"EXTRACTION AND PARTIAL PURIFICATION OF BETA AMYLASE FROM SWEET POTATO"

Dissertation Submitted by

ALPHINE THOMAS

(REGISTER NUMBER: VM20FPT003)

In partial fulfilment of the degree of

MASTER OF VOCATIONAL STUDIES

IN

FOOD PROCESSING TECHNOLOGY

ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

Affiliated to Mahatma Gandhi University, Kottayam

Under the guidance of

Dr. Prabhakumari C

Dy. Principal Scientist

Undertaken at

DEPARTMENT OF BIOTECHNOLOGY

CEPCI - CASHEW EXPORT PROMOTION COUNCIL OF INDIA, KOLLAM

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DECLARATION

I, **Alphine Thomas (VM20FPT003)** hereby declare that this project entitled **"EXTRACTION AND PARTIAL PURIFICATION OF BETA AMYLASE FROM SWEET POTATO"** is a bonafide record of the project work done by us during the course of study and that the report has not previously formed the basis for the award to us for any master degree,diploma, fellowship or other title of any other university or society.

Place: ALPHINE THOMAS

ACKNOWLEDGEMENT

The successful completion of any task would be incomplete without the mention of the people who made it possible, whose constant guidance and encouragement crowned my efforts with success.

At the outset I thank the **Almighty** for bestowing his blessings and grace which enabled the successful completion of the project. I would like to express my gratitude and sincere thanks to our esteemed Director **Rev. Sr. Emeline** CSST(St.Teresas College Ernakulam)and **Dr. Alphonsa Vijaya Joseph** (Principal,St.Teresas college Ernakulam) for providing us with the necessary support and infrastructure.

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Alphine Thomas

ABSTRACT

Enzyme activity plays a key role in each stage of plant development. Beta amylase is a starch degrading enzyme which was extracted from Sweet potato and partially purified. Beta amylase is different from alpha amylase, Beta amylase act on starch molecule from non reducing end, it yields maltose after hydrolysis of starch. Sweet potato is a rich source of Beta amylase present in tuberous root of Sweet potato .Beta amylase was extracted and precipitated by ammonium sulphate salt and partially purified by dialysis bag.Assays for the presence of Amylase were performed including starch hydrolysis confirmatory test and iodine test.

Key words: Sweet potato, Beta amylase, Extraction, partial purification

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ABBREVATIONS AND SYMBOLS

Wt - Weight

L - Liter

% - Percentage

g - Gram

µl - Microliter

ml - Milli liter

°C - degree Celsius

g/l - Grams Per Litre

V_{max} –Maximum Velocity

Hr- Hour

CHAPTER 1

1.INTRODUCTION

Enzymes are biological catalysts which regulate specific biochemical reactions. In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms. Among the industrially important enzymes, proteases and amylases are considered to be the most prominent enzymes since they are widely utilized in brewing, detergent, and food industries. Amylases are employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents. With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many new fields such as clinical, medicinal, and analytical chemistry . Starch processing, which is undertaken in two steps, involves liquefaction of the polysaccharide using bacterial α-amylase, followed by saccharification catalyzed by fungal glucoamylase. After the World War II, enzyme applications increased due to advances in industrial microbiology and biochemical engineering. Now a days, enzymes are employed in many different areas such as food, feed, detergent, textiles, laundry, tanning, as well as pharmaceuticals, cosmetics, and finechemicals industries. Industrial applications account for over 80% of the global market of enzymes. At least 50% of the enzymes marketed today are obtained from genetically modified organisms, employing genetic and protein engineering. Food enzymes are the most widely used and still represent the majorshare in enzyme market. Enzymes are now being used in various sectors of industry. They are used in detergents, paper industry, textile industry, food industry and many others industrial applications. Enzymes have been in use since ancient times and they have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders and production of cheese from milk . Among the many enzymes that are widely used α-Amylase has been in increasing demand due to its crucial role of starch hydrolysis and the applications of this hydrolytic action. The following sections elaborate on the types of amylases and their roles in enzymatic reactions.

1.1AMYLASES

Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing α-1,4glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Bernfeld, 1955; Fisher and Stein, 1960; Myrback and Neumuller, 1950). They act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved. In recent years a number of new enzymes associated with degradation of starch and related polysaccharides structures have been detected and studied (Boyer and Ingle, 1972; Buonocore et al., 1976; Griffin and Fogarty, 1973; Fogarty and Griffin, 1975).

1. The enzymes of actual or potential commercial importance of microbial origin that split α-1,4 or α-1,4 and/or α-1,6 bonds in these structures, may be divided in the following six classes (Fogarty and Kelly, 1979)

2. Enzymes that hydrolyze α-1,4 bonds and bypass α-1,6 linkages e.g. α-amylase (endoacting amylases).

3. Enzymes that hydrolyze α-1,4 and cannot bypass α1,6 linkages e.g. β-amylase (exoacting amylases producing maltose as a major end product).

4. Enzymes that hydrolyse α-1,4 and α-1,6 linkages e.g. amyloglucosidase (glucoamylase) and exoacting amylase.

5. Enzymes that hydrolyze only α-1,6 linkages e.g. pullulanase and other debranching enzymes.

6. Enzymes that hydrolyze preferentially α-1,4 linkages in short chain oligosaccharides produced by the action of other enzymes on amylose and amylopectin e.g. α-glucosidases.

7. Enzymes that hydrolyse starch to a series of nonreducing cyclic D-glucosyl polymers called cyclodextrins or Sachardinger dextrins e.g. Bacillus macerans amylase (cyclodextrin producing enzyme).

