

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

Enzymes are used in the free form or in an immobilized form in aqueous systems. Enzymes bound to a solid carrier can result in increased operational stability, enhanced activity and the possibility of continuous process in which better control of substrate and product flow can be maintained (Phillips and Poon, 1988). The recent developments in the technology of immobilized enzymes and enzyme engineering have widened the application of enzymatic processes in different industries. By enabling repeated use of enzymes and due to freedom from contamination of the product, the technology has become very popular.

At present, application of immobilized biocatalysis include (a) the production of useful compounds by stereo-specific or regiospecific bioconversion (b) the production of energy by biological processes (c) the selective treatment of specific pollutants to solve environmental problems (d) continuous analysis of various compounds with a high sensitivity and high specificity and (e) medical uses (Gerhartz, 1990). Although the industrial applications of immobilized biological systems as heterogenous biocatalysts today involves only relatively simple transformations, recent advances in live cell immobilization clearly indicate the great potential of heterogenous

biocatalysts in continuous fermentation processes (Linko, 1980). The industrial uses of immobilized enzymes and cells have been the topic of many reviews (Chibita, 1978). Immobilized enzyme technology has been employed to produce fructose containing sweet syrups from starch for more than twenty years (Linko *et al.*, 1977). Immobilized enzymes are being used in production of many pharmaceutical, food and other products. A fair number of processes using immobilized biocatalysis have been industrialized so far. Immobilized biocatalysts have been used for the production or conversion of various compounds such as amino acids, peptides and enzymes, sugars, organic acids, antibiotics, steroids, nucleosides and nucleotides, lipids, terpenoids, fuels or commodity chemicals (Gerhartz, 1990).

## **2.1 IMMOBILIZATION OF ENZYMES**

### **2.2.1 HISTORICAL DEVELOPMENT**

It has been known for a long time that enzymes in water insoluble form show catalytic activity. As early as 1908, Michaelis and Ehrenreich studied the adsorption of a range of enzymes on various solid adsorbents as a function of pH and found that adsorption is pH dependant. A few years later, Nelson and Griffin (1916) found that when invertase extracted from yeast was adsorbed on charcoal and certain other biocolloids, the adsorbed enzyme showed the same activity as native enzyme. The dependence of adsorption on the concentration of the enzyme in solution was studied by Jacoby in 1916

(Phillips and Poon, 1988). In 1940, Katchalski-katzir and coworkers were the first to realize the potential of polymer matrix bound enzymes in industrial applications (Bohak and Sharon, 1977). After the Second World War ,the poineering work of Grubhoefer and Schlieth of Heidelberg who immobilized enzymes such as carboxy-peptidase, diastase, pepsin and ribonuclease by using diazotised polyamino polystyrene resin, and Katchalski-katzir and coworkers of Israel, who carried out extensive studies on immobilization techniques and on properties of immobilized enzymes, laid the foundation of immobilizaed biocatalyst technology (Linko, 1980). These workers made extensive contributions to the understanding of immobilized enzymes. In 1969, they succeeded in industrialization of continuous process for the optical resolution of D-L-amino acids using immobilized enzymes. This was the first industrial application of immobilized enzymes in the world (Chibita *et al.*, 1972). Several enzymes have since been immobilized by adsorption, ion- and covalent binding, entrapment and microencapsulation on different carriers.

In the initial stages of the evaluation of enzymes, the major problems have been immobilization techniques and carrier development. Consequently, fundamental work has been concerned with the preparation of suitable carriers and the investigation of optimized methods (Messing, 1975, Zaborasky, 1974) resulting in preparation of special and effective immobilized enzymes. It was found that the catalytic properties of the immobilized enzymes underwent various changes during preparation (Zaborasky, 1974). Such changes have been found during the direct comparison of enzymes immobilized by different

methods with native enzymes . The present day problems of evaluating immobilized enzymes are mainly concerned with their practical applications (Reimerdes,1980). During the investigation of the hydrolysis of beta-casein by immobilized trypsin, it was found that highly hydrophobic proteolysis products interact with the enzyme beads, resulting in inactivation of the proteolytic activity by adsorptive binding of resulting peptides (Reimerdes, 1979). Several solutions to non-substrate interaction have been attempted. In lactose hydrolysis, ultrafiltration and electrodialysis have been applied to whey in order to partially remove the interfering proteins and milk salts (Pitcher, 1976).

Since the late 1960s, the science and technology aiming at more efficient utilization of enzyme activity and specificity has been called “enzyme engineering” . In this rapidly developing field, immobilized enzymes have played a very important role and the articles and patents on this subject have increased very rapidly since the late 1960s. In recent years, in order to avoid the need to extract enzymes from microbial cells or to utilize multi-enzyme systems of the cells, techniques of direct immobilization of whole microbial cells have been developed. Methods of cell immobilization, roughly parallel to those of enzyme immobilization, are classified as mechanical, chemical or ionic methods based on the mode of attachment. In mechanical immobilization, the cells are localized by means of physical barriers. In chemical immobilization, covalent bonds are formed among cells (Phillips and Poon, 1988).



### 2.1.2 ADVANTAGES AND DISADVANTAGES OF IMMOBILIZED ENZYMES

The change over from soluble or free enzymes to immobilized enzymes is associated with many advantages and disadvantages. Heterogenous biocatalysts approximate native biological enzyme systems often attached to cell structures. In many cases, the time required for enzymatic processes has been remarkably reduced and the products have become free from by-products. A number of new food and pharmaceutical applications became a reality. The decrease in the availability of petrochemical feedstocks, an increase in energy and raw material cost and the emergence of immobilized biocatalysis technology during the last four decades to provide specific stable insoluble catalysts for continuous conversions, have revived major interest and trust in biotechnology as a tool for both time and bulk chemical production (Chibita, 1978).

A shift in the pH optimum and in the action patterns of enzymes was achieved by proper immobilization. Linko *et al.* (1975) observed a change in the action pattern of  $\alpha$ -amylase bound on cyanogen bromide activated carboxy methyl cellulose, making controlled modifications of starch possible. In many cases, stability of enzymes is improved by immobilization. Immobilized enzymes are receiving increased attention because of the advantages like (a)

the stability of the enzymes is improved (b) immobilized enzyme can be tailor-made for specific use (c) enzymes can be reused (d) continuous operation becomes practical (e) better control of reaction is possible (f) reactions require less space and time (g) higher purity and yield of products is achieved and (I) there is a saving of resources and less pollution. If a support or entrapping material is used, its properties combined with those of the enzymes and the immobilization procedure dictates overall catalyst properties.

