A RESEARCH REPORT ON

"A comparative study on Extraction, Purification and Characterization of Amylase Enzyme from *Aspergillus niger* and *Aspergillus awamori*"

Submitted by:

Mr. SWAROOP G

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SWAROOP. G

DECLARATION

We hereby declare that the research report entitled "**A comparative study on Extraction**, **Purification and Characterization of Amylase Enzyme from *Aspergillus niger* and *Aspergillus awamori***" work carried out under the guidance of Dr. Sunitha and Miss Ramya K. "Wingene Bio-tech Research Lab" Bangalore and is submitted to Bangalore university , Bangalore. No candidate of any other universities has submitted thesis on this research project.

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I. INTRODUCTION

Much of the history of biochemistry is the history of enzyme research. Biological catalysis was first recognized and described in the late 1700s, in studies on the digestion of meat by secretions of the stomach and research continued in the 1800s with examinations of the conversion of starch into sugar by saliva and various plant extracts. In the 1850's Louis Pasteur concluded that fermentation of sugar into alcohol by yeast is catalyzed by "ferments". He postulated that these ferments were inseparable from the structure of living yeast cells a view called vitalism that prevailed for many years. Then in 1897 Eduard Buchner discovered that yeast extracts can ferment sugar to alcohol, proving that fermentation was promoted by molecules that continued to function when removed from the cells. Frederick W. Kuhne called these molecules enzymes.

Enzymes are essential to sustain life. It work together cooperating with vitamins and minerals and act as a catalyst to accelerate chemical reaction because most chemical reactions in biological cells would occur too slowly. Enzymes don't only act as a catalyst. When the body has an abundance of enzymes, it can protect itself and repair the damage More than 5,000 enzymes are known. To name different enzymes, one typically uses the ending -ase with the name of the chemical being transformed (substrate), e.g., lactase is the enzyme that catalyzes the cleavage of lactose. Each enzyme does one work. Therefore, 5,000 enzymes do 5,000 different works.

Amylase is an enzyme, which is produced by the microorganisms, which has many applications in today's market. Amylase is required in digestion of carbohydrates into smaller units and eventually converting them into even smaller units such as glucose. It is also involved in inflammatory reaction, such as those caused by the release of histamine and similar substances. A number of digestive enzymes including amylase are required to produce fructose in large quantities. Many detergents also contain enzymes such as amylase to remove stains.

Since Amylase is having so many industrial advantages, the study of these enzymes will be helpful in better development of human race. Hence we

concentrated on extraction and purification of enzyme amylase produced by *Aspergillus niger* and *Aspergillus awamori* by solid state and submerged state fermentation method, its characterization and application to develop a method for high production of amylase.

Amylase belongs to group, which is called amylolytic enzymes. Amylolytic enzymes represent a group of catalytic proteins of great importance to food industry. They were also the one of the first enzymes to be produced commercially by microorganism. Amylase refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) sugar and starch. Amylase digests carbohydrates (polysaccharides) into smaller disaccharide units, eventually converting them into monosaccharide such as glucose. People who are fat intolerant (can't digest fats) often eat sugar and carbohydrates to make up for the lack of fat in their diet. If their diet is excessive in carbohydrates, they may develop an amylase deficiency.

Different types of amylase are present depending on the bond they are breaking in starch molecule. They are α – amylases, β – amylases and iso – amylases etc α – Amylases are produced by the fungi, *Aspergillus niger* and *Aspergillus awamori*. Therefore α – amylases are called fungal α – amylase production utilizes strains of *Aspergillus awamori* for stationary culture with wheat bran and strains of *Aspergillus niger* for submerged, aerated, agitated culture. Fungal α – amylase is produced in significant amounts in solid culture. Wheat bran serves as the basic component of the medium.

The above project is carried out with the following objectives

1. Isolation of *Aspergillus niger* and *Aspergillus awamori* from soil.
2. Extraction of Amylase from *Aspergillus niger* and *Aspergillus awamori*.
3. Purification of Amylase.
4. Characterization of amylase.

II. REVIEW OF LITERATURE

Microbial cells produce a variety of enzymes. These enzymes are the biological catalysts for the biochemical reactions, leading to microbial growth and respiration, as well as to produce fermentation products. Enzymes are used for a variety of purposes. They are employed in three major fields.

They are

1. Laboratory
2. Industrial
3. Clinical

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The amylases constitute a large group of enzymes. They are characterized by their ability to hydrolyse 1,4-glucosidic linkages in polysaccharides. α-amylases are exoenzymes. They attack all linkages between glucose units in the starch molecules. The bond hydrolyzed is between carbon-1 and oxygen atom linked to adjacent glucose group. α-amylases vary in their effectiveness, depending on their source. The substrate for α-amylase is starch. Two types of microbial α-amylase have been recognized on termed "liquefying" and "saccharifying" α-amylases. The main difference between them is that the saccharifying enzyme produces a higher yield of reducing sugar than the liquefying enzymes.

Amylase is found in both plants and animals. Amylase has various commercial applications. Some of them are as follows:

- Amylase enzymes are used to hydrolyse the starch.
- Amylase are used in the textile and paper industries, mainly to dissolve and remove starch or to coat starch paper.

- Amylase is used in laundry to remove the spots from clothes in conjunction with amylases.
- Amylase, also used in the brewing industry in the initial breakdown of starchy substrates.
- Amylase includes α -amylase, ,β-amylase, and gluco-amylase.α-amylase convert starch into oligosaccharides and maltose, β-amylase converts starch into maltose and dextrin, gluco-amylase convert starch to glucose.

- Fungal amylase finds extensive use in the preparation of dried baby foods and cereal products.
- Amylases are used in clarification of fruit juices for jam and jelly manufacture.

  Amylases are used in the production of chocolate syrups.
- Amylases is a digestive enzyme needed to digest carbohydrates.

  Therefore, Amylase has a vast significance in day-to-day industrial fields.

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called "black mold").

Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins, but other sources disagree, claiming the latter report is based upon misidentification of the fungal species. Recent evidence suggests some true *A. niger* strains do produce ochratoxin A

Scientific classification

Domain:	Eukaryota
Kingdom:	Fungi
Phylum:	Ascomycota
Subphylum:	Pezizomycotina
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	Trichocomaceae
Genus:	Aspergillus
Species:	***A. niger***

Taxonomy

A. niger is included in *Aspergillus* subgenus *Circumdati*, section *Nigri*. The section *Nigri* includes 15 related black-spored species that may be confused with *A. niger*, including *A. tubingensis*, *A. foetidus*, *A. carbonarius*, and *A. awamori*. A number of morphologically similar species were recently described by Samson *et al*.

Pathogenicity

Plant disease

A. niger causes black mold of onions. Infection of onion seedlings by *A. niger* can become systemic, manifesting only when conditions are conducive. *A. niger* causes a common post harvest disease of onions, in which the black conidia can be observed between the scales of the bulb. The fungus also causes disease in peanuts and in grapes. The spore comes to common trees such as maple.

Human and animal disease

A. niger is less likely to cause human disease than some other *Aspergillus* species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis can occur. Aspergillosis is particularly frequent among horticultural workers who inhale peat dust, which can be rich in *Aspergillus* spores. Less commonly, it has been found on the walls of ancient Egyptian tombs and can be inhaled when the area is disturbed. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss and, in severe cases, damage to the ear canal and tympanic membrane.

Industrial uses

A. niger is cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic

acid (E574) and have been assessed as acceptable for daily intake by the World Health Organization.

Many useful enzymes are produced using industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification. α-galactosidase, an enzyme that breaks down certain complex sugars, is a component of Beano and other medications which the manufacturers claim can decrease flatulence. Another use for *A. niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for NMR analysis.

Keay L, Monser P.W., and Wildi B.S. (1970):

Keay et.al, showed that *subtilis* in Carlsberg produced by *Bacilus licheniformies* had a slightly higher optimum pH value of 10.5 for casein digestion than had *sublitis* in *novo*. The later enzyme shows the same property as an enzyme from *Bacillus subtilis* NRRL B3411 and *sublitis* in BPN, with an optimum pH 10 but higher activity than *sublitis* in Carlsberg throughout the pH range of 7-10.

Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrins, and progressively smaller polymers composed of glucose units (windish et at., 1965)

The maximum growth yields of Aspergillus niger are high when compared with other organisms. Such as penicillium chrysogenum (Manson & Rigelato 1976) – The fungal mycelium synthesized and excretes high quantities of hydrolytic exoenzymes Rainbact et al., 1981.

Starch molecules are glucose polymers linked together by the alpha, 1-4 and alpha 1-6 glycosidic bonds. These polymers exist in two basic components amylase (16-30%) and amylopectins (65-85%) Amylase is a polymer of glucose inked by alpha, 1-4 bonds,

mainly in linear chains. Amylopectin is a large highly branched polymer of glucose including alpha, 1-6 bonds at the branch points (Bailey and oils, 1986).

The effect of yeast extract for the synthesis of glucoamylase by *Aspergillus niger* in solid state fermentation (Ashok pandey et al., 1994 revealed a 20% increasing in enzyme secretion at 0.5% yeast extracts (chandha et al., 1995)

Filamentous fungi isolated from cereals were screened for their ability to produce alpha-amylase. A selected strain identified as *Aspergillus flavus* showed high enzymatic activity. A single extra cellular alpha amylase was purified to homogeneity by a starch adsorption method. *Aspergillus flavus* enzyme was mainly glucose as well as unidentified oligosaccharides Abou-Zeid-A-M et al., 1997.

Yam peel was used as a carbon to produce extracellular amylase in shake flask cultures of a thermophilic strain if *Aspergillus niger*. Peak amylase activity was obtained on the 4^{th} day and 6^{th} day fermentation, period which corresponded with the early stationary phase of the organism ufuru-G-C Akinyam-5-A; sani-A et al., 1997.

A new starch degrading enzyme activity is induced by storage of potato tubers at low temperatures. The cold induced activity was separated from other amylolytic activities in Zymograms based on iodine staining of PAGE gets containing Amylopectin. The cold induced enzyme was separated by 10^{th} exchange chromatography from other amylolytic activities. Nielson-T-H: Deiting-U et al., 1997.

Aspergillus flavus and *Aspergillus niger* produce extracellular amylase into the culture medium when grown on basal medium containing 2% (WlV) soluble starch or cassava peel as a sole carbon source On soluble starch hydrolysed min/per mg. Protein for *A.flavus* and *A niger*, respectively it is concluded that cassava peal might be a better substrate for the production of amylase by *Aspergillus flavus* than commercial soluble starch (Sani-A AWE-F-A1997).

Starch substrates constitute the major part of the human diet for most the people in the world, as well as many other animals. They synthesized naturally in a variety of plants. Some examples with high starch content are corn, potato, rice, sorghum, wheat and cassava upadak and Kottwitz et al., 1997. Enzymatic hydrolysis of starch is catalysed by alpha amylase and disbranching enzymes such pullulanase and 100wmylase (Aenie et al., 1997.)

The amylase from different sources shows a wide variety of reaction properties such as kinetic parameters, pH optimum and substrate specifically. (Terashima et al., 1997).

Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market sindhu et al., 1997.

It is desirable that alpha gelatination (100-110 c and liquefaction (80-90'c) to economical processes, therefore there has been a head for more thermophilic and thermostable alpha amylases sindhu et al., 1997.

The spectrum of amylase application has widened in many other fields, such as clinical, medical, and analytical chemistries, as well as their wide spread application in starch saccharification and in the textile, food, fermentation, paper, brewing and distilling industries Pandey et.al, 2000.

The alpha amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate being glucose residues linked through an alpha 1-1, alpha 1-4 linkage, alpha-1-6 linkage glycosidic bonds members of this family share a number of common characteristic properties (Van dermaarel et.al, 2003).

The method performance of a rapid procedure for the measurement of alpha-amylase activity in and microbial enzyme preparations. Samples were milled to pass a 0.5 mm sieve and then extracted with a buffer/salt solution, and the extracts were clarified and diluted aliquots of diluted extract (Containing alpha amylase) were incubated with substrate mixture under defined conditions of pH, temperature and time Mccleary-Barry-V: Mc Nally-Marian et.al. 2002.

Amylases can be divided into two categories exoamylases and endoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch

molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non-reducing end successively resulting in short end product (Gupta et.al. 2003).

Alpha amylases are availability of thermostable enzymes, a number of possibilities for industrial processes have emerged (itaki and Rakshit, et.al. 2003).

Conversion of agricultural and other lignocelluloses wastes to more useful products by fermentation. One of the most commonly practical methods of disposal of agricultural residue is composting, which is also a fermentation process. The transformation is carried out by micro organisms in the natural state of biological materials by the process called solid state fermentation.

