Stability studies and steady state kinetics of α-amylase from *Vigna radiata*

Thesis

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CERTIFICATE

This is to certify that the thesis entitled **"Stability studies and steady state kinetics of α-amylase from *Vigna radiata*"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF TECHNOLOGY** in **Biotechnology** of the College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, is a record of bonafide research work carried out by **Mr. Poornima Maurya, ID. No. PG/2882,** under my supervision, and no part of the thesis has been submitted for any other degree or diploma. It is further certified that the assistance and help received during the work of research have duly acknowledged.

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CERTIFICATE

We, the undersigned, members of the Advisory Committee of **Mr. Poornima Maurya, ID. No. PG/2882**, a candidate for the degree **Master of Technology** with major in **Biotechnology** agree that the thesis entitled, **"Stability studies and steady state kinetics of α-amylase from *Vigna radiata*"** may be submitted by **Mr. Poornima Maurya** in partial fulfillment of the requirements for the degree.

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ABBREVIATIONS

DNSA	:	3, 5-Di-nitro salicylic acid
Rochelle salt	:	Sodium - potassium tartrate
Vmax	:	Maximum velocity
Km	:	Michaelis – Menten constant
I_{50}	:	50% loss of activity
OD	:	Optical density

Chapter 1

INTRODUCTION

α-amylase, (endo-1,4-D glucose-D glucohydrolase; EC 3.2.1.1.) is an enzyme that hydrolyses α- bonds of large, alpha-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose. It is the major form of α-amylase found in humans and other mammals. It is also present in seeds containing starch as a food reserve, and is secreted by many fungi. α-amylase catalyzes the hydrolysis of internal α-1,4-O-glycosidic bonds in polysaccharides with retention of α- anomeric configuration in the products. α-amylases are ubiquitous in nature and have been isolated, purified and characterized from a number of animals, plant, fungal as well as bacterial sources (Kumar *et al.,* 2009). They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes and are of great significance in biotechnological applications ranging from food, fermentation, detergent, pharmaceutical, brewing and textile to paper industries due to their inherent properties, such as stability, reusability and specificity.

EC Tree of α- amylase (3.2.1.1)

└ 3 Hydrolases

└ Glycosylases

└ Glycosidases, i.e. enzymes that hydrolyse *O*- and *S*-glycosyl compounds

└ α-amylase

α-amylases are enzyme which hydrolyse starch molecule to give diverse product including dextrin and progressively smaller polymer composed of glucose units (Windish and Mhatre 1965). α-amylase acts on starch and breaking them up into sugars (hence the term saccharification). Starch is a carbohydrate source consisting of two molecules amylase and amylopectin. α-amylase is formed from chains of glucose linked α-1, 4 and amylopectin is formed from α-1, 4 linked chains of glucose with 1, 6 linked branch points. The α-amylases are enzymes that work by hydrolyzing the straight chain bonds between the individual glucose molecules that make up the starch chain. A single straight chain in starch is called an amylose. A branched starch chain (which can be considered as being built from amylose chains) is called an amylopectin.

α-amylase enzyme is drastically found in legumes including *Vigna radiate.* Pulses are an excellent source of dietary proteins and can play an important role in fulfilling requirements of rapidly increasing population. Green gram locally called as mung bean (*Vigna radiata*) belongs to the family leguminosae. Being a short duration crop and having wider adaptability, it can be grown in *kharif* as well as in summer season. The green gram foliage after harvest can be used as feed for livestock or used as green manure by ploughing in situ.

Mung bean is considered as an important legume due to its high level of protein and carbohydrate content. Its protein quality is equal or better than other legumes, like chickpea, black gram, peas, pigeon pea, etc. The initial legume seedling growth begins after imbibition of water when hydrolytic enzymes mobilize storage material accumulated in the starchy endosperm. Among these enzymes, the most abundant is α-amylase which hydrolyzes the endosperm starch into metabolizable soluble sugars. Changes in the activity of α-amylase of germinating plant seeds have been documented**.** (Sultana *et al.*, 2000)

α-amylase was the first enzyme to be discovered and isolated (Payen 1833). Although it can be derived from several sources, including plants, animals and microorganism, microbial enzymes generally meet industrial demands. α-amylase is produced endogenously in many different organisms e.g. *B. subtilis, B. stearothermophilus, B. licheniformis b*esides *Aspergillus niger, A. orzyae.* Amylases have received a great deal of attention because of their significance especially in biotechnology (Reddy *et al* 2003). α-amylase constitutes a class of industrial enzymes having approximately 25% of the enzyme market world-wide (Sindhu *et al* 1997). Many *Bacillus* species and thermostable *Actinomycetes* like *Actinomycetes thermomonospora* and *Actinomycetes thermoactinomyces* are versatile producers of amylase (Buzzini *et al* 2002). The genus *Bacillus* produces a large range of extracellular enzymes of which α-amylases and proteases are of Industrial importance. Currently two types of α-amylases glucoamylase and α- glucosidase are important for starch hydrolysis. Glucoamylase attacks - 1, 4-bonds, releasing D- glucose molecules, this enzyme also attacks α-1, 6 bonds at branching points in the amylopectin molecule but much more slowly than α-1, 4 linkages.

Objectives:

Following objectives will be fulfilled in present study:

- To isolate the α-amylase enzyme from *Vigna radiata* seeds.
- Construction of Maltose Standard Curve by DNS Method.
- To conduct the storage stability studies with respect to temperature.
- To characterize the α-amylase biochemically and to determine the values of various kinetic parameters such as, Michaelis-Menten constant *(Km), Vmax*, optimum pH, optimum temperature etc.
- To find various inhibitors of α-amylase and to determine their I_{50} values.

Chapter 2

REVIEW OF LITERATURE:

The history of α-amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff. Ohlsson suggested the classification of starch digestive enzymes in malt as alpha- and beta-amylases according to the anomeric type of sugars produced by the enzyme reaction (Gupta *et al.,* 2003). These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (Sivaramakrishnan *et al.,* 2006). Endoamylases: cleave internal alpha-1, 4 bonds resulting in a- anomeric products, exoamylases: cleave α-1, 4 or α-1, 6 bonds of the external glucose residues resulting in alpha or beta anomeric products.

α-amylase is classical calcium containing enzyme composed of 512 amino acids in a single polypeptide chain with a molecular weight of 57.6 kDa. There are five α-amylase genes clustered in chromosome 1, at location 1q21, in humans. Three of them code for salivary R-amylase, *AMY1A*, *AMY1B*, and *AMY1C*, and the other two genes *AMY2A* and *AMY2B* are expressed in the pancreas. Human salivary and pancreatic α-amylases share a high degree of amino acid sequence similarity with 97% identical residues overall and 92% in the catalytic domains.

α-amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. α-amylases from plant and microbial sources have been employed for centuries as food additives. Barley α-amylases have been used in the brewing industry. Fungal α-amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of α-amylases, microbial sources, namely fungal and bacterial α-amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Burhan *et al.,* 2003). Among bacteria, *Bacillus sp.* is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis* and but *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications (Vihinen *et al.,* 2000).

α-amylase catalyze the hydrolysis of starch via a double displacement mechanism involving the formation and hydrolysis of a covalent *β*- glycosyl enzyme intermediate by using active site carboxylic acids for it. The residues, in particular, Asp^{197}, Glu^{233}, and Asp^{300} were described to function as catalytic residues. Probably, Asp^{197} acts as nucleophile that attacks the substrate at the sugar anomeric center, forming a covalently bound reaction intermediate. In this step, the reducing end of the substrate is cleaved off the sugar skeleton. In a second step a water molecule attacks the anomeric center to break the covalent bond between Asp^{197} and the substrate, attaching a hydroxyl group to the anomeric center. In both steps Glu^{233} and Asp^{300} either individually or collectively acts as acid/base catalysts. As a consequence, the active site of human α-amylase consists of several major binding sub-sites identified through kinetic studies. The enzyme was characterized in terms of pH optimum and stability, temperature optimum and stability, activation energy, *Km* and *V*max.

α-amylase presents a three-dimensional structure capable of binding to substrate and, by the action of highly specific catalytic groups, promotes the breakage of the glycoside links. The protein contains 3 domains: A, B, and C. Domain A, which has a (β/α) barrel fold, constitutes the catalytic core domain, it contains about 280–300 residues. The catalytic triad (Asp, Glu) is present in domain A. The B domain is inserted between A and C domains and is attached to the A domain by disulfide bond. The C domain presents a β sheet structure linked to the A domain by a simple polypeptide chain and seems to be an independent domain with unknown function. The active site (substrate binding) of the α-amylase is situated in a long cleft located between the carboxyl end of both A and B domains. The calcium is situated between A and B domains and may act in stabilizing the three-dimensional structure and as an allosteric activator.

Some α-amylases, especially alkaline α-amylases are used in detergents. To some extent α-amylases are also used as digestive aids. To supplement the diastolic activity of flour and to improve digestibility of some of the animals feed ingredients. Liquefaction is a process of dispersion of insoluble starch granules in aqueous solution followed by partial hydrolysis using thermo stable amylases. In industrial process, the starch suspension for liquefaction is generally in excess of 35 (w/v) (Beazell *et al.,* 1994). The α-amylase activity is under significant influence of variegated environmental conditions

such as temperature, aerobic/anaerobic conditions (Guglielminetti *et al*., 1995). α-amylase is an enzyme which is involved in the hydrolysis of starch into maltose and is under nuclear gene control (Scandalios *et al.,* 1974).

A clinical method for the determination of α-amylase was developed by Rinderknecht, using a chromogenic substrate specific to a-amylase. This substrate is potato starch derivatized with RBB4 (Remazol brilliant blue R) commercially available as starch azure or amylopectin azure. This insoluble substrate is suspended in buffer and α-amylase action results in the solubilization of colored fragments of the starch azure. After the assay is terminated, the unreacted substrate is removed by filtering or by centrifugation and the color in solution is used as an estimation of the α-amylase activity. This method was originally developed for use in health sciences, and because animals have no β amylase (Rinderknecht *et al.,* 1967).

α-amylase is produced endogenously in many different organism e.g. *Bacillus subtilis, B. stearothermophilus, B. licheniformis.* Besides *Aspergillus niger, A.orzyae.* Today a large number of microbial amylases are available commercially and they have almost complete replaced chemical hydrolysis of starch in starch processing industry (Bernfeld *et al.,* 1955). This is because the amylase effects a rapid reduction on the length of the starch polymer. The resulting fragments are oligosaccharides that are readily soluble in water and are to short retain significant adhesive capability.

