Urease: Occurrence, inhibition and immobilization

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Abstract

Urease (*Urea amidohydrolase*; EC 3.5.1.5) was the first enzyme to be crystallized. Urease is an important enzyme present in many organisms, including plants. This enzyme plays diverse roles and mainly involved in the nitrogen metabolism. The bacterial urease has been extensively studied may be due to the simplicity of system and its significance in various pathological conditions and role in agriculture etc. Jack bean urease was the first nickel-containing enzyme to be described and it is the only nickel-containing metalloenzyme identified so far in plants. Its rapid catalysis for the hydrolysis of urea to ammonia and carbon dioxide plays an essential role in agriculture and human health. In most of the industrial, analytical, and clinical processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the exhaustion of the substrates. By mimicking the natural mode of occurrence in living cells, where enzymes for the most cases are attached to cellular membranes, the systems stabilize the structure of enzymes, hence their activities. Thus, as compared to free enzymes in solution immobilized enzymes are more robust and more resistant to environmental changes. More importantly, the heterogeneity of the immobilized enzyme systems allows easy recovery of enzyme and product, multiple reuses of enzymes, continuous operation of enzymatic processes, rapid termination of reactions and greater variety of bioreactor designs.

Keywords: urease, inhibition, immobilization, hydrogels, urea

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Introduction

In bacteria, the enzyme urease, as in other species, catalyzes the hydrolysis of urea. Urea hydrolysis in most bacteria is used as a nitrogen or carbon source for anabolic processes. However, in gastric tract-dwelling bacteria and other bacteria that encounter acid during their life cycles, urea hydrolysis contributes to their ability to survive in acid. Moreover, in gastric tract-dwelling bacteria, this process confers on them the unique ability to colonize the stomach. Although urea is the major substrate of urease, this enzyme is capable of hydrolyzing (*albeit* poorly) other substrates such as acetamide, formamide, *N*-methylurea, semicarbazide, and hydroxyurea (Dixon *et al.*, 1980). Bacterial ureases have been shown to be

important virulence determinants in the pathogenesis of many clinical conditions in human and animals. Urease is directly involved in the formation of infection stones and contributes to the pathogenesis of urolithiasis, pyelonephritis, ammonia and hepatic encephalopathy, hepatic coma and urinary catheter encrustation. Urease is known to be the major cause of pathologies induced by *Helicobacter pylori*, which allows this pathogen to survive at the low pH of the stomach during colonization and therefore plays an important role in the pathogenesis of gastric and peptic ulcers, which in some cases may progress to cancer (Moblely *et al.*, 1995). In agriculture, high urease activity causes significant environmental and economic problems by releasing abnormally large amounts of ammonia

into the atmosphere during urea fertilization (Mobley and Hausinger, 1989; Mobley *et al.*, 1995; Sirko and Brodzik, 2000).

Occurrence and Role of Urease

Many organisms, including plants, some bacteria, fungi and invertebrates, synthesize urease. Urease is a cytosolic enzyme. In most of the studied cases the majority of its activity is associated with the soluble fractions of the cells (Mobley *et al.*, 1995). Many species of *leguminosae* have high levels of urease activity in their seeds. Whereas in mammals urea is a non-toxic waste form for excess ammonia, in plants significant quantities of nitrogen flow through urea (urea is 47% nitrogen), which can be recycled only by urease action (Polacco and Holland, 1993; Sirko and Brodzik, 2000). Urease activity was found in several species of fungi. Biochemically, the best-characterized plant urease is that from jack bean (Takishima *et al.*, 1988; Riddles *et al.*, 1991; Hirai *et al.*, 1993; Karmali and Domingos, 1993). Urease from mulberry (*Morus alba*) leaves has also been purified and characterized (Hirayama *et al.*, 2000).

The best genetic data concerning plant ureases are available for soybean (Polacco and Holland, 1993; 1994). There are at least three key enzymes involved in urea metabolism in plants: arginase, urease and glutamine synthetase. The primary role of ureases is to allow the organism to use external or internally generated urea as a nitrogen source (Mobley and Hausinger, 1989; Mobley *et al.*, 1995). Significant amounts of plant nitrogen flow through urea. This compound derives from arginine and possibly from degradation of purines and ureides (Polacco and Holland, 1994). The nitrogen present in urea is unavailable to the plant unless hydrolyzed by urease. The product of urease activity - ammonia - is incorporated into organic compounds mainly by glutamine synthetase.