1.1.1Types of Amylase

α-Amylase

α-Amylase is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its activity [4]. There are 2 types of hydrolases: endo-hydrolase and exohydrolase. Endo- hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends [2]. Hence, terminal glucose residues and α-1, 6-linkages cannot be cleaved by αamylase. The substrate that α-amylase acts upon is starch. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by α-1, 4-glycosidic linkage. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by α1, 4-glycosidic linkage while branching occurs every 15- 45 glucose units where α-1, 6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. The optimum pH for activity is found to be 7.0 [4].α-Amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch. α-Amylase catalyses the first step in this process. Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures. These limitations are overcome by enzyme hydrolysis of starch to yield high fructose syrup. The use of enzymes in detergents formulations has also increased dramatically with growing awareness about environment protection. Enzymes are environmentally safe and enhance the

detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter. There are many such applications of the enzyme which is the driving force behind the research to produce this enzyme in an optimum, safe and convenient manner

β – Amylase

β-Amylase is an exo-hydrolase enzyme that acts from the nonreducing end of a polysaccharide chain by hydrolysis of α-1, 4-glucan linkages to yield successive maltose units. Since it is unable to cleave branched linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. Primary sources of β-Amylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, β-Amylase breaks down starch into maltose resulting in the sweetness of ripened fruit. The optimal pH of the enzyme ranges from 4.0 to 5.5. βAmylase can be used for different applications on the research as well as industrial front. It can be used for structural studies of starch and glycogen molecules produced by various methods. In the industry it is used for fermentation in brewing and distilling industry. Also, it is used to produce high maltose syrups

1.2APPLICATIONS OF AMYLASES

The history of the industrial production of enzymes dates back to the time when Dr. Jhokichi Takamine began the production of digestive enzyme preparation by wheat bran koji culture of Aspergillus oryzae in 1894. Industrial production of dextrose powder and dextrose crystals from starch using α-amylase and glucoamylase began in 1959. Since then, amylases are being used for various purposes. Conversion of starch into sugar, syrups and dextrins forms the major part of the starch processing industry (Marshall, 1975). The hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products and beverages. Hydrolysis of starch to products containing glucose, maltose, etc. is brought about by controlled degradation (Norman, 1978; Barfoed, 1976; Hurst, 1975; Slott and Madser, 1975). Some of the applications of amylase are:

1.2.1Liquefaction

Liquefaction is a process of dispersion of insoluble starch granules in aqueous solution followed by partial hydrolysis using thermostable amylases. In industrial processes, the starch suspension for liquefaction is generally in excess of 35% (w/v). Therefore the viscosity is extremely high following gelatinization. Thermostable α-amylase is used as a thinning agent, which brings about reduction in viscosity and partial hydrolysis of starch. Retrogradation of starch is thus avoided during subsequent cooling. The traditional thinning agent used in starch technology was acid (hydrochloric or oxalic acids, pH 2 and 140 – 150°C for 5 min). The introduction of thermostable αamylases has meant milder processing conditions. The formation of byproducts is reduced and refining and recovery costs are lowed (Greenshields and Macgrillivray, 1972; Birch and Schallenberger, 1973). In the enzymatic process the hydrolytic action is terminated when the average degree of polymerization is about 10-12. Two distinct types of thermostable αamylases are commercially available and used extensively in starch processing technology. The amylase of Bacillus amyloliquefaciens was the first liquefying αamylase used on a large scale. Later, a more heat stable enzyme from Bacillus licheniformis was introduced commercially (Madsen et al., 1973).

Liquefaction can be done by two methods: Single stage enzyme liquefaction: In 1973, Novo Ondustri A/S Copenhagen developed and patented the process. In this process, starch slurry containing 30 – 40% dry solids is prepared in the feed tank. The pH is adjusted to about 6 – 6.5 with sodium hydroxide. Calcium salts may be added if the level of free calcium ions is below 50 ppm. The liquefying enzyme is then added. The slurry is then pumped continuously through a jet cooker where the temperature is raised to 105°C by direct injection of live steam. Tremendous shearing forces are exerted on the slurry as it is pumped through the jet cooker. So in addition to the viscosity reduction action of the enzyme, some mechanical thinning also occurs. The slurry is maintained at this high temperature in the pressurized holding cell for about 5 min, after which it is discharged via a spring loaded release valve into a reaction, where enzyme action is allowed to continue for about 2 hours at 95°C. After this treatment the liquefied starch will have dextrose equivalent (DE) of 10 – 20 depending on the amount of enzyme used. DE is defined as reducing sugars expressed as dextrose and calculated as a percentage of dry substance. This process is simple energy consumption is relatively low because the maximum operating temperature is only 105°C as compared to 140 – 150°C normally used. Acid enzyme liquefaction: This is another process which takes advantage of the thermostability of B. licheniformis amylase. The enzyme is added after the starch has been cooked and cooled to 100 – 95°C. A starch slurry containing 30 – 40% dry solids is cooked at a high temperature for about 5 min. A jet cooker is used so that sufficient mechanical thinning, due to shearing takes place. The pH may be in the range 2 – 5, but if it is too low, byproduct formation will be significant. If it is too high there will be no thinning effect from the acid and there will be an increased color formation. After cooking, the slurry is flash cooled to about 100°C and the pH is set to 6 to 6.5 before the addition of enzyme. By this process the enzyme consumption is slightly reduced. The filtration properties are also improved because better fat/protein separation is achieved. There is an increase in steam consumption and hence fuel costs due to high temperature cooking. Liquefaction is the first and most important step in starch processing. The purpose is to provide a partially hydrolyzed starch suspension of relatively low viscosity which is free from by products, stable to retro gradation and suitable for further processing i.e saccharification. If the liquefaction process does not go well, problems like poor filtration and turbidity of the processed solution occurs. The most important factor for ideal liquefaction of starch is that the starch slurry which contains

suitable amount of α-amylase is treated at 105 to 107°C as quickly and uniformly as possible (Hattori, 1984). Thermostable amylase are not sufficiently heat stable to be used during liquefaction process, but they can be used as saccharifying enzymes. The most widely used enzymes in this group are the maltogenic enzymes.

1.2.2 Manufacturing of maltose

Maltose is a naturally occurring disaccharide. It chemical structure has 4-O-α-D-glucopyranosyl-D-glucopyranose. It is the main component of maltosugar syrup (Sugimoto, 1977). Maltose is widely used as sweetener and also as intravenous sugar supplement. It is used in food industries because of low tendency to be crystallized and is relatively nonhygroscopic. Corn, potato, sweet potato, and cassava starches are used for maltose manufacture. The concentration of starch slurry is adjusted to be 10 – 20% for production of medical grade maltose and 20 – 40% for food grade. Thermostable α-amylase from B. licheniformis and B. amyloliquefaciens are used.

