CHAPTER 2

LITERATURE REVIEW

2.1 Immobilization

2.1.1 Methods of Immobilization

Immobilized enzymes are defined as "enzyme physically confined or localized in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously". This definition is applicable to enzymes, cellular organelles, microbial cells, plant cells, and animal cells, that is, to all types of biocatalyst. In some cases, these biocatalysts are bound to or within insoluble supporting materials (carriers) by chemical or physical binding. In other cases, biocatalysts are freely confined to limited domains or spaces of supporting materials (entrapment).

Since the late 1960s a variety of technique have been developed for immobilization biocatalysts. Several schemes have been suggested for classifying immobilization techniques [1,11-15]. The classification that is used here is based on that of Goldstein L. and Manecke G. [14]. The techniques available for the immobilization of enzymes and other biologically active proteins can be grouped in four main classes.

2.1.1.1 Adsorption on Inert Supports or Ion Exchange Resins

Adsorption of an enzyme can be achieved by simply bringing an enzyme solution in contact with the support surface by physical, ionic and biospecific adsorption. Examples of adsorption technique are given in the work of Johnson, J. C. [16].

Physical Adsorption. Biocatalysts often bind to carrier by a physical interaction such as hydrogen bonding, hydrophobic interaction, van der Waals forces, or their combined action. Although biocatalysts are immobilized without any modification, interactions between the biocatalyst and the support are generally weak and affected by such environmental conditions as temperature, pH, or concentration of reactants. The inorganic supports are often used such as alumina, activated carbon, clay, diatomaceous earth, glass, and hydroxyapatite. Currently, serveral synthetic resin beads and natural materials (e.g. chitosan beads with micropores of controlled size) having strong adsorption capacities are available.

Ionic Binding. The binding force are ion-ion interactions and are stronger than in simple physical adsorption. Binding enzyme on a support is affected by the kind of buffers used, pH, ionic strength, and temperature. Several derivatives of cellulose and sephadex, as well as various ion-exchange resins, can be utilized for immobilization. This method has been applied for the immobilization of many enzymes because the procedure is very simple, the supports are renewable, and the enzymes are not modified.

Biospecific Adsorption. Biospecific interactions between enzymes and other molecular species (e.g. lectins or antibodies are also used for

binding. Lectins such as concanavalin A, which is capable of agglutinating red blood cells, is known to bind specifically to mono- and oligo-saccharide substituents present on cell surfaces; moroever, it specifically precipitates polysaccharides and glycoproteins from solution [17].

2.1.1.2 Entrapment within Crosslink Gels or by Encapsulation within Microcapsules, Hollow Fibers, Liposomes, and Fibers

In principles, all entrapment methods are based on the occlusion of an enzyme within a constrain in structure tight enough to prevent the protein from diffusing into the surrounding medium, while still allowing penetration of substrate. Free enzymes are entrapped within the interstitial spaces of a crosslinked, water-insoluble polymeric gel. Gel forming materials such as polysaccharides, protein, or synthetic polymers can be employed. Among the natural polysaccharides, alginate, agar, and κ-carrageenan were widely used in preparing immobilized enzymes. Entrapped biocatalysts are classified according to the following different types:

Lattice type: biocatalysts entrapped in gel matrices prepared from polysaccharides, proteins, or synthetic polymers.

Microcapsule type: biocatalysts entrapped in microcapsules of semipermeable synthetic polymers.

Liposome type: biocatalysts entrapped within liquid membranes prepared from phospholipids.

Hollow-fiber type: biocatalysts separated from the environment by hollow fibers.

Membrane type: biocatalysts separated from the spent reaction solution by ultrafiltration membranes.

The advantages of entrapping methods are that not only single enzymes but also several different enzymes, cellular organelles, and cells can be immobilized with essentially the same procedures. Biocatalyst are not subjected to serious modification and immobilization that eliminates the effect of proteases and enzyme inhibitors of high molecular mass. However, disadvantages are substrates of high molecular mass can hardly gain access to the entrapped biocatalysts and supports are not renewable. Entrapment within ultrafiltration membranes can avoid the disadvantages inherent in entrapping methods, although inactivated enzyme molecules often precipitate on the membrane suface, which results in decreased permeability to reaction solutions. The lattice type method is most widely applied for preparing immobilized biocatalyst. Several examples of this technique are mentioned in the following paragraphs.

Polyacrylamide gel has been the most widely used entrapment technique since Bernfeld, P. and Wan, J. [18] reported the entrapment of several enzymes such as trypsin, papain, amylase and ribonuclease in polyacrylamide gel. The different types of biocatalyst including cellular organelles, and microbial cells have been immobilized by this method. In a typical procedure acrylamide (AM) and N,N′-methylenebisacrylamide (MBA) are mixed with biocatalysts and polymerized in the presence of an initiator such as potassium persulfate, ammonium persulfate and an accelerator (3-dimethylaminopropionitrile (DMAPN), or N,N,N′,N′-tetraethylmethylenediamine (TEMED). The reaction is shown in Figure 2.1.

Acrylamide monomer sometimes inactivates enzymes, however, analogues or derivatives of acrylamide can also be used by this method.