Today a large number of microbial amylases are available commercially and they have almost complete replaced chemical hydrolysis of starch in starch processing industry (Bernfeld *et al.,* 1955). This is because the amylase effects a rapid reduction on the length of the starch polymer. The resulting fragments are oligosaccharides that are readily soluble in water and are to short retain significant adhesive capability. Thus, to cope up with the increasing demand, a variety of projects have been focused on increased production, activity and stability of these enzymes. The major advantage of using microorganisms for the amylase production is economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane *et al.,* 1990).

Plants are an important source of chemical constituents with potential for inhibition of α-amylase and can be used as therapeutic or functional food sources. A review about crude extracts and isolated compounds from plant source that have been tested for α-amylase inhibitory activity has been done. The analysis of the results shows a variety of crude extracts that present α- amylase inhibitory activity and some of them had relevant activity when compared with controls used in the studies (Paloma Michelle *et al.,* 2012).

α-amylases are generally assayed using soluble starch or modified starch as the substrate. Alpha amylase catalyzes the hydrolysis of α -1, 4 glycosidic linkage in starch to produce glucose, dextrin. The reaction is monitored by an increase in the reducing sugar levels or decrease in the iodine color of the treated substrate (Priest *et al.,* 1977). Physiology of α-amylase production by submerged fermentation (SmF) and solid-state fermentation (SSF) has been thoroughly investigated and is affected by a variety of physicochemical factors. Most notable among these are the composition of the growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source and nitrogen source (Fogarty *et al.,* 1979). Among different carbon, nitrogen and trace elements supplemented, glucose, peptone and calcium chloride, respectively enhanced enzyme production.

Enzyme is a glycoprotein. Its single polypeptide chain of about 475 residues has SH group and four disulfide bridges and contains a tightly bound Ca^{2+}. It exists in two forms (I &II) which have identical enzymatic properties, differing only in electrophoretic mobility. A binding site for Cl^{-1} has been reported which effect a conformational change that enhances activity. α- amylase producing microbes are generally present in degraded atmosphere. Micro-organisms perform their metabolic processes rapidly and with remarkable specificity under ambient conditions. Catalyzed by their diverse enzyme alternatives to harsh chemical technologies has led to extensive exploration of natural microbial diversity to discover enzyme which could function effectively and generate pollution free "Dream technologies" in the immediate future (Sivakumar *et al.*, 2012, Anupama and Jayaraman 2011). In medical, Blood serum amylase may be measured for purposes of medical diagnosis. A normal concentration is in the range 21-101 U/L. A higher than normal concentration may reflect one of several medical conditions, including

acute inflammation of the pancreas (concurrently with the more specific lipase), but also perforated peptic ulcer torsion of an ovarian cyst, strangulation ileus, macroamylasemia and mumps. Amylase may be measured in other body fluids, including urine and peritoneal fluid (Anupama and Jayaraman 2011; Kaur *et al*., 2012). These α-amylase enzymes account for about 30 % of the world's enzyme production (Akcan *et al*., 2011; Deb *et al*., 2013**).**

Crystalline suspensions in sodium-calcium chloride are stable for several month refrigerated. Solutions in buffered sodium chloride pH 7.0 are stable for month providing the protein concentration exceeds 0.1%. α- amylase enzymes are unstable and loss their property in various conditions like high temperature, pH, and different chemicals used in the reaction and it is still undergoing to find out the bacterial strain which produce more stable amylase enzyme with higher production rate within low cost.

An interesting α-amylase has been obtained in large quantity (2400 U/g of body weight) with specific activity (20.22 U/g proteins) from a freshwater zooplankton, Heliodiaptomus viduus (Gurney). Partially purified enzyme showed activity up to 70 ºC and demonstrated optimum activity at 30 ºC. The enzyme was active between pH 3.5 and 8.5, with maximum activity at pH 6.0. It retained its full activity at 30 ºC for 2 h, but became inactive at 60 ºC after 2 h, and at 70 ºC after 1 h. Enzyme activity was retained at 60% in 2 M NaCl after 24 h incubation, while full activity was found in 0.5 M NaCl for the same duration of incubation. Addition of metal ions like Fe^{+2}, Ba^{+2}, Co^{+2}, Ag^{+2}, and Mn^{+2} enhanced activity up to 130%-200% of the original activity, while K^{+} and Sn^{+2} caused a negligible increase in the activity. Addition of Hg^{+2} and Li^{+2} completely inhibited amylase activity, whereas Cu^{+2}, Mg^{+2}, and Pb^{+2} reduced activity to as little as 5% of original activity. Soluble starch, amylose, and amylopectin were completely digested by this amylase, whereas glycogen was hydrolyzed to a lesser extent. During hydrolysis of soluble starch, initially, maltose (G2) and maltotetraose (G4) were produced in similar magnitude, followed by a distinctly higher amount (> 80%) of maltose. Amylose was the most potential substrate with a Km value of 1.82 mg/ml. The molecular mass was 50 kDa in the Native PAGE and no multiple forms were observed (Tapan Kr. Dutta *et al.*, 2005).

The presence in milk of a-amylase was confirmed. Analyses for the enzyme in milk and fractions there for were made by measuring the rate of decrease in viscosity of starch paste with Ostwald viscosimeters. Analysis of milk and that dialyzed with various added salts revealed that the enzyme requires both calcium and chloride for activity. When milk proteins were fractionated, much of the a-amylase activity was concentrated into the lacto globulin fraction precipitated from whey at 43% saturation with (NH~), The enzyme was further concentrated by dissolving the lacto globulin in a solvent consisting of ethylene glycol, ethanol, and water in the ratio of 15:29:56 by volume, adsorbing it upon rice starch and eluting with saturated CaSe solution. A preparation with 30 times the specific activity of lacto globulin and containing 20% of the total activity of the lacto globulin from which it was prepared, had starch liquefying, dextrinizing, and saecharilying properties. The optimum pI for the enzyme was 7.4 at 34°C. The energy of activation of the catalyzed reaction, measured by starch liquefaction, was 8,500 calories per mole. The enzyme was progressively inactivated by 30-rain. Heating between 45°C and 52°C, at pH 6.4, A plot of velocity vs. velocity/substrate concentration was linear, indicating a single enzyme entity (Robert jenness *et al.*, 2005).

Bacillus species isolated from acidic soil samples of a tea farm was identified as Bacillus cereus, based on 16S rRNA sequencing and standard bacterial identification methods. Following optimization of enzyme production, the resulting α-amylase was purified by acetone precipitation and ion exchange chromatography. Consequently, thermo-stability and kinetic parameters of the purified enzyme were determined. The temperature profile of the enzyme indicated a very broad temperature range (from 10 to 70°C) with 50°C representing the optimum temperature for enzyme activity, which is different from those of the known *Bacillus* α-amylases. This enzyme was optimally active at pH 6.0 and retained 75 and 50% of its maximal activity at pH 8.0 and 9.0, respectively. It was also strongly inhibited by Zn^{+2} and partially inhibited by Ni^{+2} and ethylene diamine tetra acetic acid (EDTA). The α-amylase enzyme was found to hydrolyze starch forming various malto Oligosaccharides, such as maltose (G2) and malto pentaose (G5) as major products (Atiyeh Mahdavi *et al.*, 2010).

α- amylase is an enzyme present in our saliva and pancreatic secretion and helps in the digestion of starch by breaking it down into sugar (glucose) molecules. The sugar molecules then go into our blood stream and provide energy. Very recently it was shown that caffeine inactivates the enzyme, and the amount of inactivation is increased as the caffeine concentration is increased before it levels off. The inactivation of the enzyme results in a decreased amount of sugar formation and may benefit those who are pre-diabetic or diabetic and want to control blood sugar level. Interestingly, alcohol (ethanol) works just in the reverse manner and resists the thermal inactivation of the enzyme, and hence, provides stability resulting in an increased amount of sugar formation (Arshad Khan *et al*., 2019).

α- amylase producing thermophile bacterium isolated and identified from a hot spring in Jordan and designated as *Bacillus sphaericus* were carried out in a laboratory scale fermenter. The growth and enzyme production optimum conditions were pH 7 and 50 . The kinetic study of cellular growth indicates max, Ks, d, Yx/s and kd were 0.53 h-1, 1.1 g/l, 1.98 h, 0.44 g cell/g starch and 0.4 g/l/h, respectively. The optimum starch concentration for the enzyme production was 32 g/l and higher concentrations show substrate inhibition with inhibition constant Ki 190 mg/l. The kinetic parameters of amylase activation V*max*, and Km were 263 mole mg-1 enzyme min-1 and 0.97 mg/ml, respectively. The effect of different carbon and nitrogen sources on the cellular growth was tested (Al-Qodah *et al*., 2007). Figueira and coworkers worked on different types of amylase production medium composition by toxigenic Fungi. (Aguilar *et al*., 2000) studied on Purification and characterization of an extracellular α-amylase by *Lactobacillus manihotivoran.* They found optimum temperature and pH were 55°C and 5.5, respectively. They observed stability of α-amylase was good at pH range from 5 to 6 and when the enzyme was incubated in presence of soluble starch. They found enzyme was sensitive against temperature if the enzyme was incubated at 55°C for 1 hour.

α- amylase was produced by *Aspergillus terreus* NCFT 4269.10 using both liquid static surface (LSSF) and solid-state fermentation using pearl millet residues as substrate. The maximum production of α-amylase was noticed at 30°C incubated for 96h. The crude α-amylase was purified to electrophoretic homogeneity and characterized. Characterization of amylase confirmed that the purified α-amylase was found to be most stable at pH 5.0, 60°C temperature, and a substrate concentration of 1.25%. The enzyme was active for 40 min at 70°C with an optimum enzyme–substrate reaction time of 60 min. Amylase was compatible with all detergents tested having highest activity with Surf excel followed by Henko and Ariel. SDS and Tween 20 reduced the activity. Among the metal ions tested, the maximum α-amylase activity was attained in the presence of Ca^{2+}, followed by Mg^{2+} and Mn^{2+}. The activity of α-amylase was not considerably affected in the presence of ethylene-di-amine- tetra-acetic acid and Triton X-100. Amylase activity was accelerated in the presence of sodium lauryl sulfate and phenyl-methyl-sulfonyl fluoride did not significantly (or slightly) affect the activity and stability. Tween 20, urea (5%), and the reducing agent, β- mercaptoethanol significantly inhibited the activity of α-amylase. Owing to its noteworthy stability in the presence of detergents, additives, inhibitors, and metal ions, this α-amylase could be an impending enzyme for significant industrial exploitations (Bijay Kumar Sethi *et al.*, 2015).