It has been reported that overexpression of the pine glutamine synthetase in transgenic *South Asian J. Food Technol. Environ.*, 2(3&4):418- 431 (2016)

poplar improves the growth of the plants, probably by increasing the efficiency of nitrogen utilization (Gallardo *et al.*, 1999). On the other hand, it has been shown that increased activity of this enzyme in the roots of a legume plant, *Lotus japonicus*, leads to decreased plant biomass production, possibly due to limited nitrate uptake (Limami *et al.*, 1999). These results, which seem contradictory at first glance, might reflect the different roles of glutamine synthetase isoenzymes in different plants and/or different plant organs. They indicate the significance of this enzyme for plant nitrogen metabolism. The activity of glutamine synthetase, the amount of its product, glutamine, and possibly the availability of its substrate, ammonia, seem to be important factors controlling nitrogen metabolism and affecting plant growth (Stitt, 1999; Wiren *et al.*, 2000). Carlini and Guimarães (1981) isolated canatoxin, a highly toxic protein from jack beans that induces convulsions and death in mice and rats when injected intraperitoneally.

Canatoxin was subsequently identified as an isoform of urease (Follmer *et al.*, 2001) which can be isolated separately from jack bean urease using their different affinities for metal ions (Follmer *et al.*, 2004). Ureases from plant and microbial sources belong to a group of multifunctional proteins displaying not only the capacity for ureolysis, but also toxic effects in insects. The newly described properties of plant ureases may shed new light on the physiological roles of these proteins in the source organisms, reinforcing the possibility of plant ureases having a protective role against phytopathogens through an entirely different mechanism, unrelated to the release of ammonia. Microbial ureases are important enzymes in certain human and animal pathogenic states, in ruminant metabolism, and in environmental transformations of certain nitrogenous compounds.

Urease activity plays a central role in the pathogenesis elicited by a number of bacterial species. This enzyme plays an

important role in the pathological conditions like, urolithiasis (stone formation), catheter encrustation, pyelonephritis, ammonia encephalopathy, hepatic encephalopathy, hepatic coma, and inactivation of complement etc. In humans, *P. mirabilis* is the most common organism implicated in stone formation (Rosenstein, 1986). Microbial ureases play an important role in the nitrogen metabolism of ruminants such as cattle, sheep, and other animals that contain a forestomach (Huntington, 1986). Substantial amounts of animal-derived urea are recycled to the rumen, where ureolytic activity releases ammonia, the major source of nitrogen for most ruminal bacteria (Bryant, 1959). The microbial biomass generated is then utilized as a nutrient by the ruminant. Urease activity is widely distributed in soil and aquatic environments, where it plays an essential role in nitrogen metabolism (Bremner and Mulvaney, 1978). For example, degradative processes involving protein and nucleotide turnover require urease activity. More importantly, effective urea fertilization requires controlled ureolysis to enhance efficiency and minimize crop damage. The availability of fixed nitrogen is the major limitation to agricultural productivity.

Two major forms of fixed nitrogen in the environment are proteins and polynucleotides. These components are hydrolyzed to the amino acid and nucleotide monomers and then further degraded by a variety of enzymes. The guanidino group of arginine and the ring nitrogens of purines are released as urea, which is further hydrolyzed by urease (Varner, 1959; Vogels and vander Drift, 1976). The product ammonia can be taken up and utilized by soil microbes and plants. However, in many environments the level of available nitrogen compounds is inadequate for optimal crop production. Therefore, fertilizers are applied which can be converted to a form of nitrogen that plants can assimilate. Urea is widely used because of its low cost, ease in handling, and high nitrogen content (Beaton, 1978). *Ureaplasma* species differ from all other

mycoplasmas (class Mollicutes) by possessing urease activity (Taylor-Robinson and Gourlay, 1984). Urea hydrolysis appears to play a major role in the energy metabolism of ureaplasmas by promoting ATP synthesis through a chemiosmotic mechanism (Masover *et al.*, 1977a,b; Romano *et al.*, 1980; 1986). This rather unique energy-yielding mechanism is essential for the ureaplasmas, which are known to lack the major energy-yielding (glycolytic and arginine dihydrolase) pathways established so far for other mollicutes (Pollack, 1986).