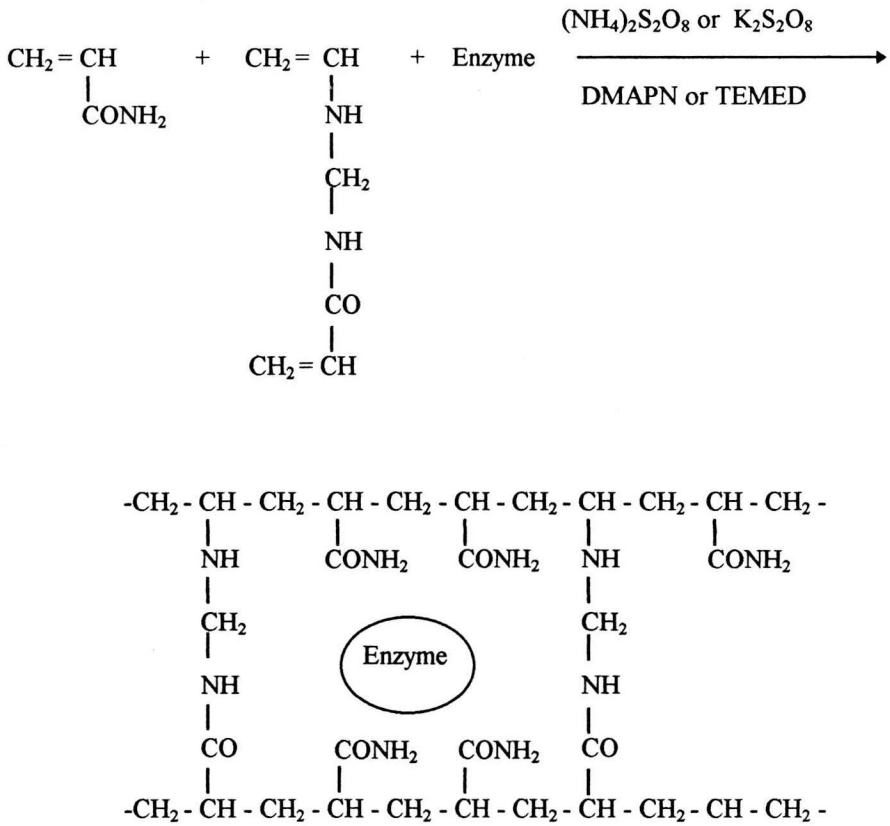

Figure 2.1 The polymerization reaction of polyacrylamide

Several natural polysaccharides such as alginate, agar and κ-carrageneen are excellent gel materials and used widely for the entrapment of various biocatalysts. Sodium alginate, which is soluble in water, is mixed with a solution or suspension of the biocatyst and then dropped into a calcium chloride solution to form water insoluble calcium alginate gels that immobilize enzymes, cellular organells, or microbial cells [19]. However, gels are gradually solubilized in the presence of calcium ion trapping reagents such as phosphate. Alumium ions or several divalent

metal ions can be substitued for calcium ions. This method is used widely for immobilization of various biocatalysts because of its simplicity and the availability of sodium alginate.

In encapsulation, enzymes were immobilized by enclosing them in membrances that are permeable to the substrate and the product. The resulting microcapsules generally have a diameter of 1-100 μm. Chang, T. M. S., who pioneered this approach, described the procedures in detail [20-21]. An emulsion is usually prepared from an organic phase and an aqueous enzyme containing phase in the presence of surfactant. The membrane-formed in the polymer, dissolved in an organic solvent, is then added. On standing, polymer precipitates on the microdroplet interface to form stable microcapsules [22]. Unmodified coenzymes or multienzyme systems can also be immobilized by microencapasulation with lipid-polyamide membranes. Microencapsulation gives the higher enzyme concentrations per unit volume of immobilized preparation. However, in all semipermeable membrane systems, the substrate diffusion rate limits the applications of microencapsulated biocatalysts. By means of a similar approach, enzymes have also encapsulated within liposomes [23]. Examples for entrapment biocatalyst are presented below :

Harrion, L. A., and Fint, K. P. [5] used immobilized neutral phosphatase enzyme by entrapment into polyacrylamide gel for the detection of oxyanions in river water. The activity of phosphatase was checked by colorimetric substrate of ρ-nitrophenyl phosphate (ρ-NPP). The oxyanions was checked by determining the vanadium occurrence as vanadate in water, although the oxidation state is dependent on ionic concentration and on pH. It is acutely toxic to fish,

lichens, and plants. The mechanism of its toxicity is believed to be an inhibition of phosphatase or ATPase function.

Park, T. G. and Hoffman, A. S. [24] entrapped *Arthrobacter simplex* cells, which covert the steriod hydrocortisone to prenisolone in thermally reversible hydrogels based on poly(N-isopropyl acrylamide) and poly(N-isopropylacrylamide-co-acrylamide) by inverse suspension polymerization. The sizes of the beads obtained as powder forms by lyophilizing were 150 to 300 μm. They also suggested that this method can be applied for immobilization of cells and enzymes.

Chibata, I., Tosa, T. and Sato, T. [25] succeeded in producing L-aspartic acid from ammonium fumarate by polyacrylamide gel entrapped *Escherichia coli* cells containing a high activity of aspartase. An active and stable preparation is obtained (half-life, 120 days) when incubated with a substrate solution for 48 hours at 30°C. This process is the first example of an industrial application of immobilized cells and the enzyme, partially purified from *E.coli* cells, is not stable even after immobilization.

Hulst, A. C., et. al [26] presented the production of immobilization of yeast (*Saccharomyces cerevisieae*) and plant cells (*Haplopappus gracilis*) in calcium alginate in large quantities. The production capacity of the nozzles used (0.5, 0.8, and 1.1 mm exit diameters) is two orders of magnitude larger than the production capacity of the conventional techniques (maximum capacity with a 1.1 mm nozzle diameter is 24 L/h). The beads size depending on frequency, nozzle diameter, and volumetric flow rate varies between 1-2 mm with a standard derivation of 3-5% for

yeast immobilization and 10-15% for plant cells. The deactivation of both yeast and plant cells is small and comparable to that found in the corresponding conventional procedures.

Moreno, J. M. and Fagain, C. O. [27] immobilized alanine aminotransferase (ALT) by entrapment in calcium alginate beads from which a synthesis was carried out. ALT has been used in biosensors for lactic acid determination and also used as a clomoca, a diagnostic marker. The thermostabilities and apparent kinetic constants (K_m) of immobilized ALT were higher than the native one, while their V_{max} values were lower. The pH optimum (7.5) was identical for both the immobilized enzyme and the native enzyme.

Yamamoto, K., Tosa, T. and Chibata, I. [28] produced L-Alanine from L-aspartic acid by κ-carrageenan entrapped cells of *Pseudomonas dacunhae* having a high activity of L-aspartate-4-decarboxylase. This process was commercialized in 1982 by Tanabe Seiyaku Co. in Japan.

Mosbach, K. and Nillsson, K. [8] immobilized various biocatalysts such as aminal cells, plant cells, bacteria, algae or fungi with a retained ability of growth by encapsulation in polymer beads. Encapsulation is carried out by adding the biomaterial to an aqueous solution of a polymer such as agar, agarose, carrageenan, chitosan, gelatin, collagen or fibrinogen and acrylamide, dispering to solution in a water-insoluble dispersion medium such as soybean oil, tri-n-butylphosphate liquid silicone, paraffin oil, or phthalic acid dibutylester.