Since enzyme may be lost by desorption, adsorbents to which enzymes are bound firmly with minimum denaturation are most suitable. When an enzyme is bound to a support enzymatic activity may be lost in several ways like (a) some enzyme molecules may be immobilized relative to the support in a configuration that completely prevents substrate access to the active site (b) a reactive group in the active site may be involved in the binding to the support; protection of the active site by a reversible inhibitor during binding activity (c) enzyme molecules on binding may be held in an inactive configuration and (d) the reaction conditions for binding may cause denaturation or inactivation (Wang *et al.*, 1979). When an enzyme is immobilized by entrapment some of it may be denatured or inactivated by reactants or products involved in the formation of entrapping matrix. Chibita *et al.* (1972) showed that the retention of the activity of aminoacylase immobilized by entrapment and binding varied greatly. Loss of enzyme activity on immobilization may be due to (a) blockage of active site (b) alteration of enzyme structure (Koshland effect) (c) protein denaturation and

(d) structural change (Sastri, 1994). When an enzyme is immobilized, the substrate has to diffuse from the bulk solution through the stationary liquid film on the surface of the support and if the support is porous, into the pores. These diffusional processes may restrict the reaction rate. When external mass transfer of the substrate to the surface is limiting the reaction rate, the flow conditions around the immobilized enzyme influence the reaction rate (Wang *et al.*, 1979). Immobilization may change the kinetics and other properties of the enzyme. Several factors such as conformational effects, steric effects, partitioning effects and mass transfer or diffusional effects are considered to cause these changes. Internal pore diffusional limitation of the reaction rate of immobilized enzymes has been demonstrated in several cases (Ravito and Kittrell, 1973). Kay and Lilly (1970) showed that when particles of DEAE cellulose to which chromotrypsin was attached were ground, there was a large increase in the reaction rate at each substrate concentration. Pore diffusional limitation was found with two enzymes glucoamylase (Marsh *et al.*, 1973) and glucose oxidase (Ravito and Kittrell, 1973) immobilized to porous glass.

Improvement of storage and thermal stability of enzymes due to immobilization was observed in many cases. There are many reports that an enzyme on immobilization showed changes in the thermal stability. Wykes *et al.* (1971) showed that the thermal stability of  $\alpha$ -amylase was increased by covalent binding of the enzyme to water-soluble polymer. Lilly (1972) showed that the amount of linking reagent used to immobilize  $\alpha$ -galactosidase to DEAE-cellulose markedly influenced the thermal stability of the immobilized

enzyme. Although the majority indicate an increase in thermal stability, there are sufficient examples of decreased stability to show that immobilization does not necessarily confer increased stability. An increase in storage and heat stability of several enzymes immobilized on several supports are reported.

Many instances were reported where the activity of the enzyme towards a high molecular weight substrate has been reduced on immobilization to a much greater extent than for the low molecular weight substrate. This is believed to result from steric hindrance by the support to access of the large molecules to the active site of the enzyme (Wang *et al.*, 1979). Since substrates such as casein and starch have molecular weights comparable with those of enzymes, they do not penetrate into entrapped enzymes and the measured activity is therefore low.

When enzymes are immobilized, shift in pH optima were observed in some cases. Goldstein *et al.* (1964) found that when trypsin was covalently bound to high polyanionic copolymers of maleic anhydride and ethylene, the pH activity profile for an ester substrate was shifted to a more alkaline pH at low ionic strength. At higher ionic strength the pH activity profile shifted back towards that of free enzyme. They proposed that the electrostatic field produced by the highly negatively - charged support caused an unequal distribution of hydrogen and hydroxyl ions between support microenvironment and external solution. Wykes *et al.* (1971) observed similar effects with many other immobilized enzymes.

Goldstein (1972) has shown that the overall reaction rate constant  $K_{cat}$  of chymotrypsin covalently bound to several polyanionic supports was considerably higher than that for the free enzyme. Conversely, the  $K_{cat}$  values with polycationic supports were lower. In addition, in some cases the shift in pH activity profile observed on enzyme immobilization was independent of ionic strength (Wang *et al.*, 1979). Shifts in pH activity profile to the alkaline region on immobilization that are almost independent of ionic strength have been reported with polytyrosyl trypsin, papain and subtilisin peptidase 'A' bound to diazotized starch derivatives (Goldstein *et al.*, 1970). Goldstein *et al.* (1964) found that the  $K_m$  (app) of immobilized trypsin acting on a positively charged ester substrate was 30 times lower than for the free enzyme at low ionic strength. Again, increasing the ionic strength abolished this effect.

Ample evidence exists, however, to conclude that enzyme immobilization can alter the intrinsic kinetics of enzyme deactivation. Several different mechanisms and explanations for this phenomena have been proposed, postulated and some have been tested experimentally (Bailey and Ollis, 1986).

Immobilized enzymes are found to generally show higher operational stabilities than free enzymes because after binding to a solid carrier optimal enzyme conformation and structure may be maintained. Immobilization may provide some protection for reactive sites on the enzymes against denaturation

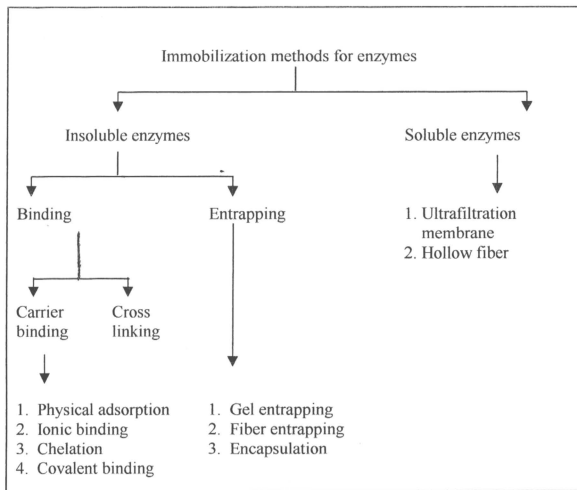
and provide easy access for co-enzymes and substrates (Phillips and Poon, 1988).

For enzymes that act on two or more different substrates, it is possible to compare the relative rates of reaction with these substrates for an enzyme before and after immobilization. For  $\beta$ -galactosidase the relative activity towards O-nitrophenylgalactoside and lactose decreased on immobilization. This was attributed to diffusional limitations since the rate of reaction with O-nitrophenylgalactoside is much greater and will therefore be reduced to a greater extent (Sharp *et al.*, 1969). For the application of immobilized biocatalysts, their screening to the desired activity and characteristic is most important. In addition, selection of the appropriate combination of supporting material and immobilization method, both of which should be suitable for each biocatalyst, is necessary. No systematic concept is available at present for design of the most appropriate method of immobilization for various biocatalysis. Optimization is carried out in general by trial and error (Gerhartz, 1990).

