobilization are key parameters in enzyme immobilization. There are five principal methods for immobilization of enzymes and cells (adsorption, covalent, entrapment, encapsulation and crosslinking) and no one method is perfect for all molecules or purposes. However, Katzbauer and Moser, 1995 represented a classification of combination between these methods.

1.4.1 Adsorption

Immobilization by adsorption is the simplest method and involves reversible surface interactions between enzyme and support material as shown in Fig. 2. The procedure of adsorption consists of mixing together the biological component(s) and a support with adsorption properties, under suitable conditions of pH and ionic strength for a period of incubation, followed by collection of the immobilized material and extensive washing to remove the unbound biological components. The first industrially used immobilized enzyme was prepared by adsorption of amino acid acylase on DEAE-cellulose (Tosa et al., 1967). Menaa et al. (2008a) reported the role of hydrophobic surfaces of nanoporous silica glasses on protein folding enhancement. The proteins were adsorbed on silica surfaces.

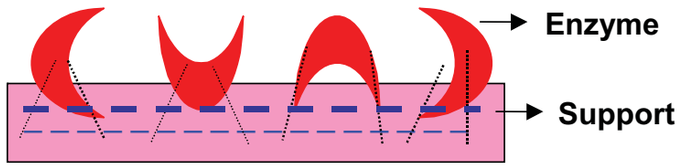


Fig. 2. Immobilization of enzymes using the adsorption technique.

Advantages of enzymes immobilized using the adsorption technique:

- Reversibility, which enables not only the purification of proteins but also the reuse of the carriers;
- Simplicity, which enables enzyme immobilization under mild conditions;
- Possible high retention of activity because there is no chemical modification (Çetinus et al., 2009);
- Cheap and quick method;
- No chemical changes to the support or enzyme occurs.

Disadvantages of enzymes immobilized using the adsorption technique:

- The immobilized enzymes prepared by adsorption tend to leak from the carriers, owing to the relatively weak interaction between the enzyme and the carrier, which can be destroyed by desorption forces such as high ionic strength, pH, etc,
- Contamination of product,
- Non-specific binding,
- Overloading on the support and
- Steric hindrance by the support.

Consequently, a number of variations have been developed in recent decades to solve this intrinsic drawback. Examples are adsorption–cross-linking; modification–adsorption; selective adsorption–covalent attachment; and adsorption–coating, etc. For more details, the reader is recommended to read the book of Cao L, 2005.

1.4.2 Covalent binding

This method of immobilization involves formation of a covalent bond between the enzyme and support material as shown in Fig. 3. Covalent bonds usually provide the strongest linkages between enzyme and carrier, compared with other types of enzyme immobilization methods. Thus, leakage of enzyme from the matrix used is often minimized with covalently bound immobilized enzymes (Cao, 2005). The bond is normally formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme.

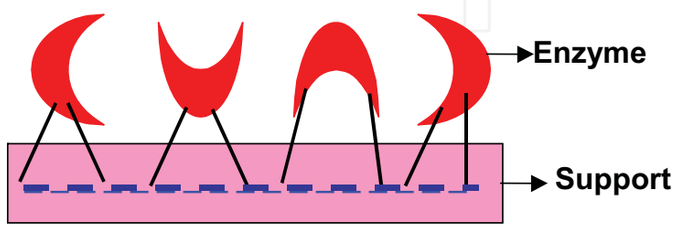


Fig. 3. Immobilization of enzymes using the covalent technique.

Multi-step immobilization is one of the technologies to enhance enzyme covalent immobilization (Xie et al., 2009). There are many reaction procedures for coupling an

enzyme to a support via covalent bond however, most reactions fall into the following categories: formation of an isourea linkage; formation of a diazo linkage; formation of a peptide bond or an alkylation reaction as shown in Table 1.

Reaction	Support – Enzyme Linkage
Diazotization	SUPPORT--N=N---ENZYME
Alkylation and arylation	SUPPORT--CH2-NH---ENZYME
	SUPPORT--CH2-S---ENZYME
Schiff's base formation	SUPPORT---CH=N---ENZYME
Amide bond formation	SUPPORT---CO-NH---ENZYME
Amidation reaction	SUPPORT---CNH-NH---ENZYME
Thiol-Disulfide interchange	SUPPORT---S-S---ENZYME
Carrier binding with bifunctional reagents	SUPPORT---O(CH2)2 N=CH(CH2)3 CH=N---ENZYME

Table 1. Different methods for covalent binding of enzymes to supports

Advantages of enzymes immobilized using the covalent technique:

- No leakage of enzyme.
- The enzyme can be easily in contact to substrate due to the localization of enzyme on support materials.
- Increase of the thermal stability.

Disadvantages of enzymes immobilized using the covalent technique:

- The cost is quite high as the good supports are very expensive (e.g. Eupergit C and Agaroses).
- Loss of enzyme activity (e.g. mismatched orientation of enzyme on the carriers such as involvement of active centre in the binding).

1.4.3 Entrapment

Immobilization by entrapment differs from adsorption and covalent binding as shown in Fig. 4 in that enzyme molecules are free in solution, but restricted in movement by the lattice structure of a gel (Bickerstaff, 1995). The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage of enzyme or cells, yet at the same time allows free movement of substrate and product. The support also acts as a barrier and can be advantageous as it protects the immobilized enzyme from microbial contamination by harmful cells, proteins, and enzymes in the microenvironment (Riaz et al., 2009).

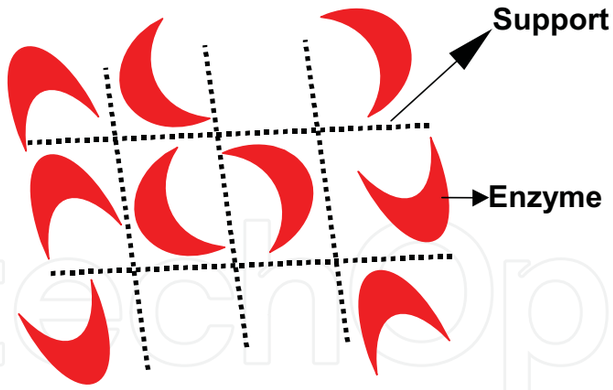


Fig. 4. Immobilization of enzyme using the entrapment technique.

Entrapment can be achieved by mixing an enzyme with a polyionic polymer material, such as carrageenan, and by crosslinking the polymer with multivalent cations, e.g. hexamethylene diamine, in an ion-exchange reaction to form a lattice structure that traps the enzymes, this is termed ionotropic gelations.

Advantages of enzymes immobilized using the entrapment technique:

- Enzyme loading is very high

Disdvantages of enzymes immobilized using the entrapment technique:

- Enzyme leakage from the support.
- Diffusion of the substrate to the enzyme and of the product away from the enzyme (diffusion limitation).