2.1.1.3 Crosslinking by Bi- or Multi-functional Reagents

The enzyme can be immobilized by crosslinking it to other enzyme molecules of an inert protein, such as albumin (co-crosslinking), and precipitating the resulting aggregate. This method can also be used in combination with a carrier, such as a membrane, where the physically adsorbed enzymes are crosslinked on the membrane surface, Bi- or multi-functional reagents, such as glutaraldehyde, toluene diazobenzidine derivatives can be used as crosslinking agents. The primary disadvantages of this method are the relatively severe reaction conditions and difficulties in controlling the reaction. Hence, activities of such enzymes are generally low.

2.1.1.4 Covalent Binding to Polymeric Supports

Accumulated experience in the field has stressed the importance of two main factors that have to be considered when choosing a method for the covalent immobilization of an enzyme: (a) the type of functional groups on the protein through which the covalent bonds with the support material are formed and hence the chemical reaction to be empolyed; (b) the physical and chemical characteristics of the support material onto which chemically reactive groups are to be grafted.

The type of functional groups on the protein through which the covalent bond with the support is to be formed should naturally be nonessential for the catalytic activity of the enzyme; morever, binding reactions that can be carried out under relatively mild conditions and in an essentially aqueous medium should be preferred. Such reactions should exhibit, under ideal conditions, relatively high

specificity toward one type of functional group on the protein and minimal side reactions with other functional groups or with the aqueous medium; in practice such a situation is seldom if ever realized. In selecting the appropriate coupling reaction and functionalized polymer to be employed for the immobilization of a given enzyme, all the avaliable information on the amino acid composition, the amino acids involved in the active site, the effects of specific chemical modifications on activity, the protection of the active site region by specific chemical agents or inhibitors, as well as the three-dimensional structure of the enzyme, when known, should be considered. The protein functional groups that can be utilized in principle for the covalent binding of enzymes to polymeric supports include: (1) amino groups, the ε-amino groups of lysine and the α-NH_2 groups of the N-terminal of the polypeptide chains; (2) carboxyl groups, the β- and γ-carboxyls; (3) phenol rings of tyrosine; (4) sulfohydryl groups of cysteine; (5) hydroxyl groups of serine, threonine, and tyrosine; (6) imidazole groups of histidine; (7) indole groups of tryptophan. In practice, most of the common covalent coupling reactions involve amino groups, carboxyls, or the aromatic rings of tyrosine and histidine.

Numerous natural and synthetic macromolecules have been explored as potential supports for enzyme immobilization; this has led to the development of a wide variety of techniques, (1) grafting of specific functional groups onto performant polymers, (2) synthesis of tailor-made polymers and copolymers devised to fulfill particular needs, and (3) the use of "parent carrier polymers," which by consecutive chemical manipulations can be transformed into the chemical species best suited for a given task. Several of the common materials support e.g.,

crosslinked polyacrylamide, crosslinked dextrans, or crosslinked copolymers of maleic acrylic, and methacrylic acids have been widely explored as supports. Enzymes immobilized by covalent binding have the following advantages: (1) because of the tight binding, they do not leak or detach from supports during utilizaton; (2) immobilized enzymes can easily come into contact with substrates because the enzymes are localized on the surface of supports; and (3) an increase in heat stability is often observed because of the strong interaction between enzyme molecules and supports. On the other hand, disadvantages of covalent binding are: (1) active structures of enzyme molecules are liable to be destroyed by partial modification; (2) strong interaction between enzyme molecules and supports often hinders the free movement of enzymes molecules, resulting in decreased enzyme activity; (3) optimal conditions of immobilization are difficult to find; (4) this method is not suitable for immobilization of cells; and (5) supports, in general, are not renewable. Hence, this principle is well-suited to expansive enzymes whose stability is significantly improved by covalent binding. Despite these disadvantages, covalent binding is often applied to the preparation of immobilized enzymes for analytical purposes. Some typical examples of covalent binding methods are described below, but many other techniques have also been reported.

Acylation Reactions. The nucleophilic (amino, hydroxyl, thiol) groups of the enzymes can attack the activated functional groups to form a cavalent (amide, peptide,or other) bond. The different surface activation process can be used: (a) Support containing carboxyl groups are converted to reactive derivatives

(e.g. acyl azide, acid chloride, acid anhydride, isocyanate, isothiocyanate, cyclic carbonate, or cyclic imidocarbonate), which can react directly with free amino functions of the proteins, (b) Condensing reagents such as carbodiimides or Woodward's reagent K (N-ethyl-5-phenylisoxazolium-3′-sulfonate) can form amide linkage between the free amino and carboxyl groups of the carrier surface and the enzyme. Some examples of enzyme immobilization by acylation reactions are presented in [6, 29-34].

Arylation and Alkylation Reactions. Free amino, phenolic, or thiol groups of and enzyme are alkylated or acrylated with an activated functional group of the support such as halide, epoxy, or vinyl sulfonyl groups. The support with hydroxyl groups (e.g. polysaccharides or minerals) can be activated by a treatment with cyanuric chloride derivatives. Another widely used support contains reactive epoxy groups. Summaries of the concerned work are given in [35-36].

Cyanogen Bromide Reactions. The cyanogen bromide method used for activation of a support having vicinal hydroxy groups such as polysaccharides, glass beads, or ceramic, with cyanogen bromide, to give reactive imidocarbonate derivatives. The subsequent reaction between the activated supports and enzyme molecules give N-substituted isourea, N-substituted imidocarbonate and n-substituted carbamate derivatives. This method has been widely used for the immobilization of various enzymes, and CNBr-activated supports, such as CNBr-activated sepharose are available. Aminated glass beads and aminated

polyacrylamide gel are also used as supports in this method. The insertion of spacers such as hexamethylenediamine is also possible, to avoid a strong interaction between enzyme molecules and supports. Interesting research works are shown in [7, 37].

Diazo Coupling Reactions. Diazo coupling is based on a coupling of the enzyme (e.g., the imidazole side chain of histidine or the phenolic group of tyrosine) to the aryldiazonium groups of the carrier. The aromatic amino groups on the surface of the carrier can be diazotized easily with nitrite in an acidic medium. An example is shown in Fillippisson, H. and Hornby, W. E. [38].