Two different strains, *Aspergillus awamori* TGDTh-4 and *A. awamori* TGP-3 over expressing a synthetic gene encoding the plant sweet protein thaumatin, showed an unfolded protein response. To facilitate protein secretion, the chaperone BiPA gene was expressed in *A. awamori* under control of the strong constitutive promoter of the *gpdA* gene. A good correlation was observed between the level of the *bipA* transcript in different strains and the amount of thaumatin secreted. Thaumatin secretion was increased 2- to 2.5-fold in transformants over expressing the *bipA* gene compared with the parental strain. Secretion of the homologous proteins α-amylase and glucoamylase was not affected by the *bipA* gene over expression. The requirement for BiPA for secretion of thaumatin was confirmed by attenuation of the endogenous *bipA* gene expression with an antisense RNA cassette. The decrease in *bipA* expression reduced the amount of secreted thaumatin up to 80% without affecting the secretion of the homologous α-amylase and glucoamylase proteins. The BiPA protein is, therefore, very important for secretion of some heterologous proteins, such as thaumatin in *A. awamori.*

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Five commercial preparations of glucoamylases (three from *Aspergillus niger*, one each from *Aspergillus foetidus* and *Aspergillus candidus*) were purified by Ultrafiltration, Sepharosegel filtration and DEAEsephadex chromatography. Two forms of the enzyme, namely glucoamylase I and glucoamylase II were obtained from the fungi except from one strain of *A. niger*. All the enzymes appeared homogeneous by electrophoresis and ultracentrifugation.

The specific activities varied between 85 and 142 units. The pH and temperature optima were between 4 and 5, and 60° C respectively. The molecular weight as determined by the sodium dodecyl sulphatepolyacrylamide gel electrophoresis ranged from 75,000 to 79,000 for glucoamylase I and 60,000 to 72,000 for glucoamylase II. Only *A. niger* gluco amylases contained phenylalanine at the N-terminal end. The amino acid composition of the enzymes was generally similar. However, *A. niger* and *A. foetidus* glucoamylases, in contrast to *A. candidus* enzymes, contained greater percentage of acidic than of basic amino acids. The enzymes contained 15 to 30% carbohydrate and 49 to 57 residues of monosaccharide per mol. *A. niger* enzymes contained mannose, glucose, galactose, xylose and glucosamine but the *A. candidus* enzyme lacked xylose and glucose and only xylose was absent in *A, foetidus* enzymes. Majority of the carbohydrate moieties were Oglycosidically linked through mannose to the hydroxyl groups of seline and threonine of the polypeptide chain.

The filamentous fungus *Aspergillus niger* is widely exploited by the fermentation industry for the production of enzymes and organic acids, particularly citric acid. We sequenced the 33.9-megabase genome of A. niger CBS 513.88, the ancestor of currently used enzyme production strains. A high level of synteny was observed with other aspergilli sequenced. Strong function predictions were made for 6,506 of the 14,165

open reading frames identified. A detailed description of the components of the protein secretion pathway was made and striking differences in the hydrolytic enzyme spectra of aspergilli were observed. A reconstructed metabolic network comprising 1,069 unique reactions illustrates the versatile metabolism of A. niger. Noteworthy are the large number of major facilitator superfamily transporters and fungal zinc binuclear cluster transcription factors, and the presence of putative gene clusters for fumonisin and ochratoxin A synthesis.

Starch degrading enzymes, viz., β-amylase, glucoamylase, and pullulanase, were purified using magnetite- alginate beads. In each case, the enzyme activity was eluted by using 1.0 M maltose. β-Amylase (sweet potato), glucoamylase (*Aspergillus niger*), and pullulanase (*Bacillus acidopullulyticus*) from their crude preparations were purified 37-, 31-, and 49-fold with 86, 87, and 95% activity recovery, respectively. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis showed single band in each case.

SATOSHI NAKAMURA and YASUYUKI OGURA have found that The reaction of glucose oxidase [EC 1.1.3.4 [EC], ß-D-glucose: oxygen oxidoreductase] obtained from *Aspergillus niger* was investigated by overall reaction kinetics as well as by the "stopped flow" and "rapid flow" methods. The experiments were carried out using D-glucose and 2-deoxy-D-glucose as substrates at pH 5.5 and at different temperatures (15°, 20°, 25°, and 30°C).The difference absorption spectrum of the enzyme (steady state minus reduced level), which was obtained by the "stopped flow" method, was the same as that of the oxidized minus reduced level. No ESR-signal was observed within 5m/sec, after mixing the oxidized enzyme solution with D-glucose or the reduced form with molecular oxygen. When D-glucose or 2-deoxy-D-glucose was used as substrate, the value of $k^{\max}_{\text{red}}$, the rate constant for reduction of the FAD moiety of oxidized enzyme at a sufficient concentration of the substrate, was almost the same as that of respective V_m/e obtained by overall reaction kinetics, (V_m/e: the maximum velocity per unit enzyme concentration). The value of K_m at 25°C for 2-deoxy-D-glucose was the same as that for D-glucose at the same temperature. The following scheme for the action mechanism of the glucose oxidase of *Aspergillus niger* was proposed to account for the data obtained.

$E_{ox}+S \rightarrow E_{ox}\ S \rightarrow E_{red}+ P$

$E_{red}+O_2 \rightarrow E_{ox}+ H_2O_2$

Where E_{ox} stands for the oxidized form of the enzyme, $E_{ox}S$ the enzyme (oxidized form)-substrate compound, E_{red} the reduced form of the enzyme, S the substrate (D-glucose or 2-deoxy-D-glucose & P the product (δ – lactone).

A crude extract of α-galactosidase obtained by fermenting Aspergillus oryzae on wheat bran was purified 35 fold by ethanol precipitation, gel filtration and ion-exchange chromatography. The final preparation was free of protease activity but contained invertase activity. The molecular weight of the enzyme was estimated as 64,000 daltons. The pH and temperature optima were 4.0 and 60°C, respectively. The enzyme was stable over the pH range 3–7.5 and at temperatures up to 55°C (pH 4.0). The K_m values for p-nitrophenyl α-Dgalactopyranoside (PNPG) and raffinose were 4.0×10^{-4}M and 1×10^{-2}M, respectively. Divalent cations were not required for activity. More than 80% of the oligosaccharides in soy milk were hydrolyzed after 3 h at 50°C using 0.113 PNPG units/ml milk.

Prakasham, Subba Rao, Sreenivas Rao, Sharma have found that eight fungal metabolic influential factors viz, soluble starch, corn steep liquor (CSL), Casein, Potassium dihydrogen Phosphate ($KH_2\ PO_4$) & Magnesium Sulphate ($MgSo_4$, $7H_2O$), pH, temperature and inoculums level were selected to optimize amylase production by A.awamori using fractional factorial design of Taguchi methodology. Significant improvement in acid amylase enzyme production (48 %) was achieved. The optimized medium composition consisted of soluble starch – 3%, CSL – 0.5%, KH_2Po_4 – 0.125%, $MgSo_4$, 7 H_2O- 0.125%, Casein – 1.5% at pH – 4.0 and at 31ºC. Optimization of components of the fermentation medium was carried out using fractional factorial design of Taguchi's L – 18 orthogonal arrays based on the influence of interaction components of fermentation; these could be classified as the least significant & the most significant at individual and interaction levels. Least significant factors of individual level have higher interaction severity index and vice versa at enzyme production in this fungal stain. The

pH of the medium & substrate (soluble starch) showed maximum production impact (60%) at optimized environment temperature and CSL were the least influential factors for acid amylase production.

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III. MATERIALS AND METHODS

- **ISOLATION OF FUNGI FROM THE SOIL.**
- **IDENTIFICATION OF ASPERGILLUS NIGER AND ASPERGILLUS AWAMORI.**
- **SCREENING FOR ENZYME PRODUCTION BY STARCH HYDROLYSIS TESTS.**
- **BATCH FERMENTATION.**
- **EXTRACTION OF ENZYME.**
- **SALT PRECIPITATION OF ENZYME USING AMMONIUM SULPHATE.**
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- **ESTIMATION OF PROTEINS BY LOWRY'S METHOD.**
- **ESTIMATION OF AMYLASE ENZYME ASSAY BY DNS METHOD.**
- **SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS.**
- **IMMOBILIZATION OF ENZYMES.**

MATERIALS AND EXTRACTION TECHNIQUES

1. STERILIZATION

All the glassware's, media used were sterilized according to microbiological techniques before the experiments.

2. COLLECTION OF SAMPLES

The protein rich soil samples with sandy texture and grey to black in color were collected from the basement of our lab in J.P. Nagar 2nd block.

3. ENUMERATION OF TOTAL HETEROTROPIC POPULATION FROM SOIL BY DILUTION AGAR PLATING TECHNIQUE

Though various methods are available to isolate and enumerate microorganisms from soil, serial dilution agar plating method or liable plate count method is one of the commonly used for the isolation and enumeration of microorganisms.

PRINCIPLE:

This method is based upon the principle that when material- containing microorganisms is cultured, each liable microorganism will develop into a colony, hence the number of colonies appearing on the plate represent the number of living organisms present in the sample.

REQUIREMENTS:

- Soil sample
- Potato Dextrose Agar media – Potato (20g) is boiled in a heating mantle and filtered in a muslin cloth and to the filtrate, Dextrose(2g) is added and pH is adjusted to 5.6 , Agar (1.8g) is added and the volume is made up to 100 ml and a pinch of antibiotic (streptomycin) is added.

Sterile Petri plates, test tubes, pipettes, inoculation loop, Distilled water.

PROCEDURE

- 9.0ml of sterile water blanks and sterile Petri plates were labeled as 10^{-1}, 10^{-2}, 10^{-3}, $10^{-4,}$ 10^{-5} ……..
- 1gm of finely pulverized, air-dried soil was added into (10^{-1}) water blank to make 1: 10 dilution.
- Vigorously the dilution was vortexed for 10 minutes to obtain uniform suspension of microorganisms. 1ml of suspension was transferred from 10^{-1} to 10^{-2} with a sterile pipette under aseptic conditions.
- Further dilutions were made by pipetting 1.0ml of suspension into additional water blanks $10^{-3,}$ 10^{-4}, 10^{-5} as prepared above
- O.1 ml aliquots each test tubes dilution was transferred to the Petri plates. Approximately 15.0ml of the cooled agar medium (45 ^{o}C) was poured into each Petri plate and the inoculums is spread by 'L' shaped rod by gentle rotation of the Petri plates.
- Upon solidification of the media, the plates were incubated in an inverted position at 37 ^{o}C for 7 – 8 days. This plating was done for each sample. After 7 – 8 days of incubation, number of colonies in each Petri plates were calculated using the formula :

$$\textbf{Organisms per gram of sample} = \frac{\textbf{Number of colonies}}{\textbf{Amount plated. Dilution}}$$

ISOLATION OF COLONIES FROM MIXED CULTURE

PURPOSE

Perform the spread plates or the streak – plate inoculation procedure for the separation of cells of a mixed culture so that discrete colonies can be isolated.

PRINCIPLE

The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculum is reduced. The diminution of the population size ensures that the individual cell will be sufficiently far apart on the surface of the agar medium to affect the separation of different species present. The following are the techniques that can be used to accomplish the necessary dilution. The streak – plate method is a rapid qualitative isolation method it is essentially a dilution technique that involves spreading of loopful of culture over the surface of an agar plate. Although many types of procedures are performed the four – way or quadrant streak is described.

PROCEDURE

- Place a loopful of culture on the agar surface in Area 1. Flame and cool the loop and drag it rapidly several times across the surface of Area 1.
- Reflame and cool the loop and turn the Petri plate to 90^{o}. Then touch the loop to the corner of the culture in Area 1 and drag it several times across the agar in Area 2. The loop should never enter Area 1 again.
- Reflame and cool the inoculation loop and again turn the Petri plate to 90^{o}. Streak Area 3 in the same manner as Area 2.
- Without reflaming the loop, again turn the Petri plate to 90^{o} and then drag the culture from the corner of Area 3 across Area 4 using a wider streak. Don't let the loop touch any of the previously streaked areas. The flaming of the loop at the points indicated is to effect the dilution of the culture so that fewer organisms are streaked in each area, resulting in the final desired separation.