α- amylase purified from Pennisetum typhoides (specific activity 578.8 U/mg proteins) was immobilized within calcium alginate beads. The required hardness and consistency of the bead was achieved at 3% sodium alginate and 5% $CaCl_2$. The percent immobilization achieved was 69%. The pH optimum of α-amylase calcium alginate bead was 7.0 without any shift from the pH optimum of soluble enzyme (7.0). The value of Km for soluble starch in calcium alginate beads is 1.57 mg/ml. The immobilized enzyme showed temperature optimum at 70°C. From the Arrhenius plot, activation energy of α-amylase calcium alginate bead was found to be 14.8kJ/mol. The α-amylase calcium alginate bead retained 67.1% activity, when incubated for 10 min at 70°C. The calcium alginate α-amylase beads when stored at 4°C in 50 mm imidazole buffer, pH 7.0 (assay buffer), retained 95% activity when stored for 90 days. The same enzyme bead could be reused to up to 20 cycles with a loss of only 30% activity (K. Kharkrang *et al.*, 2015).

α- amylase is widely used in the starch processing, food and paper industries, hydrolyzing starch, glycogen and other polysaccharides into glucose, maltose and oligosaccharides. α- amylase gene family from *Aspergillus niger* CBS513.88 encode eight putative α-amylases. The differences and similarities, biochemical properties and functional diversity among these eight α-amylases remain unknown. The eight genes were cloned and expressed in Pichia pastoris GS115 by shaking-flask fermentation under the induction of methanol. The sequence alignment, biochemical characterizations and product analysis of starch hydrolysis by these α-amylases were investigated. It is found that the eight α-amylases belonged to three different groups with the typical structure of fungal α-amylase. They exhibited maximal activities at 30–40°C except AmyG and were all stable at acidic pH. Ca^{2+} and EDTA had no effects on the activities of α-amylases except AmyF and AmyH, indicating that the six amylases were Ca^{2+} independent. Two novel α-amylases of AmyE and AmyF were found. AmyE hydrolyzed starch into maltose, maltotriose and a small amount of glucose, while AmyF hydrolyzed starch into mainly glucose. The excellent physical and chemical properties are including high acidic stability, Ca^{2+} independent and high malto-triose forming capacity make AmyE suitable in food and sugar syrup industries (J. Wang *et al.*, 2018).

Amylase is an extracellular enzyme, which is involved in the starch processing industries where it breaks starch into simple sugar constituents. Two major classes of amylases namely α-amylase and glucoamylase have been identified in microorganisms. In addition, β-amylase which is of plant origin has also been reported from few microbial strains. These amylases are usually extracellular and are widely distributed in bacteria, actinomycetes and fungi. Amylases are of ubiquitous occurrence and holding maximum market share of enzyme sales. Compared to plant and animal origins, microbial α-amylase is the most popular source of industrial α-amylase. Microbial sources of α-amylases are cost effective and appropriate for industrial demands. These microbial amylases are now available commercially and they have almost completely replaced acid hydrolysis of starch in starch processing industry because of number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps. Amylase has wide application in starch processing, baking, brewing, sugar production, textile industries and in detergent

manufacturing processes. Interestingly, the first enzyme produced industrially was an amylase. This review focuses on the production, purification, characterization and immobilization of microbial amylase (Nisha Kumari *et al.,* 2018).

A study was carried out with a newly isolated bacterial strain yielding extracellular α- amylase. The phylogenetic tree constructed on the basis of 16S rDNA gene sequences revealed this strain as clustered with the closest members of *Bacillus* sp. and identified as *Bacillus subtilis* BI19. The effect of various fermentation conditions on amylase production through shake-flask culture was investigated. Rice flour (1.25%) as a cheap natural carbon source was found to induce amylase production mostly. A combination of peptone and tryptone as organic and ammonium sulfate as inorganic nitrogen sources gave highest yield. Maximum production was obtained after 24 h of incubation at 37°C with an initial medium pH 8.0. Addition of surfactants like Tween 80 (0.25 g/L) and sodium lauryl sulfate (0.2 g/L) resulted in 28% and 15% increase in enzyme production, respectively. Amylase production was 3.06 times higher when optimized production conditions were used. Optimum reaction temperature and pH for crude amylase activity were 50°C and 6.0, respectively. The crude enzyme showed activity and stability over a fair range of temperature and pH. These results suggest that *B. subtilis* BI19 could be exploited for production of amylase at relatively low cost and time (Biplab Kumar Dash *et al.,* 2015).

Thermophilic microorganisms are known for their high temperature requirement. One outstanding attribute of these organisms is the production of thermo-stable enzymes, and because of this feature, enzymes produced by thermophiles have found a number of commercial and research applications. There is a continuous search for thermophilic micro-organisms capable of producing thermo-stable enzymes with novel characteristics. In the present study, a thermo-stable α-amylase-producing bacterial strain was isolated from soil. The isolate was phenotypically identified as Bacillus sp. Cos by adopting standard methods and was used in the production of amylase in sub-merged shake flask culture. The enzyme was purified 8-fold by cold acetone (-10 ˚C) precipitation, ion-exchange chromatography on DEAE -Sephadex (A-50) and on Bio-gel P-4 gel filtration column. The homogeneity and molecular weight were estimated to be 38.02KDa by SDS-PAGE. The enzyme was optimally active at 70 and pH7.0. It was not activated by any

of the tested metal ions but was strongly inhibited by Hg2+. The enzyme hydrolyzed some tested polysaccharides producing only glucose. The Km values (mg/ml) for glycogen, starch, amylopectin, pullulan and amylose were 0.71, 0.83, 0.83, 1.67 and 10.0, respectively. The production of only glucose from various polysaccharides by Bacillus sp. Cos thermo-stable α-amylase, its optimum pH and temperature of activity at 7.0 and 70 ̊C, coupled with its independence on metal ions for activation and stabilization suggests its applicability in starch saccharification for industrial production of glucose and fructose syrups (Opurum *et al.,* 2019).

Amylases have found applications in juice processing, starch processing, desizing of textiles, paper sizing, detergent additives, bread improvement, utilization of waste biomass for valuable products, treatment of waste water and other fermentation processes including malting barley and bakery industries. In the present investigations, amylase producing thermophilic bacterial strain M13 was isolated from spent mushroom compost. M13 isolate produced amylase activity of 61.35 IU after 72 h of incubation period at a pH of 9.0 and temperature of 45°C. It was identified as Bacillus subtilis M13 by using 16S rDNA sequence analysis and deposited in NCBI gene bank (KY962809). Further, the application of extracted amylase was evaluated for apple and kiwi juice yield and clarification as well as for the preparation of buns. An application of 1 and 0.75 per cent of amylase yielded 60 and 55 per cent apple and kiwi juice and enhanced their color, taste, flavour and overall acceptibility. Whereas, 1.25 per cent of amylase resulted in the maximum leavening activity (2.15 ml/h), loaf volume (177.43 cm^{-3}) and crumb grain (0.145%) of dough. Sensory characteristics of dough viz., color, taste, flavour and overall acceptibility were also enhanced at 1.25 per cent of amylase (Neerja Rana *et al.,* 2017).

The investigations have shown the feasibility of waste loquat kernels as substrate in solid-state fermentation for α-amylase production by Penicillium expansum MT-1. The kernels accounted for 22.5% of whole fruit (by wet weight). They were rich in protein (22.5%) and total carbohydrate (71.2%). The starch accounted for 25.6% of total carbohydrate and 36% of whole kernel, on dry weight basis. The fungus was isolated from fermented loquat kernels. Loquat kernel flour (LKF) could serve as a sole source of nitrogen and carbon for the fungus to grow and synthesize α-amylase. However, additional carbon and nitrogen sources increased the enzyme production.

Supplementation of each one of alcohols gave rise to a positive effect on the enzyme production. Optimal conditions for the production of α-amylase by the fungus on LKF were determined as initial moisture content of 70%, particle size of 1 mm, pH 6.0, incubation temperature of 30 , starch and peptone as supplements, 1 ml methanol as supplement alcohol and incubation period of 6 days. Under the optimized culture conditions, the maximum enzyme production was 1012 U/g of LKF. Usability of waste loquat kernels as substrate in microbial culture media for the production of α-amylase was investigated for the first time in the present study (Serkan erdal *et al.,* 2009).

α- amylase inhibitor was purified by buffer extraction, ammonium sulfate fractionation, CM-cellulose, and sephadex G-75 chromatography from the soaked seeds of Mucuna pruriens. The molecular weight determined by gel permeation chromatography on Sephadex G-100 and SDS-PAGE, both in the presence and absence of 2-mercaptoethanol, was found to be 27.24 kDa and 25.6 kDa, respectively. The purified Mucuna pruriens amylase inhibitor showed a specific inhibitor activity of 61.18, fold purity of 36.68, and the yield obtained was 14.01%. The purified amylase inhibitor was found to be heat-stable and retained 80.50% activity at 65°C. Inhibitor was found to have pH optima of 6.9. Hundred percent zone of inhibition was observed when added on the plated organisms of purified inhibitor. Purified amylase inhibitor was found to inhibit the activity of human salivary α-amylase. Inhibitory activity of α-amylase inhibitor against mammalian amylases could suggest its potential in treatment of diabetes and cure of nutritional problems, which results in obesity. Practical applications Purified amylase inhibitor was found to inhibit the activity of human salivary α-amylase. The potential of this inhibitory activity from α-amylase inhibitors, especially in the mammalian α-amylase, could play an important role in the management of nutritional and diabetes-related disorders. Mucuna, an underutilized legume found in tropical region and also cultivated as food by various tribal's in Asia and Africa can be used as a potential source for extraction of these beneficiary protease inhibitors, which in turn finds its applications in various human therapeutic and/or disorder management (Rajiv Pranesh Bharadwaj *et al.,* 2018).

Enzyme characteristics such as thermostability, selectivity, solvent tolerance and substrate affinity can be improved through genetic engineering based on the availability of large data on improving these characteristics. Alteration in pH stability is tedious and lacks rational approaches. However, a few reports on enhancing acid stability of α-amylases through protein engineering are available (Yang *et al.,* 2013).