## 2.2 GENERAL METHODS OF IMMOBILIZATION

Immobilized enzymes are chemically or physically attached to a water insoluble matrix, polymerised into water insoluble gel, entrapped within a water insoluble gel matrix or water insoluble microcapsule (Mosbach, 1987).

The classification of Kennedy and Cabral (1987) attempts to combine the nature of interaction responsible for immobilization with the nature of the support as shown in Fig. 2.1.



**Fig. 2.1 Classification of immobilized enzymes**

Gerhartz (1990) classified enzyme immobilization methods as:

A: Carrier binding methods

- |                           |  |
|---------------------------|--|
| (a). covalent binding     | (b) physical adsorptions                               |
| (c). electrostatic forces | (d) biospecific binding (coenzyme, antibody, cofactor) |

B: Cross-linking with bi or multifunctional reagents

- C: Entrapment in gel matrices, microcapsules, liposomes, hollow fibres, or ultrafiltration membranes (a) lattice type (b) microcapsule type
- D: Combined methods
- (a) entrapment and cross-linking

Because each method of immobilization has its own merits and demerits, selection of the technique should be based on the intended purpose including type of biocatalyst, type of reaction and type of reactor. Supporting materials should have adequate functional groups to immobilize enzymes, as well as sufficient mechanical strength, physical, chemical and biological stability and non-toxicity. Easy shaping is also important for applying immobilized enzymes to different types of reactors. Economic feasibility is of great importance (Gerhartz, 1990).

### **2.2.1 IMMOBILIZATION OF ENZYMES BY ADSORPTION**

The adsorption method is the earliest and the simplest method of enzyme immobilization. Adsorption involves bringing an aqueous solution of enzyme into contact with adsorbent surface. Biocatalysts often bind to carriers by physical interaction such as hydrogen bonding, hydrophobic interaction, Van der Waals forces or their combined action. Although biocatalysts are immobilized without any modification, interaction between biocatalyst and support is generally weak and affected by such environmental conditions as temperature or concentration of reactants (Gerhartz, 1990).



Adsorption of enzymes on a water-insoluble carrier is dependent on the variables such as pH, nature of solvent, ionic strength, quantity of enzyme and adsorbent, time and temperature (McLaren, 1954). Adsorption of enzymes onto water insoluble matrices can be attributed to an ion-exchange mechanism, or to simple physical adsorption at the external surface of a material or to physicochemical bond created by hydrophobic interactions, Van der Waals forces, hydrogen bonding etc. A close control of these variables is required for optimal adsorption and retention of activity, owing to the relatively weak binding forces between protein and adsorbent.

Adsorbents for enzymes are either organic or inorganic in nature. The most commonly used adsorbents are alumina, silica gel, bentonite, cellulose, nitrocellulose, polyacrylamide, collagen, clay, activated carbon, CM-sephadex, CM-cellulose, amberlite, nylon and chitin. Currently, several synthetic resin beads and natural materials like chitosan beads with micropores of controlled size having strong adsorption capacities are use. Phenoxyaetylated cellulose and glass beads are being used as specific supports of a hydrophobic nature. Tannins, which interact strongly with proteins, are also being applied as ligands after appropriate immobilization (Gerhartz, 1990). Adsorption followed by cross-linking with glutaraldehyde is found to stabilize the activity of immobilized enzymes.

## 2.2.2 IMMOBILIZATION BY IONIC BINDING

This method has been applied for many enzymes, because the procedure is very simple, the supports are renewable and the enzymes are not modified. The most notable example is the production of L-amino acids by aminoacylase immobilized on DEAE-sephadex (Chibita *et al.*, 1972).

Ionic binding involves formation of salt-like linkages between the enzyme's charged groups and the opposite charges on the carrier which is an ion exchanger. Van der Waals forces also play a part. Immobilization procedure is similar to the adsorption process and shares similar advantages and disadvantages. However ionic binding is stronger than physical adsorption and very little conformational changes occur with the enzyme during immobilization. Several derivatives of cellulose and sephadex, as well as various cation and anion-exchange resins are used as carriers for immobilization of enzymes by this method. DEAE-Cellulose, AE-cellulose, TEAE-cellulose, DEAE-sephadex, Amberlite, CM-cellulose etc are used commonly for immobilization of enzyme by ionic adsorption. Binding of enzymes on supports is affected by the kind of buffers used, pH, ionic strength and temperature. In general the optimal pH for enzyme activity shifts to the acid side when the enzyme is immobilized on a polycationic support and shifts to alkaline side when bound to a polyanionic support. This shift may be minimized by using solutions of high ionic strength.

Penicillin amidase (Carleysmith *et al.*, 1980) and Amyloglucosidase are immobilized on CM cellulose and DEAE-cellulose respectively (Arasaratnam *et al.*, 1994). *Bacillus strearothermophilus* pullulanase was immobilized on DEAE-cellulose (Manolov *et al.*, 1993). Pectinase (Channe and Shewale, 1995) and D-glucose isomerase were immobilized by this method on Amberlite XAD-7 respectively and on INDION 48-R (Pawar and Deshmukh, 1994).

### 2.2.3 BINDING OF ENZYMES USING METALS

Using this method, immobilization of enzymes have been carried out by activation of organic and inorganic carriers with transition metal salts. The enzyme binds with the carriers by the formation of chelate between the enzyme and the activated carriers. The carboxyl, hydroxyl, amino and thiol groups present in the enzyme take part in chelation. The transition metal salts used for activation process include  $\text{TiCl}_3$ ,  $\text{TiCl}_4$ ,  $\text{Ti}(\text{SO}_4)_3$ ,  $\text{FeCl}_2$ ,  $\text{FeCl}_3$ ,  $\text{FeSO}_4$ ,  $\text{ZrCl}_4$ ,  $\text{SnCl}_2$ ,  $\text{SnCl}_4$  and  $\text{VCl}_3$ . The supports which have been used include glass, chitin, celite, alginic acid and cellulose.

Beta-galactosidase (Brena *et al.*, 1994) have been immobilized by this method on alumina and on metal-chelated substituted gels respectively.

## 2.2.4 IMMOBILISATION BY COVALENT BINDING

Covalent binding for the immobilization of enzymes is based on the formation of a covalent bond between the enzyme molecules and carrier materials. The protein functional groups that commonly take part in the covalent binding of enzyme to the support are: (a) amino groups of lysine and N-terminal amino acids (b)  $\beta$  and  $\gamma$ -carboxyl groups of aspartic and glutamic acid and terminal acid group (c) hydroxyl groups of serine, threonine and tyrosine (d) imidazole group of histidine and (e) indole group of tryptophan.

