CHAPTER II

THEORETICAL AND LITERATURE

2.1 Starch Characteristics

Starch is a major component of foods and the most important energy source in our food supply [7]. Plants synthesize starch as a result of photosynthesis, the process during which energy from the sunlight is converted into chemical energy. Starch is synthesized in plastids founds in leaves as a storage compound for respiration during dark period. It is also synthesized in amyloplasts found in tubers, seeds, and roots, as a long-term storage compound. Glycogen is stored as a reserve carbohydrate in the liver of animals and humans [8].

Starch is a complex carbohydrate which is insoluble in water. It is a white powder, and is tasteless and odorless. Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. At the end of the polymeric chain, a latent aldehyde group is present. This group is known as the reducing end. In general, starches have the formula $(C_6H_{10}O_5)_n$, where "n" denote the total number of glucose monomer units. Starch consists of two type of molecule, amylose that constitutes about 20-30% and amylopectin about 70-80% of ordinary starch. The relative content of amylose to amylopectin depends on the source of starch (Table 2.1). Both amylopectin and amylose are polymers of glucose, and a typical starch polymer chain consists of around 2500 glucose molecules in their varied forms of polymerisation. By far the largest source of starch is corn (maize) with other commonly used sources being wheat, potato, tapioca and rice. When treated amylase with iodine solution, starch forms a deep blue colored iodine complex, while amylopectin gives a red colorization [9].

Table 2.1 The composition of various starches [10,11].

Source	Amylose (%)	Amylopectin (%)
Maize	26	74
Potato	24	76
Wheat	25	75
Tapioca	17	83
Corn	28	72
Barley	22	78
Oat	27	73
Rice	19	81
Sago	26	74
Arrowroot	21	79

2.1.1 Gelatinization

Starch is generally insoluble in water at room temperature. Because starch in nature is stored in cells as small granules which resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the molecule and with other molecules. When native starches are heated in excess water, the hydrogen bonds weaken, water is absorbed and the starch granules swell. This process makes the starch completely digestible by starch hydrolysis enzymes [11,12].

2.1.2 Liquefaction

The objective of the liquefaction is to convert a concentrated suspension of starch granule into solution dextrin and reduces the viscosity of gelatinization starch solution for convenient handling in ordinary equipment and for easy conversion to glucose by glucoamylase. Dextrins are shorter broken starch segments that form as the result of random hydrolysis of internal glycosidic bonds [11,12].

2.1.3 Saccharification

Saccharification is the process that converted starch to glucose in yields as high as possible while observing restraints bearing on economics. Using glucoamylase it is possible to convert starch almost totally to glucose. Several important technical and economic variables interact to limit condition to those allowing a maximum conversion to about 93-96% glucose when starch is converted with glucoamylase [11].

2.1.4 Amylose

Amylose is a linear polymer consisting of 500-20,000 glucose units with α-1,4- glycosidic linkage. The number of glucose residues, also indicates with the term DP (degree of polymerization), varies with botanical origin. It is one of the two components of starch, the other being amylopectin. The α-1,4- glycosidic bond promote the formation of helix structure. The structure formula of amylose is Figure 2.1. The number of repeated glucose subunits (n) can be many thoundsands. Amylose starch is less readily digested than amylopectin. However it takes up less space so is preferred for storage in plants; it is how about 80% of the starch in plants is stored. The digestive enzyme amylase works on the ends of the starch molecule, breaking it down into sugars. Iodine molecules fit neatly inside the helical structure of amylose, binding with the starch polymer, causing it to absorb certain wavelengths of light. Hence, a common test for starch is to mix it with a small amount of yellow iodine solution [8,13].

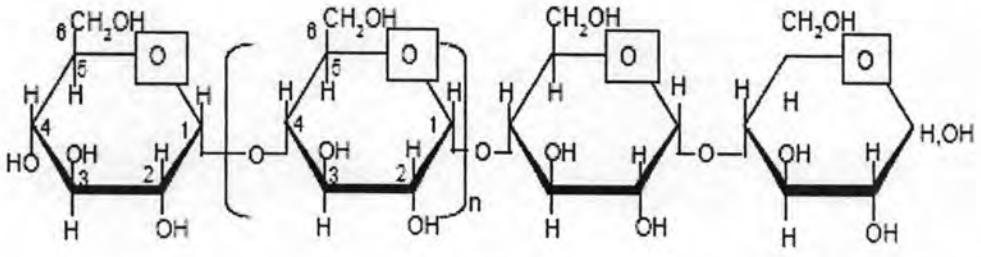

Figure 2.1 Structure of amylose

2.1.5 Amylopectin

Amylopectin is a highly branched polymer of glucose found in plants. Amylopectin consists of α-1,4 glycosodic linkage linear chains of 10-60 glucose units and α-1,6 glycosidic linkage side chains with 15-45 glucose units (Figure 2.2). Amylopectin is a much larger molecule than amylose and is heavily branched with 95% α-1,4 and 5% α-1,6 but varies with the botanical origin. The complete amylopectin molecule contain on average about two million glucose units, thereby being one of the largest molecule in nature [8,13].

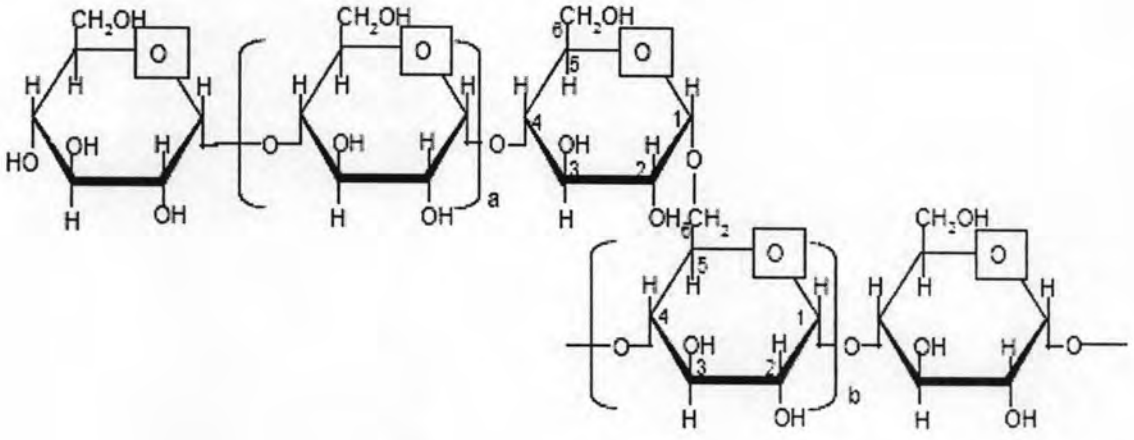

Figure 2.2 Structure of amylopectin

2.2 Protein Structure

Protein are composed of 20 amino acid which are linked together into a peptide chain by the peptide bonds, an amide linkage involving the amino group of one amino acid and the carboxyl group of another. The progressive condensation of many molecules of amino acids gives rise o an unbranched polypeptide chain. By convention, the N-terninal amino acid is taken as the beginning of the chain and the C-terminal amino acid the end of the chain. Polypeptide chains contain between 20 and 2000 amino acids residues and hence have relative molecular mass ranging between about $2x10^3$ and $2x10^6$. Generally proteins have a relative molecular mass in the range $2x10^4$ to $1x10^6$ [14].