1.2.3 Manufacture of high fructose containing syrups

High fructose containing syrups (HFCS) 42 F (Fructose content = 42%) is prepared by enzymic isomerization of glucose with glucose isomerase. The starch is first converted to glucose by enzymic liquefaction and saccharification.

1.2.4 Manufacturing of high molecular weight branched dextrins

Branched dextrins of high molecular weight are prepared by hydrolysis of corn starch with α-amylase. The extent of starch degradation to be carried out depends on the type of starch and the physical properties desired. The branched dextrins are obtained as powder after chromatography and spray drying. These are used as extender and a glozing agent for production of powdery foods and rice cakes, respectively.

1.2.5 Removal of starch sizer from textile (desizing)

In textile weaving, starch paste is applied for warping. This gives strength to the textile at weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of string due to laid down warp. After weaving the cloth, the starch is removed and the cloth goes to

scouring and dyeing. The starch on cloth is usually removed by application of α-amylase.

1.2.6 Direct fermentation of starch to ethanol

The amylolytic activity rate (Abouzied and Reddy, 1986) and amount of starch utilization and ethanol yields increase in several fold in cocultures (Van Lenen and Smith, 1968). Moulds amylases are used in alcohol production and brewing industries. The advantages of such system are uniform enzyme action in mashes, increase rate of saccharification, alcohol yield and yeast growth (Van Lenen and Smith, 1968).

1.2.7 Treatment of starch processing waste water (SPW)

Starch is also present in waste produced from food processing plants. Starch waste causes pollution problems. Biotechnological treatment of food processing waste water can produce valuable products such as microbial biomass protein and also purifies the effluent (Bergman et al., 1988; Friendrich et al., 1987; Jamuna and Radhakrishna, 1989; Kingspohn et al., 1993).

1.2.8 Other applications

Amylases, especially alkaline amylases are used in detergents. To some extent amylases are also used as digestive aids (Beazell, 1942) to supplement the diastatic activity of flour and to improve digestibility of some of the animal feed ingredients.

1.3 STARCH

Before describing the action pattern and properties of amylolytic enzymes, it is essential to discuss the features of the natural substrate, starch. Starch is a major reserve carbohydrate of all higher plants. In some cases it accounts for as high as 70% of the undried plant material. It occurs in the form of water insoluble granules. The size and shape of the granules are often characteristic of the plant species from which they are extracted. When heated in water the hydrogen bonds holding the granules together begin to weaken and this permits them to swell and gelatinize. Ultimately they form paste or dispersion, depending on the concentration of polysaccharide. Starches are produced commercially from the seeds of plants, such as corn, wheat,

sorghum or rice; from the tubers and roots of plants such as cassava, potato, arrowroot and the pith of sago palm. The major commercial source of starch is corn from which it is extracted by a wet milling process (Berkhout, 1976).

Starch is a heterogeneous polysaccharide composed of two high molecular weight entities called amylose and amylopectin. These two polymers have different structures and physical properties .Starch may be separated into its two components by addition of a polar solvent, e.g. n-butanol, to a dispersion of starch. The insoluble amylose complex can then be separated from soluble amylopectin fraction. Amylose is composed of linear chains of α-1,4 linked D-glucose residues. Hence it is extensively degraded by α-amylase. Some amylose is not totally degraded to maltose by this enzyme. Amylose has a degree of polymerization of several thousands of glucose units (Banks and Greenwood, 1975). Because of the molecular shape and structure of amylase, it is not stable in aqueous solution and retrogrades (precipitates spontaneously). This is because linear chains align themselves by hydrogen bonding and thus forms aggregates. This process is irreversible. Retrograded amylase will only dissolve in alkaline solution. Amylose has considerable viscosity in alkaline solutions due to its molecular shape. Amylase forms complex with iodine to form intense blue color and this forms the basis of a method for quantitative determination of amylase.

Amylopectin may account for 75 to 85% of most starches. It has molecular weight in excess on 107 – 108 and has a branched structure composed of chains of about 20 – 25 α-1,4 linked D-glucose residues. Amylopectin which is branched by α-1,6 linkages may contain 4 to 5% α-1,6-D-glucosidic bonds. In aqueous solutions, amylopectins are relatively stable due to branched molecules and are not able to form compact aggregates. There is no apparent relationship between the limiting viscosity number and the degree of polymerization. Due to the nature of branched structure, the iodine binding power is reduced. The branched components of starch is amylopectin which has different types of chains referred to as A, B and C chains (Fogarty, 1983)

The hydrolysis of starch may be carried out using either acid or enzyme as catalyst. Enzyme hydrolysis has several advantages: it is more specific, therefore fewer byproducts are formed, and hence yields are higher. Conditions for enzyme hydrolysis are milder therefore refining stages to remove ash and color is minimized. The enzymatic hydrolysis of starchy has been practicedon an industrial scale for many years and is gradually replacing the traditional acid hydrolysis process (Underkofler et al., 1965; Barfoed, 1976).

1.4 SWEET POTATO

Sweet potato, Botanical Name- lpomoeabatatas, is a dicotyledonous plant that belong to the family convolvulaceae .It is a perennial; plant mainly grown as an annual. This species (lpomoeabatatas) was first described by Linnaeus,(1) as convolvulus batatas. However Lamarck classified this species within the genus lpomoea. Sweet potato has skin colour runs from pink to dark red. there are 400 varieties of sweet potato. There are two different varieties of Sweet potato, firm and soft. Sweet potato is nutritious and provides energy and appreciable amount of vitamins and minerals. Approximately 80-90% of the dry matter is made up of carbohydrates which consists mainly of starch and sugars. Sweet potato are cultivated wherever there is enough water to support their growth. optimal rainfall for growth range between 750and 2000mm. The sweet potato is a warm season growing plant annually requiring 20-25℃ average temperature and full sunlight for optimal development. Sweet potato tubers are mainly an energy source due to their high carbohydrates content which account for 80-90% of the dry weight. Carbohydrates consists of starch, sugar, small amount of pectins , hemicelluloses and cellulose. Starch is the main carbohydrates and is very resistant to amylase hydrolysis. Tuber are a poor protein source, as they contain about 4% of crude protein they have low content of fiber, fat.