1.4.4 Encapsulation

Encapsulation of enzymes as shown in Fig. 5 can be achieved by enveloping the biological components within various forms of semipermeable membranes (Groboillot et al., 1994). It is similar to entrapment in that the enzyme is free in solution, but restricted in space. Large proteins or enzymes can not pass out of, or into the capsule, but small substrates and products can pass freely across the semipermeable membrane.

Many materials have been used to construct microcapsules varying from 10-100 μm in diameter. For example, nylon and cellulose nitrate have proven popular. Ionotropic gelation of alginates have proven it efficacy as well for encapsulation of drugs, enzymes and cells (Patil et al., 2010). On the nano scale level, Menaa et al., 2008b, 2009 & 2010 used Silica-based nanoporous sol-gel glasses for the study of encapsulation and stabilization of some proteins.

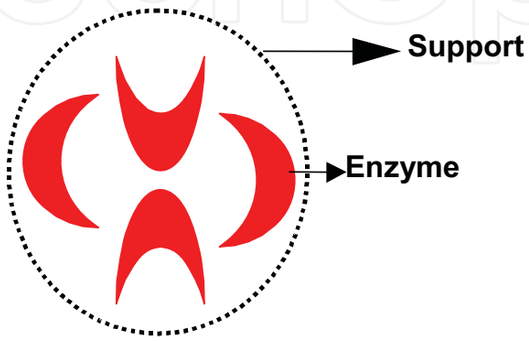


Fig. 5. Immobilization of enzymes using the encapsulation technique.

Advantages of enzymes immobilized using the encapsulation technique:

- The enzymes could be encapsulated inside the cell.
- Possibility of coimmobilization. Where cells and/or enzymes may be immobilized in any desired combination to suit particular applications.

Disdvantages of enzymes immobilized using the entrapment technique:

- The problems associated with diffusion are acute and may result in rupture of the membrane if products from a reaction accumulate rapidly.

1.4.5 Crosslinking

This type of immobilization is support-free as shown in Fig. 6 and involves joining enzyme molecules to each other to form a large, three-dimensional complex structure, and can be achieved by chemical or physical methods (Xie et al., 2009). Chemical methods of crosslinking normally involve covalent bond formation between the enzymes by means of a bi- or multifunctional reagent, such as glutaraldehyde, dicarboxylic acid or toluene diisocyanate. Flocculating agents, such as polyamines, polyethyleneimine, polystyrene sulfonates, and various phosphates, have been used extensively to cross-link cells using physical bonds. Crosslinking is rarely used as the only means of immobilization, because poor mechanical properties of the aggregates are severe limitations. Crosslinking is most often used to enhance the other methods of immobilization described.

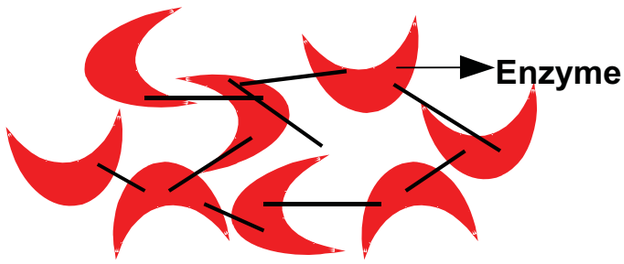


Fig. 6. Crosslinking technique.

Advantages of enzymes immobilized using the crosslinking technique:

- The immobilization is support-free.
- Cross-linking between the same enzyme molecules stabilises the enzymes by increasing the rigidity of the structure.

Disdvantages of enzymes immobilized using the crosslinking technique:

- Harshness of reagents of crooslinking is a limiting factor in applying this method to many enzymes.
- The enzyme may partially lose activity or become totally inactivated in case the cross-linking reagent reacted across the active site.

1.5 Examples of matrices and shapes for immobilization

Matrices for immobilization can be classified according to their chemical composition as organic and inorganic supports. The former can be further classified into natural and synthetic matrices as in Table 2 (Elnashar, 2005).

Organic	Inorganic
Natural polymers Polysaccharides Cellulose Dextran Starch Agar and agarose Alginate Carrageenans Chitin and chitosan Proteins Collagen Gelatin Albumin Ferritin	Minerals Attapulgite clays Bentonite Kieselgur Pumic stone Hornblend Diatomaceous earth Sand
Synthetic polymers Polystyrene Polyacrylate and polymethacrylate Polyacrylamides Hydroxyalkyl methacrylate Vinyl polymer Maleic anhydride polymer Polyethyleneglycol Aldehyde-based polymer	Fabricated materials Non-porous glass Controlled pore glass Controlled pore metal oxides Alumina catalyst Porous silica Silochrome Iron oxide Stainless steel

Table 2. Chemical classification of enzyme matrices.

The shape of the carrier can be classified into two types, i.e. irregular and regular shapes such as (A): beads; (B): fibres; (C): hollow spheres; (D): thin films; (E): discs and (F): membranes. Selection of the geometric properties for an immobilized molecule is largely dependent on the peculiarity of certain applications.

Gel disks are widely used in the literature. Researchers usually use the casting method, e.g. a Petri dish, to make a single film of gel and then cut it into disks using cork borers. Elnashar *et al*., 2005, invented a new equipment to make many uniform films in one step and with high accuracy using the equipment "Parallel Plates" as shown in Fig. 7.

Gel beads are mostly used in industries as they have the largest surface area and can be formed by many techniques such as the interphase technique, ionic gelation methods, dripping method and the Innotech Encapsulator (Danial *et al*., 2010; Elnashar *et al*., 2009a). The Innotech Encapsulator as shown in Fig. 8 has the advantage of high bead production (50 – 3000 beads per second depending on bead size and encapsulation-product mixture viscosity), which is suitable for the scaling up production on the industrial scale.

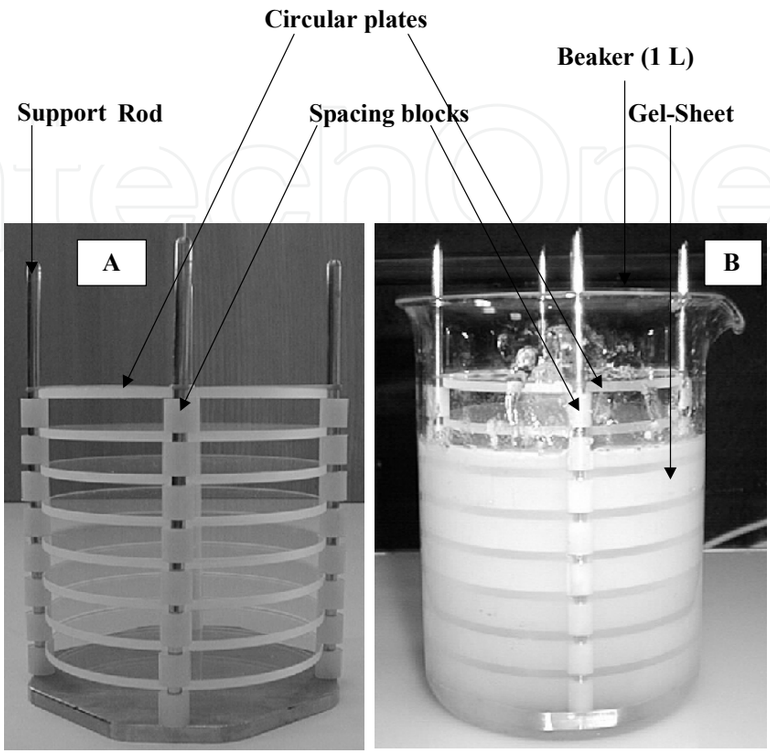


Fig. 7. Parallel plates equipment for making uniform k-carrageenan gel disks.