INOCULATION OF ASPERGILLUS NIGER AND ASPERGILLUS AWAMORI COLONIES

- The spread – plate techniques requires that a previously diluted mixture of microorganisms is used. During inoculation, the cells are spread over the surface of a solid agar medium with a sterile, L – shaped bent rod while the Petri dish is spun on a "Lazy-Susan" turntable. The step by step procedure for this technique is as follows:
- Place the bent glass rod into the beaker and add a sufficient amount of 95% ethyl alcohol to cover the lower, bent portion.
- With a sterile loop, place a loopful of culture in the center of the appropriately labeled nutrient agar plate that has been placed on the turntable. Replace the cover.
- Remove the glass rod from the beaker and pass it through the Bunsen burner flame, with a bent portion of the rod pointing downward to prevent the burning alcohol from running down your arm. Allow the alcohol to burn off the rod completely. Cool the rod for 10 to 15 seconds.
- While the Petri dish cover and spin the turntable.
- While the sterile bent rod to the surface of the agar and move it back and forth. This will spread the culture over the agar surface.
- When the turntable comes to a stop, replace the cover. Immerse the rod in the alcohol and reflame.
- The pour-plate technique requires a serial dilution of the mixed culture by means of a loop or pipette. The diluted inoculum is then added to a molten agar medium in a Petri-dish, it is mixed and allowed to solidify.

IDENTIFICATION OF ASPERGILLUS NIGER AND ASPERGILLUS AWAMORI

STAINING TECHNIQUE:

The chemical substances commonly used to stain fungi are known as dyes. Dyes are classified as natural or synthetic; the former used mainly or histological purposes while the latter are used mainly for the fungal stain preparations.

These are coal tar dyes (Alanine) dyes. Chemically a dye is defined as an organic compound containing a benzene ring with a chromophore and auxochrome group. Such dyes are acidic, basic or neutral. The acidic dyes (e.g. picric acid, acid fuchsin and cosin) are anionic and stain the cytoplasmic components of the cells which are: Low alkaline in nature. On the other hand the basic dyes (e.g. methylene blue, crystal violet or carbol fuchsin) are cationic and combine with those cellular elements which are acidic in nature (e.g. nucleic acids). Neutral stains are formed by mixing together aqueous solutions of certain acidic and basic dyes. The coloring matter in neutral stains is present in both negatively and positively charged components.

There are two kinds of staining procedures Simple and Differential simple stains employ a single dye (e.g. methylene blue, crystal violet or carbol fuchsin) and cell structures within each cell will attain the color of the stain. Differential stains require more than one dye and distinguish between structures within a cell or types of cells by staining them different colors.

LACTOPHENOL COTTON BLUE STAINING OF FUNGUS

Lactophenol Cotton Blue Stain is formulated with Lactophenol which serves as a mounting fluid and cotton blue. Organisms suspended in the stain are killed due to the presence of phenol. The high concentration of the phenol deactivates lytic cellular enzymes thus the cells do not lyse. Cotton blue is an acid dye that stains the chitin present in the cell walls of fungi

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REAGENT FORMULA

Ingredients per litre:

Phenol	200.0gm
Cotton Blue	0.5 gm
Glycerol	400.0ml
Lactic acid	200.0ml
Deionized water	200.0ml

REQUIREMENTS

24 hours old or less old cultures of *Aspergillus niger* and *Aspergillus awamori*.

Lactophenol Cotton Blue Stain.

Staining tray/clothespin, wash bottle of distilled water, Droppers, Inoculating loop, Glass slides, Blotting paper / Absorbent paper, lens paper, Bunsen burner and Microscope.

PROCEDURE

1. Place a drop of 80% alcohol on a microscope slide.
2. Place a drop of Lactophenol Cotton Blue Stain in the centre of the clean slide.
3. Remove a fragment of the fungus colony 2-3nm from the colony edge using an inoculating loop.

4. Place the fragment in the drop of the stain and tease gently. Apply a coverslip. Do not push down or tap the coverslip as this may dislodge the conidia from the conidiophores.

5. Examine the preparation under 40X magnification for the presence of characteristic mycelia and fruiting structures.

OBSERVATIONS

1. Examine the slides microscopically using oil- immersion objective.
2. Make sketches for morphology of the cultures.
3. Describe the morphology and arrangement of the cells.

SCREENING FOR AMYLASE PRODUCTION

STARCH HYDROLYSIS FOR AMYLASE ENZYME ACTIVITY:

Amylase is an exoenzyme that hydrolyses (cleaves) starch, a polysaccharide (a molecule which consists of eight or more monosaccharide molecules) into maltose, a disaccharide (double sugars, i.e. composed of two monosaccharide molecules) and some monosaccharides such as glucose. Starch is a complex carbohydrate (polysaccharide) composed of two constituents:

- AMYLOSE
- AMYLOPECTIN

Amylose is a straight chain polymer of 200 – 300 glucose units and Amylopectin is a larger branched polymer with phosphate group.

Amylase production is well known in Fungi. Amylases commercially produced from various Aspergillus species. Aspergilli are used in the initial steps in several food fermentation processes to convert starch to fermentable sugars. They are also used to partially predigest food for babies, young children to clarify fruit juices and in the manufacture of chocolate syrups. The ability to degrade starch is used as a criterion for the determination of amylase production by fungi. In the laboratory it is tested by performing the starch test to determine the presence of starch in the medium by using Iodine solution as an indicator. Starch in the presence of the iodine produces a dark blue coloration of the medium and a yellow zone around the colony or otherwise blue medium indicates amylolytic activity.

This exercise deals with starch hydrolysis test for the production of extracellular amylase by the test organism. By inoculating *Aspergillus niger* and *Aspergillus awamori* on Starch agar medium.

REQUIREMENTS

Nutrient agar slant cultures of *Aspergillus niger* and *Aspergillus awamori* , Starch agar medium:

- Starch - 2gms
- Peptone – 0.5gms
- Beef extract – 0.3 gms

- Agar – 1.5 gms
- Distilled water – 100 ml ,

Iodine solution, Sterile Petri dishes, Dropper, Inoculation Loop, Bunsen burner, and Wax marking pencil.

PROCEDURE

1. Melt the starch agar medium and cool to 45ºC and pour into the sterile Petri dishes.
2. Allow it to solidify.
3. Label each of the starch agar plate with the name of the *Aspergillus* species to be inoculated.
4. Using sterile technique, make a single streak inoculation of the each *Aspergillus* species into the centre of its appropriately labeled plate.
5. Incubate the fungal inoculated plates for 72 – 96 hours at 25ºC in an inverted position.
6. Flood the surface of the plates with Iodine solution with a dropper for 30 seconds.
7. pouf off the excess Iodine solution.

OBSERVATIONS

Examine the plates for the starch hydrolysis around the line of growth of each organism, i.e. the colour change of the medium.

MASS PRODUCTION OF ENZYME IN VARIOUS MEDIA

Mass production of the enzyme can be done either by submerged fermentation or solid substrate fermentation. In submerged fermentation, the fungus is cultivated in liquid media in the flasks for the enzyme production where as in the solid substrate fermentation, the culture is inoculated across the surface of production medium and the culture remains on the surface throughout the fermentation.

The 96 hours old *Aspergillus niger* and *Aspergillus awamori* is grown in Starch Broth medium for extracellular enzyme production.

COMPOSITION OF STARCH BROTH MEDIUM

Starch (soluble)	-	20.0g
Peptone	-	5.0g
Beef extract	-	3.0g
Distilled water	-	1000ml

The fermentation using Starch broth medium is a liquid substrate in which the culture is inoculated over the surface of the medium. After inoculation, the flask is incubated at 28^{o}C in an orbital shaker at 220 rpm for 7 – 8 days.

SALT PRECIPITATION TECHNIQUE

PRINCIPLE

Proteins are usually soluble in water because they have hydrophilic amino acids on their surfaces that attract water molecules and interact with them. Thus solubility is a function of the ionic strength and pH of the solution. Proteins have isoelectric points at which the charges of their amino acids 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 as we increase the ionic strength by adding salt, proteins will precipitate. Ammonium Sulphate is the most common salt used for this purpose because it is unusually soluble in cold buffers. Ammonium Sulphate fractionation is commonly used in research laboratories as a first step in protein purification because it provides some clued purification of proteins away from non – proteins and also separates some proteins. Ammonium Sulphate also yields precipitated protein slurry that is usually very stable.

REQUIREMENTS

- Centrifuge Tubes.
- Ammonium Sulphate.
- Magnetic Stirrer.
- Graduated Cylinder.
- Weighting Balance.
- Pipettes.
- 10mM Tris Hcl.

PROCEDURE

The broth containing enzyme amylase is centrifuged for 5 minutes at 6000 rpm in 4^{o}C. The Supernatant is collected; then filtered through Whatman Filter paper No 1; Volume is measured graduated cylinder. 80% salt cut is given to the supernatant (80ml of supernatant is collected from the solid state fermentation system. To this supernatant 41.84 g of Ammonium Sulphate is added respectively.) Ammonium Sulphate should be

added very slowly with continuous stirring of the solution on a magnetic stirrer in cold condition. And the solutions are kept for overnight incubation.

The solutions are centrifuged at 8000 rpm for 10 minutes at 4ºC. The pellet is collected and dissolved in 10mM of Tris HCl. This solution contains the enzymes precipitate by Ammonium Sulphate. Precipitate is collected by centrifugation the extract at 8000 rpm for 10min at 4ºC. The precipitate is dissolved in 10ml of 10mM of Tris HCl and subjected to dialysis.

DIALYSIS OF ENZYME

PRINCIPLE

Dialysis is a process that is based on the principle of osmosis, moving from an area of higher concentration to a lower concentration. Dialysis uses semi permeable membrane that have pores of varying molecular weight cut off's (MWCO). These pores allow smaller substances or compounds to flow between the sample and dialysate (usually a buffer with which you wish to exchange the sample buffer) while retaining the sample.

REQUIRMENTS

1. Dialysis Bag.
2. Water Bath.
3. Distilled Water.
4. 2% Sodium Bicarbonate Solution.
5. Beaker.
6. Glass rod.
7. Magnetic Stirrer.

PROCEDURE:

The Dialysis Bag is first processed to activate it. Dialysis bag of about 8 cm was boiled in 100ml of distilled water for 10 minutes. The bag is then boiled in 100ml of 2% Sodium Bicarbonate solution for 10 minutes. The bag was again boiled in 100ml distilled water for 10 minutes. Now the mouth of the bag was gently rubbed to open it. One end of the bag is tightly tied and the sample is loaded into it. After loading the sample the other end was also sealed. The dialysis bag was then suspended in a beaker containing distilled water and a magnetic bead and tied with the help of the glass rod and subjected on magnetic stirrer. The distilled water in a beaker is changed every half an hour for about three times. This setup was kept in refrigerator overnight.

ION EXCHANGE CHROMATOGRAPHY

PRINCIPLE

This form of chromatography relies on the attraction between oppositely charged particles. Many biological materials, for example amino acids and proteins have ionisable groups and the fact that they may carry a net positive or negative charge can be utilized in separating mixtures of such compounds is dependent on their pKa value and on the pH of the solution in accordance with the **Henderson – Hasselbalch equation.**

Ion exchange separations are carried out mainly in columns packed with an ion – exchanger. There are two types of ion – exchanger namely cation and anion exchangers. Cation exchangers are also called acidic ion – exchange materials because their negative charges results from the ionization of acidic groups. Anion exchangers are positively charged groups that will attract negatively charged anions. The term basic ion exchange material is also used to describe these exchangers, as positive charges generally result from the association of protons with basic groups.

The ion – exchange mechanism is thought to be composed of five distinct steps:

- Diffusion of the ion to the exchange surface. This occurs very quickly in homogenous solutions.
- Diffusion of the ion through the matrix structure of the exchanger to the exchange site. This is dependent on the degree of cross – linkage of the exchanger and the concentration of the solution. This process is thought to be the feature that controls the rate of the whole ion – exchange process.
- Exchange of ions at the exchange site. This is thought to occur instantaneously and to be equilibrium process.

CATION EXCHANGER

$$RSO^-_3...Na^+ + NH_3^+R' \leftrightarrow RSO^-_3.....NH_3^+R' + Na^+$$

Exchanger Counter ion	Charge bound	Exchanged molecule to be molecular ion	Exchanged ion

ANION EXCHANGER

$$(R)_4N^+ \ldots Cl^- + {}^-OOCR' \leftrightarrow (R)^+_4N\ldots\ldots OOCR' + Cl^-$$

The more highly charged the ionized molecule to be exchanged. The tighter it binds to be exchanger and the less readily it is displaced by other ions.

- Diffusion of the exchanged ion through the exchanger to the surface.
- Selective desorption by the eluent and diffusion of the molecule into the external eluent. The selective desorption of the bound ion is achieved by change in pH, ionic concentration or by affinity elution in which an ion that has greater affinity for the exchanger than the bound ion is introduced into the system.