Reactions with Glutaraldehyde. Glutaraldehyde, initially introduced as an intermolecular crosslinking agent to produce stable and insoluble three dimensional networks of proteins has recently found extensive uses for immobilizing enzymes onto a variety of polymeric supports. This bifunctional aldehyde can react with polymers containing primary amino groups to yield matrices containing the aldehyde function. Proteins are bound irreversibly to the glutaraldehyde treated polymer by a reaction presumably analogous to that occurring during crosslinking with the low melecular weight bifunctional reagent, Interesting researchs are given in [9-10 ,39].

Miscellaneous Methods. Several other colavent binding methods have been discribed for the immobilization of enzymes. These includes Shiff base formation, Ugi reaction, thiol-disulfide interchange, amidination, mercury enzyme

interaction and γ-radiation induced coupling. These methods have limited applicability and are reviewed in [11-13].

2.2 Protease

The classification of the proteases is based on their active centre. The four primary classes of protease are the serine, metallo, thiol and acid protease [40-41].

2.2.1 Acid Protease

These enzymes are of industrial interest in all areas where protein hydrolysis at low pH values is desired. Two applications are of major interest, namely hydrolysis of soybean protein to make soy sauce and milk coagulation in the preparation of cheese. Other applications involve improvement of the baking properties of flour, application as a digestive aid and prevention of chill haze in beer. A number of acid proteases are commerically available, but most of the preparations are only used for laboratory purposes. Industrial use is practically limited to the enzyme from *Aspergillus niger*, *Aspergillus oryzae*, *Endothiaparasitica*, *Mucor pusillus* and *Mucor miechei* [2, 42].

2.2.1.1 Protease from *Aspergillus niger*

Aspergillopeptidase A is typical acid protease and *Aspergillus niger* that can be produced by *Aspergillus Saitoi* (synonymous with *Aspergillus Pheonicis*). It has a pH optimum for protein hydrolysis at 30°C between pH 2.5 and 3.0, and it is stable in the pH range 2-5. The molecule contains 283-289 amino acid

residues (molecular weight 34,000-35,000) organized in a single peptide chain with serine as the N-terminal and alanine as the C-terminal residues. The molecule contains two cysteine residues which form a disulphide-bridge. Methionine is not present in the molecule. There is no report on carbohydrate content of the molecule.

2.2.1.2 Proteases from *Aspergillus oryzae*

Aspergillus oryzae produces acid, neutral and alkaline protease, and it has been possible to isolate several isoezymes of each type. The acid protease has an optimum pH value for a protein hydrolysis at pH 4-4.5. It is stable between pH 2.5 and 6.0, and 50% is inactivated after 10 min at 60°C. The enzyme has limited esterase activity. It activates trypsinogen, and its active centre is similar to that of pepsin.

2.2.1.3 Protease from *Mucor spp.*

The two thermophilic *Mucor* species, *Mucor pusillus* and *Mucor miehei* are important for their production of microbial rennet. Both microorganisms and enzymes are closely related, yet there are important differences in production and their application. The protease are used for milk coagulation.

(a) The acid proteases from *Mucor pusillus* has an aspartate residue in the acid centre. It consists of a single peptide chain and the molecular weight is about 30,000. Although the molecule contains two cysteine groups, disulphide bridge has been observed, and there is no carbohydrate attached to the molecule. The optimum pH value for a hydrolysis of haemoglobin is pH 4 and for

casein is pH 4.5. The enzyme is specific for peptide bonds involving aromatic side chains. It is stable in the pH range 3 to 6, and loses 90% of its activity after being heated for 15 min at 65°C and pH 5.5.

(b) The protease from *Mucor miehei* is an acid aspartate protease with a molecular weight of about 38,000. The molecule consists of a single peptide chain, and it contains about 6% carbohydrate. The enzyme is stable at 38°C between pH 3 and 6 with an optimum at pH 4.5 for both stability and activity against denatured haemoglobin.

2.2.1.4 Protease from *Endothia parasitica*

The enzyme from *E. parasitica* has a molecular weight in the range 34,000-39,000. It is a single peptide chain lacking carbohydrate. It is stable in the pH range 4.0-4.5 with an optimum value at about 4.5.

2.2.2 Metalloprotease

These enzymes are endoproteases that contain an essential metal atom, usually Zn. Their optimum pH value is around pH 7.0 so they are also called neutral proteases. Metalloproteases are found alone or together with other proteases in many microorganisms. They are formed by several *Bacillus* species, for example *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megatherium* and *Bacillus stearothermophilus*. They also found in proteases preparations from fungi, such as *Aspergillus oryzae*, where the metalloprotease occurs together with an acid and an alkaline protease. The metalloproteases from *Bacillus amyloliquefaciens* and *Bacillus thermoproteolyticus*

(thermolysin) are used for industrial purposes. The amino acid sequence of thermolysin contained 316 amino acids in a single peptide chain without disulphide bridges. The molecule is folded to form two lobes separated by a deep cleft in the bottom of which the essential zinc atom is found bound to two histidine residues and a glutamate residue. The zinc atom is essential for activity and the active site comprises six amino acids close to the zinc atom. Four calcium atoms are bound to the molecule, and they are important for the heat stability of enzyme. The properties of the neutral protease from *Bacillus amyloliquefaciens* are very close to those of thermolysin. The molecule consists of one peptide chain with 326 amino acid residues and there is an extensive homology with thermolysin. Neutal protease from *Bacillus amyloliquefaciens* assumes that the higher percentage of hydrophobic amino acid residues and the presence of four calcium atoms in thermolysin instead of two may be of importance. Metalloproteases have an optimum activity for hydrolysis of casein in the pH range 7-8 and they are stable in the pH range 5-10. The stability is increased considerably by an addition of Ca^{2+} to the reaction mixture and it is lowered by addition of sequestering agents. Strong sequestering agents, like EDTA, inhibit the enzyme by removing the zinc atom, whereas the removal of the calcium atoms only affects the stability of the enzyme. Metalloproteases hydrolyse preferentially peptides with hydrophobic side chains such as phenylalanine and leucine. They have very weak esterase activity [2, 42-43].

2.2.3 Thiol Protease

Thiol or sulfhydryl proteases contain a sulfhydryl group in the active site, are stimulated by reducing agents such as dithiothreitol and cysteine, and are inhibited by oxidizing agents and mercurials [41].