Fig. 8. Inotech Encapsulator IE-50 R

1.6 Properties of matrices for immobilization

The supports on which molecules such as enzymes, antibodies, antigens, etc will be immobilized are of great interest. The term support or media is usually understood to refer to a combination of a ligand that is firmly attached often by covalent means, and a solid insoluble matrix. These supports have to exhibit good chemical and physical stability and contain available functional groups to bind to the active molecule. To use a support for immobilization of active molecules such as enzymes, a range of fundamental properties are required, which are summarised as follows (Bickerstaff, 1995).

a. Availability of matrix from a reliable commercial source
b. Matrix has an abundance of easily derivatizable functional groups
c. Matrix has good mechanical and chemical stability
d. Matrix has good capacity for the target molecule
e. Matrix material is "user friendly"

2. Applications of immobilized molecules

2.1 Drug delivery systems

Advanced drug delivery systems (ADDS) have found applications in many biomedical fields (Lin, 2006; Pollauf and Daniel, 2006). Drug delivery is a combination of material science, pharmaceutics and biology (Pack et al., 2005). Adoption of different types of membranes in ADDS has made it possible to release drug in an optimal fashion according to the nature of a disease (Grayson et al., 2003). Examples of drug delivery systems include glucose-sensitive insulin and drug loaded magnetic nanoparticles.

2.1.1 Development of glucose-sensitive insulin

The swelling or shrinking of smart hydrogel beads in response to small changes in pH or temperature can be used successfully to control drug release, because the diffusion of the drug out of the beads depends on the gel state (Kim et al., 1995).

Drug-delivery systems in which a drug is liberated in response to a chemical signal (e.g. *insulin release in response to rising glucose concentration*) can be achieved using this system. The exposure of a glucose-sensitive insulin releasing system to glucose resulted in the oxidation of glucose to gluconic acid and thus a decrease in the pH, protonation and shrinking of the polymer, leading to an increased release of insulin. The polymer swells in size at normal body pH (pH = 7.4) and closes the gates. It shrinks at low pH (pH = 4) when the blood glucose level increases, thus opening the gates and releasing the insulin from the nanoparticles (Sona, 2010).

2.1.2 Drug loaded magnetic manoparticles

Nanotechnology offers the means to send the drugs to targeted sites, and has the drug released in a controlled manner, which reduce side effects due to lower dosage and minimize or prevent drug degradation by using pathways other than gastrointestinal. Magnetic nanoparticles are recently applied in various fields such as MRI imaging, water treatment, hyperthermia and drug delivery systems. Drug loaded magnetic nanoparticles

(DLMNP) have several advantages such as: small particle size; large surface area; magnetic response; biocompatibility and non-toxicity. DLMNP is introduced through injection and directed with external magnets to the right organ, which requires smaller dosage because of targeting, resulting in fewer side effects.
Recently, Yu et al., 2008 reported a novel In Vivo strategy for combined cancer imaging and therapy by employing thermally cross-linked superparamagnetic iron oxide nanoparticles as a drug-delivery carrier. Whereas, Kettering et al., 2009 used magnetic iron nanoparticles with cisplatin adsorbed in them for drug release in magnetic heating treatments for *cancer*.

2.2 Enzyme-Linked Immunosorbent Assays (ELISA)

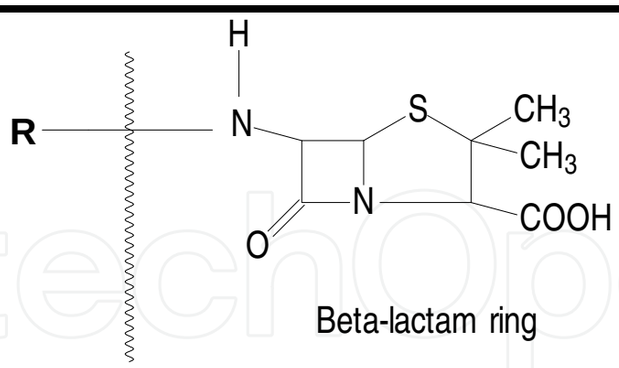
ELISA is a test used as a general screening tool for the detection of antibodies or antigens in a sample (Farré et al., 2007). ELISA technology links a measurable enzyme to either an antigen or antibody. The procedure for detection of Ab in patient's sample as follows:

- Immobilize Ag on the solid support (well)
- Incubate with patient sample
- Add antibody-enzyme conjugate
- Amount of antibody-enzyme conjugate bound is proportional to amount of Ab in the sample
- Add substrate of enzyme
- Amount of color is proportional to amount of Ab in patient's sample.

However, ELISA technique in some cases is regarded as time consuming and it needs special equipment to run the assay (not portable). Thus many techniques have been developed to fasten the process such as that of Xin et al., 2009, where he developed a chemiluminescence enzyme immunoassay using magnetic particles to monitor 17β-estradiol (E2) in environmental water samples. Another technique is using simple/rapid (S/R) test. The development of simple/rapid S/R tests has been extended from pregnancy detection of HIV antibodies in whole blood in addition to serum and plasma (World Health Organization, 2002).

2.3 Antibiotics production

Penicillins are the most widely used β-lactam antibiotics, with a share of about *19 % of the estimated world-wide antibiotic market* (Table 3) (Elnashar, 2005 & 2010).
Production of antibiotics is one of the key areas in the field of applied microbiology. The conventional method of production is in stirred tank batch reactors. Since it is a no growth associated process, it is difficult to produce the antibiotic in continuous fermentations with free-cells. But it is a suitable case for cell immobilization, since growth and metabolic production can be uncoupled without affecting metabolite yields. Therefore, several attempts have been made to immobilize various microbial species on different supports matrices for antibiotic production. The most widely studied system is the production of penicillin G using immobilized cells of Penicillium chrysogenum (Ogaki et al., 1983). In a recent study by Elnashar et al., they were successful to covalently immobilize pencillin G acylase on carrageenan modified gels with retention of 100% activity after 20 reuses (Elnashar et al., 2008).