REQUIREMENTS:

1. Column Chromatography. (2% DEAE)
2. Enzyme Sample.
3. Activation Buffer:
   - 10 ml of 25mM Tris HCl + 25mM NaCl
     0.25 ml of 1M Tris HCl + 0.25ml of 1M NaCl were mixed and made upto 10 ml with 9.5 ml of distilled water.
4. Elution Buffers:
   - **A.** 10 ml of 25mM Tris HCl + 25mM NaCl.
     0.25 ml of 1M Tris HCl and 0.25 ml of 1M NaCl were mixed and made upto 10ml using 9.5 ml of distilled water.
   - B. 10 ml of 25mM Tris HCl + 50mM NaCl.

0.25 ml of 1M Tris HCl and 0.5 ml of 1M NaCl were mixed and made upto 10 ml using 9.25 ml of distilled water.

C. 10 ml of 25mM Tris HCl + 75mM NaCl.
0.25 ml of 1M Tris HCl and 0.75 ml of 1M NaCl were mixed and made upto 10 ml using 9 ml of distilled water.

D. 10 ml of 25mM Tris HCl + 100mM NaCl.
0.25 ml of 1M Tris HCl and 1.0 ml of 1M NaCl were mixed and made upto 10 ml using 8.75 ml of distilled water.

E. 10 ml of 25mM Tris HCl + 125mM NaCl.
0.25 ml of 1M Tris HCl and 1.25 ml of 1M NaCl were mixed and made upto 10ml using 8.50 ml of distilled water.

F. 10 ml of 25mM Tris HCl + 150mM NaCl.
0.25 ml of 1M Tris HCl and 1.5 ml of 1M NaCl were mixed and made upto 10 ml using 8.25 ml of distilled water.

5. Test tubes.
6. Distilled water.

PROCEDURE

The chromatography column is packed with **DEAE cellulose** was washed using distilled water one or two times. The column was then washed with an Activation Buffer (10 ml of 25mM Tris HCl + 25mM NaCl). Then the column is filled wit Elution buffers, firstly the chromatography column is washed with

Elute 'A' (10 ml of 25mM Tris HCl + 25mM NaCl). The dialysed enzyme sample was poured into the column. The enzymes were then eluted using Elute 'B' (10 ml of 25mM Tris HCl + 50mM NaCl).The Elutants were collected in the same test tubes. The process of elution is carried out using solutions C, D, E and F. This contains the different concentrations of NaCl.

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

Protein can be estimated by different methods as described by Lowry and also by estimating the total nitrogen content. No method is 100% sensitive. Hydrolysing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry is sensitive enough to five a moderately constant value and hence largely followed. Protein content of enzyme extracts is usually determined by this method.

PRINCIPLE

The blue colour developed by the reduction of the phosphomolybdate and phosphotungstic components in the Folin – Ciocalteau reagent by the amino acid tyrosine and tryptophan present in the protein plus the colour developed by biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method.

MATERIALS

- 2% Sodium Carbonate in 0.1N Sodium Hydroxide (Reagent A).
- 0.5% Copper Sulphate ($CuSO_45H_2O$) in 1% Potassium Sodium Tartrate (Reagent B).
- Alkaline Copper Solution: Mix 50 ml of reagent A and 1ml of reagent B prior to use (Reagent C).
- **Folin – Ciocalteau Reagent** (Reagent D) – Reflux gently for 10 hours a mixture consisting of 100g Sodium Tungstate ($Na_2WoO_4.2H_2O$), 25g Sodium molybdate ($Na_2MoO_4.2H_2O$), 700 ml water, 50ml of 85% Phosphoric acid and 100 ml of concentrated Hydrochloric acid in a 1.5 L flask. Add 150g Lithium Sulphate, 50 ml of water and a few drops of Bromine water. Boil the mixture for 15 minutes without condenser to remove excess bromine. Cool, dilute to 1 litre and filter. *The reagent should have no greenish tint*.

- ***Protein Solution (Solution Stock)****:* Weigh accurately 50 mg of Bovine Serum Albumin and dissolve in distilled water and make up to 50 ml in a standard flask.

- ***Working Standard:*** Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. 1ml of this contains 200µg of protein.

PROCEDURE:

Extraction of Protein from Sample

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500mg of the sample and grind well with a pestle and mortar in 5 – 10 ml of the buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of Protein

1. Pipette out 0.1, 0.2, 0.3 ….ml of the working standard in to a series of test tubes.
2. Pipette out 0.5 ml of elutes as E_1, E_2, E_3…….. of the sample extract in to other set of test tubes.
3. Make up the volume to 1 ml in all the test tubes using distilled water. A tube with 1 ml of water serves as the Blank.
4. Add 5 ml of Reagent C to each tube including the blank. Mix well and allow standing for 10 min.
5. then add 0.5 ml of Reagent D, mix well and incubate at room temperature in dark for 30 minutes. Blue colour is developed.
6. Take the O.D. at 660nm.
7. Draw a standard graph and calculate the amount of protein in the elute sample.

Calculation

Express the amount of protein mg/g or 100g sample.

PROTOCOL

TABLE – 1

Serial no.	Working Standard	Distilled water (ml)	Reagent C	FC Reagent	OD at 660nm
1.	0.1	0.9	5ml	0.5ml	0.038
2.	0.2	0.8	5ml	0.5ml	0.071
3.	0.3	0.7	5ml	0.5ml	0.108
4.	0.4	0.6	5ml	0.5ml	0.144
5.	0.5	0.5	5ml	0.5ml	0.176
6.	0.6	0.4	5ml	0.5ml	0.222
7.	0.7	0.3	5ml	0.5ml	0.259
8.	0.8	0.2	5ml	0.5ml	0.286
9.	0.9	0.1	5ml	0.5ml	0.335
10.	1.0	------	5ml	0.5ml	0.364
11.(blank)	------	1.0	5ml	0.5ml	0.000

NOTE : After adding Reagent C to all the tubes incubate in room temperature for 10 minutes and also after adding FC Reagent incubate all the test tubes for 30 minutes and then check the O.D.

ELUTES OF *ASPERGILLUS NIGER*

TABLE - 2

Serial no.	Elutes	Distilled Water	Reagent C	FC Reagent	O.D at 660nm
E_1	0.5	0.5	5ml	0.5ml	0.022
E_2	0.5	0.5	5ml	0.5ml	0.012
E_3	0.5	0.5	5ml	0.5ml	0.013
E_4	0.5	0.5	5ml	0.5ml	0.007
E_5	0.5	0.5	5ml	0.5ml	0.004
E_6	0.5	0.5	5ml	0.5ml	0.000

NOTE : After adding Reagent C to all the tubes incubate in room temperature for 10 minutes and also after adding FC Reagent incubate all the test tubes for 30 minutes and then check the O.D.

ELUTES OF *ASPERGILLUS AWAMORI*

TABLE – 3

Serial no.	Elutes	Distilled Water	Reagent C	FC Reagent	O.D at 660nm
E_1	0.5	0.5	5ml	0.5ml	0.013
E_2	0.5	0.5	5ml	0.5ml	0.007
E_3	0.5	0.5	5ml	0.5ml	0.001
E_4	0.5	0.5	5ml	0.5ml	0.002
E_5	0.5	0.5	5ml	0.5ml	0.003
E_6	0.5	0.5	5ml	0.5ml	0.005

NOTE : After adding Reagent C to all the tubes incubate in room temperature for 10 minutes and also after adding FC Reagent incubate all the test tubes for 30 minutes and then check the O.D.

ESTIMATION OF ASSAY OF AMYLASE ENZYME BY DNS METHOD

PRINCIPLE:

Starch + H_2O alpha-amylase Reducing groups (Maltose)

UNIT DEFINITION:

One unit will liberate 1.0mg of maltose from starch in 3 minutes at pH 6.9 at 20ºC

METHOD: Colorimetric

CONDITION: Temp. = 20ºC, pH= 6.9, Absorbance at 540 nm, Light path= 1cm

PREPARATION OF REAGENTS

All reagents are prepared in Distilled water

A. *20mM Sodium Phosphate Buffer with 6.7 mM sodium chloride, pH 6.9 at 20ºC*

(Weigh accurately 119.98 mg of Sodium Phosphate, Monobasic, and Anhydrous dissolved in 80 ml of deionized water, after dissolving completely add 39.1 mg of NaCl. Adjust the Ph 6.9 at 20ºC with 1M NaOH, then return the solution to 100 ml by using distilled water.)

B. 1.0*% (W/V) soluble starch solution (starch)*

(Prepare 25 ml in Reagent A using Starch Potato soluble. Facilitate solubilization by heating /stir plate-using solution at this temperature for 15 minutes. Allow the starch solution to cool to room temperature with stirring. Return the starch solution to its original volume (25ml) by the addition of water and dispense samples for assay while stirring)

C. *Sodium Potassium Tartrate solution*

(Dissolve 12.0 grams of Sodium Potassium Tartrate, Tetrahydrate in 8.0ml of 2M NaOH. Heat directly on a heating/stirring plate using constant stirring to dissolve. Do not boil).

D. ***96mM 3, 5 Dinitrosalicylic acid solution***

(Weigh accurately 437 mg of 3, 5- Dinitrosalicylic Acid dissolved in 20 ml of deionized water. Heat directly on a heating/stir plate using constant stirring to dissolve. Do not boil).

E. *Color Reagent Solution*

(Slowly add Reagent C to Reagent D with constant stirring. Dilute to 40 ml with

deionized water. If it is not dissolved completely, the reagents should dissolve when mixed. Store the solution in an amber bottle at room temperature. The color reagent solution is stable for 6 months.)

F. *0.2% (W/V) Maltose Standard Solution*

(Prepare 10 ml in deionized water using Maltose, Monohydrate).

G. *Alpha- Amylase Solution*

(Immediately before use, prepare a solution containing 1 unit/ml of Alpha-amylase in cold deionized water).

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers:

	Test	Blank
Reagent B (Starch)	1.00	1.00
Mix by swirling and equilibrate to 20ºC. Then add:		
Reagent G (Enzyme Solution)	1.00	------
Mix by swirling and incubate exactly for 3 minutes at 20ºC. Then add:		
Reagent E (Color Reagent Solution)	1.00	1.00
Reagent G (Enzyme Solution)	-----	1.00
Cap and place in a boiling water bath for exactly 15 minutes, then cool on ice to room temperature and add:		
Deionized water	9.00	9.00

Mix by inversion and record the A 540nm for both the test and Blank using a suitable Spectrophotometer.

Aspergillus niger

TABLE - 4

Sl no.	Reagent B	Reagent G	Reagent E	Distilled water	O.D at 540nm
(Blank)	1ml	-----	1ml	9ml	0.00
$E_{1.}$	1ml	1ml	1ml	9ml	0.668
$E_{2.}$	1ml	1ml	1ml	9ml	0.490
$E_{3.}$	1ml	1ml	1ml	9ml	0.232
$E_{4.}$	1ml	1ml	1ml	9ml	0.081
$E_{5.}$	1ml	1ml	1ml	9ml	-0.04

NOTE: After adding reagent G mix the solution and incubate for 3 minutes at 20 ^{o}C and also after adding reagent E place in boiling water for 15 minutes and cool on ice to room temperature.

Aspergillus awamori

TABLE - 5

Sl no.	Reagent B	Reagent G	Reagent E	Distilled water	O.D at 540nm
(Blank)	1ml	-----	1ml	9ml	0.00
$E_{1.}$	1ml	1ml	1ml	9ml	0.731
$E_{2.}$	1ml	1ml	1ml	9ml	0.228
$E_{3.}$	1ml	1ml	1ml	9ml	0.091
$E_{4.}$	1ml	1ml	1ml	9ml	-0.040
$E_{5.}$	1ml	1ml	1ml	9ml	-0.01

NOTE: After adding reagent G mix the solution and incubate for 3 minutes at 20 ^{o}C and also after adding reagent E place in boiling water for 15 minutes and cool on ice to room temperature.

STANDARD CURVE:

A standard curve is made by pipetting (in milliliters) the following reagents into suitable containers:

Mix by inversion and record the A540nm for the standards and standard blank using a suitable spectrophotometer.