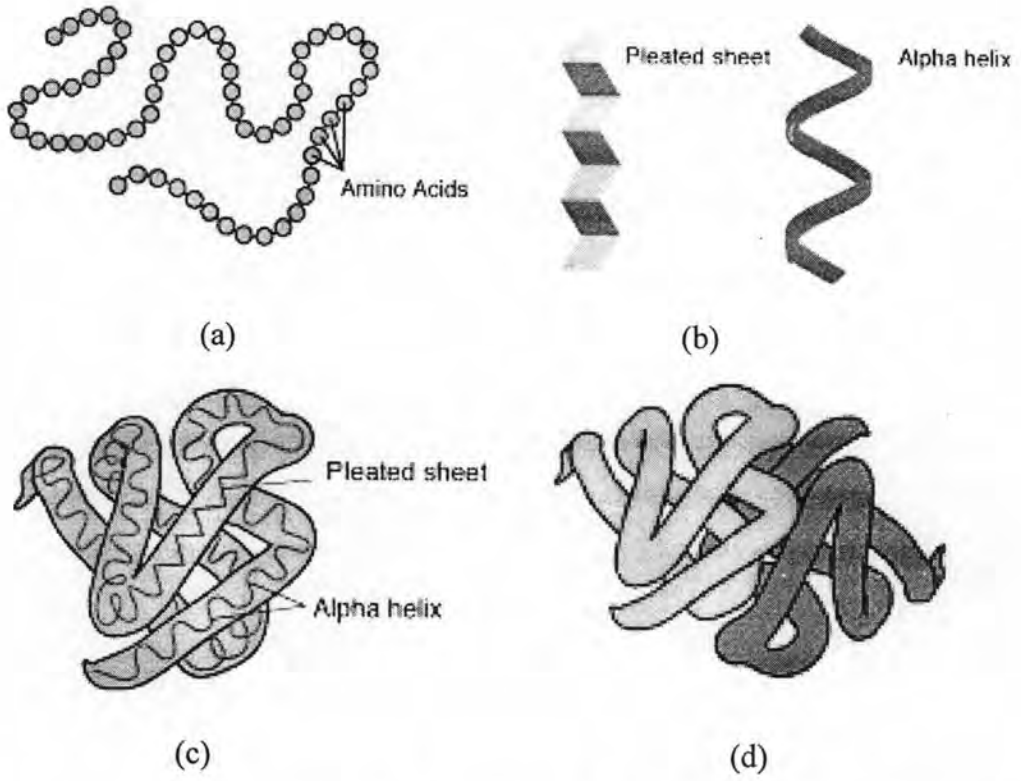

Figure 2.3 The structure of proteins. (a) Primary protein structure, (b) secondary protein structure, (c) tertiary protein structure and (d) quaternary protein structure.

Primary structure of a protein defines the sequence of amino acid residues. Secondary structure defines the localized folding of a polypeptide chain due to hydrogen bonding. It includes structures such as the α-helix and β-pleated sheet. Tertiary structure defines the overall folding of a polypeptide chain. It is stabilised by electrostatic attractions between oppositely charged ionic groups, by weak van der Waal's forces, by hydrogen bonding, hydrophobic interaction and, in some proteins, by disulphide bond formed by the oxidation of spatially adjacent sulphydryl group of cysteine residues. Quaternary structure is restriced to oligomeric protein, which consist of association of two or more polypeptide chains held together by electrostatic attraction, hydrogen bonding, van der Waal's forces and occasionally disulphide bond (Figure 2.3) [15].

2.3 Enzyme

Enzymes are protein which catalysts of biological process. Like any other catalyst, an enzyme brings the reaction catalyzed to its equilibrium position more quickly. The activity of an enzyme is determined by the enzyme concentration; substrate concentration and its availability, concentration of cofactors etc. The way in which each of these parameters affects enzyme activity is the study of enzyme kinetic.

The theory of enzyme catalyzed reaction proposed by Michaelis and Menten is based on the assumption that the enzyme and the substrate from a complex. This reaction is reversible and can be subjected to the law of mass action. The reaction proceeds as follow:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} E + P$$

Enzyme and substrate concentration are two factors that linked in the Michaelis-Menton equation:

$$\nu = \frac{V_{max}[S]}{K_m + [S]}$$

ν = the velocity (rate) of the reaction

V_{max} = $k_2[E]$ is the maximum velocity (rate) of the reaction

[S] = substrate concentration

K_m = Michaelis constant for the substrate

The value k_m can be obtained by plotting the experimentally measure reaction rate against the various substrate concentrations (Figure2.4). Two points of practical importance are obvious. Firstly, the substrate concentration giving half the maximal velocity is equal to K_m. Secondly, the maximal velocity, which is attained when each enzyme molecule is catalyzing the reaction as fast as it can, is only approached at very high (relative to K_m) concentrations of substrate [16].

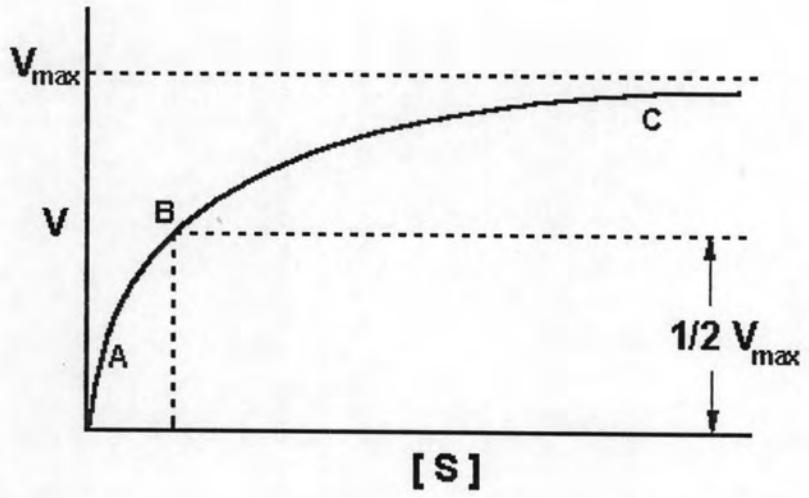

Figure 2.4 Saturation curve for an enzyme reaction showing the relation between the substrate concentration (S) and rate (v).

To avoid dealing with curvilinear plots of enzyme catalyzed reactions, biochemists Lineweaver and Burk introduced an analysis of enzyme kinetics based on the following rearrangement of the Michaelis-Menten equation. Plots of 1/v versus 1/[S] yield straight lines have a slope of K_m/V_{max} and an intercept on the ordinate at $1/V_{max}$ (Figure 2.5) [17].