Urease Inhibitors

One of the most pressing environmental issues confronting agriculture is the loss of soil nitrogen, derived from either fertilizer nitrogen or symbiotic nitrogen fixation by legumes, to the atmosphere as gases (NH_3 , N_2 and N_2O , the latter being a potent greenhouse gas) or to groundwater via leaching as NO_3^- . These losses of nitrogen not only decrease nitrogen-use efficiency, and therefore have economic implications, but just as importantly, impact on groundwater quality and contribute to greenhouse gas emissions. The nitrification and urease inhibitors can be successfully used as tools to manage these losses. Studies on inhibitors are useful to learn about how enzymes interact with their substrates; role of inhibitors in enzyme regulation; drugs if they inhibit aberrant biochemical reactions; understanding the role of biological toxins; insecticides etc (Kumar and Kayastha, 2010a; 2010b; 2010c; Kumar and Kayastha, 2012; Kumar, 2015; Lehari *et al.*, 2015; Kumar, 2016). Ammonium, present in soil is derived from several sources: directly from the mineralization of organic matter and the addition of ammonium-containing fertilizers, and, indirectly, as the result of the hydrolysis of applied urine and fertilizer urea. Under typical conditions ammonium is oxidized first to nitrite and then to nitrate (NO_3^-) by bacteria (nitrification).

Plants can utilize both ammonium and nitrate. Furthermore, under anaerobic conditions

NO_3^- nitrogen can be reduced by denitrifying bacteria to the gases N_2O and N_2 (denitrification). These gases can also be produced from ammonia and nitrite under aerobic conditions by chemical reactions-chemodenitrification. For these reasons, controlling the processes of nitrification and/or urease hydrolysis are, theoretically at least, potential tools to restrict nitrogen leaching, greenhouse gas emissions and ammonia volatilization from soils. As a consequence the nitrogen cycle should be more efficient and nitrogen use efficiency increased. It has focused therefore on the use of nitrification inhibitors in conjunction with fertilizer nitrogen and animal wastes, and urease inhibitors applied with urea-based fertilizer. Urease inhibitors restrict the conversion of urea and urine to ammonium, and hence to nitrate, in soils.

The majority of the research indicates that nitrification inhibitors, when applied to soils in conjunction with nitrogen fertilizers or animal wastes, have beneficial effects on reducing nitrate leaching and nitrous oxide emissions, and, as a result increase plant growth (Stelly, 1980; Williamson *et al.*, 1998; Merino *et al.*, 2002). The high affinity of ureases for metal ions (e.g. Hg^{2+} , Cu^{2+} , Ni^{2+} and Zn^{2+}) as well as their inhibitory effects (Hughes *et al.*, 1969) upon the ureolytic and insecticidal activities of the enzyme raises the possibility of metals being modulators of urease activities. Follmer and Carlini (2005) suggested that jack bean urease aggregation could be induced by binding of metal. The Cu^{2+} induced polymeric forms of jack bean urease have no urease or insecticidal activities, similar to aggregated forms of jack bean urease induced by freezing and thawing cycles (Follmer *et al.*, 2004). Chemical modification of histidine residues of jack bean urease by treatment with diethylpyrocarbonate decreases its affinity for Cu^{2+} and inhibits oligomerization induced by this metal, resulting in a protection of insecticidal properties of jack bean urease from the inactivation by Cu^{2+} (Follmer and Carlini, 2005). In addition, it was shown that certain ions, notably Hg^{2+} and Cu^{2+}

are inhibitors of urease activity, and it was suggested that this inhibition might involve blockage of thiol groups in the protein (Hughes *et al.*, 1969). Cu^{2+} can bind to many sites on the *K. aerogenes* urease in addition to the usual metallocenter, but most of the adventitious metal is removed by treatment with EDTA (Yamaguchi *et al.*, 1999).

Several studies hypothesized that nonproductive metal binding arises by coordination to Cys³¹⁹ localized in the mobile flap of *K. aerogenes* urease (Martin and Hausinger, 1992; Pearson *et al.*, 1997). On the other hand, Yamaguchi *et al.*, (1999) reported that the mutation Cys^{319A} does not prevent the irreversible inactivation of the enzyme by Cu^{2+} , suggesting that an affect other than blockage of thiol is involved. Cys³¹⁹ in *K. aerogenes* urease is located at the active site (in *K. aerogenes* urease, the sulfur atom is positioned 6.7 and 6.1 Å from Ni-1 and Ni-2, respectively) (Martin and Hausinger, 1992; Pearson *et al.*, 1997) and is surprisingly conserved in the amino acid sequences of both microbial and plant ureases. In addition, due to its reactivity, this cysteine is one of the most important targets for urease inhibition (Todd and Hausinger, 1991; Pearson *et al.*, 1997; Zhang *et al.*, 2006; Krajewska *et al.*, 2004).