The majority of the enzymes used at commercial scale lack adequate acid stability, thus limiting their applications. For using such enzymes, adjusting pH to their optima is required, which makes the process tedious, expensive and time consuming. In order to overcome the problems, many industries use acid-stable enzymes from fungal sources. Since these lack adequate thermostability, the enzymes get denatured when processes are carried out at elevated process temperatures (Demirjian et al., 2001; Elleuche *et al.,* 2014). In order to overcome these problems, microbes that are capable of tolerating harsh conditions could be exploited for naturally tailored enzymes that are superior to their neutronphililic counterparts for utility under harsh bioprocess conditions. It has generally been observed that the enzymes from acidophilic microbes function under their optimal growth conditions (Ferrer *et al.,* 2007), thus find several commercial applications. Furthermore, the study of these enzymes might also enable us to understand the underlying mechanisms to make them functional in extreme acidic conditions (Demirjian *et al.,* 2001). Although several acidophilic microbes have been reported, a very few acid-stable amylases have been studied in adequate detail (Matzke *et al.,* 1997 ; Sharma *et al.,* 2012).

The conventional industrial conversion of starch to glucose consists of a three-step industrial process: in the first step, 25–30% starch slurry is gelatinized in a jet cooker at 100–105°C for 5–10 min, in the second step α-amylase and Ca^{2+} (50 ppm) are added with pH adjusted to 6.5 for liquefaction, and in the last step, glucoamylase addition leads to the formation of glucose (Crabb and Mitchinson, 1997 ; Mehta and Satyanarayana, 2013). A few bottlenecks are associated with the process: firstly, gelatinization at higher temperature (100–105°C) requires high energy input. Secondly, native pH of starch slurry is around 3–4.5, thus, a pH adjustment step is required because α-amylases that are commercially available function best at pH 6–6.5. Thirdly, most of the α-amylases are

Ca^{2+}-dependent, and thus, Ca^{2+} is added during the process, which must be removed in the subsequent stages because glucose isomerase used in fructose syrup production is inhibited in the presence of Ca^{2+}. In order to make the process economical and time saving, there has been an emphasis on discovering Ca^{2+} independent, acid-stable and raw starch degrading thermostable α-amylases, which can hydrolyze raw starch at sub-gelatinization temperatures bypassing the energy intensive gelatinization, and avoiding Ca^{2+} addition and pH adjustment steps (Satyanarayana, 2010 ; Sharma *et al.*, 2016).

Despite extensive research on acidophiles, very few have been exploited for commercial purposes. (Matzke *et al.*, 1997) reported α-amylase from acidophilic bacterium *Alicyclobacillus acidocaldarius*, which is thermostable and acid stable with a molecular mass of 140 kDa, with optimum temperature and pH of 75°C and 3.0, respectively. (Bai *et al.*, 2012) reported another α-amylase from *Alicyclobacillus* sp. A4 with a molecular mass of 64 kDa and optimal activity at 75°C and pH 4.2. In still another report, an α-amylase from the acidophilic bacterium *Bacillus* sp. DR90, isolated from Dig Rostam hot mineral spring (Iran), was investigated. The enzyme was active in a wide range of pH and temperature having optimal activity at pH 4.0 and 75°C with a molecular mass of 76 kDa (Asoodeh *et al.*, 2014). (Laderman *et al.*, 1993) reported a thermo acidophilic α-amylase from *Pyrococcus furiosus* which was optimally active at ~100°C and pH 5.5–6.0. The enzyme is a homodimer with a subunit molecular mass of 66 kDa. Recently a Ca^{2+}-independent, acid-stable α-amylase (Ba-amylase) from the acidophilic bacterium *Bacillus acidicola* has been investigated in detail. This has been found to be a potential candidate for saccharification of starch at its native pH of 3.0–4.5 with $T_{1/2}$ of 25 min at 70°C (Sharma and Satyanarayana, 2010).

Amylases from acidophiles have tremendous potential to replace neutrophilic enzymes because they are active in the acidic range. Production on a commercial scale is, however, a major bottleneck because of low titres of extracellular amylases secreted by acidophilic and acid tolerant microbes. (Schwermann *et al.*, 1994) recorded maximum amylase production (90 U mL^{-1}) in the presence of maltose as compared to other carbon sources used in the production medium. (Bai *et al.*, 2012) reported a 2.3 U mL^{-1} α-amylase production in *Alicyclobacillus* sp. A4 after 48 h in the presence of starch as carbon source. A few researchers have attempted multiple strategies to increase the

extracellular acid-stable amylase titres. Improved *A. acidocaldarious* A2 strain by using UV/enrichment method for α-amylase production. A total of 11-fold increase in the enzyme titre was achieved as compared to the wild type strain (220 U mL^{-1}). (Brown *et al.*, 1990) attained a low constitutive level of α-amylase from *Pyrococcus furiosus* in the presence of simple sugars, while polysaccharides with α-1,4 linkages stimulated production. The maximum yield of extracellular α-amylase was achieved (100 UmL^{-1}) in *P. furiosus* in the presence of pullulan as a carbon source in submerged fermentation at 98°C.

A detailed investigation was carried out on the production of α-amylase by *B. acidicola* in submerged fermentation. Conventional "one-variable-at-a time" and statistical approaches have been used for optimizing the cultural parameters (Sharma and Satyanarayana, 2011). α- amylase production by *B. acidicola* was high in the presence of soluble starch (2%) as a carbon source. Among nitrogen sources tested, tryptone (HIMEDIA) [0.5%] in combination with yeast extract (0.5%) supported a high enzyme titre. The α-amylase produced by *B. acidicola* displayed a high activity (8 U/mL) at pH and 37°C after 44 h. The enzyme production was high when the cells are in stationary phase as in *Geobacillus thermoleovorans* (Uma Maheswar Rao and Satyanarayana, 2003). Further statistical approaches were employed for optimizing α-amylase production by *B. acidicola* in submerged fermentation. When the effect of 11 variables on α-amylase production was assessed using Plackett-Burman design, four variables (starch, K_2HPO_4, inoculum size, and temperature) were identified to significantly affect enzyme production. In order to control process parameters like aeration, uniform distribution of nutrients, and heat and oxygen transfer for α-amylase production by *B. acidicola*; fermentation was carried out in a 7 L laboratory fermentor. A reduction in fermentation time for attaining the peak was recorded; this could be due to improvement in mixing of nutrients and the control of dissolved oxygen (Kumar and Satyanarayana, 2007). Further, a 2.9-fold enhancement in enzyme production was attained due to fed-batch fermentation as compared to that in the initial unoptimized medium (3.5 U mL^{-1}) (Sharma and Satyanarayana et al., 2016).

Production of α-amylase by solid state fermentation was also attempted in order to find the prospects of using a wide range of agro-industrial residues as substrates (Babu and Satyanarayana *et al.,* 1995). A peak in enzyme production was reached in 72 h, when 10 g of wheat bran was used in 250 mL Erlenmeyer flasks as reported for the production of α-amylase by *Bacillus coagulans*. In contrast, 5 g wheat bran per 250 mL flask was used for α-amylase production by *B. amyloliquefaciens*. Alpha amylase production declined, with no growth and enzyme production below water activity 0.85, indicating that the bacterium is desiccation sensitive. Supplementation of wheat bran with ammonium sulfate supported a high enzyme titre. The addition of nitrogen sources to the solid substrates had been shown to enhance the production of various enzymes including α- amylase in solid state fermentation (SSF) (Pedersen and Nielsen, 2000). Moisture (substrate: water, 1:3.5), starch (2.9%) and ammonium chloride (0.38%) supported a high enzyme titre in *B. acidicola*. Statistical optimization of α-amylase production in SSF using response surface methodology led to 5.6-fold [28 ± 2.3 U/g dry bacterial bran (DBB)] increase in the titre as compared to unoptimized conditions (5 ± 1.1 U/g DBB) (Gangadharan *et al.,* 2006).

Acid-stable α-amylase gene from *Bacillus* sp. DR90 was successfully cloned in *E. coli* BL21 and expressed as an intracellular active protein. After induction, specific activity was around 600 U/mg (Asoodeh *et al.,* 2010). (Matzke *et al.* 1997) cloned acid-stable α-amylase gene from *Alicyclobacillus acidocaldarius* in *E. coli*. The enzyme was intracellular and the optimum temperature for recombinant acid-stable α-amylase was slightly lower than that of the native enzyme. Extracellular acid-stable α-amylase encoding gene of *P. furiosus* (PFA) was cloned and expressed in *E. coli* (Dong *et al.,* 1997). The recombinant acid-stable α-amylase was mainly expressed in the form of insoluble inclusion bodies. An improved purification method was developed by (Wang *et al.,* 2007). The solubilization of the inclusion bodies was achieved by treatment at 90°C for 3 min in Britton–Robinson buffer at pH 10.5. After solubilization, a total of 58,000 U/g wet cells yield was obtained. In another study, (Peng *et al.,* 2016) co-expressed PFA with chaperones in *E. coli*. Both chaperonin and a small heat shock protein (sHSP) increased the solubility of PFA to a certain degree, while pre-folding seemed to be the most efficient that increased the enzyme activity to about 60,000 U g^{-1} wet weight over

that of 5,000 U g^{-1} wet weight without chaperone. (Wang *et al.*, 2016) produced soluble PFA by expressing PFA in *B. amyloliquefaciens*. The yield of PFA was 2,000 U mL^{-1} of supernatant and 2,714 U mL^{-1} of total culture. (Zhu *et al.*, 2017) expressed PFA in *Nicotiana tabacum* and found that plant produced PFA forms functional aggregates with an accumulation level up to 3.4 g kg^{-1} fresh weight. The aggregates were functional without requiring refolding. As stated above, several attempts have been made to increase acid-stable α-amylase titres. Intracellular accumulation and inclusion body formation make them impractical for industrial applications (Grzybowska *et al.*, 2004; Wang *et al.*, 2007).

A truncated 1,441 bp acid-stable α-amylase gene encoding 479 amino acid α-amylase of *B. acidicola* was successfully cloned and expressed in active form in *E. coli*. Various approaches have been developed for efficient secretion of proteins such as increasing the permeability of the outer membrane chemically (adding EDTA, glycine, and Triton X-100) and by enzymatic (lysozyme) treatments. The purified recombinant α-amylase was active at pH 4.0 and 60°C, and retained all characteristics like that of the native α-amylase.