Sweet potato got over 400% of our daily need for vitamin A in one medium spud as well as loads of fiber and potassium. Many studies have suggested that the increasing consumption of plant foods like Sweet potato decreases the risk of obesity, diabetes, heart disease and healthy all mortality while promoting a healthy complexion, increased energy and overall lower weight.Sweet potatoes are considered low on the glycemic index scale and recentresearch suggests they may reduce episodes of low blood sugar and insulin resistance in people with diabetes. Sweet potato roots contained high amount of extractable amylolytic enzyme and use the outer tissue extract to hydrolyze starch in Sweet potato mashes. In addition to high starch content Sweet potato roots have a high content of `Amylase`. The native amylase have important and well documented influence in Sweet potato storage and processing. Amylase play an important role in digestion of starch and the combined action of α-and β-amylase is more effective than the action of α –β amylase alone. Starch is an important industrial compound and its by product have extensive uses in industries such as of food,pharmaceutics and other

Nutritional quality of Sweet Potato

Nutritional value per 100 gm

Energy	360 kJ
Carbohydrates	20.1 g
Starch	12.7 g
Sugar	4.2 g
Dietary Fibre	3.0 g
Fat	0.1 g
Protein	1.6 g
Vitamin A	709 µg

Table 1 Nutritional value of sweet potato

AIM AND OBJECTIVES

AIM:

To extract and partially purify β-Amylase from Sweet Potato.

OBJECTIVES:

- Extraction of crude enzyme from cleaned and grinded sweet potato.
- Partial purification of the crude enzyme.
- Assays for the presence of Amylase.

CHAPTER 2

2. REVIEW OF LITERATURE

2.1 Amylolytic Enzymes and its importance

Amylolytic enzymes form a large group of enzymes operating on starch and related oligo- and polysaccharides. The three best known amylases are α-amylase, β-amylase and glucoamylase (rarely γ-amylase). Since the starch or pullulan and glycogen belong to the important sources of energy for microorganisms, plants and animals, amylolytic enzymes are produced by a great variety of living systems (Vihinen and Mäntsäla 1989)

Despite the fact that they have related function (they all catalyse the cleavage of the α-glucosidic bonds in the same substrate), structurally and mechanistically they are quite different (Janeček 2000a).

2.2 β-Amylases

The polyglucan homopolysaccharide i.e. starch, is the major constituent of human diet. It is a storage polysaccharide and is composed of single type of monosaccharide called glucose at different anomeric conformation. This starch serves as energy source in all the genera by its breakdown into simple sugar, by a group of enzymes (Husain 2017).

β-Amylases are the enzymes hydrolyzing α,1-4 glycosidic linkages in starch and related polysaccharide and are widely distributed in plants as well as microbes. β-Amylase is a member of family 14 of glycosyl hydrolases and catalyzes the hydrolysis of alternate α-1,4-glycosidic linkages in starch and related polysaccharide starting from the non-reducing end. The enzyme releases maltose, continuing until the entire chain is cleaved or the enzyme encounters blockage by a physical or chemical irregularity in the chain. Unlike many other enzymes, the term ‘beta’ does not imply to the linkage present in the substrate, but the inversion of configuration which results during β-amylase action on starch (Henrissat 1991)

β-Amylase is also known to promote various less known reactions which follow other stereochemical paths. Thus, though the enzyme does not hydrolyze maltose (Genghof et al. 1978), it does convert the β-anomer of maltose to maltotetraose to a small extent by a condensation reaction, that is the reversal of maltotetraose hydrolysis (Hehre et al. 1969).

β-Amylase further catalyzes the slow irreversible hydration of maltal, an enolic glycosyl donor lacking α- or β-anomeric configuration, to form β-2- deoxymaltose (Kitahata et al. 1991).

Kuhn (1924) classified the saccharogenic amylase of malt as a β-amylase on the basis of its ability to convert starch into the β-anomeric form of maltose.

Ohlsson (1930), discovered another amylase which yielded β-mannose and named it as βamylase. Hopkins et al. (1948) were first to study the kinetics of β-amylase on potato amylose. In the 1960s and 1970s, various techniques in the purification of sweet potato β-amylase were improved (Nakayama and Amagase 1963; Takeda and Hizukuri 1969).

Cloning of the enzyme was successfully carried out in E. coli by Yoshida and Nakamura (1991).

Amino acid sequence and primary crystal structure of the tetrameric enzyme were determined in 1993 (Cudney and McPherson 1993)

The evolution of β-amylases was studied by Pujadas et al. (1996), who discovered the enzyme to be an example of stingy divergence illustrated by signature structural motifs. Finally, in 2001, a simple purification method using affinity precipitation was developed (Teotia et al. 2001).

The 3D structures derived from X-ray crystallography of β-amylases are known for sweet potato (Cheong et al. 1995), soybean (Mikami et al. 1993, 1994), barley (Mikami et al. 1999a), Bacillus cereus (Mikami et al. 1999b) etc.

The open-close movement of the flexible loop (residues 96–103 in SBA) is known to play an important role in the catalytic procedure. The aforementioned amino acid residues are important for catalysis and binding of the substrate and well-conserved between plant and bacterial enzymes, however only 29% identical residues are found between B. cereus β-amylase and SBA in the core domain. The flexible loop in the closed position helps to protect the reaction center from solvent, and assists an ordered water molecule adjacent to Glu380 to provide the steric outcome of the hydrolysis/hydration reaction. This structural arrangement explains the role of α-cyclodextrin and maltose as competitive inhibitors of β-amylase (Mikami et al. 1993, 1994).