Immobilization of an enzyme by covalent coupling to a support material involves only functional groups of the enzyme, that are not essential for its catalytic reaction and thus the active center of the enzyme is unaffected by the coupling reagent at that experimental condition.

Coupling via amino groups on the protein has been carried out with supports containing acylation groups such as acylazide (Ogumtinerin and Reilly, 1980; Blassberger *et al.*, 1978) or acid anhydride (Zingaro and Uziel, 1970).

Trypsin, papain, pepsin on cellulose (Beddows and Mirauer, 1980), have been immobilized by this method.

Enzymes immobilized by covalent binding have thus the following advantages (a) because of the tight binding, they do not leak or detach from supports during utilization (b) immobilized enzymes are localized on the surface of supports and (c) an increase in heat stability is often observed because of the strong interaction between enzymes molecule and support (Gerhartz, 1990).

The selection conditions for immobilization by covalent binding is more difficult than in the case of physical adsorption and ionic binding. Certain enzymes are extremely sensitive to changes in pH and ionic strength.. The conditions necessary for their successful immobilization can sometimes completely abolish their activity and the covalent method can give derivatives of low catalytic efficiency on high molecular weight substrates, caused mainly by steric repulsion of the macromolecules. Enzymes immobilized by covalent binding thus may have the following disadvantages (a) active structures of enzyme molecules liable to be destroyed by partial modification (b) strong interaction between enzyme molecules and supports often hinders the free movement of enzyme molecules, resulting in decreased enzyme activity ( c) optimal conditions of immobilization are difficult to find and supports in general, are not reusable (Gerhartz, 1990).

## 2.2.5 IMMOBILIZATION BY CROSSLINKING

This method is based on the formation of covalent bonds between the enzyme molecules by means of bi or multifunctional reagents, leading to three dimensional, crosslinked enzyme aggregates which are completely insoluble macromolecules. This method involves the addition of appropriate amount of cross linking agent to an enzyme solution under conditions which gives rise to the formation of multiple covalent bonds. One of the reagent's functional groups forms a covalent linkage with the support, the other functional group or groups may then be used to bind an enzyme (Goldstein, 1974; Inman and Hornby, 1972; Kay and Lily, 1970). The commonly used multifunctional reagents for the immobilization of enzymes are diazobenzidine, diazobenzidine-2,2'-disulphonic acid, diazobenzidine-3,3'-dianisidine, diazobenzidine-3-3'-dicarboxylic acid, 4-4'-diisothiocyanatobiphenyl-2,2'-disulphonic acid, 4'4'-difluoro-3,3'-dinitrophenylsulfone, 1,5-difluoro-2,4-dinitrobenzene, trichloro-s-triazine, toluene-2, 4-diisothiocyanato, glutaraldehyde, N,N1-hexamethylene bisiodoacetamide, hexamethylene diisocyanate. Glutaraldehyde, a bifunctional reagent, is the most popular cross-linking agent.

The functional groups of enzymes participating in the reaction include the  $\alpha$ -amino group at the amino terminus, the  $\alpha$ -amino group of lysine, the phenolic group of tyrosine, the sulphydryl group of cysteine and the imidazole group of histidine. The crosslinking of enzymes depends on factors such as

the concentration of the enzyme and the crosslinking agent, pH and ionic-strength of the solution, temperature and the time of reaction. Penicillin amidase (Carleymith *et al.*, 1980), catechol 1,2-dioxygenase (Neujahr, 1980), glucoamylase (Norouzian and Jaffar, 1993), lipase (Wang and Ruckenstein, 1993), NADH-cytochrome 65 reductase (Yil dirim *et al.*, 1994) have been immobilized by this method.

Immobilized enzymes can also be prepared by adsorption of the protein on surface active water insoluble supports followed by crosslinking with multifunctional reagents. Penicillin acylase on polyacrylonitrile fiber (Ishimura and Suga, 1992) was immobilized by this method.

## **2.2.6 ENTRAPMENT METHOD**

The entrapment method is based on confining enzymes in the lattice of a polymer matrix or enclosing the enzyme in a semipermeable membrane. The entrapment method is classified into gel entrapment, fiber entrapment and microencapsulation. The advantages of entrapment methods are that not only single enzymes but also several enzymes, cellular organelles and cells can be immobilized with essentially same procedures.

### **2.2.6.1 GEL ENTRAPMENT**

Entrapment of enzymes is brought about either by forming a cross-linked polymeric network around the enzyme molecules or by placing the enzyme inside a polymeric substance and then crosslinking the polymer chains. Polyacrylamide is the most commonly used polymer for immobilization of enzymes. The other polymers used are dimethyl acrylamide, polyvinyl alcohol, 2-hydroxyl ethylacrylate, collagen, gelatin, agar, calcium alginate and carrageenan.

Wheat phytase (Khare *et al.*, 1994), gamma-glutamyl transpeptidase (Gotoh *et al.*, 1994), phenol oxidase (Palmieri *et al.*, 1994) have been immobilized by this method.

Less inactivation of enzyme occurs as polymerization can be carried out in frozen state, various shapes of the entrapped enzyme is prepared by varying the type of container, it requires small amount of enzyme and no chemical modification of the enzyme is expected and consequently, the enzymes intrinsic properties are not changed.

### **2.2.6.2 FIBER ENTRAPMENT**

This method involves immobilizing enzymes by entrapment within microcavities of the fiber. Biocatalysts are separated from the environment



by hollow fibers. The polymer most commonly used in this procedure is cellulose acetate. High surface area for enzyme immobilization can be obtained by using very fine fibers and multienzyme immobilization systems can be developed. Fiber entrapment is limited to low molecular weight substrates. Inactivation of enzymes may occur because of the necessity of using water-immiscible liquid as polymer support coagulants.

### **2.2.6.3 MICROENCAPSULATION OF ENZYMES**

This method is based on enclosing enzymes within semipermeable polymer membranes. The preparation of enzyme microcapsules requires extremely well controlled conditions. Microencapsulation of enzymes is classified into four categories namely phase separation, interfacial polymerization, liquid-surfactant membranes and liquid drying methods. In this process biocatalysts are entrapped in microcapsules of semipermeable synthetic polymers.

$\beta$ -D-galactosidase (Waliack and Carbonell, 1975), alcohol dehydrogenase, malate dehydrogenase (Campbell and Chang, 1976), urease (Mogensen and Vieth, 1973), hexokinase and pyruvate kinase (Campbell and Chang, 1975) were immobilized by this method.