TABLE - 6

Sl no.	Reagent F	Distilled water	Reagent E	Distilled water	O.D at 540nm
(Blank)	0.0ml	2.0ml	1ml	9ml	0.00
1.	0.2ml	1.8ml	1ml	9ml	0.19
2.	0.4ml	1.6ml	1ml	9ml	0.57
3.	0.6ml	1.4ml	1ml	9ml	0.58
4.	0.8ml	1.2ml	1ml	9ml	0.80
5.	1.0ml	1.0ml	1ml	9ml	0.97

NOTE: After adding reagent E mix the solution and incubate for 15 minutes then cool at room temperature

CALCULATION:

ΔA540 nm standard = A 540nm Std – A540 nm Std Blank

0.097 - 0.00 = 0.97

Plot the ΔA540 of the standards vs. milligrams of Maltose.

Sample Determination:

ΔA540 sample = A540 Test -A540 Blank

0.668 - 0.00 = 0.668 (*A.niger*)

0.731 – 0.00 = 0.731 (*A.awamori)*

Determine the milligrams of Maltose liberated using the standard curve.

$$\text{Units/ml enzyme} = \frac{(\text{mg of maltose released})\ df}{(1)} = 1960 \text{ units/ml}$$

df = Dilution factor

1= Volume (in milliliter) of enzyme used

For solid samples (if you are putting dilution factor in above formula, then there is no need to put, mg solid/ml enzyme in below formula)

Units/mg solid = Units/ml enzyme

mg solid/ml enzyme

Units/mg protein = Units/ml enzyme

mg protein/ml enzyme

SODIUM DODECYL SULPHATE POLYACRYLAIDE GEL ELECTROPHORESIS (SDS-PAGE)

The analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly the strongly anionic detergent Sodium Dodecyl Sulphate (SDS) is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is always is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS – polypeptide complexes migrate through polyacrylamide gels in accordance with the size of polypeptide. At saturation, approximately 1.4g of detergent is bound per gram of polypeptide. By using markers of known molecular weight of the polypeptide chains.

SDS polyacrylamide gel electrophoresis is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gel. The SDS – polypeptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when an electric current is passed between the electrodes. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone on the surface of the resolving gel. The ability of the discontinuous buffer systems to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS poly-acryl amide gels.

The sample and the stacking gel contain Tris-HCl (pH 6.8), the upper and lower buffer reservoirs contain Tris-Glycine (pH 8.3) and the resolving gel contains Tris HCl (pH 8.8). All components of the system contain 0.1% SDS. The chloride ions in the sample and stacking gel from the leading edge of the moving boundary and the trailing edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient which sweeps the polypeptides from the sample and deposits them on the surface of the resolving gel. There, the higher pH of the resolving gel favors the

ionization of Glycine and the resulting Glycine ions migrate through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. Freed from the moving boundary, the SDS – poly – acrylamide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size by sieving.

Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross – linked by a bifunctional agent such as N, N' - Methylene – Bisacrylamide. The effective range of separation of SDS – poly – acrylamide gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross – linking.

EFFECTIVE RANGE OF SEPERATION OF SDS POLYACRYLAMIDE GELS.

Acrylamide concentration (%)	Linear range of separation (kD)
15	12 – 43
10	16 – 68
7.5	36 – 94
5.0	57 – 212

Molar ratio of Bisacrylamide: Acryl amide = 1:29.

Cross links formed from Bisacrylamide adds rigidity and tensile strength to the gel and form pores through which the SDS polypeptide complexes must pass.

The sieving properties of the gel are determined by the size of the pores, which is a function of the absolute concentrations of acrylamide and Bisacrylamide used to cast the gel.

PREPARATION OF POLYACRYLAMIDE GELS

***Role of reagents involved*:**

REAGENTS

- Acrylamide and N, N'- Methylene Bisacrylamide: A stock solution containing 29% (w/v) acrylamide and 1% (w/v) N, N' Methylene Bisacrylamide should be prepared in deionized, warm water to assist the dissolution of the Bisacrylamide. Check that the pH of the solution is 7.0 and store the solution in dark bottles at room temperature. Fresh solutions should be prepared every few months.
- Sodium Dodecyl Sulphate (SDS): A 10% (w/v) stock solution should be prepared in deionized water and stored at room temperature.
- Tris Buffers for the preparation of resolving and stacking gels: It is essential that these buffers to be prepared with Tris base. After the Tris base has been dissolved in deionized water, the pH of the solution should be adjusted with HCl.
- TEMED (N, N, N', N' – Tetramethylenediamine): TEMED accelerates the polymerization of polymerization of acrylamide and Bisacrylamide by catalyzing the formulation of free radicals from ammonium persulphate.
- Ammonium persulphate: Ammonium persulphate provides the free radicals that drive polymerization of acrylamide and Bisacrylamide. A small amount of a 10% (w/v) stock solution should be prepared in deionized water and stored at 4ºC. Ammonium persulphate decomposes slowly and fresh solutions should be prepared weekly.
- Tris – Glycine Electrophoresis Buffer: This buffer contains 25mM
  Tris base, 250 mM Glycine (Electrophoresis grade), pH 8.3, 0.1% SDS. A 5X

  stock can be made by dissolving 15.1g of Tris base and 94g of Glycine in 900ml of deionized water. Then 50ml of a 10% (w/v) stock solution of electrophoresis grade SDS is added and the volume is adjusted to 1000ml with water.

CASTING OF SDS – POLYACRYLAMIDE GELS

- Assemble the glass plates according to the apparatus manufacturer's instructions.
- Determine the volume of the gel mold (this information is usually provided by the manufacturer). In an Erlenmeyer flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel. Polymerization will begin as soon as the TEMED has been added. Without delay swirls the mixture rapidly.
- Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel (the length of the teeth of the comb + 1cm). Using a Pasteur pipette carefully overlay the acrylamide solution with 0.1% SDS (for gels containing > 8% acrylamide) or isobutanol (for gels containing > 10% acrylamide). Place the gel in a vertical position at room temperature.
- Large – scale isolation method. After polymerization is complete (30 minutes), pour off the overlay and wash the top of the gel several times with deionized water to remove any unpolymerized acrylamide. Drain as much fluid is possible from the top of the gel and then remove any remaining water with the edge of the paper towel.
- Prepare the Stacking gel as follows: In disposable plastic tubes, prepare the appropriate volume of solution containing the desired concentration of acrylamide. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to the next step.
- Pour the stacking gel solution directly on to the surface of the polymerized resolving gel. Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Place the gel in a vertical position at room temperature.
- While the stacking gel is polymerizing, prepare the samples by heating them to 100ºC for 3 minutes into 1X SDS gel – loading buffer to denature the proteins.

TABLE - 7

1X SDS gel loading buffer	
50mM Tris HCl (pH 6.8)	1.2 ml
100Mm Dithiothreitol, β Mercaptoethanol	0.95 ml
2% SDS (Electrophoresis grade)	2 ml
0.1% Bromophenol Blue	0.5 ml
10% Glycerol	1 ml

1X SDS gel loading buffer lacking Dithiothreitol, β Mercaptoethanol can be stored at room temperature. Dithiothreitol, β Mercaptoethanol should be then added just before the buffer is used from 1 M stock.

- After polymerization is complete (30 minutes) remove the Teflon comb carefully. Wash the wells immediately with deionized water to remove any unpolymerized acrylamide. Mount the gel in the electrophoresis apparatus. Add Tris – Glycine electrophoresis buffer to the top and bottom reservoirs. Remove the bubbles that are trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe.
- Load up to 15 µl of each of the samples in the predetermined order into the bottom of the wells. Load an equal volume of 1X SDS gel loading buffer into any wells that are unused.

- Attach the electrophoresis apparatus to an electric power supply (the positive electrode should be connected to the bottom buffer reservoir). Apply a voltage of 8 V/cm to the gel. After the dye front has moved into the resolving gel, increase the voltage to 50 V/cm and run the gel until the Bromophenol blue reaches the bottom of the resolving gel (about 4 hours). Then turn off the power supply.
- Remove the glass plates from the electrophoresis apparatus and place them on a paper towel. Using a spatula, dry the plates apart. Mark the orientation of the gel

by cutting a corner from the bottom of the gel that is closest to the left most well (slot 1).

IMPORTANT: Do not cut the corner from gels that are to be used for western blotting.

- The gel can now be fixed stained with Coomassie – Brilliant Blue, fluorographed or autoradiographed or used to establish a western blot.

STAINING OF SDS – POLYACRYLAMIDE GELS

Polypeptides separated by SDS polyacrylamide gels can be simultaneously fixed with methanol; glacial acetic acid and stained with Coomassie Brilliant Blue R250, a Triphenylmethane textile dye also known as Acid Blue 83. The gel is immersed for several hours in a concentrated methanol / acetic acid solution of the dye and excess dye is then allowed to diffuse from the gel during a prolonged period of destaining.

- Dissolve 0.15g of Coomassie Brilliant Blue R250 in 90 ml of methanol: water (1:1 v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatman No.1 filter to removes any particulate matter.
- Immerse the gel in at least 5 volumes of staining solution and place on a slowly rotating platform for a maximum of 4 hours at room temperature.
- Remove the stain and save it for future use. Destained the gel by soaking it in the methanol/acetic acid solution (step 1) without the dye on a slowly rocking platform for 4 – 8 hours, changing the destaining solution three or four times.
- More thoroughly the gel is destained, the smaller the amount of protein that can be detected by staining with Coomassie Brilliant Blue. After destaining gels may be stored indefinitely in water containing 20% glycerol.

IMMOBILIZATION OF ENZYME

The technique of enzyme immobilization has revolutionized the prospects of enzyme application in industry. Immobilization is defined as 'the imprisonment of a biocatalyst in a distinct phase that allows exchange with, but is separated from bulk phase in which substrate, effector or inhibitor molecules are dispersed and monitored'. In other words, an immobilized enzyme is physically entrapped by chemical means to an inert and usually insoluble matrix, where it can act upon its natural substrate. The immobilization of enzyme is performed under mild and controlled conditions, so that the enzymes retain their tertiary and quaternary structures, which are necessary for their activity.

The common use of crude enzyme preparation in the production of wine, cheese or in tanning to obtain the desired product is known for long time. Their use as catalysts was limited, however due to their limited availability, instability and the consequent high cost. This limitation has been largely overcome by the immobilization of enzyme on a support, a phenomenon reported for the first time by **J.M. Nelson** and **E.G. Griffin** (1916). They reported that immobilization of invertase on charcoal\alumina without loss of activity.

Advantages of using immobilized enzymes over free enzymes:

- Because of its binding with a matrix the immobilized enzyme has better stability.
- Its efficiency is better.
- The enzyme can be recovered at the end of the reaction and can be used repeatedly.
- Some manipulations of the catalysed reactions are better in immobilized form. For example, the reaction can be stopped rapidly by removing the enzyme from the reaction solution.

REQUIREMENTS:

2% Sodium Alginate – 10 ml

3M $CaCl_2$ - 50 ml

PROCEDURE:

The reagent Calcium Chloride was poured in to the Petri dish. The enzyme solution was mixed with the Sodium Alginate solution due to which the enzyme gets entrapped into the Alginate gel and cannot escape any permeation. Using a pipette, the enzyme entrapped in the gel was taken and released into the Calcium Chloride solution drop wise. Since the two phases or solutions are not miscible the enzyme in the gel formed bead – like round structures floating on top of the Calcium Chloride solution. To make the beads visible more clearly, dye was added.

RESULTS

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

TABLE - 1

Serial no.	Working Standard	Distilled water (ml)	Reagent C	FC Reagent	OD at 660nm
1.	0.1	0.9	5ml	0.5ml	0.038
2.	0.2	0.8	5ml	0.5ml	0.071
3.	0.3	0.7	5ml	0.5ml	0.108
4.	0.4	0.6	5ml	0.5ml	0.144
5.	0.5	0.5	5ml	0.5ml	0.176
6.	0.6	0.4	5ml	0.5ml	0.222
7.	0.7	0.3	5ml	0.5ml	0.259
8.	0.8	0.2	5ml	0.5ml	0.286
9.	0.9	0.1	5ml	0.5ml	0.335
10.	1.0	------	5ml	0.5ml	0.364
11.(blank)	------	1.0	5ml	0.5ml	0.000

NOTE : After adding Reagent C to all the tubes incubate in room temperature for 10 minutes and also after adding FC Reagent incubate all the test tubes for 30 minutes and then check the O.D.

ELUTES OF *ASPERGILLUS NIGER*

TABLE - 2

Serial no.	Elutes	Distilled Water	Reagent C	FC Reagent	O.D at 660nm
E_1	0.5	0.5	5ml	0.5ml	0.022
E_2	0.5	0.5	5ml	0.5ml	0.012
E_3	0.5	0.5	5ml	0.5ml	0.013
E_4	0.5	0.5	5ml	0.5ml	0.007
E_5	0.5	0.5	5ml	0.5ml	0.004
E_6	0.5	0.5	5ml	0.5ml	0.000

NOTE : After adding Reagent C to all the tubes incubate in room temperature for 10 minutes and also after adding FC Reagent incubate all the test tubes for 30 minutes and then check the O.D.