Chemical evaluation revealed that one disulfide bridge and one sulfhydryl group exist in B. cereus β-amylase and B. polymyxa β-amylase, whereas plant enzyme contains several sulfhydryl groups (Uozumi et al. 1991).

The structure of the core domain of B. cereus β-amylase is similar to that of soybean β-amylase, except for the two novel maltose binding sites in the L4 region and in the C-terminal domain. The property of B. cereus β-amylase to digest raw starch can be attributed to these two maltose-binding sites (Oyama et al. 1999).

2.3 Distribution and Functional Properties of β-Amylase

β-Amylases are not ubiquitous in nature and have been purified from a number of plant, bacterial, fungal sources, but absent in animal kingdom. β-Amylase is synthesized by various plants like soybean (Mikami et al. 1994), sweet potato (Cheong et al. 1995), barley (Mikami et al. 1999b), rye seeds (Sadowski et al. 1993), alfalfa (Medicago sativa) tap roots (Doehlert et al. 1982), Arabidopsis sp. (Laby et al. 2001), rhizome of Calystegia sepium (Van Damme et al. 2001).

Various bacterial strains (aerobic and anaerobic) like B. cereus, B. megaterium, B. polymyxa, Clostridium thermosulfurogenes, Thermoactinomyces sp. (Ray 2004; Ray and Nanda 1996) etc. were found to synthesize β-amylases. Cloning and amino acid sequencing was done in many strains of Bacillus sp. among which the cloned β-amylase gene from Bacillus cereus var. mycoides was found to have 1638 bp for coding (Yamaguchi et al. 1996).

Early work on highly active crude enzyme preparations led to the opinion that these enzymes are simple proteins, with no evidence for requirement of cofactors, metal ions or non-protein active groups (Bernfeld 1955).

2.4 Sources of β-Amylases

Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Fungal sources are mostly terrestrial isolates such as Aspergillus species. Amylases from plant and microbial sources are employed for centuries as food additives (Mabel et al., 2006) .

Barley amylases are used in Brewing industry. Fungal amylases are widely used in preparation of oriental foods (Popovic et al., 2009) . Fungal and bacterial amylases are mainly used for industrial applications due to their cost effectiveness, consistency, less time and space requirement for production and ease of process optimization and modification (Ellaiah et al., 2002) .

Among bacteria Bacillus sp. is widely used for the production of amylases. Species like B.subtilis, B. stearothermophilus, B. licheniformis, and B. amyloliquefaciens are known to be good producers of alpha amylase. Similarly filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including alpha amylases (Juliana et al., 2011)

2.5 Extraction and Purification

Sorel Tchewonpi Sagu(2015): The stems of Abrus precatorius were used to extract a beta-amylase enriched fraction. A three phase partitioning method and a Doehlert design with 3 variables (ratio of crude extract/t-butanol, the ammonium sulphate saturation and pH) were used. The data was fitted in a second-order polynomial model and the parameters were optimized to enrich beta-amylaseExperimental responses for the modulation were recovery of activity and the purification factor. The optimal conditions were: a ratio of crude extract/*t*-butanol of 0.87 (v/v), saturation in ammonium sulphate of 49.46% (w/v) and a pH of 5.2. An activity recovery of 156.2% and a purification factor of 10.17 were found. The enriched enzyme was identified as a beta-amylase and its molecular weight was 60.1 kDa. K_m and V_{max} values were 79.37 mg/ml and 5.13 U/ml, respectively and the highest

activity was registered at a temperature of 70 °C and a pH between 6 and 6.5. A significant stabilization of the beta-amylase was observed up to 65 °C.

Kirti Rani(2012): β-Amylases from a variety of germinating pulses (Vigna radiata, Cicer arietinum (black), Vigna mungo, Glycine max, Cicer arietinum(white) were extracted and characterized for their optimum pH, temperature, time of incubation, substrate concentration, effect of CaCl2 as well as their thermal stability. Various pulses were germinated and then extracted with Phosphate buffer (pH 7.0). The amylase assay was done by Dinitrosalicylic acid method (DNS). Optimum pH was found in the range of 5.5 to 8.5 and Thermal stability of pulses of β-Amylase was found up to 72ºC . As well as 6% of CaCl2 was good activator to enhance the enzyme activity with optimum conditions.

Padma Singh and Palavi Kumari (2016): The selected soil samples were obtained (10 gm) from Roorkee, Haridware district, Uttarakhand state, India. The collected soil samples were labeled as banana field (A), potato field (B) and sugarcane field (C) respectively. Soil samples were then serially diluted in normal saline and plated on sterile nutrient agar plates. The colonies obtained from higher dilutions were subjected to Gram staining and starch hydrolysis test. All isolated colonies were screened by activity zone techniques with iodine solution. Out of 10 bacterial strains, only 5 bacterial colonies showed positive results for amylase production and out of 5, Bacterial strains, B3 showed widest diameter i.e. 8 mm was selected for further study.

Fasiku Samuel (2015):Peels of sweet potato (Ipomoea batatas) were buried in the soil for 14 days and the isolates associated with the degradation of the peels were obtained using standard microbiological procedures. The bacterial isolates obtained were screened for amylolytic and cellulolytic activities under different pH and temperatures as parameters and optimized for enzyme production. Sixteen (16) bacterial isolates were obtained and characterized and screened for amylase and cellulase production. Bacillus pumilus has the highest frequency of occurrence (18.75%) followed by B. subtilis (12.50%). After 24 to 48 h of incubation, B. pumilus produced highest concentration of amylase at 55°C, pH 6 (5.4 U/mL) while B. subtilis had the best cellulase production of 0.75 U/mL at 55°C, pH 7. B. pumilus and Bacillus subtilis produced the highest amylase and cellulase concentrations and seem to be the potential sources ofthese enzymes for industrial application.

2.6 Applications of β-Amylase

High purity maltose obtained by crystallizing maltose syrups is utilized in pharmaceutical industry for the manufacture antibiotics, vaccines, maltitol etc. Anhydrous crystalline maltose is used as a desiccant (Nehete et al. 1992).