## 2.3 CARRIERS FOR ENZYME IMMOBILIZATION

The major components of an immobilized enzyme systems are the enzyme, the support and the mode of interaction of the enzyme with the support. The support may be a membrane, a water-insoluble solid or a polymer matrix. A suitable support will enhance the performance of an immobilized enzyme system, therefore an ideal support should have the following properties (i) Large surface area and high permeability (ii) Sufficient functional groups for enzyme attachment under nondenaturing conditions (iii) Hydrophilic character (iv) Water insolubility (v) Chemical-thermal and biological stability (vi) Mechanical strength (vii) High rigidity and suitable particle form (viii) Resistance to microbial attack (ix) Reusability (x) Toxicological safety and (xi) Low or justifiable price.

Carriers can be classified according to their chemical composition as organic and inorganic supports or carriers and the former can be further classified into natural and synthetic carriers. The inorganic carriers can be classified into mineral and synthetic material carriers. Cellulose, dextran, agarose and starch are the most important polysaccharide carriers used as matrices for immobilization of enzymes. The other polysaccharide includes chitin, chitosan, pectic acid, alginic acid and carrageenan. Carriers in use are classified as shown in Table 2.1.

**Table 2.1 Classification of carriers**

Natural polymers	Minerals	Synthetic polymers	Synthetic materials
a. Polysaccharides	Attapulgate clays	Polystyrene	Non porous glass
Cellulose	Bentonite	Polyacrylates	Controlled pore
Starch	Kieselghur	Polymethacrylates	glass
Dextran	Pumice stone	Polyamides	Controlled pore
Agar & agarose	Vermiculite	Hydroxyalkyl-	metal glass
Alginate	Montmorillinite	methacrylates	Metals
Carrageenan	Sand	Glycidyl	
Chitin & chitosan	Sepiolite	Methacrylates	
		Maleic anhydride	
		polymers	
		Vinyl and allyl	
		polymers	
		Polyacrylamide	
b. Proteins			
Collagen			
Gelatin			
Albumin			
Silk			
c. Carbon material			
Activated carbon			

Trypsin (Drobnik *et al.*, 1982) have been immobilized covalently on cellulose beads.

Starch is the least suitable polysaccharide carrier for enzyme immobilization owing to its ease of degradability by microorganisms. Starch has been used in the form of microparticles (Beddows *et al.*, 1986). Dextran is a linear, water soluble polysaccharide. Sephadex gels have been used as supports of immobilized enzymes (Chibata, 1978). Agar and agarose are

resistant to microbial degradation. Agar is a complex water soluble polysaccharide naturally occurring as a mixture of at least two polysaccharides. The gelling agent among them is the agarose. Trypsin (Blanco and Guisan, 1988) have been immobilized on agarose aldehyde.

Urease (Iyengar and Rao, 1979), Inulinase (Kim and Rhee, 1989), pullulanase (Hisamatsu and Yamada, 1989), gastric proteases (Han and Shahidi, 1995) and glucooligosaccharide oxidase (Lin *et al.*, 1996) have been immobilized on chitin. Glutaminase (Koseko *et al.*, 1994) has been immobilized on chitin and chitosan. Urease was immobilized on chitosan membrane (Krajewska, 1991).

Cholinesterases (Kuznetsova and Nikol Skaya, 1995),  $\beta$ -galactosidase (Sungur and Akbulut, 1994) and polyphenol oxidases (Nighojkar *et al.*, 1995) have been immobilized on gelatin .

Silk is a natural protein and its physical and chemical properties make it useful as a support glucose oxidase (Liu *et al.*, 1995) and peroxidase (Liu *et al.*, 1995) have been immobilized on silk fibroin membranes.

Carbon materials are used as supports for immobilization of enzymes because of their low cost, mechanical strength and are obtainable in several forms. Among carbon materials, activated carbon has been widely used because of its high porosity. Chymotrypsin was immobilized on carbon SKN<sup>-2P</sup>

(Sevast' yanova and Davidenko, 1993). Urease was immobilized on activated carbon (Kibarer, 1994).

Synthetic carriers used in immobilization of enzymes are polyvinyls, polyacrylates, polystyrene and copolymers based on maleic anhydride and ethylene or styrene polyamide, polyaldehyde and polypeptide. Synthetic polyamides produced as condensation polymers of diamines and dicarboxylic acids (or their diacid chlorides) are known as nylons. Different types of nylons differ only in the number of methylene groups in the repeating structure. They are available in a number of forms including powders, fibers, hollow fiber tubes and membranes and they offer a number of advantages as supports for enzyme immobilization including mechanical strength, resistance to microbial attack and hydrophobicity. The inertness of the polymer backbone requires its activation in order to increase its binding capacity. This can be achieved by partial hydrolysis of polyamide followed by activation of the resultant amino or carboxyl groups introduction of reactive centres by o-alkylation.

Xanthine oxidase have been immobilized on polyaniline silicone support (Nadruz *et al.*, 1996), protease on ethylene acrylic acid copolymer (Emi and Murase, 1990), penicillin acylase on polyacrylonitrile fiber (Shimura and Suga, 1992), glucose oxidase on syndiotactic polyvinyl alcohol (Iwamoto *et al.*, 1995).

Clay, bentonite, diatomaceous earth, pumice stone, sand, alumina, aluminosilicate, controlled pore glass, hydrous titanium oxide, magnetic iron oxide, nickel, stainless steel, zirconium hydroxide, titanium, vermiculite and montmorillonite are some of the inorganic materials on which enzymes have been immobilized by adsorption and covalent binding.

Inorganic carriers have many advantages over organic polymers (Kennedy and White, 1986), (i) high mechanical strength, (ii) thermal stability, (iii) resistance to organic solvents (iv) resistance to microbial attack, (v) ease of handling, (vi) long shelf life and (vii) easy regenerability.

Magnetic iron oxide, nickel, stainless steel, sand, clay are few examples of nonporous inorganic carriers. Cellulase (Shimizu and Ishihara, 1987) have been immobilized on alumina. Acetylcholinesterase (Baum *et al.*, 1972) and Trypsin (Konecny and Sieber, 1980) were immobilized on glass beads. Trypsin was immobilized on sand (Puvanakrishnan and Bose, 1980). Trypsin,  $\alpha$ -chymotrypsin,  $\alpha$ -amylase and glutamate dehydrogenase (Nemat Gorgani and Karimian, 1986), were immobilized on silica, alkaline phosphatase on glass (Pande *et al.*, 1996). Enzyme immobilized on inorganic substrate include lipase on silica glass (Kawakami and Yoshida, 1994), trypsin, chymotrypsin, papain and pepsin on porous zirconia (Huckel *et al.*, 1996), lignin peroxidase on controlled-pore glass beads (Presnell *et al.*, 1994).