ELUTES OF *ASPERGILLUS AWAMORI*

TABLE – 3

Serial no.	Elutes	Distilled Water	Reagent C	FC Reagent	O.D at 660nm
E_1	0.5	0.5	5ml	0.5ml	0.013
E_2	0.5	0.5	5ml	0.5ml	0.007
E_3	0.5	0.5	5ml	0.5ml	0.001
E_4	0.5	0.5	5ml	0.5ml	0.002
E_5	0.5	0.5	5ml	0.5ml	0.003
E_6	0.5	0.5	5ml	0.5ml	0.005

NOTE : After adding Reagent C to all the tubes incubate in room temperature for 10 minutes and also after adding FC Reagent incubate all the test tubes for 30 minutes and then check the O.D.

ESTIMATION OF ASSAY OF AMYLASE ENZYME BY DNS METHOD

Aspergillus niger

TABLE - 4

Sl no.	Reagent B	Reagent G	Reagent E	Distilled water	O.D at 540nm
(Blank)	1ml	-----	1ml	9ml	0.00
$E_{1.}$	1ml	1ml	1ml	9ml	0.668
E_{2}.	1ml	1ml	1ml	9ml	0.490
E_{3}.	1ml	1ml	1ml	9ml	0.232
E_{4}.	1ml	1ml	1ml	9ml	0.081
E_{5}.	1ml	1ml	1ml	9ml	-0.04

NOTE: After adding reagent G mix the solution and incubate for 3 minutes at 20 ^{o}C and also after adding reagent E place in boiling water for 15 minutes and cool on ice to room temperature.

Aspergillus awamori

TABLE – 5

Sl no.	Reagent B	Reagent G	Reagent E	Distilled water	O.D at 540nm
(Blank)	1ml	-----	1ml	9ml	0.00
$E_{1.}$	1ml	1ml	1ml	9ml	0.731
E_{2}.	1ml	1ml	1ml	9ml	0.228
E_{3}.	1ml	1ml	1ml	9ml	0.091
E_{4}.	1ml	1ml	1ml	9ml	-0.040
E_{5}.	1ml	1ml	1ml	9ml	-0.01

NOTE: After adding reagent G mix the solution and incubate for 3 minutes at 20 ^{o}C and also after adding reagent E place in boiling water for 15 minutes and cool on ice to room temperature.

Standard curve

TABLE – 6

Sl no.	Reagent F	Distilled water	Reagent E	Distilled water	O.D at 540nm
(Blank)	0.0ml	2.0ml	1ml	9ml	0.00
1.	0.2ml	1.8ml	1ml	9ml	0.19
2.	0.4ml	1.6ml	1ml	9ml	0.57
3.	0.6ml	1.4ml	1ml	9ml	0.058
4.	0.8ml	1.2ml	1ml	9ml	0.80
5.	1.0ml	1.0ml	1ml	9ml	0.97

NOTE: After adding reagent E mix the solution and incubate for 15 minutes then cool at room temperature

V. DISCUSSION

The genus ***Aspergillus,*** through worldwide distribution is more prevalent in tropical countries. Conidia of *Aspergillus* are always present in the air and cause contamination in laboratory cultures of fungi. *A.niger* is called as 'weed of laboratory'. The various species are of great importance because of their harmful as well as useful activities. When *Aspergillus* infects lungs (Pulmonary Aspergillosis), the symptoms resemble tuberculosis. In England, in the year 1960, about 100,000 Turkey poults died by eating groundnuts infected with *Aspergillus flavus*. A toxin called **aflatoxins,** produced by *A. flavus* was responsible for the poultry deaths. Aflatoxins are however harmless to human beings. Studies of *Aspergillus niger* are used in the manufacture of citric acids, gluconic and itaconic acids species of *Aspergillus* are used for hydrolysis of starch into glucose in alcoholic fermentation by yeast. *Aspergillus nidulans* is the historical fungus in which the parasexual phenomenon was discovered in 1952 by Pontercorvo and Roper.

Species of *Aspergillus* are found in any conceivable habitat because of the bizarre enzymes that they produce. They cause spoilage of stored products such as grains and food stuffs – including jams and jellies, salted meat, fish and leather.

Alpha*-amylase** is used as a food enzyme for the hydrolysis of starch in the starch, sugar and alcoholic beverage industries. Genencor's *alpha*-amylase is produced with the use of a non-genetically modified strain of ***Bacillus stearothermophilus.

***Alpha*-amylases** have been approved and used for many years in food manufacture. There are currently a number of approved sources of alpha-amylases listed as processing aids in Standard 1.3.3 – Processing Aids. The Applicant contends that this *alpha*-amylase derived from *Bacillus stearothermophilus* has advantages over other approved enzymes in that it has greater thermal stability, produces a different sugar profile and is not derived from genetically modified organisms.

The purpose of this assessment is to determine whether it is appropriate to amend the code to permit the use of *alpha*-amylase derived from *Bacillus stearothermophilus* as a processing aid.

The only regulatory options considered were to approve or not approve this application. Approval of the use of this enzyme has advantages for food manufacturers by providing a different source of the *alpha*-amylase enzyme; one, which has greater thermal stability and produces a different sugar profile. There are no significant disadvantages to food manufacturers, consumers or government agencies.

Public comment on the Initial Assessment Report for this application was sought from 21 August till 2 October 2002. Three submissions were received with all supporting approval of the use of the enzyme – subject to an appropriate safety assessment as part of the Draft Assessment.

Public comment was sought on the Draft Assessment Report from 18 December 2002 till 12 February 2003. Two submissions were received, both of which supported the application.

The Final Assessment Report concludes that approval of the use of *alpha* - amylase derived from *Bacillus stearothermophilus* as a processing aid is technologically justified and does not raise any public health and safety concerns.

The draft variation to Standard 1.3.3 – Processing Aids of the Code, thereby giving approval for the use of *alpha*-amylase derived from *Bacillus stearothermophilus* as a processing aid is recommended for the following reasons.

- There are no public health and safety concerns associated with the use of this enzyme.
- The use of the *alpha*-amylase enzyme derived from *Bacillus stearothermophilus* technologically justified since it has a role in food manufacturing, primarily with starch hydrolysis. This enzyme has greater thermostability and a different sugar profile.

- The source organism (*Bacillus stearothermophilus*) has a long history of safe use.
- The *alpha*-amylase enzyme has a history of safe use for many years in Australia and New Zealand.
- The enzyme *alpha*-amylase derived from *Bacillus stearothermophilus* complies with the specifications for enzyme preparations in Food Chemicals Codex (4th Edition, 1996) and the Joint Expert Committee on Food Additives (JECFA) Compendium of Food Additives Specifications, Vol. 1, Annex 1, FAO 1992, (updated in Addendum 9, 2001).
- The proposed draft variation to the Code is consistent with the section 10 objectives of the FSANZ Act.
- The regulation impact assessment has concluded that the benefits of permitting use of the enzyme outweigh any costs associated with its use.

VI. SUMMARY

Amylase is the unique class of enzymes, since they are the immense physiological as well as commercial importance. They possess both degradative and synthetic properties. Since amylases are physiologically necessary, they occur ubiquitously in animals, plants and microbes. However microbes are gold mine of amylases and represent the preferred sources of enzymes in view of their rapid growth, limited space required for their cultivation and ready accessibility to genetic manipulation. Studies were carried out to purify and characterize the amylases from Aspergillus niger and Aspergillus awamori to improve the strain of amylase production. The fungal isolates were inoculated in various media for the mass production of amylases. The broths that showed maximum amylase activity were chosen and purified to homogeneity by salt and **solvent precipitation**, **Ion exchange chromatography** and **SDS – PAGE**. Molecular weights of amylases were determined by SDS – PAGE.

Immobilization of enzymes enables their efficient and continuous use. The rationale behind immobilization is the easy separation of product from the biocatalyst. Enzymes may be immobilized by adsorption, covalent binding, entrapment and membrane confinement, each method having its pros and cons. Adsorption is quick, simple and cheap but may be reversible. Covalent binding is permanent but expensive. Entrapment is generally applicable but may cause diffusional problems. Membrane confinement is a flexible method but expensive to set up.

Immobilization of enzymes may have a considerable effect on their kinetics. This may be due to structural changes to the enzyme and the creation of a distinct microenvironment around the enzyme. The activity of an immobilized enzyme is governed by the physical conditions within this microenvironment not those prevalent in the bulk phase. The immobilization matrix affects the partition of material between the product phase and the enzyme phase and imposes restrictions on the rate of diffusion of material.

Some effects of enzyme immobilization are seen to be beneficial whilst others are detrimental to the economics of their use.

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- **www.whfreeman.com**

APPENDIX

LAB RULES:

The following basic steps should be observed at all times to reduce the ever-present microbial flora of the lab environment:

1. On entering the lab, place books and enter paraphernalia in specified locations and never on the bench top.
2. Keep doors and windows closed during the lab session to prevent contamination of air currents.
3. At the beginning and termination of each lab session, wipe bench tops with a disinfectant solution.
4. Do not place contaminated instruments such as inoculation loop, needles and pipette tips on bench tops.
5. On completion of lab session place all cultures and materials in the disposal area as designated by the instructor.

To prevent accidental injuries and infections, the following instructions have to be followed at all times:

1. Wash your hands with liquid detergents and dry them with paper towels upon entering and prior to leaving the lab.
2. Wear the paper cap or tie back long hairs to minimize the exposure to open flames.
3. Wear a lab coat or apron while working in the lab to prevent clothing from contamination or accidental discoloration by staining solutions.
4. Do not wear daily use footwear; maintain a pair of separate and clean slippers exclusively for laboratory usage.
5. Never apply cosmetics or put on contact lenses in the lab.

6. Smoking, eating & drinking are strictly prohibited.
7. To prevent accidents and prevent contamination of yourself & the environment, carry cultures in test tube racks while moving around the lab.
8. Report accidental cuts or burns to the instructor immediately.
9. Never pipette by mouth any broth cultures or chemical reagents.
10. Speak quietly and avoid unnecessary movements in lab.

INSTRUMENTS USED

- **AUTOCLAVE**
- **COOLING CENTRIFUGE**
- **EPPENDORF TUBES**
- **HEATING MANTLE**
- **ION EXCHANGE CHROMATOGRAPHY**
- **LAMINAR AIR FLOW**
- **MAGNETIC STIRRER**
- **pH METER**
- **SDS – PAGE UNIT**
- **SPECTROPHOTOMETER**

AUTOCLAVE

An **autoclave** is a pressurized device designed to heat aqueous solutions above their boiling point to achieve sterilization. It was invented by Charles Chamberland in 1879. The term **autoclave** is also used to describe an industrial machine in which elevated temperature and pressure are used in processing materials.

Introduction

Under ordinary circumstances (at standard pressure), liquid water cannot be heated above 100 °C in an open vessel. Further heating results in boiling, but does not raise the temperature of the liquid water. However, when water is heated in a sealed vessel such as an autoclave, it is possible to heat liquid water to a much higher temperature. As the container is heated the pressure rises due to the constant volume of the container (see the ideal gas law). The boiling point of the water is raised because the amount of energy needed to form steam against the higher pressure is increased. This works well on solid objects; when autoclaving hollow objects, however, (hypodermic needles, tools, etc.), it is important to ensure that all of the trapped air inside the hollow compartments is vacuumed out.

Simple autoclaves use a single pulse pre-vacuum, while a modern day autoclave has fractioned pre-vacuum that pulls the air out in several stages to achieve 100% steam penetration in the sterilization process.

Autoclaves are widely used in microbiology, medicine, veterinary science, dentistry and metallurgy. The large carbon-fiber composite parts for the Boeing 787, such as wing and fuselage parts, are cured in large autoclaves.

Autoclaves in medicine

A medical autoclave is a device that uses steam to sterilize equipment and other objects. This means that all bacteria, viruses, fungi, and spores are inactivated. However, in 2003

scientists discovered a single-celled organism, Strain 121 that survives traditional autoclave temperatures. Prions, like those associated with Creutzfeldt-Jakob disease, also may not be destroyed by autoclaving.

Autoclaves are found in many medical settings and other places that need to ensure sterility of an object. They were once more common, but many procedures today use single-use items rather than sterilized, reusable items. This first happened with hypodermic needles, but today many surgical instruments (such as forceps, needle holders, and scalpel handles) are commonly single-use items rather than reusable.