The methylotrophic yeast *Pichia pastoris* has emerged as an important production host for extracellular production of proteins for both basic research and industrial applications. Codon usage analysis of acid-stable α- amylase revealed the feasibility of its expression in *Pichia pastoris*. In order to increase extracellular production of acid-stable α-amylase, *Ba-amylase* was cloned and expressed in *P. pastoris* under dual promoters (*GAP* and *AOX*) and fused with α-factor secretion signal peptide. Mixed fed batch and high cell density cultivation experiments were performed which led to 15- and 7- fold higher extracellular enzyme titres than that of the wild type *B. acidicola* and recombinant *E. coli*, respectively. The recombinant acid-stable Ba-amylase purified from *P. pastoris* was biochemically characterized, which revealed kinetic properties and thermostability of glycosylated acid-stable Ba-amylase to be similar to those of the recombinant acid-stable Ba-amylase expressed in *E. coli*. The engineered Ba-amylase (Ba-Gt-amylase) was also cloned and expressed in *P. pastoris*. The combination of multiple transformations and post-transformational vector amplification (PTVA) and high

cell density cultivation in fermenter led to a very high production (750 U/mL) of the chimeric Ba-Gt-amylase (Cregg *et al.,* 2009; Spohner *et al.,* 2015).

Although the adaptation of acid stable enzymes to low pH has not been explored in greater detail, one explanation for pH stability has been offered from the modeling of α-amylases from *B. acidicola* and other acid-stable amylases. A detailed investigation revealed that the acid stability and activity at acidic pH could be attributed to the surface charge density and amino acid composition of these proteins. A prominent feature of acid stable α-amylases is the excess of glutamic and aspartic acid (D + E) residues on their surface as compared to their closest relatives. Moreover, the enzyme contains less positively charged amino acid residues (K + R + H) than their neutrophilic counterparts that leads to reduced positive charge density at the surface of the protein (Reed *et al.,* 2013). This effect was interpreted as follows: if the proteins were to possess a large content of positively charged residues (K + R), the positive charges at the surface will repel each other, leading to unfolding of the protein. On the other hand, protonation of the negatively charged group increases at lower pH that leads to reduction in the negative charge, which aids in stabilizing proteins in acidic conditions. If such proteins were to possess a large excess of negative groups, unfolding might also occur above the isoelectric point of the protein due to disruption of stabilizing structural interactions. In order to be stable and active in a broad pH range, these groups of proteins must have reduced number of D + E residues, which is compensated by an increase in the number of polar residues (Matzke *et al.,* 1997; Schäfer *et al.,* 2004; Reed *et al.,* 2013).

These characteristics were also found in other proteins. (Huang *et al.,* 2005) reported a high number of acidic residues on the surface of proteins, which causes repulsion due to excess negative charges resulting instability of proteins at high pH. However, a few exceptions have been found to this rule. (Schäfer *et al.,* 2004) reported thermo acid stable maltose-binding protein from *Alicyclobacillus acidocaldarius*. This protein has higher content of basic residues exposed on its surface, while most acidic residues are buried in the interior. As a consequence, this protein has a highly positive surface charge. Change in the pH activity of the amylases is depends the pKa of the catalytically important residues which are known to be influenced by the electrostatic field. It has been hypothesized that slight change in the pKa values of the catalytic

residues can change the pH activity profile of the enzyme (Nielsen *et al.,* 1999; Nielsen and Borchert, 2000). The pKa of catalytic residues in the active site can be altered by mutating selected residues that can alter the hydrogen bonding network, solvent accessibility or change in the net charge of the molecule. This can be explained as follows: active site residues must be in a catalytically competent protonation state for the enzyme to be active. The proton donor (Glu) is required to be protonated, while the nucleophile (Asp) must be negatively charged. If an α-amylase is stable over the entire pH range, it is feasible that the pH-activity profile can be changed if the pKa value of either the nucleophile or the proton donor is changed. Typically, charged residues are inserted in the vicinity of the titrable group to change the immediate environment of the active site of enzymes. In several cases, it has been observed that pH ± activity profiles shifted in the opposite direction as compared to the shift predicted from electrostatic calculations. This strongly suggests that electrostatic effects cannot be the best method to alter the optimum pH for enzymes. Secondary structural content does not appear to vary greatly in different amylases, which suggests that it is not a contributing factor (Wind *et al.,* 1998; Nielsen *et al.,* 1999).

Acid-stable α-amylases appear to degrade starch essentially by the same mechanism as neutrophilic α-amylases despite their distinguishing characteristics. This contention is based on the deduction that the α-amylase from acid-stable and neutrophilic members conserved the same charge at the catalytic active site. Moreover, multiple amino acid alignments and site directed mutagenesis revealed that acid-stable amylases conserved same catalytic residues (Asp^{189}, Glu^{320}, and Asp^{401}) like that of their neutrophilic counterparts. Hence, the acid stability of amylases has minor effect on their catalytic sites.

Several methods of protein engineering are employed for improving acid stability of thermostable α-amylases to make them suitable for industrial applications, although success achieved so far is negligible. (Nielsen *et al.,* 1999) modified thermostable α-amylase from *Bacillus licheniformis* by using site directed mutagenesis, which was predicted to change the pKa values of the catalytic residues. The observations suggested that pH ± activity profiles of mutants which change the net charge on the molecule were significantly different from the wild-type pH ± activity profile. The differences were,

however, difficult to correlate with the electrostatic field changes calculated. In another study, two amino acids of α-amylase from *B. licheniformis* were substituted (Leu^{134} to Arg and Ser^{320} to Ala) for acid tolerance, and the mutated gene was expressed in *Bacillus subtilis* WB600. The α-amylase variants were found to be more acid tolerant than the native protein. The optimum pH and stable pH range of the mutein (mutated protein) were 4.5 and 4.0–6.5, as compared to 6.5 and 5.5–7.0 as the optimum pH and pH stability range of the native protein. It has been postulated that mutations changed the net charge on the substituted residues, which influenced the pKa values of catalytically important amino acid residues (Liu *et al*., 2012).

Yang and coworkers engineered amylase from *Bacillus subtilis* for improving protein stability and catalytic efficiency under acidic conditions by site-directed mutagenesis (Yang *et al.,* 2013). Based on the analysis of 3-D structure model, four basic histidine (His) residues (His^{222}, His^{275}, His^{293}, and His^{310}) in the catalytic domain were replaced with acidic aspartic acid (Asp) residues. The acid stability of the enzyme was significantly enhanced after mutation. It has been observed that the hydrogen bonds and salt bridges increased after mutation around the catalytic domain. The higher pKa of Asp was responsible for destabilizing the protonated form of Glu^{250}, resulting in a decrease of the pKa value of Glu^{250}. These changes around the catalytic domain have been suggested for improvement in protein stability and catalytic efficiency at low pH. Similar efforts have also been made for improving the pH stability of amylases by protein engineering (Shaw *et al.,* 1999 ; Liu *et al.,* 2012).

Despite a few strategies reported in the literature for improving acid stability of proteins (Yang et al., 2013), there is no universal strategy that aids in engineering pH-activity profiles. Therefore, further research efforts are needed to find other factors that can contribute to the acid stability of proteins. Investigations have shown that it is possible to increase the thermostability, but not so with acid-stability. Therefore, instead of increasing acid-stability of already existing thermostable enzymes, a better option is to improve the thermostability of acid-stable enzymes. Since α-amylase from *B. acidicola* is stable in acidic conditions with moderate thermostability, an attempt has thus been made to improve thermostability. Several chimeras were constructed with the addition of amino

acids at N- and C-terminal ends of acid-stable Ba-amylase from the α-amylase of *Geobacillus thermoleovorans* (Gt-amylase). All chimeras were successfully expressed in active form in *E. coli*. Among all chimeras, one chimera displayed higher thermostability and specific activity as well as catalytic efficiency without change in its acid stability and pH optimum for activity. Increase in starch binding capacity of the chimeric α-amylase was observed in comparison with that of the wild type. The adsorption of chimeric α-amylase to raw starch suggests that the hydrolysis of raw starch can occur at high temperatures without energy-intensive gelatinization step, which brings down the energy consumption for starch saccharification. Furthermore, the end products of raw starch hydrolysis by the chimera suggested that the addition of residues did not alter the catalytic activity. The chimeric α-amylase was fused with acid-stable glucoamylase of *Aspergillus niger* through a linker peptide for saccharifying starch in a single step. The kinetic properties of the fused enzyme supported its suitability in raw starch saccharification in acidic conditions of native starch that liberates glucose besides maltodextrins as the major starch hydrolysis products. The fused chimeric enzyme can, therefore, be a practical option for the cost effective saccharification of raw starch. Engineering multidomain enzymes that are capable of catalyzing two or more reactions is a potential strategy to reduce enzyme costs in industrial processes because multiple catalytic properties in a single polypeptide simplify production and purification process (Fan *et al.*, 2009 ; Ribeiro *et al.*, 2011).

Calcium is known to stimulate α-amylases and has also been implicated in enhancing their thermostability (Savchenko *et al.*, 2002). Acid-stable Ba-amylase was found to be a calcium-independent. A few Ca^{2+}-independent α-amylases have also been reported earlier (Babu and Satyanarayana, 1993; Asoodeh *et al.*, 2010), which were considered to be useful in industrial starch saccharification. In general, most of the α-amylases possess conserved calcium ion binding sites, which are positioned at the interface between domains A and B, and play a major role in its stability and activity (Boel *et al.*, 1990 ; Linden et al., 2003). *Bacillus* α-amylases have been reported to have three Ca^{2+} ions and one Na^{+} ion and a metal triad bridge (calcium-sodium-calcium). This metal triad is important for maintaining the compact protein structure and provides thermal stability to the enzyme (Linden *et al.*, 2003). Calcium ion helps in salting out of

hydrophobic residues in the protein, resulting in the formation of a compact structure that enhances stability. Whenever calcium ions are detached, amylases lose their stability, while its restoration recovers the stability (Boel *et al.*, 1990). Sequence analysis revealed that acid-stable Ba-amylase comprises three calcium binding sites despite the fact that Ba-amylase does not show any calcium-dependent activity. Since the calcium binding site is far away from the active site residues, it has been postulated that the role of calcium ions is in maintaining the structure rather than in catalysis. A second theory suggests that calcium is loosely bound, which is replaced by other metal ions such as Na^+. These theories get support from the investigations on other calcium-independent enzymes (Nonaka *et al.*, 2003).

Extremophiles are potent sources of extremozymes, which display a high stability under extreme bioprocess conditions (Elleuche *et al.*, 2014).The biocatalysts from extremophiles have been shown to be useful in industrial bioprocesses. Only a few extremozymes, however, found their way to the market (e.g., thermostable DNA polymerases from *Thermus aquaticus* and *Pyrococcus furiosus*). There is a tremendous potential for acid stable enzymes from acidophiles to revolutionize existing industrial processes and to make many novel applications possible (Mehta and Satyanarayana, 2016; Sharma *et al.*, 2016).