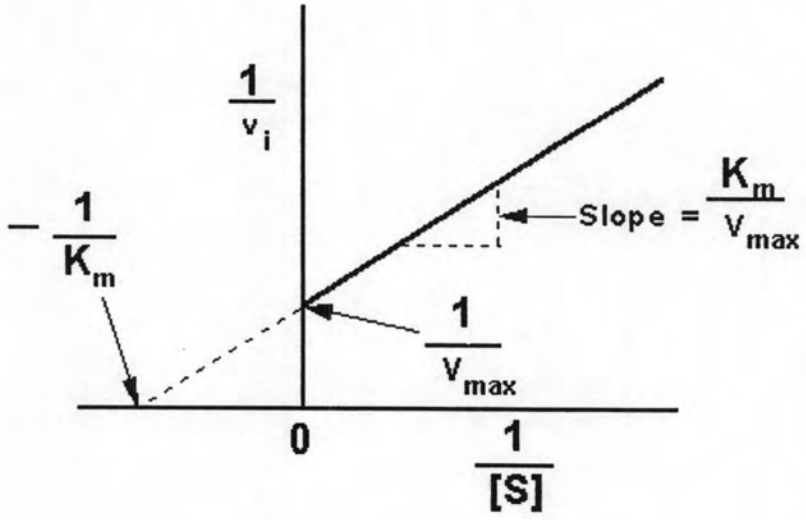

Figure 2.5 The Lineweaver-Burk Plot

2.3.1 Starch Hydrolyzing Enzyme

Starch can be digested by hydrolysis, catalyzed by enzyme call amylase. Amylases are enzymes that catalyze the hydrolysis of starch to give diverse products including dextrins and progressively smaller polymers composed of glucose unit. They are extensively distributed in nature and widely employed in industry, particularly the food industry in the liquefaction of starch and starch-containing raw materials. Enzyme hydrolysis has several advantages to offer. It is more specific and therefore products with a particular carbohydrate distribution can be tailor-made. The conditions under which hydrolysis take place are also milder, so that fewer by-products are formed [10]. The starch hydrolyzing enzyme may be classified into three main groups, as follow

2.3.2 Endoamylase

α-Amylase (EC 3.2.1.1, α-1,4-Glucan-4-Glucanohydrolase) is distributed widely in microorganisms and belong to the class of endoamylase. α-Amylase hydrolyses by randomly cleaving the internal α-1,4-glycosidic linkage in amylose and amylopectin and related polysaccharides but α-1,6-glycosidic in branched polymers are not attacked. Depending on the source, the properties and mechanisms of different amylase vary widely. Hydrolysis of amylose by α-amylase causes its conversion into maltose and maltotriose, initially. Hydrolysis of maltotriose, which is a poor substrate, follows in a second stage reaction. Hydrolysis of amylopectin by α-amylase also yields glucose and maltose in addition to a series of branched α-limit dextrins. These dextrins of four or more glucose residues contain all the α-1,6- glucosidic linkages of the original structure. With amylopectin or glycogen, the second stage of α-amylase degradation involves slow hydrolysis of maltotriose as well as slow hydrolysis of specific bonds near the branch points of α-limit dextrins.

As the name suggest, endo-amylase hydrolyze the bonds located in the inner regions of the substrate. This result in a simultaneous rapid decrease in the viscosity of starch solution and a rapid decrease in the iodine-staining power of amylase [10,18,19].

2.3.3 Exoamylase

β-amylase (EC 3.2.1.2, α-1,4-glucanmaltohydrolase) is exoamylase that hydrolyze maltose residues at the α-1,4 glycosidic linkage from the nonreducing end of the starch chain. The designation dose not relate to the configuration of the glycosidic linkage that is hydrolyze, but rather to the free hydroxyl of the cleavage product. β-Amylase dose not split α-1,6 glycosidic linkages. The β-amylases occur in the different plant and microorganism with various characteristic.

γ-Amylase (EC 3.2.1.3, amyloglucosidase, α-1,4 glucan-glucohydrolase, Glucoamylase) is an exohydrolase hydrolyze α-1,4 and α-1,6 glycosidic linkage from non reducing end of amylose, amylopectin and relate polysaccharides. The end product of glucoamylase reaction is β-D-glucose. It also hydrolyses α-1,6 and α-1,3 linkages although at a much slower rate than α-1,4 linkage [20,21].

2.3.4 Debranching Enzyme

Debranching enzyme hydrolyse 1,6-α branch points in amylopectin, glycogen and certain branched maltodextrins and oligosaccharides. These enzyme may be devided into three groups according to their substrate specificity are amylo-1,6-glucosidases (dextrin 6-α-D glucosidase) found in higher organisms, the microbial pullulanases (pullulan-6-glucanohydrolase) and the isoamylases (glycogen-6-glucanohydrolase). A representation of amylase hydrolysis is shown in Figure 2.6 [10,19].

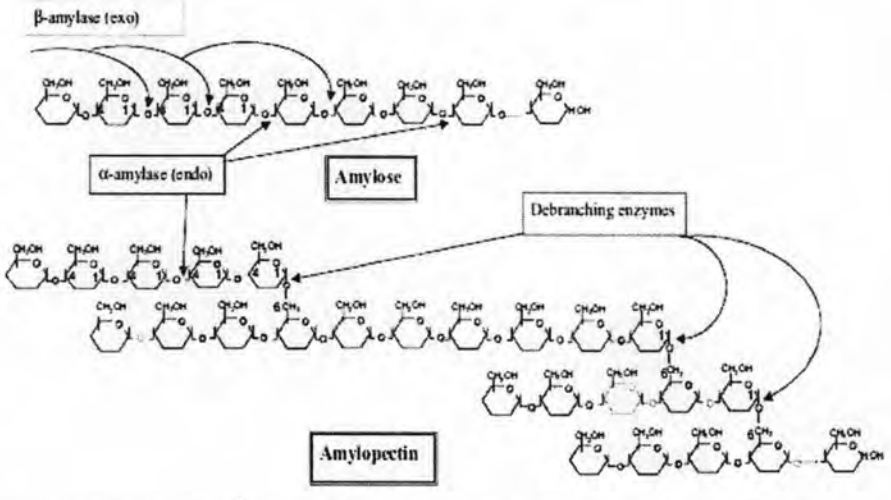

Figure 2.6 Action pattern of hydrolytic enzymes on amylose and amylopectin.

2.4 Factors effect on amylase activity

2.4.1 Temperature

An increase in temperature increases the rate of all chemical reaction, including those catalysed by enzymes, but it also increases the rate of denaturation of enzyme protein. Denaturation also occurs more readily with pure solutions of enzyme than with impure. The optimum is generally between 40°C to 60°C. The lowest optimum temperature is reported to be 25-30°C for *Fusarium oxysporum* and the highest of 100°C and 130°C from archaebacteria. The temperature at which inactivation begin is characteristic for every enzyme. In industry, the optimum temperature range for a given enzyme reaction is that at which the enzyme is still just sufficiently stable [7,21,22].