2.2.4 Serine Protease

This group of enzymes was often referred to as the "alkaline protease". They have an alkaline pH optimum. The microbial serine proteases are usually very stable and have a strong proteolytic activity with a low specificity. Serine proteases are inhibited by diisopropylfluorophosphate (DPF) or phenylmethylsulfonyl fluoride (PMSF). There are three types of alkaline protease, commonly referred to as Subtilisin Carlberg, Subtilisin Novo and protease from *Alkalophilic Bacillus Species* [2, 42, 44-49].

2.2.4.1 Subtilisin Carlberg

This enzyme was discovered in 1947 by Linderstrom Lang and Ottesen at the Carlsberg Laboratory. The commercial utilization of the enzyme started in 1960 when it was discovered that the enzyme had excellent properties for use in detergents. Subtilisin Carlsberg is now the most widely used detergent protease. Subtilisin Carlsberg is produced by *Bacillus licheniformis*. The enzyme consists of a single peptide chain with 274 amino-acid residues and cystine or cysteine residues are absent, no disulphide is formed. The tertiary structure is spherical with a diameter of about 4.2 mm. The active site is formed by the residues: serine 221, histidine 64, and

aspartate 32. The enzyme is stable at pH 5-10 at 25°C, and up to ~50°C for 1 h at pH 8.5. Over 80% of the activity is maintained in the pH range 8-11. The enzyme does not depend on Ca^{2+} for stability and will maintain the stability in the presence of sequestering agents such as tripolyphosphates and ethylenediaminetetraacetic acid. Inactivation of the enzyme is rapid at pH values below 4 or above 11.5 and at temperatures above 70°C. Oxidizing agents such as hydrochlorite and hydrogen peroxide, destroy the enzyme rapidly, but are stabilized by hydrogen peroxide, such as in sodium perborate, it does not harm the enzyme activity. The specificity is broad; only 30-35% of the peptide bonds in casein will not be hydrolyzed by the enzyme. Subtilisin Carlberg has performed excellently in detergents. The use of detergents enzyme depends on washing habits. Subtilisin Carlsberg is a durable and inexpensive protease, it may be used in many fields where proteolysis under alkaline conditions is needed, for instance, in preparation of protein hydrolysates for food or feed purposes [2, 42, 44-46].

2.2.4.2 Subtilisin Novo (Subtilisin BPN)

Subtilisin Novo or Subtilisin BPN is produced by *Bacillus amyloliquefaciens*. This new species is separated from *B. subtilis*, because the strains used for an industrial enzyme production have many properties in common, primary the properties of their extracellular enzymes which are different from those of the neotype of *Bacillus subtilis*, the Marburg strain. *Bacillus amyloliquefaciens* from a number of other enzymes of commercial interest, including α-amylase, β-glucanase, neutral proteinase, and hemicellulase. The latter two enzymes are unstable and are

usually present in low concentrations in the final preparation if no special precautions are taken. Subtilisin Novo contains 275 amino acid residues in the sequence. There is extensive homology with Subtilisin Carlsberg. Only 58 of 275 amino residues differ. There is no cystein residue in the molecule, hence no disulphide bridges. The N-terminal is alanine and C-terminal is glutamine. The active site comprises serine (221), histidine (54), and aspartate (32). Activity and stability at different pH values and temperature are similar to those of Subtilisin Carlsberg. The enzyme has a limited application, mainly in a combination with α-amylase in detergents [2, 42, 45, 47].

2.2.4.3 Proteases from *Alkalophilic Bacillus Species*

In 1960s, the commercial success of the detergents proteases initiated a search for alkaline proteases that would act as better detergent proteases than subtilisin Carlsberg. The properties looked for were primarily good stability under washing conditions, i.e. pH 9-10, temperature above 50°C, and the presence of the surfactant and sequestering agents. These so called *alkalophilic bacilli.* The organisms used in the preparation of the new protease may be characterized as alkalophilic counterparts to *Bacillus subtilis* of *Bacillus licheneformis.* These alkaline proteases are serine proteases like the subtilisin. They are single peptide chains free from disulphide bridges and carbohydrates. The N-terminal residue is alanine as in the subtilisins. The specificity for peptide bond hydrolysis is broad and the enzymes have an esterase activity. The molecular weight is 20,000-30,000 and the isoelectric point about 11. The enzymes are stable and active in the pH range 6-12 and they may

be used at temperatures over 60°C. These proteases are valuable enzymes for incorporating into detergents, and because of their good stability at high pH values they have made a new process for enzymic dehairing possible [2, 42].

The literature reviews for production and properties of alkaline proteases from various microorganisms were shown elsewhere [48-61].

2.3 Acrylamide Polymers

2.3.1 Acrylamide Monomers

Acrylmide (2-propenamide) is a white, crystalline solid. The important physical properties of acrylamide are summeried in Table 2.1. Acrylamide exhibits a good thermal stability and a long shelf life. In the absence of light, the pure sample can withstand heating at 80°C for 24 h with little or no polymer formation. When heated above its melting point, however, acrylamide may undergo vigorous polymerization with evolution of heat. Precautions should be taken when handing even small quantities of molten acrylamide. In addition to the solid form, a 50% aqueous solution of acrylamide is available commercially. The solution is stabilized by oxygen and small content (25-30 ppm based on acrylamide) of cupric ion. Several other types of stabilizers, such as ferric ion, nitrite, and ethylenediamine (EDTA), etc., can also be used to stabilize aqueous acrylamide solutions. The only effect of oxygen is to increase the induction period. Iron complexes of cyanogen or thiocyanogen are useful stabilizers for aqueous salt-containing acrylamide solutions [4].

2.3.2 Analytical and Test Methods for Monomer

Among various techniques developed for an analysis of acrylamide, the bromate-bromide method is the most commonly used one. Refractive index measurement is a convenient way to determine acrylamide concentration in water within the 20-60% monomer concentration. Several other methods were applied for determining the residual monomer in water or in the aqueous and nonaqueous dispersed phase polymeric system [62- 67]. In this work, the method of Husser, E.R et al [65] is applied for checking the residual acrylamide during the reaction at any interval time. This method gives data from which percentage conversion of monomer to polymer as a function of time can be constructed.