Urease Immobilization

Enzymes may be immobilized by a variety of methods, which may be broadly classified as physical, where weak interactions between support and enzyme exist, and chemical, where covalent bonds are formed with the enzyme (Kennedy and Cabral, 1983; Bullock, 1995;). To the physical methods belong: (i) containment of an enzyme within a membrane reactor, (ii) adsorption (physical, ionic) on a water-insoluble matrix, (iii) inclusion (or gel entrapment), (iv) microencapsulation with a solid membrane, (v) microencapsulation with a liquid membrane, and (vi) formation of enzymatic Langmuir-Blodgett films. The chemical immobilization methods include: (i)

covalent attachment to a water-insoluble matrix, (ii) cross-linking with use of a multifunctional, low molecular weight reagent, and (iii) co-cross-linking with other neutral substances, e.g. proteins. Urease has been immobilized on a variety of carriers such as on starch, alginate, silica, chitosan or agarose (Kumar *et al.*, 2005; Kumar *et al.*, 2009; Swati *et al.*, 2007).

Numerous other methods which are combinations of the ones listed or original and specific of a given support or enzyme have been devised. However, no single method and support is best for all enzymes and their applications. This is because of the widely different chemical characteristics and composition of enzymes, the different properties of substrates and products, and the different uses to which the product can be applied. Besides, all of the methods present advantages and drawbacks. Adsorption is simple, cheap and effective but frequently reversible, covalent attachment and cross-linking are effective and durable, but expensive and easily worsening the enzyme performance, and in membrane reactor-confinement, entrapment and microencapsulations diffusional problems are inherent. Consequently, as a rule the optimal immobilization conditions for a chosen enzyme and its application are found empirically by a process of trial and error in a way to ensure the highest possible retention of activity of the enzyme, its operational stability and durability.

This immobilization on a variety of carriers is well documented in the literature: There is adsorption on raw starch (Ray *et al.*, 1994), entrapment in alginate (Kokufuta *et al.*, 1988), immobilization on ion exchanger (Roy and Hedge, 1987), silica beds (Germain and Crichton, 1980), porous chitosan (Obha *et al.*, 1979) or agarose (Sheffield *et al.*, 1995). To date, several immobilized enzyme-based processes have proved economic and have been implemented on a larger scale, mainly in the food industry, where they replace free enzyme-catalyzed processes, and in the manufacture of fine specialty chemicals and pharmaceuticals,

particularly where asymmetric synthesis or resolution of enantiomers to produce optically pure products are involved (Kennedy and Cabral, 1983; Bullock, 1995). The scale of the processes ranges from about 10^6 tons per year for high-fructose corn syrup, arguably one of the most commercially important immobilized enzyme-based processes, to about 10^2 tons per year for enantiopure L-DOPA (van de Velde *et al.*, 2002). Immobilization on a conducting matrix (e.g., a metal electrode) is generally difficult but is desirable in biosensor application. Several enzyme biosensors have been designed and constructed using various kinds of immobilization techniques. Works dealing with chemically modified electrodes have made possible the permanent chemical modification of various electrode materials (Watkins *et al.*, 1975). For example, gold electrodes have been covalently modified using thiol compounds (Katz *et al.*, 1997).

Glassy carbon is not an attractive material for enzyme immobilization as its chemical inertness towards coupling reagents is very high. Enzyme adsorptions on graphite or activated carbon followed by glutaraldehyde (Liu *et al.*, 1975) or soluble carbodiimide (Cho and Bailey, 1977; 1979) cross-linking have been reported. Covalent immobilization of the glucose oxidase enzyme onto graphite powder by carbodiimide treatment was reported (Gorton, 1995). The direct covalent binding of the enzyme by carbodiimide coupling onto glassy carbon after initial activation by electrochemical oxidation was reported by Bourdillon *et al.* (1980).