2.4.2 pH

Enzymes are very sensitive to change in pH and function best over a very limited range with a definite pH optimum. The effects of pH are due to changes in the ionic state molecules. These alterations in charge will affect substrate binding and the result rate of reaction. Over a pH range, these effects will be reversible but extremes of acidity or alkalinity often cause serious distortion of protein structure and result in permanent denaturation. The generally optimum pH of amylases varies from 4 to 12. Amylases from most bacteria and fungi have pH optimum in the acidic to neutral range. Amylase from *Aspergillus niger* showed an acidic pH optimum of 8. In contrast to alkaline amylase with optimum pH 9-10.5 reported from *Bacillus sp.* This factor is of prime importance when choosing an enzyme for an industrial process [7,22].

2.4.3 Substrate specificity

As same as the other enzymes, the substrate specificity of amylase varies from microorganism to microorganism. In general, amylase displays highest specificity towards starch followed by amylose, amylopectin, cyclodextrin, glycogen and maltrotriose [23].

2.4.5 Effects of metal ions

Various metal ions activate or inhibit the catalytic activity of enzymes. In addition to substrates and coenzymes many enzymes require non protein or, in some case, protein compounds to be fully active. Enzyme activation by many inorganic ions has been adequately described. The activating ion may be involved directly in the reaction by complexing the coenzyme or cosubstrate. In other cases, the ion is part of the enzyme and either act as a stabilizer for the active conformation or participates directly at the active site [21].

2.5 Amylase source

Although the hydrolytic profile of α-amylase and β-amylase on starch are very different (they are endo- and exo- acting enzymes respectively), amylase from different sources have different profiles of hydrolysis. However, enzymes derived from different sources will be subject to genetic variation in terms of structure and associated variation with respect to activity (even if the active site is heavily conserved). The amylase from *Aspergillus fumigatus* was, for example, found to be especially active against native starch granules when compared to porcine pancreatic and Bacillus species enzymes. In terms of α-amylase derives from a particular source, twenty eight Bacillus strains have been investigated and it was found that only strains of *Bacillus stearothermophilus* and *Bacillus amylolyticus* secreted amylase which high activity towards native starch granules [8].

2.6 Endophytic Fungi

Endophytic fungi are microorganism which spends the whole or parts of its life cycle colonizing inter and intracellular spaces of stems, petioles, roots and leaves inside the healthy tissues of the host plant (Figure 2.7).

Symbiotic associations between fungi and photosynthetic organisms are both ancient and ubiquitous. The relationship the endophyte and its host plant many range from latent phytophatogenesis to matualistic symbiosis. Endophytes are generally considered to be matualists because the fungus subsists entirely on the resources of the host. The fitness of an endophytic symbiotic that has lost or limited opportunities

for contagious spread by spores depends largely on the fitness of the host plant. The host benefits include improved tolerance to heavy metal, increase drought resistance, reduced herbivory, insect feeding on the host plant, systemic resistance against pathogen and generally enhanced growth.

Endophytic fungi are obviously a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential. For example, the anti cancer from taxol compound, peramine was shown to be toxic to insects, ergot alkaloids were demonstrated later to be neurotoxic to insects and mammal herbivores. Many endophytes have the ability to synthesize a variety of extracellular enzymes. Such enzymes including pectinase, esterases, cellulases, lipase, protease, phosphatase and amylase [5,6].

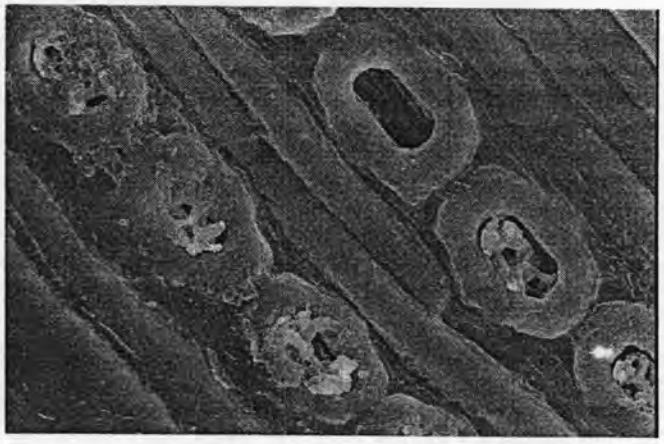

Figure 2.7 Endophytic fungi that growth with stroma of leave

In 2000, Yetti Marida *et al.* isolated endophytic fungi from forest tree in Malaysia. Four newly isolated were previously screened from the 52 endophytic fungi strains for its high raw starch degrading activity on raw sago starch. Four newly endophytic fungi identified as *Gibberella pulicaris, Acremoium sp., Synnematous sp.* and *Nodilusporium sp.* These fungi were qualitatively compared for their ability to hydrolyze raw sago starch granule after 7 days of growth on Czapex Dox agar. The widest clear zone was produced by *Acremonium sp.* which cell growth and raw sago starch degrading enzyme production reached their optimum of pH 5.0 and incubation temperature of 30°C [5].

Yetti *et al.* studied the production of and amylase by *Gibberella pulicaris* too. Amylase was obtained from *Gibberella sp.* have the highest enzyme activity (85 unit $mg.^{-1}$ protein). Optimum amylase activity is found in pH 5.5 and optimum temperature 40°C [24].

Table 2.2 Source microorganism and properties of starch hydrolyzing enzyme

Enzyme	Microorganisms	Enzyme properties		References
		Optimum pH	Optimum temperature (°C)	
amylase	*Aspergillus oryzae*	4.5-5.0	50-55	[25]
	Aspergillus niger	4.0	50-55	[26]
	Aspergillus ochraceus	5.0	-	[27]
α-amylase	*Bacillus subtilis*	6.0	55	[28]
	Bacillus licheniformis	7.5	60-75	[29]
	Bacillus flavothermus	5.5-6.0	60	[30]
	Bacillus sp.	7.0	70	[31]
	Clostridium acetobutylicum	5.6	45	[25]
	Thermococcus profundus	5.5	80	[32]
	Thermobifida fusca	7.0	60	[33]
	Thermonospora curvata	5.5-6.0	65	[34]
	Nocardiopsis sp.	5.0	70	[35]
	Aspergillus flavus	6.2	35	[36]
	Thermomyces lanuginosus	4.6-6.6	70	[37]
	Tricoloma matsutake	5.0-6.0	60	[38]
	Scytalidium thermophilum	6.0	60	[39]
β-amylase	*Bacillus sp.*	7.5	50	[40]
	Clostridium thermosulphurogenes	5.5	75	[40]
	Clostridium thermosulfurogenes	5.8-6.0	60	[40]
	Syncephalastrum racemosum	5.0	60	[41]

Table 2.2 Source microorganism and properties of starch hydrolyzing enzyme

γ-amylase	*Streptosporangium sp.*	4.5	70	[42]
	Aspergillus fumigatus	7.0	35	[1]
	Aspergillus niger NRRL 330	4.5-6.5	50	[43]
	Acremonium sp.	5.5	55	[44]
	Thermomyces lanuginosus	4.4-5.6	70	[37]

2.7 Industrial Applications of Amylase

Amylase plays an important role in the degradation of starch. They are produced commercially in bulk from microorganism and represent about 25-30% of the world enzyme market, in the second after protease [37]. These enzymes are very useful in present day biotechnology to variety of fields such as food production, fermentation and detergent textile to paper industry. Today a large of microbial amylases is available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. Several different amylase preparations are available with various enzyme manufactures for specific use in varied industries. Various applications of amylases are dealt here in brief [7,23].