2.3.3 Polymerization of Acrylamide

Acrylamide and related monomer have been polymerized by free radical and ionic initiators. The aqueous free radical initiated systems is the most common for acrylamide. The aqueous phase may be homogeneous or heterogeneous. Polymerizations in aqueous solution are initiated by thermal or redox activated peroxide systems, by ultrasonic waves, by high energy radiation (γ-rays), or by simply melting the monomer in the absence of water.

Table 2.1 Physical properties of acrylamide

Property	**Value**
Appearence	white crystalline substance
molecular weight	71.08
melting point,°C	84.5 ± 0.3
equilibrium moisture content, particle size 45 mesh, at 22.8°C, 50% RH	1.7 g of water/kg of dry acrylamide
density, kg/m^3 at 30°C	1.122×10^3
solubilities, g/100 cm^3 solvent at 30°C	
acetone	63.1
benzene	0.346
carbon tetrachloride	0.038
chloroform	2.66
methanol	155
water	215.5

2.3.3.1 Free Radical Polymerization

2.3.3.1.1 Aqueous Polymerization

The two classes of the aqueous polymerization system of acrylamide polymer are in solution, and dispersed phase solution polymerization.

(a) Solution polymerization

Polymerization of acrylamide can be either homogeneous when the polymer is soluble in the solvent used or sedimentary, whereby the polymer formed in the polymeric solution is separated out as a final product. Molecular weights of polymers produced by the precipitation polymerization are much lower than those obtained under homogeneous conditions. Moreover, the powdered polymers obtained by the precipitation method are readily caked, which hinders their storage and subsequent use. From this point of view, synthesis of polymer in suspension and emulsion appeares to be a more preferable process.

(b) Dispersed Phase Solution

Inverse suspension and inverse emulsion polymerizations can be operated to produce macroscopic "beads" or submicron sized particles.

Inverse Suspension Polymerization. The system is consisted of the dispersion of concentrated aqueous monomer solutions (containing water-soluble initiators and the other necessary additions) in an appropriate organic solvent (nonmixing with water) in the presence of protecting colloids. After the termination of stirring, the polymer precipitated from the organic dispersion phase. Molecular weights of the polymers formed by a suspension technique approach those of materials synthesized in aqueous solutions.

Inverse Emulsion polymerization. The aqueous solution of monomer that is dispersed with the aid of surface active substances in organic solvents is immiscible with water. In contast to suspension polymerization, the emulsion process involves the use of initiators, which is not soluble in the aqueous phase. The particles formed are much lower as compared to the suspension process. Emulsion allows, in principle, the production of high molecular weight polymers at a high polymerization rate.

2.3.3.1.2 Solid-State Polymerization

Acrylamide may be polymerized in the solid state through the use of ionizing radiation. Crystals may be irradiated continually during the course of reaction at 0-60°C, or they may be exposed to γ rays at -78°C, removed from the source, and then allowed to polymerize at a higher temperature. Many of the radicals formed at very low temperature survive on warming, whereupon their nucleate polymerization occurs. The out-of-source reaction is slow, but it continues for a long time, owing to restrictions on normal termination steps imposed by the matrix. After a limiting conversion is reached at one temperature, chain ends are still reactive and polymerization can be resumed by warming to a higher temperature. Some chain transfer to monomer occurs, but the growing chains do not react easily with oxygen or with most other small molecules.

2.3.3.1.3 Polymerization in Other Systems

Several less common methods of polymerization have been described. Polymer may form in molten monomer and will do so rapaidly if the melt is exposed to UV light or to other sources of free radicals. Polymer is insoluble in the melt and, owing to an imide formation, may be insoluble in water.

Electrolysis of acrylamide solutions produces two products. Finely divided polyacrylamide separated in the anode compartment, and soluble nylon-3 is produced at the cathode. Dimethylformamide containing an electrolyte is a suitable reaction medium.

2.3.3.1.4 Anionic Polymerization

The hydrogen transfer polymerization of acrylamide to give crystalline poly(β-alanine) or (Nylon-3) occurs in aprotic or basic solvents such as toluene or pyridine in the presence of a metal alkoxide, metal alkyl, or other strong base and an inhibitor for vinyl polymerization. Polymers made in this way have molecular weights on the order of 10^5 or higher. Two fractions can be isolated from many preparations, one soluble in water and the other insoluble. The water-soluble fraction contains some primary amide groups and a few carboxyl groups which are now believed to be located at the ends of short branches originating at secondary amide linkages in the main chain. Nylon-3 has long been of interest for textile fiber applications. Melt spinning is unsuitable because the polymer melts with decomposition, but attractive fibers have been made by wet spinning.

2.3.4 Copolymers

2.3.4.1 Copolymerization

Acrylamide copolymerizes easily with many vinyl monomers. Reaction rates and molecular weights tend to be lower than those obtained in acrylamide homopolymerization. Acrylamide content in the copolymer is often higher than that of the feed. Some of the most widely used comonomers are:

Weak acids: Acrylic acid, methacrylic acids

Strong acids: Styrene sulfonic, ethylene sulfonic, and 2-acrylamido-2-methyl-propanesulfonic acids

Base: Dimethylaminoethylmethacrylate, dimethylamino ethylacrylate, diallyldimethylammonium chloride and quaternary salts of these bases [4].

In this work, the methacrylic acid was selected as a comonomer with acrylamide. Methacrylic acid is a moderately strong carboxylic acid. The dissociation constants is 2.2×10^{-5}. Physical properties of methacrylic acid are shown in the Table 2.2. Methacrylic acids is a type of alkene acid in which one of the vinyl hydrogen atoms has been replaced by a carboxyl group. This functional group may display an electron withdrawing ability, an inductive effect of the electron deficiency carbonyl carbon atom, and an electron releasing effect by resonance stability involving the electrons of the carbon-oxygen double bond. Therefore, these compounds react readily with electrophilic, free radical and nucleophilic agents. Free radical initiated polymerization of the double bond is the most common reaction [68].