An extracellular amylase was purified upto homogeneity from the culture broth of Bacillus licheniformis SKB4 by a combination of 80% $(NH_4)_2SO_4$ precipitation, DEAE cellulose column chromatography, and sephadex G-100 gel filtration technique. The purified alpha amylase exhibited specific activity of 827 U/mg with 64.8% yield having a molecular weight of 60 kDa and retained 80% of its original activity in the presence of 5 M NaCl solution. Starch is the best substrate (100%) for enzymatic digestion followed by amylopectin, potato starch, corn starch, amylose and glycogen. The non-inhibitory effect of Beta-specific inhibitor p-chloromercuro benzoate and iodoacetamide (10 mm), rapid blue loss percentage of starch-iodine complex and formation of Alpha-anomeric products of starch hydrolysis indicated that the purified enzyme was an endo attacking Alpha amylase. This enzyme hydrolyzes starch to malto oligosaccharides like materials maltotriose (G3), maltotetrose (G4), maltopentose (G5) at an early stage of the reaction

which were further hydrolyzed to maltose. This salt-tolerant and malto-oligomer producing alpha amylase has commercial value, especially in the food industry (Saptadip Samanta *et al.,* 2017).

Most of the extracellular enzymes of acidophilic bacteria and archaea are stable at acidic pH with a relatively high thermostability. There is, however, a dearth of information on their acid stability. Although several theories have been postulated, the adaptation of acidophilic proteins to low pH has not been explained convincingly. This review highlights recent developments in understanding the structure and biochemical characteristics, and production of acid-stable and calcium-independent α-amylases by acidophilic bacteria with special reference to that of Bacillus acidicola (Deepak Parashar *et al.,* 2018).

Studies on extracellular enzymatic activity profiles in yeast and yeast like strains isolated from tropical environment. (Haq *et al*., 2003) suggested the formulation of medium for production of alpha-amylase by *Bacillus licheniformis. (*Akpan and Adelaja 2004) worked on production and stabilization of amylase preparations from rice bran solid medium. (Reshmi *et al*., 2006) worked on the enhancement of activity and stability of α-amylase immobilized on alumina. (Sivaramakrishnan *et al.,* 2006) were studied various microbial sources for the extracellular production of amylase enzyme. (Buzzini and Martini *et al*., 2002) Isolate a novel raw starch digesting α-amylase bacteria *Bacillus* sp. YX-1: they worked on the purification and characterization of amylase enzyme. Maximum α- amylase activity (53 U mL^{-1}) was obtained at 45°C after 44 h of incubation. The enzyme was purified using ammonium sulfate precipitation, ion exchange and gel filtration chromatography, and showed a molecular weight of 56 kDa by SDS-PAGE. This enzyme activity was maximum pH 5.0, and optimum active temperature was at 40-50°C. (De Souza and Magalhães *et al.,* 2010) studied about the application of microbial α-amylase in different types of industries.

Stability in higher temperature at 105°C and pH 11.0 (Devi *et al*., 2012) worked on Immobilization of purified alpha-amylase enzyme which was produced by *Bacillus sp*. They immobilized the enzyme in sodium alginate and studied the stability and activity of immobilized enzyme beads in different pH and temperature. At 60°C and pH

6 the enzyme was incubated for 10 minutes showed maximum enzymatic activity (Jamrath *et al*., 2012). Production of amylases and proteases by *Bacillus caldolyticus* from food industry wastes. In this work, thermo-stable a-amylase and neutral proteases were produced using the thermophilic strain *Bacillus caldolyticus* (Mojsov *et al*., 2012). Studied about different amylases and their industrial application. He studied the way by which amylase enzyme is used in different industries like detergent, beverage, leather, pharmaceutical and paper industries (Akkaya *et al*., 2012).

Asoodeh and coworkers made studies on constituents of media for higher amylase production and purified amylase with different chromatographic technique (Asoodeh *et al*., 2013). They found optimum pH and temperature is 6-7 and 60 - 80°C respectively. They also analyzed the effect of different ions and it was observed that enzyme activity was increased by Ba^{2+}, Fe^{2+} and Mg^{2+}, and decreased by Hg^{2+} and Zn^{2+}, while it was not affected by Na^{+}, K^{+}, phenyl methyl sulfonyl fluoride and β-mercaptoethanol. Ca^{2+} and EDTA did not have significant effect on the enzyme activity and thermal stability. (Kumar *et al*., 2013) worked on thermo-stable α-amylase enzyme production, purification and characterization from *Bacillus laterosporus*. (Abdel-Fattah *et al*., 2013) analyzed the effect of temperature and pH on purified enzyme and found maximum activity in at optimal conditions of temperature 60°C and pH 7. They found no effect in the activity of enzyme in presence of Ca^{2+} ions and EDTA and in presence of Mg^{2+} ions, SDS and β-mercapto-ethanol activity was reduced. Isolate *Bacillus subtilis* for the production, purification and characterization of α-amylase. There result showed that the partially purified enzyme has specific activity of 0.144±0.019 U/mg, these was increase of 33.5 times than the raw enzyme extract. The optimum pH of the purified enzyme was 6.0, but the enzyme can work in the pH range of 5.0 – 9.0. The optimum temperature of the enzyme was 60°C. (Sundarram and Murthy *et al*., 2014) has studied on the different technique, methods and process parameters for amylase production. They studied the different enzymatic activity determination methods and their applications.

The specific measurement of a-amylase activity in crude plant extracts is difficult because of the presence of f8-amylases which directly interfere with most assay methods. Methods compared in this study include heat treatment at 70°C for 20 min, $HgCl_2$ treatment, and the use of the amylase specific substrate starch azure. In comparing alfalfa (Medicago sativa L.), soybeans (Glyjine max IL.l Merr.), and malted barley (Hodum vsdgare L.), the starch azure assay was the only satisfactory method for all tissues. While amylase can liberate no color alone, over 10 International units per ml amylase activity has a stimulatory effect on the rate of color release. This stimulation becomes constant (about 4-fold) at amylase activities over 1,000 International units per ml. Two starch azure procedures were developed to eliminate .8-amylase interference: (a) the dilution procedure, the serial dilution of samples until -amylase levels are below levels that interfere; (b) the, B-amylase saturation procedure, addition of exogenous f8-amylase to increase endogenous f8-amylase activity to saturating levels. Both procedures yield linear calibrations up to 0.3 International units per milliliter. These two procedures produced statically identical results with most tissues, but not for all tissues. Difference between the two methods with some plant tissues was attributed to inaccuracy with the dilution procedure in tissues high in amylase activity or inhibitory effects of the commercial fi-amylase. The amylase saturation procedure was found to be preferable with most species. The heat treatment was satisfactory only for malted barley, as a-amylases in alfalfa and soybeans are heat labile. Whereas $HgCI_2$ proved to be a potent inhibitor of f8-amylase activity at concentrations of 10 to 100 micro molar, these concentrations also partially inhibited a-amylase in barley malt. The reported a-amylase activities in crude enzyme extracts from a number of plant species are apparently the first specific measurements reported for any plant tissues other than germinating cereals (Douglas c. doehlert. *et al*., 1982).

The α-amylase produced by *Brevibacillus borstelensis* R1 has a number of applications in many fields such as bakery industry, food preparations, automation dish washing, ethyl alcohol dual fermentation, sago and rice industrial effluent treatment, sewage water treatment, fodder production, laundry and textile industries. The effect of inhibitors ($AgNO_3$, $HgCl_2$, EDTA, $CuSO_4$, L-Glutamic acid and $ZnCl_2$) on partially

purified α-amylase activity from *Brevibacillus borstelensis* was studied. All the inhibitors inhibited the activity when compared with the control. The percentage of α-amylase activity retained after treating with 0.5M of L-Glutamic acid, $HgCl_2$, $AgNO_3$, $ZnCl_2$, $CuSO_4$ and EDTA was 31.59%, 27.79%, 24.58%, 23.69%, 20.19% and 19.03% respectively (Suribabu K. *et al*., 2014).

Studies have evaluated the inhibitory effects of plant-based extracts (grape seed, green tea, and white tea) on α-amylase and α-glucosidase activity, glucosidases required for starch digestion. The abundant flavan-3-ol monomers (catechins) in these extracts were also tested for their inhibitory potential and evaluated against the pharmacological glucosidase inhibitor, acarbose. To evaluate relative potency of these extracts and catechins, the concentrations required for 50% and 90% inhibition of enzyme activity were determined. Maximum enzyme inhibition was used to assess an inhibitor's relative efficacy. Results showed that grape seed extract strongly inhibited both α-amylase and α-glucosidase activity, with equal and much higher potency, respectively, than acarbose. While tea extracts and individual catechin 3-gallates were less effective inhibitors of α-amylase, they were potent inhibitors of α-glucosidase. Our data show that plant extracts containing catechin 3-gallates are potent inhibitors of α-glucosidase, and suggest that procyanidins found in grape seed extract strongly inhibit α-amylase activity (Meltem Yilmazer-Musa. *et al*., 2015).

Diabetes mellitus is a metabolic disease characterized by hyper-glycaemia resulting from either defect in insulin secretion, insulin action, or both. Though, there exists many therapeutics for the treatment of diabetes, Plant based drugs are generally considered safe and are much effective. However, traditional usage of medicinal plants for the treatment of diabetes lacks scientific validation. Enzyme inhibitors have potential role in many areas of disease control and treatment and this review focuses on the plants which possess outstanding anti-diabetic property which could be possibly investigated further for the presence of alpha-amylase inhibitor (K. Alagesan *et al.,* 2012)

Inhibition of α-amylase, enzyme that plays a role in digestion of starch and glycogen, is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity, as well as, dental caries and periodontal diseases. Plants are an important source of chemical constituents with potential for inhibition of α-amylase and can be used as therapeutic or functional food sources. A review about crude extracts and isolated compounds from plant source that have been tested for α-amylase inhibitory activity has been done. The analysis of the results shows a variety of crude extracts that present αamylase inhibitory activity and some of them had relevant activity when compared with controls used in the studies. Amongst the phyto-constituents that have been investigated, flavonoids are one of them that demonstrated the highest inhibitory activities with the potential of inhibition related to number of hydroxyl groups in the molecule of the compound. Several phyto-constituents and plant species as α-amylase inhibitors are being reported in this article. Majority of studies have focused on the anti-amylase phenolic compounds (Paloma Michelle de Sales. *et al*., 2012).