2.7.1 Bread and Baking industry

The baking industry has made use of these enzymes for hundreds of year to manufacture a wide variety of high quality products. Both type of amylase, α-amylase and β-amylase, are use in baking. β-Amylase is generally available in sufficient quantity in the grain, but α-amylase is often deficient and must be supplemented. The amount of additional enzyme must be sufficient for desired gas production, volume control and color. It must not be added in excess because this can result in excessive dextrin formation, leading to loaf stickiness, dark color and possibly insufficient product strength. The α-amylase supplement can be derived from malted flour however fungal α-amylase is commonly used because this α-amylase

and β-amylase are mesophile and cease activity as the temperature approaches and pass through the gelatinization point [21,23].

2.7.2 Starch liquefaction and saccharification

The major market for α-amylases used in the production of starch hydrolysates such as glucose and fructose. Starch is converted into high fructose corn syrup (HFCS). Because of their high sweetening property, these are use in huge quantities in the beverage industry as sweetener for soft drinks. The process requires the use of highly thermostable α-amylase for starch liquefaction. The use of enzyme in starch liquefaction is well established and has been extensively reviewed [23].

2.7.3 Textile Desizing

Production processes for textiles introduce a considerable strain on the warp during weaving. The yarn must, therefore be prevented from breaking. For this purpose a removable protective layer is applied to the threads. The materials that are used for this size layer are quiet different. Starch is a very attractive size, because it is cheap, easily available in most regions of the world, and it can be removed easily. Good desizing of starch sized textiles is achieved by the application of α-amylase, which selectively remove the size and do not attack the fibers. It also randomly cleaves the starch into dextrins that are water soluble and can be removed by washing. The use of amylases in warp sizing of textile fibers for manufacturing fibers with great strength has been reported [23].

2.7.4 Paper Industry

The use of amylase for the production of low viscosity, high molecule weight starch coating of paper is reported. The use of amylases in the pulp and paper industry is in the modification of starched for coated paper. As for textiles, sizing of paper is performed to protect the paper against mechanical damage during processing. It also improves the quality of the finished paper. Starch is added to the paper in the size press and paper picks up the starch by passing through two rollers that transfer the starch slurry [23].

2.7.5 Detergent Application

Amylase now comprise as one of the ingredients of modern compact detergents. For use in powder detergents, amylase are formulated with fillers and binding agent into small beads or granules, sometimes surrounded with the layer of inert material such as a wax. Furthermore, the particles must dissolve rapidly when the wash liquor is prepared. Nowadays, 90% of all liquid detergents contain amylase and the demand for amylase for automatic dishwashing detergents is growing [21,23].

2.8 Protein Purifiacation

2.8.1 Protein Precipitation

High concentration of variety of salts including sulphates, sulphites and phosphate can be used to precipitate proteins but each fraction produced still consist of a mixture of proteins and usually requires further purification. Excessive denaturation of the protein is avoid by the used of low temperature.

Proteins are usually soluble in water solutions because they have hydrophilic amino acids on their surfaces that attract water molecules and interact with them. This solubility is a function of the ionic strength and pH of the solution. Proteins have isoelectric points at which the charges of their amino acid side groups balance each other. If the ionic strength of a solution is either very high or very low proteins will tend to precipitate at their isoelectric point. The solubility is also a function of ionic strength and as you increase the ionic strength by adding salt, proteins will precipitate (Figure 2.8). Ammonium sulfate is the most common salt used for this purpose because it is unusually soluble in cold buffers. Ammonium sulfate fractionation is commonly used in research laboratories as a first step in protein purification because it provides some crude purification of proteins away from non-proteins and also separates some proteins [15,22].

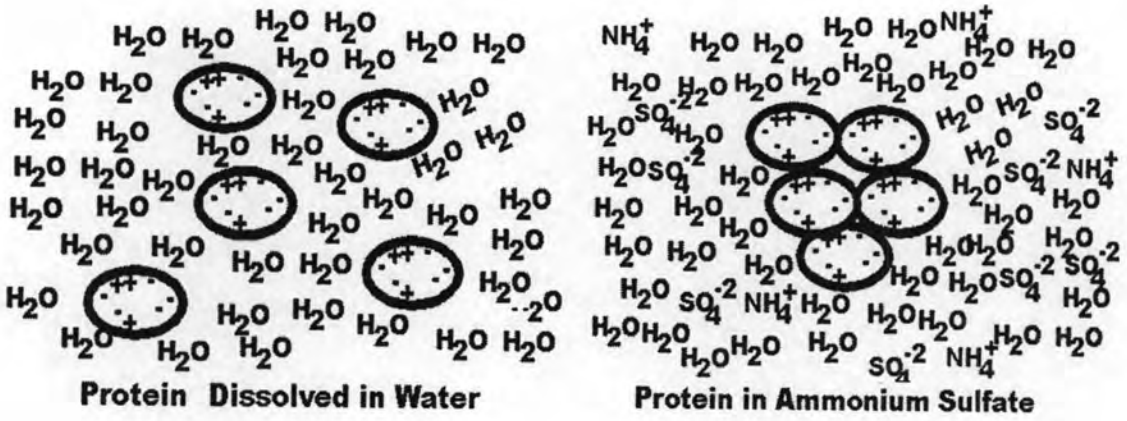

Figure 2.8 Protein precipitation with ammonium sulphate

2.8.2 Ion Exchange Chromatography

Ion Exchange Chromatography (IEC) is one of the most widely used techniques for separation of protein base on charge-charge interactions between the proteins in sample and the charges immobilized on the resin. Ion exchange chromatography can be subdivided into cation exchange chromatography, in which positively charged ions bind to a negatively charged resin; and anion exchange chromatography, in which the binding ions are negative, and the immobilized functional group is positive (Figure 2.9).