B-Amylase is also known to inhibit retrogradation of starch. The enzyme shortens the α-1,4-linkage in the straight chain starch molecule utilizing its exo-type activity and ultimately reducing intermolecular association of the straight chain portion of 28raphem, which is known as the main cause for starch retrogradation (Das and Kayastha 2018).

Immobilized enzymes are used in various reactor configurations, permitting an easy control of the reaction, avoiding contamination of the product by the enzyme (this is highly relevant in food technology), and permitting their reuse over many reaction cycles (Khan et al. 2011; Barbosa et al. 2013).

For reasonable and efficient usage of β-amylase, the enzyme has been immobilized on various conventional supports like chitosan coated PVC, chitosan/ PVP blend (Srivastava et al. 2015), acrylic carriers (Bryjak 2003), phenyl boronate agarose (Viera et al. 1988), etc

Recently, a new β-amylase was isolated from Pergularia tomentosa and immobilized onto a matrix of titanium dioxide-based hybrid materials incorporated to cellulose acetate butyrate and copolymer of acrylonitrile and acrylamide, which was found to be a highly active biocatalyst at Ph 7.0, 70 C and thermal-stability at 60 C (Lahmar et al. 2018)28

Nanostructures have gained attention particularly for features such as high surface to volume ratio, desired aqueous suspending ability, small size, abundant oxygen containing surface functionalities and designable function (Singh and Kayastha 2014; Singh et al. 2014; Torres-Salas et al. 2011)

β-amylase from various sources have been reported to be immobilized on 28rapheme oxide (Srivastava et al. 2014), 28 rapheme oxide carbon nanotubes, iron oxide nanoparticles (Fe3O4), molybdenum sulfide (MoS2) (Das et al. 2017, 2018a, b), modified magnetic nanoparticles ZnFe2O4@SiO2-NH2 (Rasouli et al. 2016).

CHAPTER 3

3.MATERIALS AND METHODS

3.1 Sample collection

Sweet Potato was collected from the local markets of kochi and was cleaned,peeled and cut into small pieces

3.2 Sample Preparation

250 kgs of clean Sweet potatoes were taken and peeled ,the skin of.sweet potatoes were cut into small pieces and transferred into blender and grinded for 10 minutes with 40ml of cold 20mM sodium phosphate buffer saline.

Constituents	Weight
Sodium hydrogen orthophosphate	1.104 g
Disodium hydrogen phosphate	4.273 g
Distilled water	160 mL

Table 2 Phosphate Buffer Constituents (Ph 6, 20 mm)

3.3 Extraction of crude enzyme

Grinded material was filtered with muslin cloth. Filtered material was transferred into centrifuged tubes and was centrifuged at 12000 rpm for 20 min at 4°C. After centrifugation supernatant was collected and pellets were discarded. This procedure was repeated for two times.

Fig 1 Grinded Sample

Fig 2 Filtered Sample

3.3 Partial purification of crude enzyme

Ammonium sulphate (0.47g/ml) was added in supernatant and kept it for overnight. Next day again solution was centrifuged at 12000rpm. for 20 min. Supernatant was discarded and pellets were suspended in 20 mM phosphate buffer. This collected solution was kept in an activated dialysis membrane for one day. Dialysis bag was kept in sucrose solution for one hour. Enzyme was collected from dialysis bag.

3.4 Starch Hydrolysis Confirmatory Test

Partially purified enzyme was confirmed by the presence of of β-amylase, To identify the activity of β-amylase a petridish with starch agar was prepared and autoclaved .The plate was incubated at 37^{o} C for 48 hours. After incubation a clear zone of hydrolysis should be observed on the addition of iodine solution.

Constituents	Weight
Beef Extract	0.075 g
Soluable starch	0.25 g
Agar	0.375 g
Peptone	0.125 g
Distilled Water	25 mL

Table 3 Starch Agar Constituents

Fig 3 Starch agar

Fig 4 Streaked Agar Plate

3.5 Iodine Test

1 ml of starch solution and crude enzyme was taken in a test tube and one drop of iodine solution was added to the test tube .The precipitate should turn blue black in colour which indicates the presence of starch.

CHAPTER 4

RESULTS

Extraction and partial purification of crude enzyme from grinded sweet potato

Extraction and partial purification of enzyme was done by ammonium sulphate precipitation.

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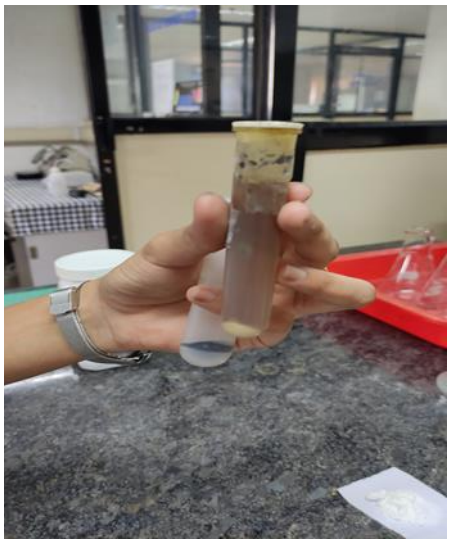

Fig. 5 Pellets collected after crude enzyme extraction

Fig.6 Crude enzyme supernatant

ASSAYS FOR AMYLASE ACTIVITY

Starch Hydrolysis Test

Clear zone was present after the addition of iodine solution to the starch agar plate after the incubation of the plate at 35^0C for 48 hours.