Table 2.2 Physical properties of methacrylic acid

Property	**Value**
molecular weight	86.10
melting point,°C	14
boiling point, 101 kPa	159-163
vapor pressure, 25°C, kPa	0.13
density, 25°C, kg/m^3	1.015×10^3
refractive index, 25°C	1.4588
viscosity, 25°C, mPa.s	1.3
surface tension, 25°C, (mN/m)	26.5
solubility in water	miscible

2.3.4.2 Graft and Block Polymerization

Acrylamide and its derivatives have been grafted onto a numberous substrate for the purposes of increasing hydrophilicity, altering crystallinity, reducing susceptibility to degradation especially by microorganisms, or providing a reactive site. Grafts are initiated by chemical free radical sources, ultraviolet light with a photosensitizer, or X-rays. Substrates include celluloses, polysaccharides, proteinaceous materials, polyolefins, poly(vinyl chloride), poly(vinyl alcohol), acrylics, polyesters, polyamides, urethanes, and clays. Peroxidation of the substrate prior to grafting or catalyst systems which react at the desired site of the graft such as cerium(IV) or iron-peroxide systems usually produce more grafts and less

homopolymer than do homogeneous catalyst systems. Block copolymers of acrylamides are rare. They may be prepared by a mechanical or ultrasonic degradation of polymer in the presence of acrylamide monomer or by incorporating a reactive end group in the base polymer. The graft and block copolymers are less convenient to prepare than random copolymers. The random copolymers are therefore more important technologically.

2.3.5 Physical Properties of Polyacrylamide

Polyacrylamide isolated by freeze drying is a white, fluffy, noncrystalline solid, but when precipitated from solution and dried, it is glassy and partially clear. Films prepared by casting on glass plates are clear, hard, and brittle. Physical properties of solid polyacrylamide are listed in Table 2.3.

2.3.6 Analysis of Polymers

2.3.6.1 Chemical Methods

Elemental analysis of polyacrylamide is done by conventional procedures. Kjeldahl nitrogen measures total nitrogen and if all the nitrogen present is the amide nitrogen, the result can be combined with a carbon analysis to measure the degree of hydrolysis.

Table 2.3 Physical properties of solid polyacrylamide

Property	Value
density, 23°C, kg/m^3	1.302×10^3
critical surface tension, mN/m	30-40
glass transition temp, °C by DTA	165-185
approx softening temp, °C	210
approx weight loss by TGA, °C	initial loss: 290°C
	70% loss: 430°C
	98% loss: 555°C
gases evolved on heating	NH_3 below 300°C
	H_2, CO, NH_3 above 300°C

Carboxyl content may be determined by conductometric titration with dilute sodium hydroxide of a sample after a conversion by passage through a cation exchange resin. Alternatively, especially in the presence of electrolytes, falling at pH 3.3 and 7.0. This procedure works best at low levels of hydrolysis. At high levels of hydrolysis (ca. 30%) results are low. The entire titration curve should be obtained from samples of uncertain identity. Both these procedures measure monomeric as well as polymeric carboxylic acids.

2.3.6.2 Rheological Methods for Molecular Weights Determination

The intrinsic viscosity-molecular weight relationship has been determined for homopolymer and copolymer of polyacrylamide made with free radical catalysts[69-71]. Mark-Houwink constants have been summaried [72]. The moleculare weight distributions of polyacrylamide have been studied by GPC [73-74].

2.3.6.3 Spectroscopic Methods

Spectral examination of polyacrylamides will usually reveal functional groups rather than amide indicative of comonomers, partial hydrolysis of the amide function, or the presence of β-alanine groups rather than pendant amide. The infrared spectra obtained in mull, film, or pellet mounts are observed. In the visible and near UV regions polyacrylamide shows no absorbance but there is a weak absorbance which increases in intensity with decreasing wavelength in the region below 240 nm. The monomer having a much higher extinction coefficient is independent of the molecular weight of the polymer. Nuclear magnetic resonance spectra of polyacrylamide show amide proton resonance and rapid acid or base catalyzed exchange of the protons with the water.

2.3.7 Applications

(a) Paper Manufacture. In the papermaking process, fiber, pigments, and other additives are retained as a sheet on the wire screen while large volumes of water pass through the screen and, at least in part, are recycled. Polyacrylamides are

added just prior to the sheet-forming step to avoid loss through the screen of pigment particles and fiber fines.

(b) Water Treatment. Manufacturers offer a variety of cationic, anionic, and nonionic acrylamide polymers for use as flocculants and filtration aids. Nonionics may require the assistance of an electrolyte to serve as an activator.

(c) Mining. The mining industries typically use huge quantities of water. Valuable solids must be recovered from the slurries and wastewater must be clarified for reuse or disposal. Polyacrylamides have been used successfully to promote rapid settling and mechanical dewatering of concentrates and tailings. These polymers permit higher production rates, reduce processing costs, lower capital costs for new equipment, reduce water consumption, and assist in meeting environmental standards.

(d) Oil Recovery. Large amounts of oil remain trapped in oil-bearing rock after primary and secondary recovery methods are exhausted. Much of this trapped oil can be forced out by injection of solutions containing partially hydrolyzed polyacrylamide. The amount of polymer required annually for enhanced oil recovery is expected to grow substantially and, according to some projections, could become enormous.

(e) Other Applications. Paper, water treating, mining, and oil recovery are by far the principal uses of polyacrylamide, but several other uses have been mentioned. Examples mentioned include:

Textile agents. Warp-sizing agent for polyester, cotton, and other fibers; creaseproofing of cotton; grafting agent to increase hydrophilicity of synthetic fibers.

Friction reduction (flow promotion). Reduction of liquid friction in pipelines; creaseproofing of cotton, grafting agent to increase hydrophilicity of synthetic fibers.

Viscosity enhancer and gelling agent. Improve effciency in fire fighting; latex viscosity control; gelation of hazardous wastes, explosives, and deodorants.

Adhesives. Component of industrial adhesives; denture adhesives; pigment used in metal casting.

Medical. Components of contact lenses and other hydrogels, matrix for controlled-release agents.

Research. Gel for electrophoretic separation of proteins; chromatographic column material.

2.3.8 Economic Aspects

Acrylamide monomer and polymers are produced in the United States, Europe, Japan, and the Russian Area. A list of producers compiled from the best information available is listed [72]. Forecasts of the polyacrylamide market in western Europe for the 1993-2000 period were estimated in Table 2.4. This represents an average price of $3,000 per ton. A revenue increase of 21.8 percent overall is expected for the 1993-2000 period. Revenues in 2000 are anticipated to be $243.7

million on a volume of about 81.2 millon tons [75]. Selling prices for polyacrylamide vary widely depending on product form, real polymer content, molecular weight, and other factors.