Penicillin core structure

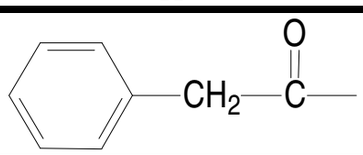
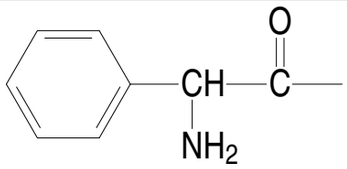
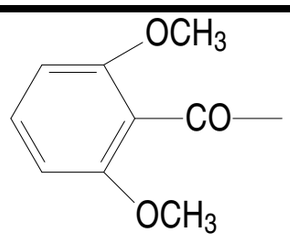
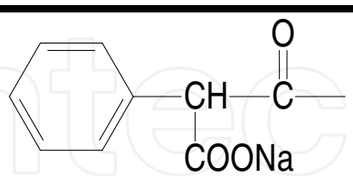
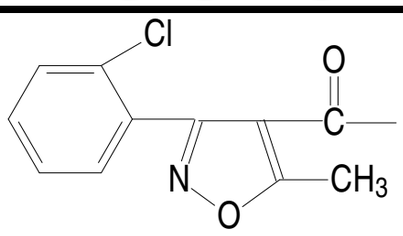
R =	Penicillin Variant
H -	6-Amino-penicillanic acid (6-APA)
	Penicillin G
	Ampicillin
	Methicillin
	Carbenecillin
	Cloxacillin

Table 3. Some semi-synthetic penicillins and naturally produced penicillin G.

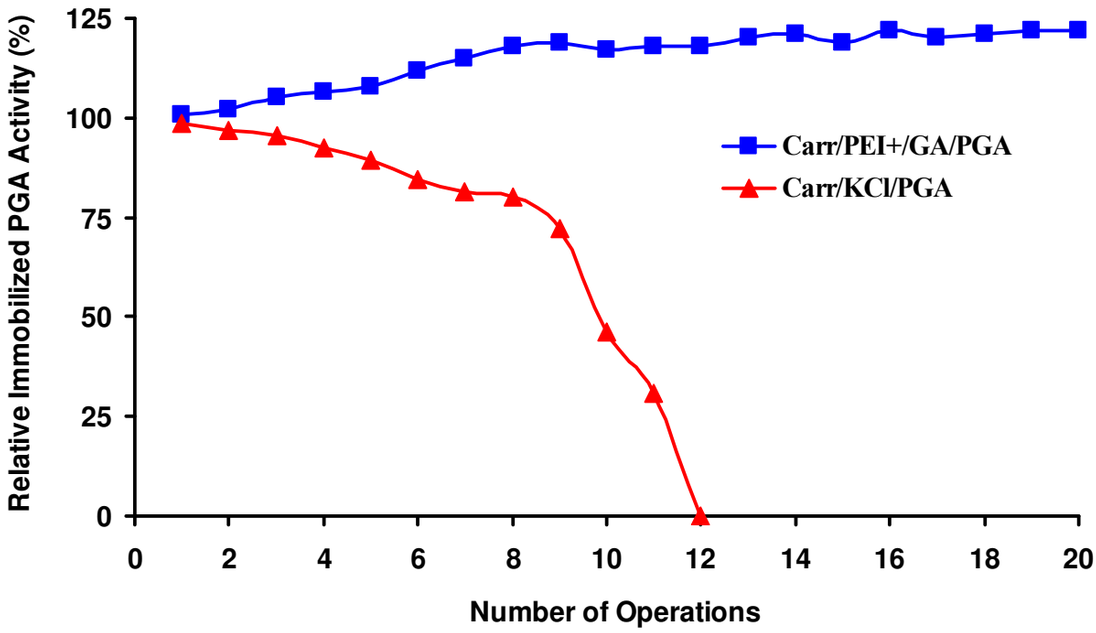


Fig. 9. Operational stability of immobilized PGA. Using modified gel (Carr/PEI+/GA) and unmodified gel (Carr/KCl) for immobilization of PGA.

2.4 Medical applications particularly in therapy

Medical applications of immobilized enzymes include (Piskin, 1993) diagnosis and treatment of diseases, among those enzyme replacement therapies, as well as artificial cells and organs, and coating of artificial materials for better biocompatibility. Examples of potential medical uses of immobilized enzyme systems are listed below. For more applications, readers are encouraged to read the review article of Soetan et al., 2010, where he reviewed the biochemical, biotechnological and other applications of enzymes.

- Asparaginase (3.5.1.1) for leukemia
- Arginase (3.5.3.1) for cancer
- Urease (3.5.1.5) for artificial kidney, uraemic disorders
- Glucose oxidase (1.1.3.4) for artificial pancreas
- Carbonate dehydratase (4.2.1.1) and catalase (1.11.1.6) for artificial lungs
- Glucoamylase (3.2.1.3) for glycogen storage disease
- Glucose-6-phosphate dehydrogenase (1.1.1.49) for glucose-6-phosphate dehydrogenase deficiency
- Xanthine oxidase (1.1.3.22) for Lesch–Nyhan disease
- Phenylalanine ammonia lyase (4.3.1.5) for phenylketonuria
- Urate oxidase (1.7.3.3) for hyperuricemia
- Heparinase (4.2.2.7) for extracorporeal therapy procedures

In addition to the above applications, we will focus the light on some important applications as solving the problem of lactose Intolerant people, production of fructose for diabetics and for people on diet regimen, and treatment of rheumatoid arthritis and joint diseases.

2.4.1 Solving the problem of lactose intolerant people

β-galactosidase is widely used in milk industries for hydrolysis of lactose to glucose and galactose. Lactose is the main carbohydrate contained in milk at a concentration between 5% and 10% (w/v) depending on the source of milk (Ordoñez, 1998). Lactose could also be found in whey permeate at higher concentrations. The consumption of foods with a high content of lactose is causing a medical problem for almost 70% of the world population, especially in the developing countries, as the naturally present enzyme (β-galactosidase) in the human intestine, loses its activity during lifetime (Richmond & Stine, 1981). Undigested lactose in chyme retains fluid, bacterial fermentation of lactose results in production of gases, diarrhoea, bloating and abdominal cramps after consumption of milk and other dairy products.

Unfortunately, there is *no cure to "lactose intolerance"*. This fact, together with the relatively low solubility and sweetness of lactose, has led to an increasing interest in the development of industrial processes to hydrolyze the lactose contained in dairy products (milk and whey) with both the free and immobilized conditions (German, 1997). The studies have shown that glucose and galactose, the two monosaccharides hydrolosates of lactose (products hydrolyzed from lactose), are four times sweeter than lactose, more soluble, more digestible (Sungur & Akbulut, 1994), and can be consumed by 'lactose intolerant' people. Immobilized β-galactosidase on thermostable biopolymers of grafted carrageenan were studied recently by Elnashar and Yassin and interesting results were obtained (Elnashar and Yassin, 2009a, 2009b).