Natural products are considered as potential sources of pharmaceutical agents and/or as sources of lead compounds in drug development. In this study, two species of plant seeds were analyzed which are: Radish (*Raphanus sativus L*.) Rape seed (*Brassica napus L*.) The present study was conducted to evaluate the quantity of phytochemicals present, antioxidant activity, α-amylase inhibitory activity, and antibacterial activity of those two edible seeds. The total flavonoids in the plant extract were estimated using the aluminum chloride colorimetry method. Antioxidant activity was analyzed by DPPH (2,2-diphenyle-1-picryl-hydrazylehydrate) assay. Alpha-amylase inhibitory activity was determined by modified method described by the Worthington Enzyme Manual. The antibacterial activity was carried out by disc diffusion assay. The results revealed that the methanolic extract of rapeseed (1000µl) extract exhibited the highest radical scavenging activity with IC50 value of 323.30 mg/ml. Radish seed showed the highest alpha amylase inhibition activity with IC50 value of 1332.99µg/ml. Both seeds showed significant antibacterial activity against six pathogenic bacterial species. Results obtained in this study indicated the promising medical potential of these two plant species (Bibek Khatiwada. *et al*., 2018).

It explores the natural compounds and the potential α-amylase and α-glucosidase inhibitory actions of stem extracts. The aqueous stem extract was selected from other extracts (ethanol, acetone, petroleum ether and chloroform) for the in vitro study of anti-diabetic activity by alpha amylase and alpha glucosidase inhibitory assays. The stem extract was also analyzed by gas chromatography mass spectrometry to identify the natural chemical components. Phytochemical analysis of aqueous stem extract showed major classes of secondary metabolites such as phenols, flavonoids, alkaloids, terpenoids, tannins, saponins. The total flavonoid, terpenoid, and tannin contents were quantified as 19.82±0.06 mg QE/g, 96.2±0.20 mg/g and 11.25±0.03 mg TAE/g respectively. The percentage inhibition of assays showed maximum inhibitory effects (59.46±0.04% and 68.51±0.01%) at a concentration of 100 mg/ ml. The IC50 values of stem extract was found to be 73.56 mg/mL and 80.90 mg/mL for alpha amylase and alpha glucosidase inhibition. Fifteen chemical constituents were found by GC-MS analysis. This study suggests the aqueous stem extract of Salacia oblonga might be considered as potential source of bio active constituents with excellent anti-diabetic activity (Gladis Raja Malar. *et al*., 2018).

Chapter 3

MATERIALS AND METHODS

All amylase, irrespective of their source, have identical structure of their active site and therefore exhibit similar catalytic mechanism. Amylases are enzyme which hydrolyse starch molecule to give diverse product including dextrin and progressively smaller polymer composed of glucose units. Alpha amylase acts on starch and breaking them up into sugars (hence the term saccharification). Starch is a carbohydrate source consisting of two molecules, amylose and amylopectin. Amylose is formed from chains of glucose linked by α-1, 4-linkage and amylopectin is formed from α-1, 4-linked chains of glucose with 1, 6 linked branch points. The amylases are enzymes that work by hydrolyzing the straight chain bonds between the individual glucose molecules that make up the starch chain.

These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, and textile to paper industries, starch syrup industry, distillery and detergent industry. In present studies, the *vigna radiata* amylase was characterized biochemically with respect to various kinetic parameters viz. steady state kinetics, stability studies, inhibition studies. Such studies are important for refinement of the overall understanding of the catalytic mechanism of amylase and have application in industries.

Collection of sample:

Seeds of *vigna radiata* were bought from the local market of Modipuram, Meerut.

Chemicals:

Mercuric chloride ($HgCl_2$), Rochelle salt (sodium - potassium tartrate), DNSA (Di-nitro salicylic acid), Starch (substrate), Maltose, Ethanol, monosodium phosphate, disodium phosphate, sodium chloride and Sodium hydroxide (NaOH) were purchased from Himedia. All solutions were prepared in double distilled water (DDW). Unless and otherwise mentioned, each experimental point represent the mean of three determinations.

Preparation of reagent:

Extraction buffer:

For the preparation of extraction buffer (0.2 M sodium phosphate buffer, pH-7.0), 0.345g NaH_2PO_4 was dissolved in 20ml of DDW, 0.445g $NaHPO_4$ was dissolved in 20ml of DDW and 1.46g NaCl was dissolved in 10ml of DDW, and the final volume was made to 50ml with pH 7.0.

Assay buffer:

Sodium phosphate buffer (0.05 M, pH 7.0) was used during this study. Enzyme was diluted with the same buffer during activity assay as required.

Substrate solution:

1% (w/v) Starch solution was prepared by dissolving 1g starch in 100ml of DDW with continuous boiling for 2-3min on hot plate. Solution was cooled and filtered. Filtrate was used as substrate solution.

3,5-dinitrosalicylic acid [DNSA]:

About 1g of DNSA was dissolved in 50ml of DDW (0.5% w/v). To this solution about 30g of sodium potassium tartrate tetra-hydrate was added in small lots. On addition the solution turned to milky yellow in color. Then 20ml of 2N NaOH was added, which changed the solution to transparent orange yellow in color. The final volume was made to 100ml with DDW. This solution was stored in an amber colored bottle.

Maltose solution:

Maltose solution (0.002M) was prepared by dissolving 0.1g of maltose in 100ml of DDW.

Rochelle salt solution:

40% (w/v) Rochelle salt solution was prepared by dissolving 40g of sodium potassium tartrate in small amount of DDW and pH was adjusted to 6.5 with a final volume of 100ml.

Extraction of enzyme:

During extraction of α-amylase all operations were carried out at 4°C. Following steps were performed in extraction of enzyme.

a) Sodium phosphate buffer (0.05M, pH 7.0) was used during this study. Enzyme was diluted with the same buffer during activity assay. For enzyme activity, the DNSA method was followed.

b) Mung bean was used as a source material for the extraction of α-amylase. 20g seed of mung bean were soaked in 50ml of DDW for 5-6 hour at room temperature and seeds were spread over the blotting paper in a petri dish overnight.

c) Alpha-amylase was extracted from the germinating seeds of mung bean. The seeds were washed and homogenized with motor and pestle in phosphate buffer.

d) A four layered muslin cloth (cheese cloth) was then used to filter and remove cell debris from the slurry.

e) Next, the filtrate obtained was centrifuged at 10,000 rpm for 15min at 4°C.

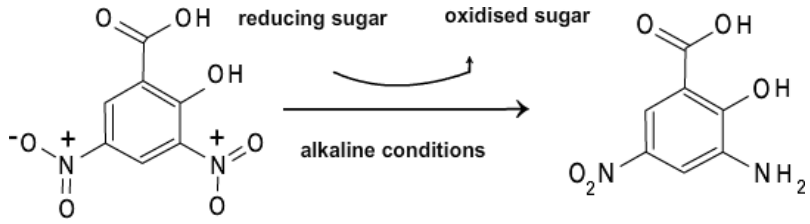
f) A clear supernatant containing mung bean α-amylase was obtained and was stored at 4°C till further use.

Construction of Maltose Standard Curve by DNSA Method

Maltose is a disaccharide made up of two subunits of glucose monomers. It is also called malt sugar. It is present in germinating grain, in a smaller amount in corn syrup, and also is a product of the partial hydrolysis of starch. Maltose is considered as an important constituent in making fermented barley which is used to brew beer.

Maltose is a reducing sugar. Maltose can be used as a standard for estimating reducing sugar in unknown samples. Constructing a standard curve / graph for maltose helps us to estimate concentration of reducing sugars present in an unknown sample and for determining the activity of α-amylase. The standard curve for maltose is usually constructed using 3, 5-dinitro salicylic acid (DNSA) as the reagent. Maltose reduces the pale yellow colored alkaline 3, 5-

dinitro salicylic acid (DNSA) to the orange- red colored, 3 amino – 5, nitro salicylic acid. The intensity of the color is proportional to the concentration of



maltose present in the solution [as per Beer Lambert's law]. This intensity change in color was measured using a UV Spectrophotometer as the absorbance at 540nm wavelength. Wave length was set to 540nm because it is the region where orange-red color absorbs. A series of solutions containing varying concentrations of maltose was prepared in test tubes and a known quantity of DNSA is added to each. These test tubes, was then heated on a water bath for 5min, Then 1ml of Rochelle salt solution was added in each test tube. The test tubes were cooled to room temperature, after taking them out of the water bath. Final volume in each test tube was made up to 10ml with DDW. Their optical densities were measured on a U.V-1800 Simadzu UV Spectrophotometer. A graph was then plotted with amount of maltose on X-axis and the observed optical density at Y-axis. The plot thus obtained was called a maltose standard curve.

Procedure:

- From the standard solution of maltose, different concentrations (0.36mg, 0.76mg, 1.08mg, 1.44mg, and 1.80mg) were made in five separate test tubes.
- A test tube containing a blank solution was also prepared.
- Using DDW, the volume was brought up to 2ml in each test tube, including the test tube containing the blank solution.
- 2ml of DNSA reagent was added to each tube and was covered with aluminum foil.
- The content in the test tubes were heated in a boiling water bath for 5 minutes.

- Then 1ml of Rochelle salt solution was added in each test tube.
- The test tubes were cooled to room temperature, after taking them out of the water bath.
- Final volume in each test tube was made up to 10ml with DDW.
- Absorbance was then recorded at λ_{max} = 540 nm. A graph was plotted as maltose concentration vs absorbance.

Alpha-amylase activity assay:

Following reagents were used for activity assay:

- Extraction buffer (0.05M sodium phosphate buffer; pH 7.0.)
- 1% w/v substrate solution (1gm starch in 100ml DDW)
- 0.05M sodium phosphate buffer, pH 7.0 was used as assay buffer.
- 40% w/v Rochelle salt solution was prepared as mentioned earlier.

Following steps were performed:

A) 5 test tubes were taken with one blank and 1ml of starch solution in every test tube was added.
B) 0.1 ml of enzyme was added in each test tube except blank.
C) Now all test tubes were incubated at room temperature (37^{o}C) for 15min.
D) The reaction was stopped by adding 2ml of DNSA in every test tube.
E) Next, all the test tubes were kept in boiling water bath for 5min.
F) 1ml of Rochelle salt solution was added in every test tube.
G) The test tubes were cooled under running tap water.
H) The final volume in every test tube was then made-up to 10ml with DDW.
I) Further, the absorbance was recorded at λ_{max} = 540 nm.
J) A blank without enzyme was run side by side and correction was applied for the same.

An enzyme unit has been defined as the amount of α-amylase required to liberate 1**µmol** of maltose per min under our test conditions.