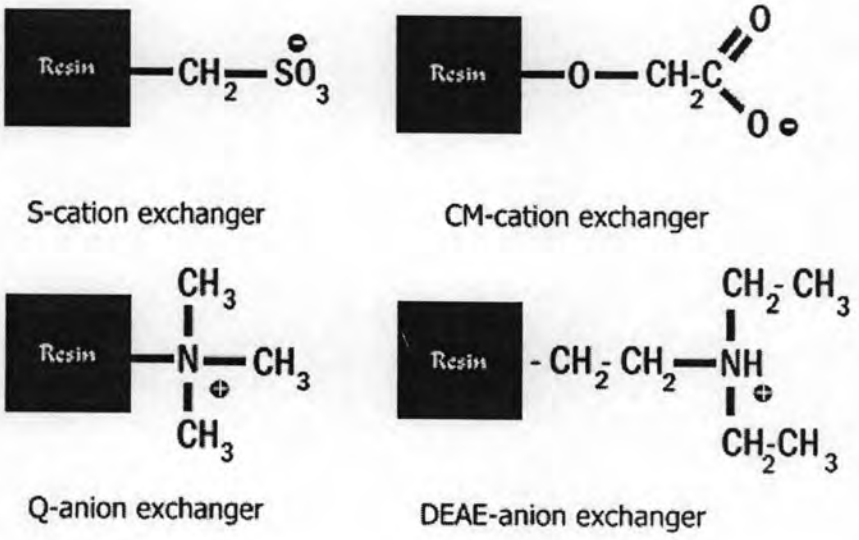

Figure 2.9. Ion exchange resin with functional group structure

Proteins differ from one another in the proportions of the charged amino acids that they contain. So protein will differ in net charge at a particular pH. This difference is exploited in ion–exchange chromatography, where the protein bound on to a resin bearing charge groups of the opposite sign. Proteins with the same charge as the resin pass through the column, after which bound proteins released from the column by gradually increasing the strength of salt ions in the buffer passing through the column or by gradually changing the pH of the eluting buffer. These ion compete with the protein for binding to the resin, the more weakly charged protein being eluted at the lower salt strength and the more strongly charged protein being elute at higher salt strength (Figure 2.10) [22,15].

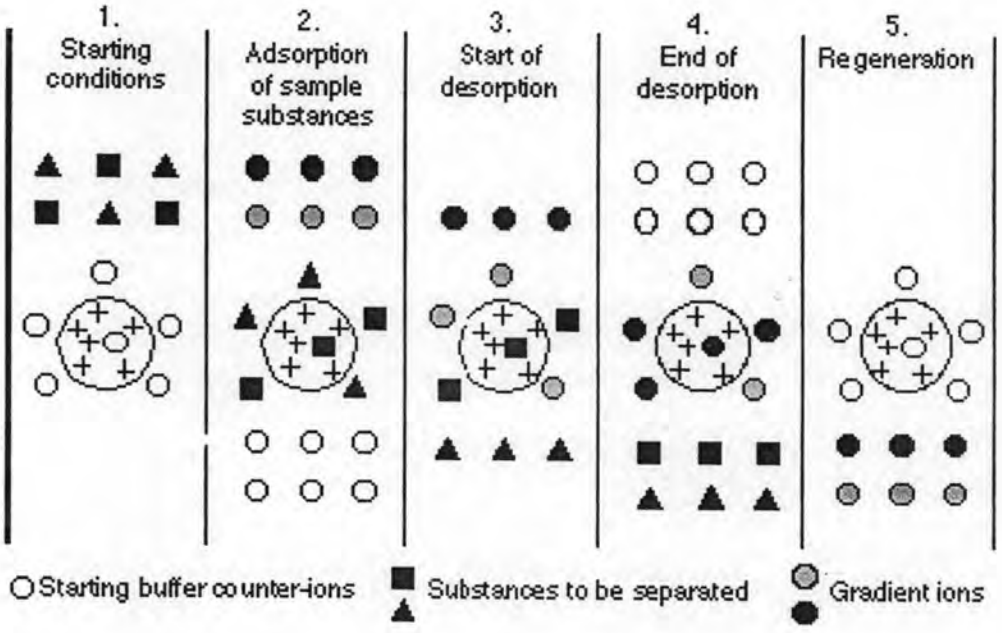

Figure 2.10 The of ion exchange chromatography (salt gradient elution)

2.8.3 Gel Filtration Chromatography

Gel filtration chromatography is a separation based on size. It is also called molecular exclusion or gel permeation chromatography. In gel filtration chromatography, the stationary phase consists of porous beads with a well-defined range of pore sizes. It is generally used to separate biological molecules, and to determine molecular weights and molecular weight distributions of polymers. The stationary phase for gel filtration is said to have a fractionation range, meaning

that molecules within that molecular weight range can be separated. Proteins that are small enough can fit inside all the pores in the beads and are said to be included. These small proteins have access to the mobile phase inside the beads as well as the mobile phase between beads and elute last in a gel filtration separation. Proteins that are too large to fit inside any of the pores are said to be excluded. They have access only to the mobile phase between the beads and, therefore, elute first (Figure 2.11) [15].

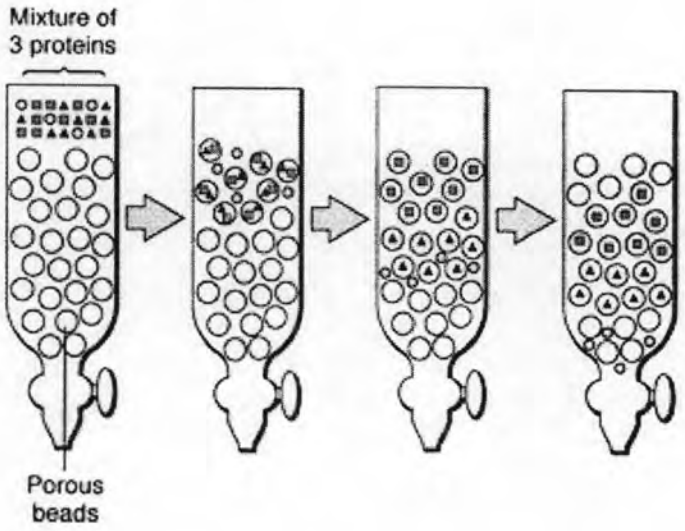

Figure 2.11 Separation of different sized molecules by gel filtration chromatography

2.8.4 Electrophoresis

Electrophoresis, in all it's from, has a major application in the separation of proteins because charge and molecular size are important to both electrophoresis separation and to protein structure. Many important biological molecule, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations or anions. Under the influence of an electric field this charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

Acrylamide is use in technique such as Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), where the smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size.

Cross-linked polyacrylamide gel are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of N,N'-methylenebisacrylamide (normally referred to as 'bis'-acrylamide). Note that bis-acrylamide is essentially two acrylamide molecyles link by amethylene group, and is used as a cross-linking agent. Acrylamide monomer is polymerized in a head-to-tail fashion into long chain and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way a cross-linked matrix of fairly well-defined structure is formed (Figure 2.12).