Immobilization on Hydrogels: Hydrogels are superabsorbent (they can contain over 99% water) natural or synthetic polymers. Hydrogel is a network of polymer chains that are water-insoluble, sometimes found as a colloidal gel in which water is the dispersion medium. Gelling agents are materials used to thicken and stabilize liquid solutions, emulsions, and suspensions. They dissolve in the liquid phase as a colloid mixture that forms an internal structure giving

the resulting gel an appearance of a solid matter, while being mostly composed of a liquid.

Typical gelling agents include natural gums, starches, pectins, agar-agar and gelatine. Often they are based on polysaccharides or proteins. Examples include alginic acid, sodium alginate, potassium alginate, calcium alginate (polysaccharides from brown algae); agar (a polysaccharide obtained from red seaweeds); carrageenan (a polysaccharide obtained from red seaweeds); locust bean gum (a natural gum from the seeds of the Carob tree); pectin (a polysaccharide obtained from apple or citrus-fruit); gelatine (made by partial hydrolysis of animal collagen).

Calcium alginate is just as widely used as polyacrylamide. Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather, polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 70% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous. If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

Alginate, commercially available as alginic acid, sodium salt, commonly called sodium alginate, is a linear polysaccharide normally isolated from many strains of marine brown seaweed and algae, thus the name alginate. The copolymer consists of two uronic acids: D-mannuronic acid (M) and L-guluronic acid (G). Because it is the skeletal component of the algae it has the nice property of being strong and yet flexible. Alginic acid can be either water

soluble or insoluble depending on the type of the associated salt. The salts of sodium, other alkali metals, and ammonia are soluble, whereas the salts of polyvalent cations, e.g., calcium, are water insoluble, with the exception of magnesium. Polyvalent cations bind to the polymer whenever there are two neighboring guluronic acid residues. Thus, polyvalent cations are responsible for the cross-linking of both different polymer molecules and different parts of the same polymer chain. The process of gelation, simply the exchange of calcium ions for sodium ions, is carried out under relatively mild conditions. Because the method is based on the availability of guluronic acid residues, which will not vary once given a batch of the alginate, the molecular permeability does not depend on the immobilization conditions. Rather, the pore size is controlled by the choice of the starting material. The ionically linked gel structure is thermostable over the range of 0-100 °C; therefore heating will not liquefy the gel.

However, the gel can be easily redissolved by immersing the alginate gel in a solution containing a high concentration of sodium, potassium, or magnesium. Maintaining sodium: calcium $\leq$ 25: 1 will help avoid gel destabilization. In fact, it is recommended by alginate vendors to include 3 mM calcium ions in the substrate medium. On the other hand, citrate or phosphate pH buffers cannot be effectively used without destabilizing the alginate gel. Alginate is currently widely used in food, pharmaceutical, textile, and paper products. The properties of alginate utilized in these products are thickening, stabilizing, gel-forming, and film-forming. Alginate polymers isolated from different alginate sources vary in properties. Different algae, or for that matter different part of the same algae, yield alginate of different monomer composition and arrangement. There may be sections of homopolymeric blocks of only one type of monomer (-M-M-M-) (-G-G-G-), or there may be sections of alternating monomers (-M-G-M-G-M-).

Different types of alginate are selected for each application on the basis of the molecular weight and the relative composition of mannuronic and guluronic acids. For example, the thickening function (viscosity property) depends mainly on the molecular weight of the polymer; whereas, gelation (affinity for cation) is closely related to the guluronic acid content. Thus, high guluronic acid content results in a stronger gel. The enzyme-immobilization by entrapment using an alginate gel is fast, mild and low-cost, and this method is used with a wide range of biocatalysts. However, the matrix has some disadvantages because alginate gel is hydrophilic and dissolves in highly concentrated electrolyte and phosphate buffer solutions. The gel network is so soft that a low molecular weight enzyme such as protease leaks out of the gel network.

Chitin and chitosan are natural polyaminosaccharides (Hudson and Smith, 1998), chitin being one of the world's most plentiful, renewable organic resources. A major constituent of the shells of crustaceans, the exoskeletons of insects and the cell walls of fungi where it provides strength and stability. Chemically, chitin is composed of β (1 $\rightarrow$ 4) linked 2-acetamido-2-deoxy- β -D-glucose units (or *N*-acetyl-D-glucosamine) (Peter, 1995), forming a long chain linear polymer. It is insoluble in most solvents. Chitosan, the principal derivative of chitin, is obtained by *N*-deacetylation to a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine.