## 2.4 PROPERTIES OF UREASE

Urease is the first enzyme in the world crystallized by (Sumner, 1926). He had shown that the enzyme was a pure protein devoid of any organic cofactor and that it contained neither iron nor manganese. Some 50 years after its crystallization, it was shown to be the first example of the nickel (II) metalloenzyme (Dixon *et al.*, 1975). Urease is an absolutely specific enzyme and the enzyme catalyzes the hydrolysis of the substrate urea to carbamate and ammonia as the initial products (Gregory, 1987).

The major physicochemical properties of the enzyme are summarized in Table 2.2 (Zerner, 1991). It was observed that the subunit weight of the polypeptide of 840-aminoacid residues is 90,777 (Mamiya *et al.*, 1985). This corresponds to a molecular weight of 545, 365 for the hexameric molecule which contains 12g-atoms of nickel.

**Table 2.2:** Some physicochemical properties of urease (Zerner, 1991)

Property	Value
Molecular weight	545,365 (sequence data + Ni <sup>2+</sup> content)
Molecular weight	590,000 ± 30,000 (equilibrium ultra centrifugation)
Equivalent weight	96,600 ± 1000
Polypeptide mol weight	95,000 ± 5,000 (equilibrium ultra centrifugation)
Subunit mol. weight <sup>a</sup>	~93,000 (PAG-SDS electrophoresis)
Subunit mol. weight <sup>b</sup>	~96,000 (PAG-SDS electrophoresis)
Subunit mol. weight <sup>c</sup>	0.734 cm <sup>3</sup> g <sup>-1</sup> (gravimetric)
Partial specific volume	0.738 cm <sup>3</sup> g <sup>-1</sup> (amino acid analysis)
Diffusion coefficient (D <sub>20,w</sub> )	3.27 ± 0.32 × 10 <sup>-7</sup> cm <sup>2</sup> s <sup>-1</sup>
Sedimentation coefficient (S <sub>20,w</sub> )	19.35 S
ε <sub>280</sub> (gross)	62,000 m <sup>-1</sup> cm <sup>-1</sup> at 280 nm <sup>d</sup>
A 1%, cm (net)	6.20 at 280nm
Specific activity	93 (mkat liter <sup>-1</sup> )/A <sub>280</sub>
Nickel content	2.00 ± 0.12 ions per subunit

<sup>a</sup>In 0.05M Tris H<sup>+</sup>Cl<sup>-</sup> buffer, pH 7.4 (0.15M in KCl, 1 mM in EDTA, 6.0M in guanidinium chloride, 0.1M in 2-mercapto ethanol)

<sup>b</sup>Sample dialyzed into the buffer in footnote (a) for 20 h at 280°C

<sup>c</sup>Sample preequilibrated at 100°C for 2 min in 0.05 M Tris H<sup>+</sup>Cl<sup>-</sup> buffer, pH 8.0 [1% (w/v) in SDS, 1% (v/v) in 2-mercapto ethanol]

<sup>d</sup>Based on the 96.6 kDa subunit

There are several sources of urease. Based on source they are known as jack bean ureases, soybean ureases, soil ureases and bacterial ureases. Jackbean ureases of type III, VI, IX, VII are available. Type III of the jackbean urease is the commonly used urease. Type III has a specific activity of 33.6 units per mg protein, optimum pH range between 6.9 and 7.2, optimum temperature range between 62 and 65°C and kinetic constant  $k_m = 5, 01 \text{ mmol/dm}^3$  (Krajewska *et al.*, 1990; Pozniak *et al.*, 1995). The soybean urease is reported to have an optimum pH of 7.0 and optimum temperature of 40°C (Krysteva *et al.*, 1991).



Ureases obtained from plant leaves are reported to have an optimum pH of 7.5 and optimum temperature of 70°C. Some of the values of Michaelis constant,  $K_m$  and maximum initial velocity  $V_{max}$  of urease in various plant materials are given (Frankenberger and Tabatabai, 1982).

**Table 2.3:**  $K_m$  and  $V_{max}$  values of ureases obtained from different sources

ppwplant Source	Urease	
	$K_m$ , mM	$V_{max}$
Alfalfa	0.69	52
Corn	0.77	70
Oat	0.74	68
Reed canary grass	0.72	123
Rye grass	1.63	33
Sudan grass	0.38	39

Soil ureases are found in soils intracellularly (like enzymes present in living cells, dead cells or cell debris) and extracellularly (like free enzymes, enzymes bound to inorganic and organic colloids). Ureases in soils are reported to have an optimum pH of 9.0 (Lester soil) and 9.5 (Tama soil) and optimum temperature of 70°C. The  $K_m$  values of soil urease is between 1.77 and 2.90mM (Lai and Tabatabai, 1992).

Bacterial ureases such as urease of *Proteus rettgeri* was reported to have an optimal pH of 7.5 and optimal temperature of 55°C. The  $K_m$  value was found to be  $7.1 \times 10^{-2}M$  (Plaza *et al.*, 1971). Urease extracted from the mixed rumen bacterial fraction of bovine rumen contents have been reported

to have an optimum pH of 8.0.  $K_m$  and  $V_{max}$  of  $8.3 \times 10^{-4}M$  and  $3.2 \pm 0.25$  mmol of urea/h/mg respectively (Mahadevan *et al.*, 1977)

## 2.5 STUDIES ON IMMOBILIZATION OF UREASE

Immobilization of urease with various supports, the different coupling techniques and its effects on the immobilized enzymes compared to the free enzyme were studied and discussed.

Jackbean urease was immobilized onto collagen-poly (Glycidyl methacrylate) graft copolymer by Ragunath *et al.*, (1984). Results showed that the maximum binding occurred at pH 8.5 where 16.45 mg enzyme was bound to 100mg matrix. The immobilized urease showed an activity of about 80% of the free enzyme. Free urease showed a temperature optimum at 60°C whereas immobilized urease had an optimum temperature of 70° C.

In another interesting study by Karube *et al.*, (1976), jackbean urease was immobilized onto spiropyran collagen membrane in the dark and under visible light. Results showed that the activity of the urease - spiropyran collagen membrane in the dark being 2 times greater than that under visible light at pH 6.0. The activity in the dark was about 30% of that of free urease. The optimum pH of the urease-spiropyran collagen membrane under visible light was 6.8 which was the same as that of free urease. The pH versus activity curve shifted in the acidic direction in the dark. Similar results reported by

Karuber and Suzuki (1972), showed that the shape of the pH - activity curve of the urease collagen membrane was similar to the free urease with the optimum pH of immobilized urease being between 5 to 7. Their findings also showed that the relative activity of the urease collagen membrane was 51% of that of the free urease and there was no decrease in the activity of the entrapped urease after 10 days of storage at 20°C.