Because damp heat is used, heat-labile products (such as some plastics) cannot be sterilized this way or they will melt. Some paper or other products that may be damaged by the steam must also be sterilized another way. In stovetop autoclaves, items should always be separated to allow the steam to penetrate the load evenly.

Chemiclave

Unlike the humid environment produced by conventional steam, the unsaturated chemical vapor method is a low-humidity process. No time-consuming drying phase is needed, because nothing gets wet. The heat-up time is shorter than for most steam sterilizers, and the heaters stay on between cycles to minimize warm-up time and increase the instrument turnover.

Autoclave quality assurance

Sterilization bags often have a "sterilization indicator mark" that typically darkens when sterilization temperatures have been reached. Comparing the mark on an unprocessed bag to a bag that has been properly cycled will show an obvious visual difference.

There are physical, chemical, and biological indicators that can be used to ensure an autoclave reaches the correct temperature for the correct amount of time.

Chemical indicators can be found on medical packaging and autoclave tape, and these change color once the correct conditions have been met. This color change indicates that the object inside the package, or under the tape, has been autoclaved sufficiently. Biological indicators include Attest devices. These contain spores of a heat-resistant bacterium, *Goebacillus stearothermophilus.* If the autoclave does not reach the right temperature, the spores will germinate, and their metabolism will change the color of a pH-sensitive chemical. Physical indicators often consist of an alloy designed to melt only after being subjected to 121 °C for 15 minutes. If the alloy melts, the change will be visible.

In addition to these indicators, autoclaves have temperature and pressure gauges that can be viewed from the outside.

There are certain plastics that can withstand repeated temperature cycling greater than the 121 °C required for the autoclaving process. PFA is an example.

Some computer-controlled autoclaves use an F_0 (F-naught) value to control the sterilization cycle. F_0 values are set as the number of minutes of equivalent sterilization at 121 °C (e.g.: F_0 = 15 min.). Since exact temperature control is difficult, the temperature is monitored, and the sterilization time adjusted accordingly.

COOLING CENTRIFUGE

A **centrifuge** is a piece of equipment, generally driven by a motor that puts an object in rotation around a fixed axis, applying force perpendicular to the axis. The centrifuge works using the sedimentation principle, where the centripetal acceleration is used to separate substances of greater and lesser density. There are many different kinds of centrifuges, including those for very specialized purposes.

Theory

Protocols for centrifugation typically specify the amount of acceleration to be applied to the sample, rather than specifying a rotational speed such as revolutions per minute. The acceleration is often quoted in multiples of g, the acceleration due to gravity at the Earth's surface. This distinction is important because two rotors with different diameters running at the same rotational speed will subject samples to different accelerations. The acceleration can be calculated as the product of the radius and the square of the angular velocity.

History and predecessors

English military engineer Laval (1707-1751) invented a whirling arm apparatus to determine drag, and **Antonin Prandl** invented the first centrifuge in order to separate cream from milk to make churning butter much easier.

Types and uses

There are basically four types of centrifuge:

- Tabletop/clinical/desktop centrifuge or micro centrifuge
- High-speed centrifuge
- Cooling centrifuge
- Ultra-centrifuge

Biology and biochemistry

Simple centrifuges are used in biology and biochemistry for isolating and separating biomolecules, cell organelles, or whole cells. They vary widely in speed and capacity. They usually comprise a rotor containing two, four, six, or many more numbered wells within which centrifuge tips may be placed.

The rotor is covered by a plastic cover. The cover is usually interlocked to prevent the motor from turning the rotor when it is open, and from allowing the cover to be opened before the rotor stops for several minutes. The cover protects the user from being injured by touching a rapidly spinning rotor. It also protects the user from fragments in case the rotor fails catastrophically.

To ensure that the rotor does not turn unbalanced, it must be balanced by placing samples or blanks of equal mass opposite each other. Since most of the mass is derived from the solvent, it is usually sufficient to place blanks or other samples of equal volume. As a safety feature, some centrifuges may stop turning when wobbling is detected.

The centrifuge tips are usually made of plastic or glass; they may vary in capacity from tens of milliliters, to much smaller capacities used in micro centrifuges used extensively in molecular biology laboratories. These micro centrifuges typically accommodate micro centrifuge tubes with capacities from 250 µl to 2.0 ml.

The ultracentrifuge was invented in 1925 by **Theodor Svedberg**, which by use of very high acceleration, and allowing the observation of sedimentation rates for macromolecules, allowed for the determination of their approximate molecular weights. Svedberg's 1926 Nobel Prize in Chemistry was earned for work made possible using his ultracentrifuge. Isotope separation

Other centrifuges, the first being the Zippe-type centrifuge, are used to separate isotopes, and these kinds of centrifuges are in use in nuclear power and nuclear weapon programs.

Gas centrifuges are used in uranium enrichment. The heavier isotope of uranium (uranium-238) in the uranium hexafluoride gas tend to concentrate at the walls of the centrifuge as it spins, while the desired uranium-235 isotope is extracted and concentrated with a scoop selectively placed inside the centrifuge. It takes many thousands of centrifuges to enrich uranium enough for use in a nuclear reactor (around 3.5% enrichment), and many thousands more to enrich it to atomic bomb-grade (around 90% enrichment).

Commercial applications

- Standalone centrifuges for drying (hand-washed) clothes - usually with a water outlet.
- Centrifuges are used in the attraction Mission: SPACE, located at Epcot in Walt Disney World, which propels riders using a combination of a centrifuge and a motion simulator to simulate the feeling of going into space.
- In soil mechanics, centrifuges utilize centrifugal acceleration to match soil stresses in a scale model to those found in reality.
- Large industrial centrifuges are commonly used in water and wastewater treatment to dry sludge. The resulting dry product is often termed **cake**, and the water leaving a centrifuge after most of the solids have been removed is called **centrate**.

Use and safety

The load in a laboratory centrifuge must be carefully balanced. Small differences in mass of the load can result in a large force imbalance when the rotor is at high speed. This force imbalance strains the spindle and may result in damage to centrifuge or personal injury.

Centrifuge rotors should never be touched while moving, because a spinning rotor can cause serious injury. Modern centrifuges generally have features that prevent accidental contact with a moving rotor.

Because of the kinetic energy stored in the rotor head during high speed rotation, those who have experienced the loss of a rotor inside of an ultracentrifuge compare the experience to having a bomb explode nearby.

Calculating relative centrifugal force (RCF)

Relative centrifugal force is the measurement of the force applied to a sample within a centrifuge. This can be calculated from the speed (RPM) and the rotational radius (cm) using the following calculation.

$$\mathbf{g = RCF = 0.00001118 \times r \times N^2}$$

where

g = Relative centrifuge force
r = rotational radius (centimeter, cm)
N = rotating speed (revolutions per minute, r/min).

EPPENDORF TUBES

These are miniature test tubes with safety lids. They are made up of heat resistant, non-corrosive, non-reactive plastic. These Safe – Lock Eppendorf tubes ensure absolute safety while working with expensive or toxic samples, radioactive substance or DNA. The 0.5 ml, 1.5 ml & 2.0ml test tubes have volume graduations and etched making surfaces.

Special features:

1. Easy- open, easy- close design provides convenient & reliable sample preparation, centrifugation and storage.
2. Tubes can be autoclaved when open (121°C, 20 minutes).
3. The lid hook prevents the tube from opening automatically (e.g. during temperature control processes).
4. Rated to 25,000 X g for excellent mechanical stability during centrifugation.
5. They have volume graduations, economically priced etched marking on surfaces and are sterile.

Applications:

1. Recombinant DNA technology.
2. Medical, pharmaceutical and food industries.
3. PCR techniques, Molecular biology & cell technology.

HEATING MANTLE

Heating mantles are used to heat flasks containing a sample. Heating mantles are used in place of Bunsen burners or hot plates when the sample is an organic liquid. Organic liquids have a tendency to burst into flames when exposed to direct heat, or release flammable organic vapors are heavier than air, which can explode when contacting open flames or heating coils. Mantles have an enclosed heating system, which is designed to avoid these dangers. Heating mantles are available in either fabric or rigid constructions. Fabric mantles are flexible, and can be molded to accommodate a wide variety of flask shapes and sizes. The holding receptacles within rigid heating mantles are shaped either for a particular size flask, or simply have a large orifice that can be filled with sand to hold various size flasks. These sand baths serve to also provide a more uniform heating of the sample.

Fabric mantles are generally cheaper then rigid mantles, although they are susceptible to damage from spilled liquids. The covering fabric layered can be torn or frayed, by exposure to hot liquids or corrosives, exposing the wiring beneath. This can lead to a fire hazard.

Heating mantles produce their heat by converting AC voltage. The intense energy draw needed to generate the high degree of required heat, can blow fuses or melt socket wiring. For this reason, heating mantles should never be plugged directly into a wall socket.

Heating mantles are available in the following configurations: mantle and controller unit, mantle only, or controller only. Mantle and controller devices include both the mantle itself and the device used to controller it. This configuration may be either a single unit; a mantle with an integral controller or it may be comprised of two separate parts, sold together. Mantle only devices are just the actual container for holding a flask; there is no temperature controller. Controller only devices are not mantles per se. They are simply the instrumentation board for controlling the heating of a mantle.

ION EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography (or *ion chromatography*) is a process that allows the separation of ions and polar molecules based on the charge properties of the molecules. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids, with the experimental solution to be separated collectively known as the ***analyte***. It is often used as a first step in protein purification.

History

Ion methods have been in use since 1850, when **H. Thompson** and **J. T. Way**, researchers in England, treated various clays with ammonium sulfate or carbonate in solution to extract the ammonia and release calcium. In 1927, the first **zeolite** mineral column was used to remove interfering calcium and magnesium ions from solution to determine the sulfate content of water. The modern version of IEC was developed during the wartime Manhattan Project. A technique was required to separate and concentrate the radioactive elements needed to make the atom bomb. Researchers chose adsorbents that would latch onto charged transuranium elements, which could then be differentially eluted. Ultimately, once declassified, these techniques would use new IE resins to develop the systems that are often used today for specific purification of biologicals and inorganics. In the early 1970s, ion chromatography was developed by Hamish Small and co-workers at Dow Chemical Company as a novel method of IEC usable in automated analysis. IC uses weaker ionic resins for its stationary phase and an additional neutralizing stripper, or suppressor, column to remove background eluent ions. It is a powerful technique for determining low concentrations of ions and is especially useful in environmental and water quality studies, among other applications.

The Dow Chemical Company technology was acquired by Durrum Instrument Corp. (maker of the Durrum_D-500), which later formed a separate business unit for its new IC products, naming it Dionex (**D**ow **Ion Ex**change). Dionex

Corporation was incorporated in Sunnyvale, California in 1980, and, led by A. Blaine Bowman, purchased the Dionex assets.

Principle

Ion exchange chromatography retains analyte molecules based on coulombic (ionic) interactions. The stationary phase surface displays ionic functional groups that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography:

- Cation exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group such as a phosphoric acid.
- Anion exchange chromatography retains negatively charged anions using positively charged functional group such as a quaternary ammonium cation.

Separating Proteins

Proteins have numerous functional groups that can have both positive and negative charges. Ion exchange chromatography separates proteins according to their net charge, which is dependent on the composition of the mobile phase. By adjusting the pH or the ionic concentration of the mobile phase, various protein molecules can be separated. For example, if a protein has a net positive charge at pH 7, then it will bind to a column of negatively-charged beads, whereas a negatively charged protein would not. By changing the pH so that the net charge on the protein is negative, it too will be eluted.

Elution by changing the ionic strength of the mobile phase is a more subtle effect - it works as ion from the mobile phase will interact with the immobilized ion in preference over those on the stationary phase. This "shields" the stationary phase from the protein (and vice versa) and allows the protein to elute.

Typical Technique

A sample is introduced, either manually or with an autosampler, into a sample loop of known volume. A buffered aqueous solution known as the mobile phase carries the sample from the loop onto a column that contains some form of stationary phase material. This is typically a resin or gel matrix consisting of agarose or cellulose beads with covalently bonded charged functional groups. The target analytes (anions or cations) are retained on the stationary phase but can be eluted by increasing the concentration of a similarly charged species that will displace the analyte ions from the stationary phase. For example, in cation exchange chromatography, the positively charged analyte could be displaced by the addition of positively charged sodium ions. The analytes of interest must then be detected by some means, typically by conductivity or UV/Visible light absorbance.

In order to control an IC system, a Chromatography Data System (CDS) is usually needed. In addition to IC systems, some of these CDSs can also control Gas Chromatography (GC) and HPLC systems.