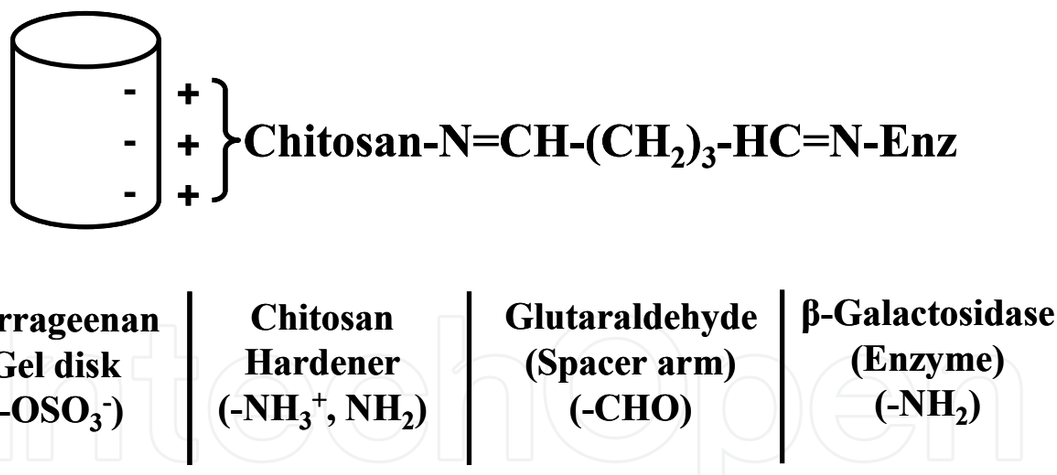


Fig. 10. Carrageenan coated chitosan followed by glutaraldehyde (GA) as a spacer arm to immobilize covalently β-galactosidase (Enz) to the chitosans amino groups via Schiff's base formation.

2.4.2 Fructose for diabetics and for people on diet regimen

People on diet regimen and patients suffering from diabetes are highly recommended to consume fructose rather than any other sugar. Fructose can be produced from starch by enzymatic methods involving α-amylase, amyloglucosidase, and glucose isomerase, resulting in the production of a mixture consisting of oligosaccharides (8%), fructose (45%),

and glucose (50%) (Gill et al., 2006). However, separation of fructose from this high content fructose syrup is costly and thus makes this method uneconomical. In industries, inulinases are used to produce 95% of pure fructose after one step of the enzymatic hydrolysis of inulin. Industrial inulin hydrolysis is carried out at 60 °C to prevent microbial contamination and also because it permits the use of higher inulin substrate concentration due to increased solubility., Elnashar et al., have succeeded recently to produce a thermostable inulinolytic immobilized enzyme, which would be expected to play an important role in food and chemical industries, in which fructose syrup is widely applied (Elnashar et al., 2009; Danial et al., 2010).

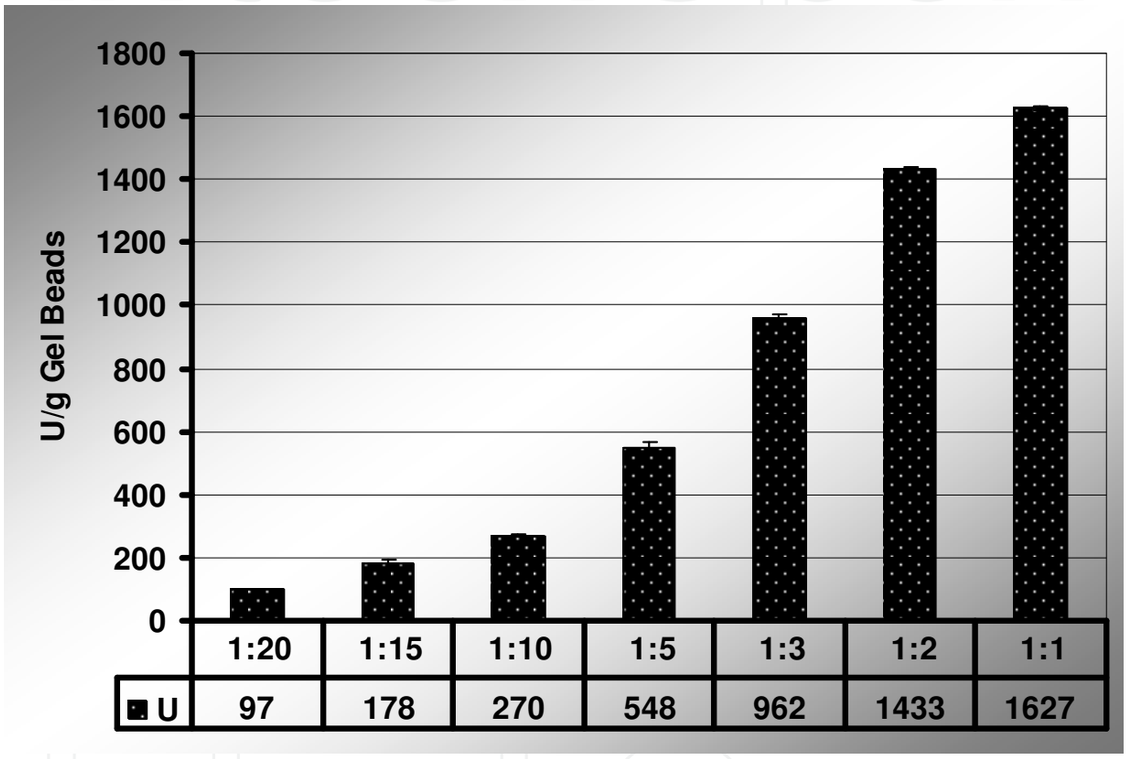


Fig. 11. Optimization of the enzyme loading capacity using grafted alginate beads prepared by the one-step method. Where 0.5g of the gel beads were soaked in 5 ml of 1:20, 1:15, 1:10, 1:5, 1:3, 1:2 and 1:1 dil enzyme in 0.1 M acetate phosphate buffer at pH 4.8 for 16 h.

2.4.3 Treatment of rheumatoid arthritis and joint diseases

Superoxide dismutase (SOD) and catalase (CAT) have been encapsulated in biodegradable microspheres (MS) to obtain suitable sustained protein delivery (Giovagnoli, 2004). A modified water/oil/water double emulsion method was used for poly (D,L-lactide-co-glycolide) (PLGA) and poly (D, L-lactide) PLA MS preparation co-encapsulating mannitol, trehalose, and PEG400 for protein stabilization. SOD release from PLGA MS may be potentially useful for long-term sustained release of the enzyme for the treatment of rheumatoid arthritis or other intra-articular and joint diseases (inflammatory manifestation).