Chitin and chitosan can be chemically considered as analogues of cellulose, in which the hydroxyl at carbon-2 has been replaced by acetamido and amino groups, respectively. Chitosan is insoluble in water, but the presence of amino groups renders it soluble in acidic solutions below pH about 6.5. It is important to note that chitin and chitosan are not single chemical entities, but vary in composition

depending on the origin and manufacture process. Chitosan can be defined as chitin sufficiently deacetylated to form soluble amine salts, the degree of deacetylation necessary to obtain a soluble product being 80-85% or higher. Commercially, chitin and chitosan are obtained at a relatively low cost from shells of shellfish (mainly crabs, shrimps, lobsters and krills), wastes of the seafood processing industry (Hudson and Smith, 1998). Basically, the process consists of deproteinization of the raw shell material with a dilute NaOH solution and decalcification with a dilute HCl solution. To result in chitosan, the obtained chitin is subjected to *N*-deacetylation by treatment with a 40-45% NaOH solution, followed by purification procedures. Chitosan possesses distinct chemical and biological properties (Hudson and Smith, 1998). In its linear polyglucosamine chains of high molecular weight, chitosan has reactive amino acid hydroxyl groups, amenable to chemical modifications (Peter, 1995; Kurita, 2001; Dutta *et al.*, 2002). Additionally, amino groups make chitosan a cationic polyelectrolyte ($pK_a \approx 6.5$), one of the few found in nature. This basicity gives chitosan singular properties: chitosan is soluble in aqueous acidic media at pH < 6.5 and when dissolved possesses high positive charge on $-\text{NH}_3^+$ groups, it adheres to negatively charged surfaces, it aggregates with polyanionic compounds, and chelates heavy metal ions. Both the solubility in acidic solutions and aggregation with polyanions impart chitosan with excellent gel-forming properties. Along with unique biological properties that include biocompatibility, biodegradability to harmless products, non-toxicity, physiological inertness, remarkable affinity to proteins, hemostatic, fungistatic, antitumoral and anticholesteremic properties, chitin and chitosan, as yet underutilized, offer an extraordinary potential in a broad spectrum of applications, which are predicted to grow rapidly once the standardized chitinous materials become available. Crucially, as bio- and biodegradable polymers

chitin/chitosan materials is eco-friendly, safe for humans and the natural environment.

As enzyme immobilization supports chitin- and chitosan based materials are used in the form of powders, flakes and gels of different geometrical configurations. Preparation of chitosan gels is promoted by the fact that chitosan dissolves readily in dilute solutions of most organic acids, including formic, acetic, tartaric and citric acids, to form viscous solutions that precipitate upon an increase in pH and by formation of water-insoluble ionotropic complexes with anionic polyelectrolytes. In this way chitosan gels in the form of beads, membranes, coatings, capsules, fibers, hollow fibers and sponges can be manufactured. Commonly, different follow-up treatments and modifications are applied to improve gel stability and durability. The methods of chitosan gel preparation can be broadly divided into four groups (Hudson and Smith, 1998; Dutta *et al.*, 2002): (i) Solvent evaporation method is mainly used for the preparation of membranes and films. A solution of chitosan in organic acid is cast onto a plate or an electrode tip and allowed to dry, if possible at elevated temperature (ca. 65°C). Upon drying the membrane/film is normally neutralized with a dilute NaOH solution and cross-linked to avoid disintegration in solutions of pH < 6.5. (ii) If an acidic chitosan solution is mixed with alkali, an increase in pH results in precipitation of solid chitosan. This method is known as neutralization method and is exploited to produce chitosan precipitates, membranes, fibers, but foremost spherical beads of different sizes and porosities. These are obtained by adding a chitosan solution dropwise to a solution of NaOH. Following the preparation, the beads are commonly subjected to cross-linking. (iii) In this method, an acidic chitosan solution is subjected to straightforward cross-linking by mixing with a cross-linking agent, which results in gelling. To obtain gel membranes, the chitosan solution cast on a plate is immersed in a cross-linking bath, and to obtain beads the solution is added dropwise

therein. (iv) By virtue of the attraction of oppositely-charged molecules, chitosan, owing to its cationic polyelectrolyte nature, spontaneously forms water-insoluble complexes with anionic polyelectrolytes (Dumitriu and Chornet, 1998). The anionic polyelectrolytes used include alginate, carrageenan, xanthan, various polyphosphates and organic sulfates or enzymes themselves (Wei *et al.*, 2002). The method is known as ionotropic gelation method (or coacervation) and is utilized chiefly for the preparation of gel beads, which is achieved by adding an anionic polyelectrolyte solution dropwise into an acidic chitosan solution.