Similarly, other studies have also been reported to have optimum pH similar to that of the free urease. Jackbean urease which was immobilized on kaolinite and montmorillonite and the mineral constituents of two lower surface soils after silanization of the support surfaces by 3-aminopropyltriethoxysilane showed that the optimal pH value of immobilized urease was similar to that of free urease (7.0) except for montmorillonite (8.5). It was also reported that the  $K_m$  values of immobilized urease (25.1 - 60.8 mM) were in the same order of magnitude as that of free urease (29.4mM). The optimum temperature of immobilized urease (60°C) was also the same as that for free urease (Lai and Tabatabai,1992).

Other findings on immobilization of urease on soil dominants have also been reported. The work by Tarafdar and Chhonkar 1982, involved the adsorption of soybean urease on kaolinite, bentonite and vermiculite. Their work showed that maximum adsorption of urease occurred at pH 6.5 for kaolinite and bentonite and at pH 7.5 for vermiculite. A continuation of this study by Tarafdar (1987), led to more findings such as an increase in

temperature from 25°C to 45°C resulted in more sorption of the enzymes. It was also reported that the kaolinite dominant soils adsorbed least amounts of enzymes as compared to illite and montmorillonite dominant soils. These inorganic carriers have been reported to have many advantages over organic polymers. In one study, urease was covalently coupled to glass with an amino-functional silane coupling agent by Weetall and Hersh (1969), and was employed continuously in a column over long periods without detectable losses in enzymatic activity.

In a different study by Bollmeier and Middleman (1974), urease was immobilized within a glutaraldehyde crosslinked gelatin film. Their findings showed that the immobilized urease was subjected to deactivation at 22°C. Similarly, further studies on immobilization of urease within a crosslinked gelatin film was conducted by Bollmeier and Middleman (1979), which further indicated that the immobilization procedure did led to some loss of activity when urease were immobilized within a crosslinked gelatin film. In contrast to these findings, a study by Sungur and Elcin (1992), using Carboxymethylcellulose (CMC)-gelatin as carrier for urease immobilization revealed that the activities of the immobilized enzymes were found to be stable for at least 2 months with 16-24 usages. Effect of pH on relative activities of free enzyme and immobilized enzyme were investigated. The optimum pH value found for free enzyme was 8.0 and in the case of immobilized enzyme, the optimum value shifted to pH 7.0. Other important findings from this study were that the relative activities of immobilized enzyme decreased by

increasing urease concentration. When urease prepared from *Cajanus indicus*, has been immobilized with glutaraldehyde on treated chitin, the immobilized urease activity remained constant beyond the enzyme : carrier ratio of 8:4 (2:1). Final activity retention was reported to be about 20-30% of the added enzyme units and corresponded to the binding of 1.0-1.5mg protein/100mg chitin (Iyengar and Rao, 1979). The optimum pH of native and immobilized urease were 7.2 and 6.3, respectively. The native urease had an optimum temperature around 45°C whereas the urease bound to chitin had an optimum temperature of 70°C. In another study, Jackbean Urease (Type III) was covalently immobilized on glutaraldehyde pretreated chitosan membranes by Krajewska *et al.*, (1990). Deacetylation of chitin in concentrated sodium hydroxide gives chitosan which was used in this study. The immobilized enzyme retained 94% of its original activity and the protein content of the membrane were 0.0507 mg cm<sup>-2</sup>. The chitosan - urease ( $K_m = 26.4$  mmol/dm<sup>3</sup>) exhibited  $K_m$  values about 5 times higher than that of the free urease ( $K_m = 5.01$  mmol/dm<sup>3</sup>), while the maximum reaction rate,  $V_{max}$  was slightly higher for the immobilized enzyme (0.337 mmoles UREA/s g Protein) compared to the free enzyme (0.226 mmoles UREA/s g Protein).

Similarly high Michaelis constant ( $K_m$ ) values for immobilized systems have been reported. Studies by Medeira (1977), with urease entrapped in egg lecithin liposomes, led to a change of the apparent Michaelis constant from about 68mM (free enzyme) to about 167mM (entrapped enzyme). However, the  $V_{max}$  value did not change appreciably. Similar result were reported by

May and Li, (1972) for jackbean urease immobilized onto hydrocarbon-based liquid surfactant membranes. The apparent  $K_m$  value determined for immobilized jackbean urease (0.18M) was approximately 50 times as large as the value obtained for free urease ( $3.4 \times 10^{-3}$ ).

In a different study, spherical microcapsules nylon enclosing solutions of Jackbean urease V which was encapsulated both in the presence and absence of a foreign protein haemoglobin, followed Michaelis-Menten kinetics with optimum activity occurring at lower pH values (5.0 and 5.8) than for the native enzyme (6.5 and 7.0). The  $K_m$  values have been reported to have increased upon encapsulation with the value of 3.2 mM for encapsulated enzymes and 2.72 mM for the native enzyme (Sundaram, 1973). Further studies were reported by Krajewska *et al.*, (1989), using Jackbean urease immobilized on nylon membrane which led to a change of the Michaelis constant from 5.00 mM for the free enzyme to 7.67mM for the immobilized enzymes. The immobilized and free urease had optimum pH between 6.9 and 7.0. However, the immobilization did not protect the enzyme against heat inactivation at 70°C in this study.

In contrast to those findings, when urease had been immobilized on o-alkylated nylon tubes, the apparent  $K_m$  decreased and  $V_{max}$  increased compared to the free enzyme (Onyezili and Onitiri, 1981). Studies have also been reported to have the free and bound urease behaving similarly with respect to the kinetic parameters ( $V_{max} = 10 \times 10^{-5} \text{ mmol NH}_3\text{s}^{-1}$  and  $K_m = 6.6$

$\times 10^{-3}\text{M}$ ), pH optimum (7.0), when jackbean urease (IV) was adsorbed on hexadecyltrimethyl-ammonium (HDTMA)-smectite by hydrophobic bonding (Body and Mortland, 1985). However, thermal stability and resistance to proteolysis were greatly decreased for this study.