2.5 Non medical applications of immobilized enzymes

2.5.1 Treatment of pesticide-contaminated waste

Application of pesticide in agriculture serves to lower the cost of production, increase crop yields, provide better quality produce and also reduce soil erosion. Although pesticides are toxic and have adverse effect on human health and the environment, their use is inevitable in many cases as an effective means of controlling weeds, insect, and fungus, parasitic and rodent pests. One of the most important technologies to be applied for this approach is immobilized enzyme. The immobilized enzyme is capable of breaking down a range of pesticide-contaminated waste as organophosphate insecticides (Horne et al., 2002; Sharmin et al., 2007).

2.5.2 Neutralizing dangerous chemical gases or vapors

The use of immobilized enzymes in the national security arena has shown to be promising. For example, they could include infiltrating items such as air filters, masks, clothing, or bandages with the concentrated immobilized enzymes to neutralize dangerous chemical gases or vapors (Ackerman and Lei, 2008).

2.6 Purification of proteins

Protein purification is an important objective in industrial enzymes in order to increase the enzyme's specific activity and to obtain an enzyme in its pure form for a specific goal. Affinity ligands is the most used technique for purification of target molecules as it can reduce the number of chromatographic steps in purification procedures to one or two steps. Immobilization of affinity ligands to an insoluble support can be a powerful tool in isolation of particular substances (e.g. protein) from a complex mixture of proteins. Some examples of affinity ligands are immobilized carbohydrate-binding proteins and immobilized metal ions. Another technique for protein purification is using Electric field gradient focusing (EFGF). For more information on the principles and methods of protein purification, readers should refer to the handbook "Purifying challenging proteins: principles and methods" in 2007.

2.6.1 Immobilized carbohydrates-binding proteins

Purification of proteins could be performed using immobilized carbohydrates such as mannose, lactose and melibiose. For example, immobilized lactose on sepharose 4B™ will be selective for purification of lactase from a mixture of other proteins. More information on this technique can be found in the book "Immobilized affinity ligand techniques" (Hermanson et al., 1992).

2.6.2 Electric Field Gradient Focusing (EFGF)

Electric field gradient focusing is a member of the family of equilibrium gradient focusing techniques (e.g gel electrophoresis). It depends on an electric field gradient and a counter-flow to focus, concentrate and separate charged analytes, such as peptides and proteins. Since analytes with different electrophoretic mobilities have unique equilibrium positions, EFGF separates analytes according to their electrophoretic mobilities, similar to the way isoelectric focusing (IEF: electrophoresis is a pH gradient where the cathode is at a higher pH value than the anode) separates analytes according to isoelectric points. The constant

counter flow is opposite to the electrophoretic force that drives the analytes. When the electrophoretic velocity of a particular analyte is equal and opposite to the velocity of the counter flow, the analyte is focused in a narrow band because at this position the net force on it is zero.

However, EFGF avoids protein precipitation that often occurs in IEF when proteins reach their isoelectric points and, therefore, can be applied to a broad range of proteins. Sun (2009) in his Ph.D. thesis demonstrated that protein concentration exceeding 10,000-fold could be concentrated using such devices.

2.7 Extraction of biomolecules using magnetic particles

The traditional methods for biomolecules purification such as centrifugation, filtration, and chromatography can today be replaced by the use of magnetic particles. They are reactive supports for biomolecules capturing. Their use is simple, fast, and efficient for the extraction and purification of biomolecules. In the biomedical Weld, numerous publications deal with the use of magnetic particles for biomolecule extraction (Delair and Meunier, 1999), cell sorting (Kemshead et al., 1985), and drug delivery (Langer, 1990). Magnetic beads are widely used in molecular biology (Andreadis and Chrisey, 2000), medical diagnosis (Myrmel et al., 2000), and medical therapy (Kemshead et al., 1985).

The major application concerns the extraction of biomolecules such as proteins (Ding and Jiang, 2000), antibodies, and nucleic acids (Rouquier and Tracks, 1995). Magnetic beads carrying antibodies are also used for specific bacteria (Kemshead et al., 1985) and virus captures (Myrmel et al., 2000). Krupey in 1994 patented a method for virus capture process. The method was based on interactions between viruses and anionic polymers, leading to the precipitation of complexes by charge neutralization. After the capture step, viruses were extracted by centrifugation. At the current time, to our knowledge, only one method using magnetic beads has been published recently (Ifiata et al., 2003). In these studies, some DNA and RNA viruses were concentrated more than 100 and 1000 times, respectively, using polyethyleneimine (PEI)1-conjugated magnetic beads.

2.8 Heavy metals removal

Heavy metal pollution is an environmental problem of worldwide concern. Several industrial wastewater streams may contain heavy metals such as; Pb, Cr, Cd, Ni, Zn, As, Hg, Cu, Ag. Traditionally, precipitation, solvent extraction, ion-exchange separation and solid phase extraction are the most widely used techniques to eliminate the matrix interference and to concentrate the metal ions. Many materials have been used to remove them such as sorbents (Abdel Hameed and Ebrahim, 2007) (e.g. silica, chitosan, sponge, etc) and biosorbents (Shareef, 2009) (e.g. immobilized algae).

Biosorbents: can be defined as the selective sequestering of metal soluble species that result in the immobilization of the metals by microbial cells such as cyanobacteria. It is the physicochemical mechanisms of inactive (i.e. non-metabolic) metal uptake by microbial biomass. Metal sequestering by different parts of the cell can occur via various processes: complexation, chelation, coordination, ion exchange, precipitation, reduction. Size of immobilized bead for metals removal is a crucial factor for use of immobilized biomass in bio-sorption process. It is recommended that beads should be in the size range between 0.7 and 1.5 mm, corresponding to the size of commercial resins meant for removing metal ions.

Abdel Hameed and Ebrahim, 2007 in their review article, has revealed some of the immobilized algae on different matrices that have potential in heavy metals removal due to its high uptake capacity and abundance.

2.9 Production of biosensors

Biosensors are chemical sensors in which the recognition system utilizes a biochemical mechanism (Jianrong et al., 2004). A biosensor is a sensing device made up of a combination of a specific biological element and a transducer. The "specific biological element" such as antibodies, (Rodriguez-Mozaz et al., 2004) enzymes (Nistor, 2002), bacteria (Philp et al., 2003; Petanen and Romantschuk, 2002) and DNA (Marrazza et al., 1999) recognizes a specific analyte such as pollutions (toxicity caused by pesticides, phenols, mercury, arsenic, etc) and the changes in the biomolecule are usually converted into electrical signal (which is in turn calibrated to a certain scale) by a transducer.

2.10 Production of biodiesel

The idea of using biodiesel as a source of energy is not new (Sawayama et al., 1995), but it is now being taken seriously because of the escalating price of petroleum and, more significantly, the depletion of fossil fuels (oil and gas) within the next 35 years and the emerging concern about global warming that is associated with burning fossil fuels (Gavrilescu and Chisti, 2005). Biodiesel is much more environmentally friendly than burning fossil fuels, to the extent that governments may be moving towards making biofuels mandatory (Biodiesel review, 2006). The global market survey of biodiesel has shown a tremendous increase in its production.