Table 2.4 Polyacrylamide Market: Volume and Revenue Forecasts (Western Europe)

	1990	1993	1995	1997	2000
Volume (000 Tons)	62,900	66,700	71,450	76,539	81,225
Revenues ($ Million)	189.0	200.1	214.4	229.6	243.7

Note: Percentage revenue increase 1993-2000: 21.8% [75].

The reviews of preparation and characterization of polyacrylamide are mentioned below:

Cohen, Y., Ramon, O., Kopelman, I. J., and Mizrahi, S. [76] characterized the physical and structure properties of acrylamide gels by osmotic deswelling, mechanical compression, and X-ray scattering. These properties vary considerably with the concentration of the crosslinking agent (bisacrylamide) at a fixed monomer concentration.

Hunkeler, D. [76] polymerized acrylamide at high concentrations (25-50 wt%) at temperatures between 40 and 60°C with potassium persulfate as an initiator. The rate of polymerization was found to be proportional to the monomer concentration to the 5/4th power. Limiting the rate order is invariant to the acrylamide concentration up to its solubility limit in water. Limitation of coversion is also observed and is reciprocally related to the initial monomer concentration.

Zhou, W. J., Yao, K. J., and Kurth, M. J. [77] polymerized superabsorbent copolymers of acrylamide, sodium allylsulfonate, sodium acrylate, and N,N′-methylenebisacrylamide, using potassium persulfate/N,N,N′,N′-tetramethyl ethylenediamine as the initiator. The influences of synthesis variables (monomer concentration, temperature, initiator concentration and pH) on the polymerization conversion (%yield) and swelling properties were studied. These gels should have a potential application as superabsorbents.

Klein, J., and Conrad, K. D. [70] synthesized polyacrylamide in water and in a water-methanol mixture. The sample was investigated and characterized for intrinsic viscosity, osmotic pressure, refractive index, light scattering sedimentation coefficients and differential thermogravimetry. The molecular weight was also determined.

Kurenkov, V. F., and Myagchenkov, V. A. [3] analyzed various heterogeneous acrylamide polymerizations including precipitation, inverse suspension and inverse emulsion type reactions and considered some technological problems of the process.

Kurenkov,V. F. and Abramova, L. I. [79] discussed the homogeneous polymerization of acrylamide in solution by free radical polymerization. Various methods of polymerizations are considered. Kinetic parameters of polymerization and the molecular mass of the polymer are shown to depend on the nature of the solvent, pH of the medium, complexing agent, surfactant, viscosity, temperature, and side reactions.

Kawaguchi, H. [80] reported the copolymerization of acrylamide and methacrylic acid that produced microspheres with uniformly sizes distribution. The microspheres swelled to become a shrunk mass at a transition temperature of 32°C and the particle size expands by about 6 times. The cost of copolymerization of acrylamide-methacylic acid was lower than that for N-isopropylacrylamide-methacrylic acid. This technique can be applied to the manufacture of a broad range of drug delivery systems.

2.4 Definition and Terminology

2.4.1 Enzymatic Activity (units)

The activity of an enzyme is expressed as microgram of L-tyrosine liberated per gram of the enzyme (free or immobilized enzymes) per minute. The activity of enzyme can be calculated by eq. 2.1.

$$\text{Activity (units)} = \frac{(A_s - A_c) \times V}{S \times t \times W} \qquad \text{---------- (2.1)}$$

where : A_s = The absorbance of the sample at 280 nm

A_c = The absorbance of the control at 280 nm

V = The total volme (5 cm^3)

S = The slope from the calibration curve of L-tyrosine absorption at 280 nm

t = The incubation time (20 min)

W = The weight of free or immobilized enzyme (g)

2.4.2 Percentage Conversion

Conversion of a monomer is defined as the extent of the monomer, which is changed to become a polymer. For this particular case, the conversion of acrylamide to become polyacrylamide and copolymerized of acrylamide and methacrylic acid to become poly(acrylamide-co-methacrylic acid) are referred to as the percentage conversion (eq. 2.2).

$$\%\text{Conversion of monomer} = \frac{\text{Polymer formed}}{\text{Monomer charged}} \times 100 \quad \text{---------- (2.2)}$$

The conversion of monomer can be checked indirectly by determining the residual monomer in the reaction. The total residual monomer in the reaction can be calculated by eq. 2.3.

$$\text{Total Residual Monomer} = \frac{P_a \times V_m \times V_b \times V_t}{S_a \times V_i \times V_d \times V_s} \quad \text{..... (2.3)}$$

where: P_a = Peak area of residual acrylamide from HPLC chromatogram

V_m = Volume of the preparation sample solution for injecting to HPLC (10 cm^3)

V_b = Volume of the dispersed polymer phase after diluting with carbonate-bicarbonate buffer solution of pH 10.5 (9 cm^3)

V_t = Total volume of the reaction (246 cm^3)

S_a = Slope of calibration curve of acrylamide monomer

V_i = Injection volume to HPLC (5 x 10^{-3} cm^3)

V_d = Dispersed polymer solution volume that is used to prepare the sample solution for injection to HPLC (2 cm^3)

V_s = Sampling volume of dispersed polymer solution in any interval time from the reaction (4 cm^3)

The percentage conversion can be calculated by eq. 2.4.

$$\%\text{Conversion of monomer} = \frac{(\text{Monomer charged} - \text{Residual monomer})}{\text{Monomer charged}} \times 100 \quad \text{--- (2.4)}$$

2.4.3 Percentage of Enzymatic Immobilization

Percentage of enzymatic immobilization is defined as the quantity of enzyme that can be entrapped on the polymer beads. The percentage of immobilization is calculated by the following equation:

$$\%\ \text{Enzymatic immobilization} = \frac{A_i \times W_t}{A_f} \times 100 \quad \text{---------- (2.5)}$$

where: A_i = Activity of immoilized enzyme in 1 gram of polymer

A_f = Total activity of free enzyme that is added in the reaction

W_t = Total weight of polymer formed (gram)

2.4.4 Water Absorption

Water absorption of polymer is defined as the amount of water that can be absorbed by the polymer beads. The water absorption is calculated by eq. 2.6.

$$\text{Water Absorption} = \frac{W_s}{W_d} \qquad \text{-------- (2.6)}$$

where: W_s = The weight of the polymer beads after being equilibrated in deionized water

W_d = The weight of the dry polymer beads