Applications of Immobilized Enzymes: Areas of present and potential future applications of immobilized enzyme systems other than industrial (shown in the Table 1.1) include: laboratory scale organic synthesis, and analytical and medical applications (Kennedy and Cabral, 1983; Bullock, 1995; van de Velde *et al.*, 2002). Having been shown to be able to catalyze reactions not only in aqueous solutions but also in organic media, enzymes offer great potential for assisting organic synthesis (Carrea and Riva, 2000). They can simplify the chemical procedures by reducing the number of synthetic steps, they can enhance the purity of the products, and most importantly, they can catalyze regio- and stereoselective synthesis giving, otherwise unobtainable compounds with the desired properties.

In analytical applications immobilized enzymes are used chiefly in biosensors (Guilbault, 1983; Srivastava and Kayastha, 2001) and to a lesser extent, in diagnostic test strips (Reddy *et al.*, 2004). Biosensors are constructed by integrating biological sensing systems, e.g. enzyme(s), with transducers. These obtain a chemical signal produced by the interaction of the biological system with an analyte and transduce it into a measurable response. Different kinds of transducers have been employed in biosensors, *viz.*, potentiometric, amperometric, conductometric, thermometric, optical and piezo-electric, most of

the current research being placed on the first two. Enzymes for the most cases are immobilized either directly on a transducer's working tip or in/on a polymer membrane tightly wrapping it up.

In principle, due to enzyme specificity and sensitivity biosensors can be tailored for nearly any target analyte, and these can be both enzyme substrates and enzyme inhibitors. Advantageously, their determination is performed without special preparation of the sample. Meeting the demand for practical, cost-effective and portable analytical devices,

enzyme-based biosensors have enormous potential as useful tools in medicine, environmental in situ and real time monitoring, bioprocess and food control, and in biomedical and pharmaceutical analysis. Their use, impaired as yet by not quite satisfactory reliability, is predicted to become widely accepted once their storage and operational stabilities have been improved. From a practical and commercial point of view, four of the sensors, namely glucose, lactate, urea and glutamate have been widely used (Wilson and Hu, 2000).

Table 1: Important industrial applications of immobilized enzyme systems (Krajewska, 2004)

Enzyme (EC number)	Substrate	Product
Glucose isomerase (5.3.1.5)	Glucose	Fructose (high-fructose corn syrup)
β -Galactosidase (3.2.1.23)	Lactose	Glucose and galactose (lactose-free milk and whey)
Lipase (3.1.1.3)	Triglycerides	Cocoa butter substitutes
Nitrile hydratase (4.2.1.84)	Acrylonitrile	Acrylamide
	3-Cyanopyridine	Nicotinamide
	Adiponitrile	5-Cyanovaleramide
Aminoacylase (3.5.1.14)	D,L-Amino acids	L-Amino acids (methionine, alanine, phenylalanine, tryptophan, valine)
Raffinase (3.2.1.22)	Raffinose	Galactose and sucrose (raffinose-free solutions)
Invertase (3.2.1.26)	Sucrose	Glucose/fructose mixture (invert sugar)
Aspartate ammonia-lyase (4.3.1.1)	Ammonia + fumaric acid	L-Aspartic acid (used for production of synthetic sweetener aspartame)
Thermolysin (3.4.24.27)	Peptides	Aspartame
Glucoamylase (3.2.1.3)	Starch	D-Glucose
Papain (3.4.22.2)	Proteins	Removal of "chill haze" in beers
Hydantoinase (3.5.2.2)	D,L-Amino acid hydantoins	D,L-Amino acids
Penicillin amidase (3.5.1.11)	Penicillins G and V	6-Aminopenicillanic acid (precursor of semi-synthetic penicillins, e.g. ampicillin)
β -Tyrosinase (4.1.99.2)	Pyrocatechol	L-DOPA

Medical applications of immobilized enzymes include (Kennedy and Cabral, 1983; Bullock, 1995) 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. Offering a great potential in this area, real application of immobilized enzymes has as yet suffered from serious problems from their toxicity to the human organism, allergenic and immunological reactions as well as from their limited stability *in vivo*.

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