Biodiesel is made by chemical combination of any natural oil or fat with an alcohol such as methanol and a catalyst (e.g. lipases) for the transesterification process. Transesterification is catalyzed by acids, alkalis (Meher et al., 2006) and lipase enzymes (Sharma et al., 2006). Use of lipases offers important advantages as it is more efficient, highly selective, involves less energy consumption (reactions can be carried out in mild conditions), and produces less side products or waste (environmentally favorable). However, it is not currently feasible because of the relatively high cost of the catalyst (Fukuda et al., 2001).

On the industrial level, a number of methods for the immobilization of lipases on solid supports have been reported (Pedersen and Christensen, 2000). Commercially available lipases are supplied both as lyophilised powders, which contain other components in addition to the lipase (Salis et al., 2005). The immobilized lipases most frequently used for biodiesel production are lipase B from Candida Antarctica (Chang et al., 2005). This is supplied by Novozymes under the commercial name Novozym 435® (previously called SP435) and is immobilized on an acrylic resin. The Mucor miehei commercial lipase (Lipozyme IM60 – Novozym) immobilized on a macroporous anionic exchange resin has also been extensively used for the same purpose (De Oliveira et al., 2004).

2.11 Life detection and planetary exploration

Analytical techniques based on mass spectrometry have been traditionally used in space science. Planetary exploration requires the development of miniaturized apparatus for in situ life detection. Recently, a new approach is gaining acceptance in the space science

community: the application of the well-known, highly specific, antibody–antigen affinity interaction for the detection and identification of organics and biochemical compounds. Antibody microarray technology allows scientists to look for the presence of thousands of different compounds in a single assay and in just one square centimeter. The detection of organic molecules of unambiguous biological origin is fundamental for the confirmation of present or past life.

Preservation of biomarkers on the antibody stability under space environments, smaller biomolecules, such as amino acids, purines, and fatty acids, are excellent biomarkers in the search for life on Mars, but they may be much less resistant to oxidative degradation. Recent work by Kminek and Bada, 2006 showed that amino acids can be protected from radiolysis decomposition as long as they are shielded adequately from space radiation. They estimated that it is necessary to drill to a depth of 1.5 to 2 m to detect the amino acid signature of life that became extinct about three billion years ago. A microfabricated capillary (Barron, 2008) electrophoresis device (kind of new immobilization technology) for amino acid chirality determination was developed for extraterrestrial exploration (Hutt et al., 1999). Recently, antibody microarray, a new immobilization technology that kept the stability of antibody under space environment allowed it to be applied for planetary exploration Exomars mission, 2005.

3. Recent advances in supports and technologies used in enzyme immobilization

In the search for suitable supports for enzyme immobilization, it was found that physical and chemical properties (e.g. pore size, hydrophilic/hydrophobic balance, aquaphilicity and surface chemistry) of support could exert effect on enzyme immobilization and its catalytic properties (Cao et al., 2003). Thus there was a need for new immobilization techniques/supports to avoid such shortcomings (Xie et al., 2009). The following are some examples of the recent carriers and technologies used for enzyme immobilization.

3.1 New carriers used in immobilization

3.1.1 Mesoporous support

Over the last few years, mesoporous support such as silica and silicates having pore size of 2–50 nm has been developed and being considered as one of the most promising carriers for enzyme immobilization (Chen et al., 2007b; Kim et al., 2007; Rosales-Hernandez et al., 2007; Wang et al., 2007; Wang et al., 2008a). The exploitation of novel carriers that enable high enzyme loading and activity retention has become the focus of recent attention (Boller et al., 2002). The large surface areas and greater pore volumes of these materials could enhance the loading capacity of an enzyme and the large pores in the support facilitate transport of substrate and product (Chong and Zhao, 2004).

Functional mesoporous material resulted in exceptionally high immobilization efficiency with enhanced stability, while conventional approaches yielded far lower immobilization efficiency (Lei et al., 2002). Additionally, the increase in the thermal stability of immobilized enzyme indicated that protein inside a confined space could be stabilized by some folding forces which did not exist in proteins in bulk solutions (Wang et al., 2008b). Confinement of the support nanopore could be similar to the macromolecular crowding (Cheung and Thirumalai, 2006), and could also stabilize the enzyme at high temperature.

Nanoporous gold (Szamocki et al., 2007) and nanotube (Chen et al., 2001; Wan et al., 2008) have also been used to immobilize enzymes. Most of the obtained immobilized enzymes were used in the electrode preparation and biosensor applications. The modified porous gold electrode shows an overall increased signal, and therefore a better detection limit and higher sensitivity when used as sensors.

3.1.2 Magnetic hybrid support

The use of magnetic supports for enzyme immobilization enables a rapid separation in an easily stabilized fluidized bed reactor for continuous operation of enzyme. It can also reduce the capital and operation costs (Bayramoglu et al., 2008). Due to the functionalization (Dyal et al., 2003) of enzyme and its suitable microenvironment, magnetic materials were often embedded in organic polymer or inorganic silica to form hybrid support (Liu et al., 2005). Recently, because of the low enzyme loading on the conventional magnetic beads (Liu et al., 2005), further attention was paid to the magnetic mesoporous support (Sadasivan and Sukhorukov, 2006). Magnetite mesoporous silica hybrid support was fabricated by the incorporation of magnetite to the hollow mesoporous silica shells, which resulted in the perfect combination of mesoporous materials properties with magnetic property. The produced hybrid support has shown to improve the enzyme immobilization (Kim et al., 2005).

3.2 New technologies for enzyme immobilization

3.2.1 Single enzyme nanoparticles

In the field of industrial enzymes, there is a great research for improving the enzyme stability under harsh conditions. As an innovative way of enzyme stabilization, "single-enzyme nanoparticles (SENs)" technology was rather attractive because enzyme in the nanoparticle exhibited very good stability under harsh conditions (Hegedus and Nagy, 2009; Yan et al., 2006). Kim and Grate (2003) have developed armored SENs that surround each enzyme molecule with a porous composite organic/ inorganic network of less than a few nanometers thick. They significantly stabilized chymotrypsin and trypsin and the protective covering around chymotrypsin is so thin and porous that a large mass transfer limitation on the substrate could not take place.

Yan et al. (2006) provided a simple method that yields a single enzyme capsule with enhanced stability, high activity and uniformed size. The 2-step procedure including surface acryloylation and in situ aqueous polymerization to encapsulate a single enzyme in nanogel to provide robust enzymes for industrial biocatalysis. The immobilized horseradish peroxidase (HRP) exhibited similar biocatalytic behavior (Km and kcat) to the free enzyme. However, the immobilization process significantly improved the enzyme\s stability at high temperature in the presence of polar organic solvent.