Fig .7 Starch agar plate before zone formation Fig. 8 Clear zone of hydrolysis after iodine addition

Iodine test

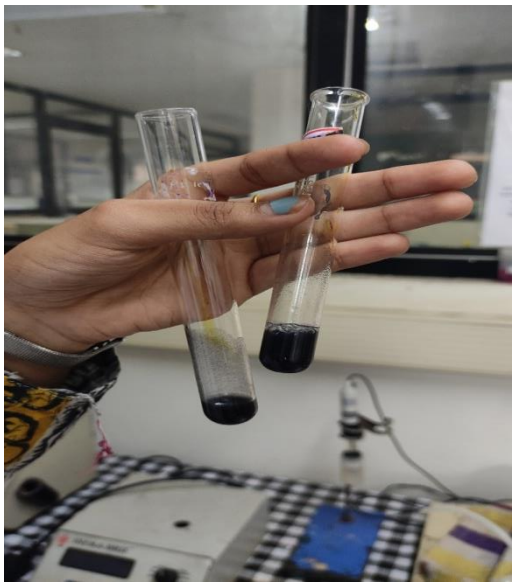

Fig .9 precipitate turned blue black indicating presence of starch

DISCUSSION

At present there are more than about 30 different amylolytic and related enzymes (Janeèek, 1997). Degradation of starch is essentially performed by the four groups of enzymes (GuzmánMaldonado & Paredes-López, 1995): endoand exo-amylases acting primarily on α-1,4-linkages, debranching enzymes attacking mainly the α1,6-linkages, and cyclodextrin glycosyltransferases that degrade starch by catalysing mainly cyclisation and disproportionate reactions . Endoamylases cleave only the α-1,4-bonds in starch in the inner regions of the starch molecule by passing the α-1,6-branching points of amylopectin (Vihinen & Mäntsälä, 1989). The αamylase is the best known endoamylase. It causes a rapid loss of viscosity of the starch solution. These enzymes are often divided according to degree of hydrolysis of substrate into two categories: liquifying (30–40%) and saccharifying (50–60%). This division is widely used to describe the properties of α-amylases (Vihinen & 606 Mäntsälä, 1989). Thus the products of endoamylases are oligosaccharides of varying lengths. Exoamylases also cleave the α-1,4-bonds, e.g. β-amylase , but some of them are able to attack the α-1,6-bonds, e.g. glucoamylase . These enzymes act externally on substrate bonds from the non-reducing end of starch and hence produce only low molecularweight products from starch, e.g. maltose and glucose, respectively (Wind, 1997). Pullulanase and isoamylase (EC may be the examples of debranching enzymes. Both are specific for α-1,6-bonds in starch (amylopectin) and related polysaccharides and branched limit dextrins. According to the inability or ability to degrade also the α-1,4- glucosidic bonds, pullulanases are classified into two categories (Wind, 1997): pullulanase I and pullulanase II, respectively. Pullulanase type II is usually referred to as α-amylase-pullulanase or amylopullulanase. However, to make it clear the specificity should be proved which enzyme it refers to. The fourth group of starch-degrading enzymes are the cyclodextrin glycosyltransferases . They produce cyclodextrins from starch, the rings which are composed of 6, 7 or 8 glucose units bound by α-1,4- bonds (Pócsi, 1999). The CGTases catalyse intraand intermolecular reaction of glycosyl transfer (Svensson & Sgaard, 1993).

The best known amylolytic enzymes are α-amylase, β-amylase and glucoamylase. As has been indicated above, however, these three amylases are quite distinct from both functional and structural points of view. This furthermore implies that there exists a rather long evolutionary distance between them (Janeèek, 1994a). They constitute their own

independent families with no sequence similarities (Pujadas et al., 1996; Coutinho & Reilly, 1997; Janeèek, 1997). Thus in the sequence-based classification of glycoside hydrolases developed almost 10 years ago (Henrissat, 1991), α-amylases, β-amylases and glucoamylases were found in the families 13, 14 and 15, respectively. Both β-amylases and glucoamylases are found in the only one sequence-based family, family 14 and family 15, respectively, (Henrissat & Bairoch, 1993). With regard to α-amylases, however, they should be considered as the α-amylase family with many various specificities covering their main family 13 together with two related families 70 and 77 established recently (Coutinho & Henrissat, 2000). Moreover, there are some α-amylases and related enzymes that have been found to exhibit no sequence similarities with the main family 13 and thus placed into the different family 57 (Henrissat & Bairoch, 1996). All this means that the amylases (and related enzymes) belonging to families 13 (plus 70 and 77), 14 and 15 differ from each other by their amino acid sequences, i.e. also by their threedimensional structures. α-Amylases, β-amylases and glucoamylases use furthermore different reaction mechanisms and catalytic machineries for cleaving the glucosidic bonds (Henrissat & Davies, 1997; Kaneko et al., 1998).

Enzymes capable of hydrolysing starch and related saccharides are produced by both prokaryotes and eukaryotes, i.e. by organisms belongingenzymes are known to be of animal, plant and microbial origins. Since degradation of starch usually requires co-operation of several amylolytic enzymes, starch-degrading organisms often have several amylolytic activities (Vihinen & Mäntsälä, 1989). On the other hand, not all the living organisms able to utilise starch and/or related saccharides always produce all the enzymes necessary for complete degradation of these substrates

α-Amylases and related enzymes (e.g. αglucosidases, pullulanases) as well as glucoamylases have been reported to occur in a wide variety of organisms, especially of microorganisms (Fogarty, 1983; Vihinen & Mäntsälä, 1989). They are produced also by animals and plants (Janeèek & Balá, 1992; Pandey, 1995). On the other hand, β-amylases have been found to be distributed in higher plants and some microorganisms only (Adachi et al. 1998; Mikami et al., 1999)

Due to the improvement of the industrial starch degradation process there has been a great interest in extremely thermostable amylolytic enzymes, especially in glucoamylase involved in the second step, i.e. in conversion of starch dextrin to glucose (Legin et al., 1998; Reilly, 1999). In the first step, degradation of starch into the limit dextrin, the highly

thermostable α-amylase from Bacillus licheniformis is used at about 95◦C (Legin et al., 1998). The used glucoamylase, which is produced by filamentous fungi, works at 60◦C (Reilly, 1999). Having thermostable glucoamylase suitable for the industrial use would reduce the cost of the starch degradation process by reducing the process to a single step. Since Archaea were found to be a good source of hyperextremostable enzymes (Woese, 1987), many efforts have been aimed at finding, isolation and biochemical characterisation of amylolytic enzymes, especially glucoamylase, of archaeal origin. Unfortunately, although there were found a lot of α-amylases and related enzymes produced by various archaebacteria (Sunna et al., 1997; Bauer et al., 1998; Niehaus et al., 1999; Léveque ^ et al., 2000), archaeal glucoamylase was reported only as a putative protein derived from the complete genome sequencing of the methanogenic archaeon Methanococcus jannaschii (Bult et al., 1996). It is remarkable that from the evolutionary point of view, archaeal hyperthermostable α-amylases were found to be most closely related to their plant counterparts with thermostability around 40◦C (Janeèek et al., 1999). The putative glucoamylase from M. jannaschii representing archaeal glucoamylases, which is at present a subject of biochemical characterisation (Reilly, 1999), seems to be related to eubacterial counterparts (Coutinho & Reilly, 1997). However, since no sequence of a plant glucoamylase has been available, the image of glucoamylases relationships cannot be completed.