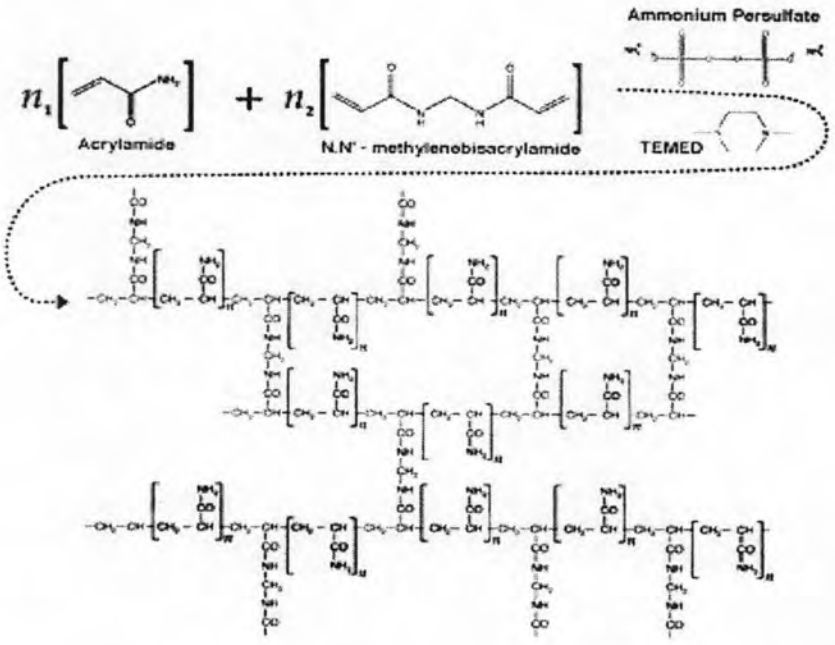

Figure 2.12 The formation of a polyacrylamide gel from acrylamide and bis-acrylamide

The polymerization of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulphate and the base N,N,N',N'-tetramethylenediamine (TEMED). TEMED catalyses the decomposition of the persulphate ion to give a free radical (i.e. a molecule with an unpaired electron). Free radicals are highly reactive species due to the presence of an unpaired electron that need to be paired with another electron to stabilise the molecule, forming a single bond by sharing its unpaired electron with one from the outer shell of the monomer molecule. This therefore produces a new free radical molecule, which is equally reactive and will attack a further monomer molecule. In this way long chains of acrylamide are built up, being cross-linked by the introduction of the occasional bis-acrylamide molecule into the growing chain. Oxygen removes free radicals and therefore all gel solutions are normally degassed prior to use.

Acrylamide gels are defined in term of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentrations of both the acrylamide (%T) and bis-acrylamide (%C).

$$\%T = \frac{\text{acrylamide(g)} + \text{bis-acrylamide(g)}}{100\ \text{ml}} \times 100\%$$

$$\%C = \frac{\text{bis-acrylamide(g)}}{\text{acrylamide(g)} + \text{bis-acrylamide(g)}} \times 100\%$$

Acrylamide gels are conventionally characterized by two values (%T and %C) where %T is the total weight percentage of the monomers (acrylamide + biscarylamide cross-linker) and %C is the proportion of cross-linker as a percentage of the monomer [15,22].

2.8.4.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analysis protein mixtures qualitatively. It is particularly useful for monitoring protein purification and, because the method is base on the separation of protein according to size, the method can also be used to determine the relative molecular mass of proteins. Protein can be dissociated into thier constituent polypeptide chain by the detergent SDS after the reduction of any disulphide bonds. The SDS binds to the polypeptide chain producing a rod shaped complex, the length of which is dependent upon the molecular weight of protein. The molecular sieving effect of polyacrylamide gel results in a relative mobility which is inversely relate to the size of the complex [45].

2.8.4.2 Native polyacrylamide gel electrophoresis (Native-PAGE)

Native polyacrylamide gel electrophoresis (Native-PAGE) it is necessary to use non-denaturing condition. In native gel, polyacrylamide gels are again used but the SDS is absent and the proteins are not denatured prior to loading. Protein separate according to their different electrophoretic mobilities and the sieving effects of the gel. It not possible to predict the behaviour of a given protein in Native gel, because the range of different charges and sizes of proteins in a given protein mixture, good resolution in achieve. The enzyme of interest can be identified by incubating the gel in an appropriate substrate solution such that a colored product is produced at the site of the enzyme [15,45].

2.9 Mass Spectrometry

Mass spectrometers can be divided into three fundamental parts, namely the ionisation source, the analyzer and the detector. The sample has to be introduced into the ionisation source of the instrument. Once inside the ionisation source, the sample molecules are ionized, because ions are easier to manipulate than neutral molecules. These ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass (m) -to-charge (z) ratios (*m/z*). The separated ions are detected and this signal sent to a data system where the *m/z* ratios are stored together with their relative abundance for presentation in the format of a *m/z* spectrum [46].

2.9.1 Ionization Method

The fundamental challenge to the application of mass spectrometry to any class of analyte is the production of gas-phase ions of those species, and difficulties in producing gas-phase ions can prevent mass spectrometric analysis of certain classes of molecules. This situation was once the case with protein and peptides. The first techniques that were applied, electron ionization and chemical ionization, are two step processes in which the analyte is vaporized with heat and ionization occurs once the analyte is in the gas-phase. This vaporization step limited mass spectrometric sequencing experiment to the analysis of small peptides, usually to a maximum of 4 to 5 amino acid. Further, these peptides had to be derivatized to minimize polarity and to give them sufficient volatility. The analysis of proteins was simply not possible, and similar problems were encountered with classes of polar molecule.

The ionisation methods used for the majority of biochemical analyses are Electrospray Ionisation (ESI) and Matrix Assisted Laser Desorption Ionisation (MALDI) [46].

2.9.1.1 Matrix-Assisted Laser Desorption Ionisation (MALDI)

Matrix Assisted Laser Desorption Ionisation (MALDI) is achieved in two steps. In the first step of ionization, the peptide to be analyzed is dissolved in solvent containing small organic molecules in solution, called a matrix, and has a strong absorption at the laser wavelength and placed on a probe or stage for the mass spectrometer. This mixture is dried before analysis and any liquid solvents use in preparation of the solution removed. The result is a solid solution deposit of analyze-doped matrix crystals where the analyze molecules are embedded throughout the matrix so that they are completely isolate from one other.

The second step involves ablation of bulk portions of this solid solution by intense pulses of laser for a short duration. Indeed, the irradiation by the laser induces rapid heating of the crystals by the accumulation of a large amount of energy in the condenses phase through excitation of the matrix molecules the rapid heating causes localized sublimation of the matrix crystals and expansion of the matrix into the gas phase, entraining intact analyze in the expanding matrix plume. Little internal energy is transferred to the analyze molecules and they may be cooled during the expansion process (Figure 2.13) [47].