Polymethylglutamate (PMG), a synthetic polypeptide, was used as a new carrier to immobilized urease by Minamoto and Yugari, (1980). The enzyme was immobilized on PMG in various forms, such as film, fiber, beads and silicon tube. The retained activities of the immobilized enzyme were excellent (more than 95%). Apparent optimal pH and  $K_m$  values of the bound enzymes (7.2 and 16mM) were almost the same as those of native enzyme (7.1 and 11mM). Heat stabilities of the resulting immobilized enzyme on the PMG-coated glass beads packed in a column, was found to retain its activity more than 80.9% of the initial value, even after the occasional use for a year. The synthetic polymers used for the immobilization of urease by Miyama *et al.*, (1982), showed that immobilized enzyme retained 50% of the original activity after 120 days at 4°C. Immobilized urease was reported to have higher heat stability than the native urease .

Jackbean Urease (Type III) was immobilized with bovine serum albumin and glutaraldehyde on polyurethane foam support of 7 to 15  $\mu\text{m}$  thickness by Huang and Chen, (1992). Their findings showed that the residual apparent activity of urease after immobilization was about 50%. The optimum values of  $V_{\text{max}}$  and  $K_m$  for free urease were obtained to be 47.2  $\mu\text{mol}$

$\text{NH}_3/\text{min.mg}$  urease and 19.2 mM, respectively, whereas the optimum apparent values of  $V_{\text{max}}$  and  $K_m$  for the immobilized urease were 24.9  $\mu\text{mol}$   $\text{NH}_3/\text{min.mg}$  urease and 19.8 mM, respectively. It was also found that the optimum pH value of urease was shifted from 7.2 to 7.0 after immobilization. The immobilized enzyme had excellent storage stability and good hydrodynamic property. Similar retained activity was reported by Krajewska *et al.*, (1990). Their studies involved the immobilization of jackbean urease (IX) on aminated butyl acrylate-ethylenedimethacrylate co-polymer. The immobilized urease retained 56% of the activity of the enzyme.

Polyaldehyde gels that were used to immobilize urease by Epton *et al.*, (1974), were reported to retain 29.6% and 19.9%, respectively, of the free solution activity. On storage for 6 months in buffer suspension, the immobilized urease retained 48% and 14% of its original activity, respectively. Urease entrapped in egg lecithin (Medeira, 1977) lost 50% of the original activity after twenty days at room temperature and the activity of the free enzyme was completely lost in eleven days. The entrapment procedure was reported to have protected the enzyme against heat inactivation.

Soybean urease was bound to synthetic membranes containing 10% acrylamide units and activated with formaldehyde at pH 7.5 and 45°C (Krysteva *et al.*, 1991). The membranes stored at 4°C in a moist state showed no change in their activity for 6 months. Results indicated that the pH optimum for the immobilized urease was pH 8.5 compared to pH 7.0 by the



free enzyme. The optimum temperature of the immobilized enzyme was 45°C and 40 C for the free enzyme. About 0.125 mg of urease was bound per cm<sup>2</sup> of the membrane. It was found that binding enzymes to synthetic membranes containing acrylamide group, through the of N-hydroxymethyl groups, possessed several advantages with respect to the activation of the membrane in a one-step reaction with cheap accessible reagent, high operative stability of the immobilized enzymes and long shelf life of the membrane. Studies by Jiugao *et al.*, (1994), showed that urease s immobilized on periodase oxidized starch retained their activities when stored at room temperature. Results also showed that the pH value was 6.0 for immobilized urease. The optimum temperature reported was 15°C and the immobilization was most effective when the reaction time was 24 hours . The Km value of the immobilized enzyme was 0.029 mol/L, while Km value of the native enzyme was 0.025 mol/L.

Jackbean Urease (Type III) has been immobilized on modified polysulphone membranes (Pozniak *et al.*, 1995). The properties of the immobilized urease were investigated and compared to those of the free enzyme. The value of Michaelis constant Km of the immobilized urease (22.11 mM) was about 4.4 times higher than free enzyme. The maximum activity of free urease was in the pH range of 6.9 - 7.2, whereas the pH range for maximum activity of the immobilized urease was 5.0 - 7.0. This range was wider than that of the free urease, indicating that immobilization increased the pH stability of the enzyme. The free urease had an optimum temperature of

about 62°C, whereas the optimum temperature of the immobilized urease was shifted to 70°C. The stabilities during storage of urease at 25°C and 4°C were also investigated. The free urease stored at 25°C lost its activity within 12 days while the immobilized enzyme retained about 40% of its initial activity after 25 days. Immobilized urease stored at 4°C retained 75% of its initial activity even after 30 days. After 17 reuses the immobilized urease retained 60% of its initial activity. The membrane lost about 30% of its initial activity within 5 hours of continuous process. The enzyme activity then decreased gradually with time; after 140 hours about 50% of the activity still remained.

## **2.6 APPLICATION OF IMMOBILIZED UREASE**

Immobilized enzymes are widely used in chemical processes, analytical and clinical applications because of their inherent advantages associated with their uses.

The determination of urea in blood or urine is an important diagnostic test routinely carried out in clinical medicine. Bailey (1978), suggested an electrochemical monitoring systems for urea. These electrode systems consist of immobilized urease, that is urease immobilized in a gel layer and fixed on the surface of an ammonia electrode. Following this, in 1982, Okada *et al.*, developed ammonia sensor applied to an urea sensor for the determination of ammonium ions in human urine. The urea sensor consisted of an urease-collagen membrane and the sensor is inserted into a sample solution

containing urea. Urea is hydrolyzed to the ammonium ion and carbon dioxide by the immobilized urease in the collagen membrane and the ammonium ion is determined by the sensor.

Auto Analyser<sup>®</sup> systems, provides a simple means of routine continuous flow analysis. By linking together a number of such immobilized enzyme tubes, containing different enzymes, multiple analyses are performed on a single sample. A simpler system, suitable for the analysis of smaller numbers of samples has been devised by Sundaram (1979). An immobilized urease pipette, in which the urease was immobilized on the inner surface of a nylon tube attached to the disposable tip of an adjustable volume pipette, is used for analysis. For example, urea in sera by holding the sample in the pipette (known as an Impette) for a fixed time prior to expulsion and determination of the products of the reaction.

Krajewska and Zaborska (1989) used a membrane-immobilized urease for removal of urea from blood in artificial kidney, for blood detoxification or in the dialysate regeneration system of artificial kidneys.

The concentration of urea in the serum is an important parameter for the diagnosis of renal disease in which urease, in tandem with electrochemical transduction is extensively used as the selective biosensory recognitive element of the determination of urea. Other transducers currently employed for

quantitating the urease/urea interaction include thermistors, ISFETs, flurometers and spectrophotometers (Kallury *et al.*, 1993).