3.2.2 Enzymatic immobilization of enzyme

The use of green chemistry rather than using harsh chemicals is one of the main goals in enzyme industries to avoid the partial denaturation of enzyme protein. An emerging and novel technology is to fabricate solid protein formulations (Tanaka et al., 2007; Wong et al., 2008). As model proteins, enhanced green fluorescent protein (EGFP) and glutathione S-transferase (GST) were tagged with a neutral Gln-donor substrate peptide for MTG (Leu-

Leu-Gln-Gly, LLQG-tag) at their C-terminus and immobilized onto the casein-coated polystyrene surface (Tanaka et al., 2007).
Luciferase (Luc) and glutathione-S-transferase (GST) ybbR-fusion proteins were immobilized onto PEGA resin retaining high levels of enzyme activity using phosphopantetheinyl transferase (Sfp) mediating site-specific covalent immobilization (Wong et al., 2008). In general, the Sfp-catalyzed surface ligation is mild, quantitative and rapid, occurring in a single step without prior chemical modification of the target protein.

3.2.3 Microwave irradiation

The use of porous supports for immobilization of enzymes is difficult to distribute because of diffusion limitations (Buchholz, 1979) and they often remain only on external channel (Chen et al., 2007a). For enzymes having large dimensions, such as penicillin acylase (PA), the mass transfer is even slower. The immobilization of such enzyme to porous materials can prove tedious using conventional techniques (Van Langen et al., 2002).
Wang et al., 2008b & 2009a have recently succeeded to immobilize papain and PA using the adsorption technique into the mesocellular siliceous foams (MCFs) using microwave irradiation technology. Reaction time of 80 and 140 s were enough for papain and PA to attach on the wall of MCFs, respectively. The activities of papain and penicillin acylase immobilized with microwave-assisted method were 779.6 and 141.8 U/mg, respectively. In another experiment, macromolecules crowding was combined with small molecular quenching to perfect microwave-assisted covalent immobilization (Wang et al., 2009a).

3.2.4 Photoimmobilization technology

In the field of *immobilization of biomolecules*, potential applications of photoimmobilization using nitrene groups could take place. Nitrene groups have a property of insertion into C-H bond. When photoreactive polymer and horseradish peroxidase or glucose oxidase are exposed to ultraviolet (UV) light at 365 nm, the reactive nitrene immobilizes the protein molecules in 10 to 20 min through covalent bonding (Naqvi and Nahar, 2004). Horseradish peroxidase (HRP) and glucose oxidase (GOD) have been immobilized onto the photoreactive cellulose membrane by the ultraviolet and sunlight Kumar and Nahar (2007). They found that sunlight intensity required for optimum immobilization was 21,625 lux beyond which no appreciable increase in immobilization was observed. Moreover, sunlight exposure gave better immobilization compared to 365 nm UV light.

3.2.5 Ionic liquids

Ionic liquids, the green solvents for the future, are composed entirely of ions and they are salts in the liquid state. In the patent and academic literature, the term “ionic liquid” now refers to liquids composed entirely of ions that are fluid around or below 100 °C (e.g. ethanolamine nitrate, m.p. 52-55 oC). The date of discovery of the "first" ionic liquid is disputed, along with the identity of the discoverer. Room-temperature ionic liquids are frequently colorless, fluid and easy to handle (Rogers and Seddon, 2003).
Versatile biphasic systems could be formed by controlling the aqueous miscibility of ionic liquid (Gutowski et al., 2003). Based on a biphasic catalytic system where the enzyme is immobilized into an ionic liquid (IL), Mecerreyes and co-workers (Rumbau et al., 2006) have reported a new method which allows recycling and re-using of the HRP enzyme in the

biocatalytic synthesis of PANI. The HRP enzyme was dissolved into the IL 1-butyl-3-methylimidazolium hexafluorophosphate and the IL/HRP phase acts as an efficient biocatalyst and can be easily recycled and reused several times. Due to the immiscibility between the IL and water, the immobilized HRP could be simply recovered by liquid/ liquid phase separation after the biocatalytic reaction (Sheldon et al., 2002; van Rantwijk et al., 2003). Although this new method is faster and easier than the classical immobilization of HRP into solid supports, it is not widely applied in the industries because of the ionic liquids' expenses. However, according to Taubert, A (2005), there will be a bright future for ionic liquids by using inorganic materials synthesis.

4. Recommendation for the future of immobilization technology

At present, a vast number of methods of immobilization are currently available. Unfortunately, there is no a universal enzyme support, i.e. the best method of immobilization might differ from enzyme to enzyme, from application to application and from carrier to carrier. Accordingly, the approaches currently used to design robust industrial immobilized enzymes are, without exception, labeled as “irrational”, because they often result from screening of several immobilized enzymes and are not designed. As a consequence, some of the industrial enzymes are working below their optimum conditions.
Recently, Cao L. (2005) in his book "Carrier bound immobilized enzymes" tackled this problem as he surmised that the major problem in enzyme immobilization is not only the selection of the right carrier for the enzyme immobilization but it is how to design the performance of the immobilized enzyme.
The author of this review article is suggesting from his point of view as he is working in that field for the last ten years to follow these steps in order to get to this goal in the shortest time:

1. build a data base containing all information on the available biomolecules (enzymes, antibodies, etc) and carriers (organic, inorganic, magnetic hybrid, ionic liquids, etc) then
2. use the dry lab (bioinformatics) to validate the probability of success and the efficiency of the immobilization process then
3. starting the experiment in the wet lab.

The author believes that if this strategy could be performed, we should expect immobilized molecules working at their optimum conditions, with higher stability and efficiency, which will save money, time and effort for the prosperity of human being.

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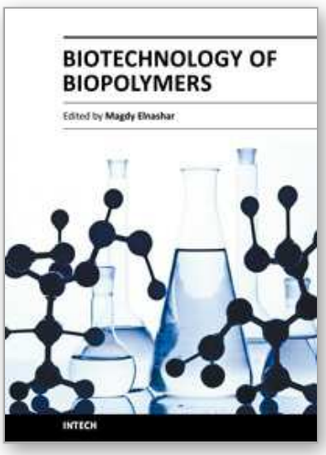

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The book "Biotechnology of Biopolymers" comprises 17 chapters covering occurrence, synthesis, isolation and production, properties and applications, biodegradation and modification, the relevant analysis methods to reveal the structures and properties of biopolymers and a special section on the theoretical, experimental and mathematical models of biopolymers. This book will hopefully be supportive to many scientists, physicians, pharmaceutics, engineers and other experts in a wide variety of different disciplines, in academia and in industry. It may not only support research and development but may be also suitable for teaching. Publishing of this book was achieved by choosing authors of the individual chapters for their recognized expertise and for their excellent contributions to the various fields of research.

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