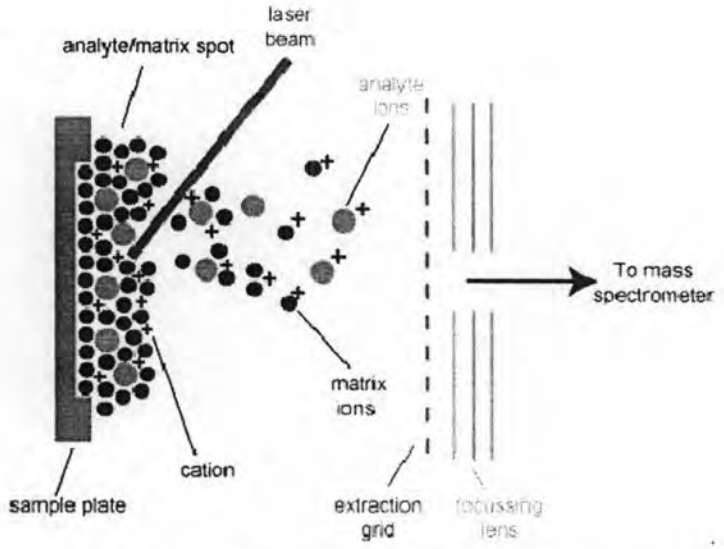

Figure 2.13 A schematic diagram of the mechanism of MALDI

2.9.1.2 Electrospray Ionization

In the electrospray ionization of peptides, an acidic, aqueous solution that contains the peptides is sprayed through a small-diameter needle. A high, positive voltage is applied to this needle to produce a Taylor cone from which droplets of the solution are sputtered. Protons from the acidic conditions give the droplets a positive charge, causing then to move from the needle towards the negatively charged instrument. During the course of this movement, evaporation reduce the size of the droplets until the number and proximity of the positive charges split the droplet into a population of smaller, charged droplets. This evaporation process can be aided by a flow of gas-typically nitrogen-and heat. The evaporation and droplet-splitting cycle repeats until the small size and charging of the droplet desorbs protonated peptides into the gas-phase, can be directed into the mass spectrometer by appropriate electric fields (Figure2.14) [46].

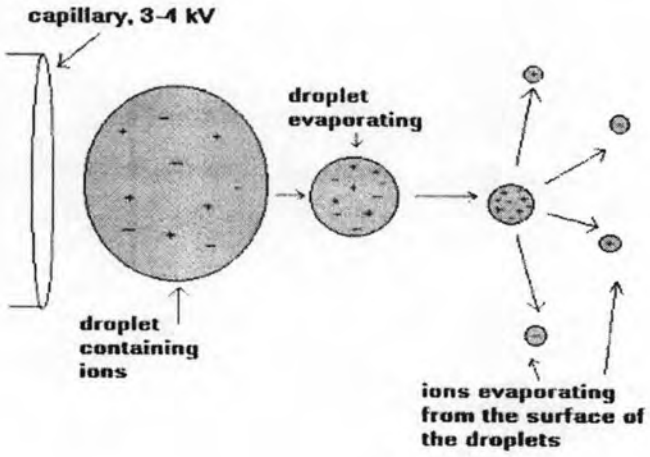

Figure 2.14 The electrospray ionisation process

2.9.2 Mass analyzer

Mass analysis in mass spectrometers determines the *m/z* of ions derived from the analyte. The unit for *m/z* is the Thomson although many sciencetist use "*m/z*" as a unit-less ratio. One must take care with the interchangeable use of "mass" and "*m/z*" because these values will not be the same for any ion that is multiply charged. It is critical to remember this distinction when considering any mass spectrum, including a production spectrum, particular when electrospray ionization is being used [46].

2.9.2.1 Quadrupole mass filters

The quadrupole mass analyzer is a mass filter. The generally constructed using four solid cylindrical rads, of circular cross-section, to which are applied both direct current (DC) and radiofrequency (RF) voltages. The only component of motion of ions along the linear *z*-axis of the filter is that derived from the injection velocity. Both the DC and RF fields cause the ion to under go complicated motion in the *x-y* plane. This together with the component of motion in the *z*-direction, results in the ion following complicated trajectories through the quadrupole filter. For the given set of field conditions, only certain trajectories are stable, allowing ions of specific mass to be transmitted through the collector/detector. Ions whose mass determines that they travel along unstable trajectories are not transmitted, hence the term filter (Figure 2.15) [15].

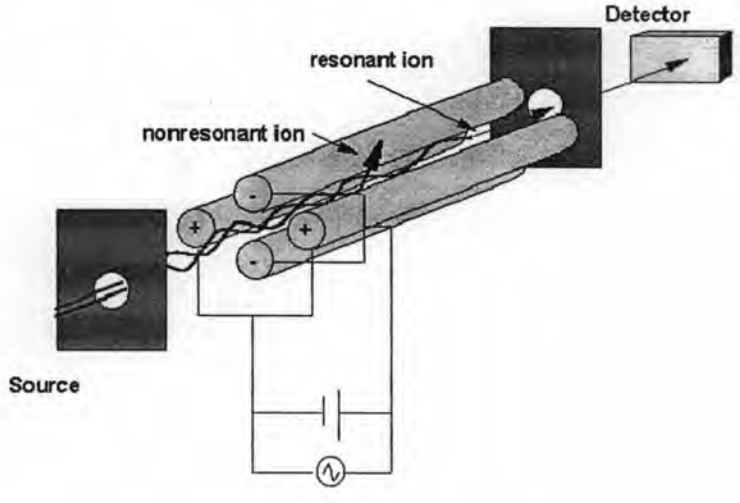

Figure 2.15 Quadrupole mass filter

2.9.2.2 Time-of-flight

A time of flight mass spectrometer measures the mass-dependent time it takes ions of different masses to move from the ion source to the detector. This requires that the starting time (the time at which the ions leave the ion source) is well-defined. Therefore, ions are either formed by a pulsed ionization method (usually matrix-assisted laser desorption ionization, or MALDI), or various kinds of rapid electric field switching are used as a 'gate' to release the ions from the ion source in a very short time [47].

2.9.3 Tandem Mass Spectrometry

Tandem Mass Spectrometry (MS-MS) is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. This information can then be pieced together to generate structural information regarding the intact molecule. Tandem mass spectrometry also enables specific compounds to be detected in complex mixtures on account of their specific and characteristic fragmentation patterns. A tandem mass spectrometer is a mass spectrometer that has more than one analyser, in practice usually two. The two analysers are separated by a collision cell into which an inert gas (e.g. argon, xenon) is admitted to collide with the selected sample ions and bring about their fragmentation.

The basic of Tandem Mass Spectrometry for peptides sequence is product or daughter ion scanning the first analyser is used to select user-specified sample ions arising from a particular component; usually the molecular-related (i.e. $(M+H)^+$ or $(M-H)^-$) ions. These chosen ions pass into the collision cell, are bombarded by the gas molecules which cause fragment ions to be formed, and these fragment ions are analysed such as separated according to their mass to charge ratios, by the second analyser. All the fragment ions arise directly from the precursor ions specified in the experiment, and thus produce a finger print pattern specific to the compound under investigation. This type of experiment is particularly useful for providing structural information concerning small organic molecules and for generating peptide sequence information [15,46].