LAMINAR AIR FLOW

Contamination is a major problem f aced by microbiologist in routine procedure such as inoculation of media, distribution of sterile media, sub culturing of organism should be performed under aseptic conditions. This is done in laminar air flow, which is placed in a sterile room. The room is closed so as to prevent any current or graft of air from entering and causing contamination. The ultraviolet lamp present in the laminar air flow sterilizes the chamber. These filters usually remove 99% of all particles, including micro- organisms having a diameter of over 0.3 micrometer. The air entering surgical units and specialized treatment facilities such as burnt units is filtered to exclude micro-organisms. In some hospital wards, such as the ones for respiratory disease and in certain pharmaceutical filling room. The air is recirculated through **HEPA** filters to ensure its purity.

The **HEPA** filters are fixed in an instrument called laminar air flow bench. The technique involves sucking in of air of the room and blowing out the air through the layer of HEPA filters with uniform velocity and in parallel flow lines. Both horizontal and vertical laminar air flow systems are available. The advantage of the system is that in laboratory works involving inoculation, transfer of culture and opening of lyophilized culture, need not be done in closed chamber or inoculating hood, instead the operation can be done on the platform provided with laminar air flow unit, making it very easy to handle.

MAGNETIC STIRRER

A **magnetic stirrer** consists of a small bar magnet (or stir bar), which is normally coated in a plastic such as **PTFE** and a stand or plate containing a rotating magnet or stationary electromagnets creating a rotating magnetic field. Often, the plate can also be heated. During operation of a typical magnetic stirrer, the bar magnet (or flea) is placed in a vessel containing a liquid to be stirred. The vessel is set on top of the stand, where the rapidly rotating magnetic field causes the bar magnet to rotate.

Arthur Rosinger of Newark, New Jersey, U.S.A. obtained US Patent 2,350,534, titled Magnetic Stirrer on 06 June 1944, having filed an application therefore on 05 October 1942. Mr. Rosinger's patent includes a description of a coated bar magnet placed in a vessel, which is driven by a rotating magnet in a base below the vessel. Mr. Rosinger also explains in his patent that coating the magnet in plastic or covering it with glass or porcelain makes it chemically inert.

The plastic-coated bar magnet was independently invented in the late 1940s by Edward McLaughlin, of the Torpedo Experimental Establishment (TEE), Greenock, Scotland, who named it the 'flea' because of the way it jumps about if the rotating magnet is driven too fast.

An even earlier patent for a magnetic mixer is US 1,242,493, issued 09 October 1917 to Richard H. Stringham of Bountiful, Utah, U.S.A. Mr. Stringman's mixer used stationary electromagnets in the base, rather than a rotating permanent magnet, to rotate the stirrer.

The first multipoint magnetic stirrer was developed and patented by Salvador Bonet of SBS Company in 1977. He also introduced the practice of noting the denomination of stirring power in "liters of water", which is a market standard today.

Magnetic stirrers are preferred over gear-driven motorized stirrers in chemical research because they are quieter, more efficient, and have no moving parts to break or wear out

(other than the simple bar magnet itself). Due to the small size, the stirring bar is more easily cleaned and sterilized than other stirring devices.

Magnetic stirrers solve two major problems with using motorized stirrers. Firstly, motorized stirrers use lubricants, which can contaminate the vessel and the product. Secondly, in motorized stirrers, the sealing of the connection between the rotating shaft of the stirrer and the vessel can be a problem, especially if a closed system is needed (e.g. due to OSHA, or environmental regulations, or because a process works only if oxygen, water or dust is absent).

The magnetic stirrer also has its drawbacks: the limited size of the stirring bar means it can only be used for lab size experiments. In addition, viscous liquids or thick suspensions are extremely difficult to stir using this method, although there are some models with special magnets to overcome this problem.

pH METER

A **pH meter** is an electronic instrument used to measure the pH (acidity or basicity) of a liquid (though special probes are sometimes used to measure the pH of semi-solid substances, such as cheese). A typical pH meter consists of a special measuring probe (a glass electrode) connected to an electronic meter that measures and displays the pH reading.

A simple pH meter with its probe immersed in a mildly alkaline solution. The two knobs are used to calibrate the instrument.

The Probe

The pH probe measures pH as the activity of hydrogen ions surrounding a thin-walled glass bulb at its tip. The probe produces a small voltage (about 0.06 volt per pH unit) that is measured and displayed as pH units by the meter. For more information about pH probes, see glass electrode.

The Meter

The meter circuit is fundamentally no more than a voltmeter that displays measurements in pH units instead of volts. The input impedance of the meter must be very high because of the high resistance—approximately 20 to 1000 MΩ (Megohms see ohm) —of the glass electrode probes typically used with pH meters. The circuit of a simple pH meter usually consists of operational amplifiers in an inverting configuration, with a total voltage gain of about -17. The inverting amplifier converts the small voltage produced by the probe (+0.059 volt/pH in basic solutions, -0.059 volt/pH in acid solutions) into pH units, which are then offset by 7 volts to give a reading on the pH scale. For example:

- At neutral pH (pH 7) the voltage at the probe's output is 0 volts. 0 * 17 + 7 = 7.
- At alkaline pH, the voltage at the probe's output ranges from > 0 to +0.41 volts (7 * 0.059 = 0.41). So for a sample of pH 10 (3 pH units from neutral), 3 * 0.059 = 0.18 volts), the output of the meter's amplifier is 0.18 * 17 + 7 = 10.

- At acid pH, the voltage at the probe's output ranges from -0.7 volts to < 0. So for a sample of pH 4 (also 3 pH units from neutral, but in the other direction), 3 * -0.059 = -0.18 volts, the output of the meter's amplifier is -0.18 * 17 + 7 = 4.

The two basic adjustments performed at calibration (see below) set the gain and offset of the inverting amplifier.

Calibration and Use

Calibration with at least two, but preferably three, buffer solution standards is usually performed every time a pH meter is used, though modern instruments will hold their calibration for around a month. One of the buffers has a pH of 7.01 (almost neutral pH) and the second buffer solution is selected to match the pH range in which the measurements are to be taken: usually pH 10.01 for basic solutions and pH 4.01 for acidic solutions (It should be noted that the pH of the calibration solutions is only valid at 25°C). The gain and offset settings of the meter are adjusted repeatedly as the probe is alternately placed in the two calibration standards until accurate readings are obtained in both solutions. Modern instruments have completely automated this process and only require immersing in each solution once, or at worst, twice.

The calibration process correlates the voltage produced by the probe (approximately 0.06 volts per pH unit) with the pH scale. After calibration, the probe is rinsed in distilled, deionized water to remove any traces of the buffer solution, blotted with a clean tissue to absorb any remaining water which could dilute the sample and thus alter the reading, and then quickly immersed in the sample. Between uses, the probe tip, which must be kept wet at all times, is typically kept immersed in a small volume of storage solution, which is an acidic solution of around pH 3.0. Alternatively, the pH 7.01 calibration solution can be

used, but this results in a need for more frequent calibration. In an emergency, tap water can be used, but distilled or deionised water must *never* be used for longer-term probe storage as the relatively ionless water 'sucks' ions out of the probe, which degrades it.

Occasionally (about once a month), the probe should be cleaned using pH-electrode cleaning solution; generally a 0.1-Msolution of Hydrochloric Acid (HCl) is used, having a pH of about 1.

SDS – PAGE UNIT

SDS – PAGE is the most widely used method for qualitative analyzing any protein mixture, monitoring protein purity and to determine their molecular weights. It is based on the separation of protein according to their size and locating them by binding to a dye.

The analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly the strongly anionic detergent **Sodium Dodecyl Sulphate (SDS)** is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is always is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS – polypeptide complexes migrate through polyacrylamide gels in accordance with the size of polypeptide. At saturation, approximately 1.4g of detergent is bound per gram of polypeptide. By using markers of known molecular weight of the polypeptide chains.

SDS polyacrylamide gel electrophoresis is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gel. The SDS – polypeptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when an electric current is passed between the electrodes. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone on the surface of the resolving gel. The ability of the discontinuous buffer systems to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS poly-acryl amide gels.

The sample and the stacking gel contain Tris-HCl (pH 6.8), the upper and lower buffer reservoirs contain Tris-Glycine (pH 8.3) and the resolving gel contains Tris HCl (pH 8.8). All components of the system contain 0.1% SDS. The chloride ions in the sample and stacking gel from the leading edge of the moving boundary and the trailing edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient which sweeps the polypeptides from the sample and deposits them on the surface of the resolving gel. There, the higher pH of the resolving gel favors the ionization of Glycine and the resulting Glycine ions migrate through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. Freed from the moving boundary, the SDS – poly – acrylamide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size by sieving.

Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross – linked by a bifunctional agent such as **N, N' - Methylene – Bisacrylamide**. The effective range of separation of SDS – poly – acrylamide gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross – linking.

SPECTROPHOTOMETER

In physics, **spectrophotometry** is the quantitative study of electromagnetic spectra. It is more specific than the general term electromagnetic spectroscopy in that spectrophotometry deals with visible light, near-ultraviolet, and near-infrared. Also, the term does not cover time-resolved spectroscopic techniques.

Spectrophotometry involves the use of a spectrophotometer. A **spectrophotometer** is a photometer (a device for measuring light intensity) that can measure intensity as a function of the color, or more specifically, the wavelength of light. There are many kinds of spectrophotometers. Among the most important distinctions used to classify them are the wavelengths they work with, the measurement techniques they use, how they acquire a spectrum, and the sources of intensity variation they are designed to measure. Other important features of spectrophotometers include the spectral bandwidth and linear range.

Perhaps the most common application of spectrophotometers is the measurement of light absorption, but they can be designed to measure diffuse or specular reflectance. Strictly, even the emission half of a luminescence instrument is a kind of spectrophotometer.

There are two major classes of spectrophotometers; single beam and double beam. A double beam spectrophotometer measures the ratio of the light intensity on two different light paths, and a single beam spectrophotometer measures the absolute light intensity. Although ratio measurements are easier, and generally stabler, single beam instruments have advantages; for instance, they can have a larger dynamic range.

Visible-region spectrophotometers

Visible region 400-700nm spectrophotometry is used extensively in colorimetry science. Ink manufacturers, printing companies, textiles vendors, and many more, need the data provided through colorimetry. They usually take readings every 20 nanometers along the visible region, and produce a spectral reflectance curve. These curves can be used to test

a new batch of colorant to check if it makes a match to specifications. Traditional visual region spectrophotometers cannot detect if a colorant has fluorescence. This can make it impossible to manage color issues if one or more of the printing inks are fluorescent. Where a colorant contains fluorescence, a bi-spectral fluorescent spectrophotometer is used. There are two major setups for visual spectrum spectrophotometers, d/8 (spherical) and 0/45. The names are due to the geometry of the light source, observer and interior of the measurement chamber. Scientists use this machine to measure the amount of compounds in a sample. If the compound is more concentrated more light will be absorbed by the sample.

components: 1. The light source shines through the sample. 2. The sample absorbs light. 3. The detector detects how much light the sample has absorbed. 4. The detector then converts how much light the sample absorbed into a number.

Spectroradiometers

Spectroradiometers, which operate almost like the visible region spectrophotometers, are designed to measure the spectral density of illuminants in order to evaluate and categorize lighting for sales by the manufacturer, or for the customers to confirm the lamp they decided to purchase is within their specifications.

components: 1. The light source shines onto or through the sample. 2. The sample transmits or reflects light. 3. The detector detects how much light was reflected from or transmitted through the sample. 4. The detector then converts how much light the sample transmitted or reflected into a number.

UV and IR spectrophotometers

The most common spectrophotometers are used in the UV and visible regions of the spectrum and some of these instruments also operate into the near-infrared region as well.

Spectrophotometers designed for the main infrared region are quite different because of the technical requirements of measurement in that region. One major factor is the type of photosensors that are available for different spectral regions, but infrared measurement is also challenging because virtually **everything** emits IR light as thermal radiation, especially at wavelengths beyond about 5 μm.

Historically, spectrophotometers use a monochromator to analyze the spectrum, but there are also spectrophotometers that use arrays of photosensors and, especially in the IR, there are spectrophotometers that use a Fourier transform technique to acquire the spectral information in a technique called Fourier Transform Infrared.

The spectrophotometer measures quantitatively the fraction of light that passes through a given solution. In a spectrophotometer, a light from a lamp in a near-IR/VIS/UV spectrophotometer (typically a deuterium gas discharge lamp) is guided through a monochromator, which picks light of one particular wavelength out of the continuous spectrum. This light passes through the sample that is being measured. After the sample, the intensity of the remaining light is measured with a photodiode or other light sensor, and the transmittance for this wavelength is then calculated.