The properties of amylolytic enzymes produced by extremophilic microorganisms (of both bacterial and archaeal origins) can be found in several recent reviews (e.g. Sunna et al., 1997; Bauer et al., 1998; Niehaus et al., 1999; Léveque ^ et al., 2000). Hyperthermostable amylolytic enzymes may expand the horizons of new frontiers in microbiology, biochemistry and biotechnology (Zeikus et al., 1998). It is worth mentioning that some of the hyperthermophiles contain the amylases and related enzymes that are at the molecular level different from their ordinary counterparts. This was the case of the thermostable α-amylases from thermophilic bacterium Dictioglomus thermophilum (Fukusumi et al., 1988) and the hyperthermophilic archaeon Pyrococcus furiosus (Laderman et al., 1993), the sequences of which exhibited no obvious homology to usual α-amylases from the family 13, and thus a new family 57 of glycoside hydrolases was established . Similarly, Galichet & Belarbi (1999) reported a new α-glucosidase gene from a hyperthermophilic archaeon Thermococcus hydrothermalis that is also different from usual α-glucosidases. In addition to amylases from hyperthermophilic archaea, a special interest has been evoked by studies on the amylolytic enzymes from hyperthermophilic bacterium Thermotoga maritima (Liebl et al., 1997; Bibel

et al., 1998), since this bacterium is capable of cell division at temperatures up to 90◦C. Very recently a CGTase (which is a member of the α-amylase family 13; from the hyperthermophilic archaeon Thermococcus sp. B1001 was isolated, purified and characterised (Tachibana et al., 1999; Yamamoto et al., 1999) and also sequenced (Fujiwara et al., 1999).

For β-amylases and glucoamylases there have been substantially less reports on their thermostability. The most thermostable β-amylase can be the one from thermophilic bacterium Clostridium thermosulfurogenes (Kitamoto et al., 1988) with 75◦C as the optimal temperature for its activity (Hyun & Zeikus, 1985). With regard to glucoamylases, the one from thermophilic fungus Thermomyces lanuginosus (Mishra & Maheshwari, 1996), reported to be fully active at 60◦C for 7 hours can be considered as the most thermostable glucoamylase

The amylase, which is an important enzyme employed in the starch processing industries forthe hydrolysis of polysaccharide .Amylase has vast industrial applications. The enzymes in Sweet potato are affected by various factors such as pH, temperature and metal ions. .Based on the present work, it can be speculated that β-amylase can be employed for industrial purpose.

CHAPTER 5

SUMMARY AND CONCLUSION

Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing α-1,4- glucosidic bonds of amylose, amylopectin, glycogen and their degradation products

In this present study Beta-amylase was extracted from sweet potato and partially purified

Sweet potato a extract could be used of a low cost for the estimation and determination of starch in food stuffes in place of important amylolytic enzyme. More research and development is still required to refine the extract and homogeneity of Sweet potato amylolytic extract.

For the efficient production of β-amylases at lower production cost, with high purity. In future three dimensional structures of more thermostable and kinetically favorable β-amylases will be determined in order to get a clear picture of structure-function relationship and molecular mechanism of this multidomain protein. Combined with site-directed mutagenesis, the role of important residues for catalysis and adaptive parameters of raw starch digesting, plant based β-amylases will add on useful information. Moreover, future fast kinetic experiments on different β-amylases shall provide a detailed mechanism of folding and unfolding transitions.

Nano-structured metal-oxide based amylase sensors should be developed with high sensitivity, fast response time, and stability/shelf-life for various biotechnological applications. In future, efforts should be made to construct different maltose biosensors using these ultrasensitive nanoparticles and explore its implementations in pharmaceutical and industrial processes. The sensors will be schemed by co-immobilization of β-amylase, α-amylase and α-glucosidase on suitable supports. Besides, each step of manufacture process of proposed maltose biosensor shall be monitored employing spectroscopic and microscopic tools like scanning electron microscopy (SEM), transmission electron microscopy (TEM) with energy dispersive X-ray analysis (EDX), X-ray photoelectron spectroscopy (XPS), fourier transform infrared spectrometry (FTIR), Raman spectroscopy, fluorescence imaging, atomic force microscopy (AFM) and surface enhanced Raman scattering (SERS) techniques.

Moving forward in the future, food, pharmaceutics and starch based industries will be the major targets for these biosensors and biocatalysts.

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APPENDICES

APPENDIX 1

Sl. No:	Equipment
1	**Centrifuge** • A centrifuge is a laboratory device that is used for the separation of fluids, gas or liquid, based on density. • Separation is achieved by spinning a vessel containing material at high speed; the centrifugal force pushes heavier materials to the outside of the vessel. • There are various types of centrifuges, depending on the size and the sample capacity. • A centrifuge works by using the principle of sedimentation: Under the influence of gravitational force (g-force), substances separate according to their density

APPENDIX 2

- **Phosphate Buffer (Ph 6, 20 Mm)**

Constituents	Weight
Sodium hydrogen orthophosphate	1.104 g
Disodium hydrogen phosphate	4.273 g
Distilled water	160 mL

- **Starch Agar Plate**

Constituents	Weight
Beef Extract	0.075 g
Soluable starch	0.25 g
Agar	0.375 g
Peptone	0.125 g
Distilled Water	25 mL