Storage stability:

3.7.1 Temperature stability studies:

Mung bean alpha-amylase was stored separately at two different temperatures and assayed for enzyme activity at every 5 days intervals. The enzyme was stored at 4^{o}C and 37^{o}C (room temperature) for 50 days.

Steady state kinetics:

Determination of optimum pH:

The assay buffers of different pH values (4, 5, 6, 7, 8, 9, 10, and 11) were prepared and the activity assay was performed. Eight test tubes were taken and 1.0ml of starch solution was added in every test tube. The pH of solution was maintained (4, 5, 6, 7, 8, 9, 10 and 11) with the help of diluted HCl and NaOH. 0.1 ml enzyme was added in every test tube. Then 900 µL assay buffer was added. Further the test tubes were incubated at room temperature (37^{o}C) for 15min. The reaction was stopped by adding 2ml of DNSA solution in every test tube. Tubes were kept in boiling water bath for 5min. Then 1.0ml of Rochelle salt was added in each test tube. The tubes were cooled under running tap water and the volume was make-up to 10.0ml with the help of DDW. Absorbance was measured at 540nm. The percent relative activity was plotted against pH.

Determination of optimum temperature:

The effect of temperature on alpha-amylase activity was studied, by varying the temperature 20^{o}C to 90^{o}C in a multi-temperature water bath (TC-120, Grant instrument, England) during the activity assay. The various values of temperature chosen were 20, 30, 40, 50, 60, 70, 80, and 90^{o}C. The activity assay was performed at each of the indicated temperatures separately. Eight test tubes were taken and 1.0ml of starch solution was added in every test tube. Further, 0.1 ml enzyme was added in every test tube. 900 µL assay buffer was then added. All the tubes were kept for incubation at different temperature (20, 30, 40, 50, 60, 70, 80 and 90^{o}C) for 15min. After then the reaction was stopped by adding 2.0ml of DNSA solution in every test tubes. All tubes were kept in boiling water bath for 5min. Then after 1.0ml of Rochelle salt solution was added in every test tube. The tubes were cooled under running tap water and the final volume was made-up to 10.0ml of every test tube with the help of DDW. Absorbance was measured at 540nm. The percent relative activity was plotted against temperature.

Effect of substrate concentration on alpha-amylase activity:

To study the effect of substrate concentration on *vigna radiata* α-amylase, the experiment was carried out. The values of K_m and V_{max} were determined by measuring the amount of substrate converted into product by the enzyme in a unit time interval. The various parameters viz; enzyme concentration, temperature and incubation time were fixed at 5x dilution, 37ºC and 15min time, respectively while the substrate concentration varied in the range of 100mg–1000mg. Ten test tubes were taken and 1.0ml of starch solution (substrate) was added with different concentration (100mg, 200mg, 300mg, 400mg, 500mg, 600mg, 700mg, 800mg, 900mg and 1000mg). Further, 0.1 ml enzyme was added in every test tube. 900 µL assay buffer was then added. All tubes were kept for incubation at 37ºC (room temperature) for 15min. The reaction was stopped by adding 2.0ml of DNSA solution in every tube. All the tubes were kept in boiling water bath for 5min. Then 1.0ml Rochelle salt solution was added in every tube. Now the tubes were cooled under running tap water and the final volume was made to 10.0ml with DDW. Absorbance was taken at 540nm and Lineweaver-Burk graph was plotted.

Inhibition studies:

α-amylase inhibition with $HgCl_2$:

The stock solution of $HgCl_2$ was prepared in 0.05M sodium phosphate buffer, pH-7.0 and were suitably diluted for experiments. The activity assay was performed at standard conditions in the presence of varying concentration of inhibitors. Appropriately diluted alpha-amylase was mixed with varying concentration of the inhibitor during the activity assay. Ten test tubes were taken and 1.0ml of starch solution was added in every test tube. Further, 0.1 ml enzyme was added in every test tube. 900 µL assay buffer containing the indicated concentration of inhibitor was then added. The tubes were kept for incubation at room temperature for 15min. The reaction was stopped by adding 2.0ml of DNSA solution in every tube. Then all the tubes were kept in boiling water bath for 5 min. Then 1.0ml of Rochelle salt solution was added in every tube. Again the tubes were cooled under running tap water and the final volume was made to 10.0ml in every tube with DDW. Absorbance was taken at 540nm. Percent residual activity was plotted against inhibitor concentration and I_{50} value was calculated.

Alpha-amylase inhibition with $CuSO_4$:

The stock solution of $CuSO_4$ was prepared in 0.05M sodium phosphate buffer, pH-7.0 and were suitably diluted for experiments. The activity assay was carried out at standard conditions in the presence of varying concentration of inhibitors. Ten test tubes were taken and 1.0ml starch solution was added in every test tube. Further, 0.1 ml enzyme was added in every test tube. 900 µL assay buffer containing the indicated concentration of inhibitor was then added. The tubes were kept for incubation at room temperature for 15min. The reaction was stopped by adding 2.0ml of DNSA solution in every tube. All the tubes were kept in boiling water bath for 5min. Then 1.0ml Rochelle salt solution was added in every tube. The tubes were cooled under running tap water and the final volume was make-up to 10.0ml with DDW. Absorbance was taken at 540nm. Percent residual activity was plotted against inhibitor concentration and I_{50} value was calculated.

Chapter 4

RESULTS AND DISCUSSION

α-amylase was the first enzyme to be discovered and isolated (Payen *et al*., 1833). Although it can be derived from several sources, including plants, animals and microorganism, microbial enzymes generally meet industrial demands. Green gram locally called as Mung bean belongs to the family *leguminaceae.* A large number of microbial amylases are available commercially and they have almost complete replaced chemical hydrolysis of starch in starch processing industry (Bernfeld *et al*., 1955). Ohlsson suggested the classification of starch digestive enzymes in malt as α and β-amylases according to the anomeric type of sugars produced by the enzyme reaction (Gupta *et al.,* 2003). These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (Sivaramakrishnan *et al.,* 2006). α-amylase may be measured in other body fluids, including urine and peritoneal fluid (Anupama and Jayaraman 2011; Amutha and Priya 2011; Akcan 2011; Kaur *et al*., 2012). These α-amylase enzymes account for about 30 % of the world's enzyme production (Akcan 2011; Malle *et al*., 2012; Deb *et al*., 2013).

The objective of present investigation was to extract α-amylase from *mung bean*, commonly available in India and to undertake its studies, mainly steady state kinetics and inhibition studies.

Construction of Maltose Standard Curve by DNSA Method

Maltose is a reducing sugar. Constructing a standard curve / graph for maltose helps us to estimate concentration of reducing sugars present in an unknown sample and for determining the activity of α-amylase enzyme. Standard curve of maltose usually constructed using 3, 5-Dinitro salicylic acid (DNSA) as the reagent. Maltose reduces the pale yellow colored alkaline 3, 5-Dinitro salicylic acid (DNSA) to the orange- red colored, 3- amino, 5- nitro salicylic acid. The intensity of the color is proportional to the concentration of maltose present in the solution. Wavelength is set to 540nm because it is the region where orange-red color absorbed. A series of solutions containing varying concentrations of maltose are prepared. These solutions

heated on a water bath for few minutes and their optical densities were measured. Standard maltose curve was obtained when absorbance was plotted against maltose concentration. The calibration curve is follows a straight line path and thus give a linear relation between absorbance and maltose concentration (Fig.4.1).

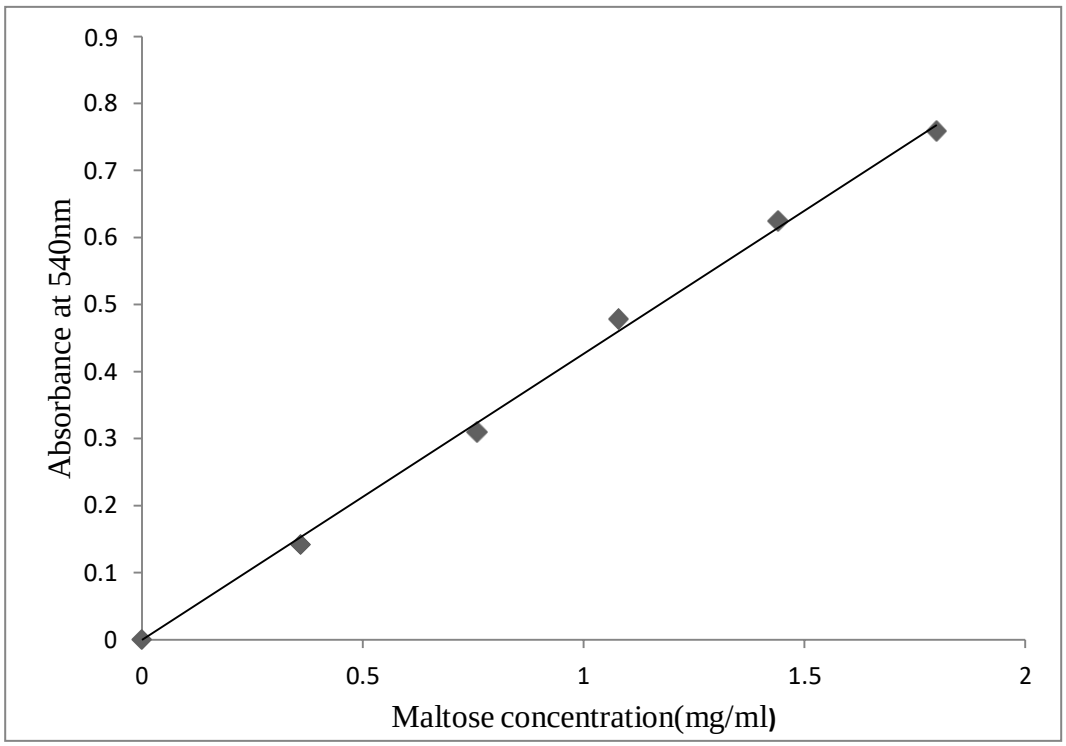


Fig.4.1. Construction of Maltose Standard Curve by DNSA method. Each experimental point represents the mean of three determinations.

Storage stability studies:

α-amylase was stored for the indicated time period (days) at 4°C (cold conditions) and 37°C (room temperature) in order to find appropriate temperature conditions to store the α-amylase for prolonged duration while retaining the maximum enzyme activity. The plot of percent residual activity versus the number of days gave a $t_{1/2}$ of 40 days for α-amylase stored at 4°C (Fig.4.2). When stored at 37°C, the activity of mung bean α-amylase decreases drastically with time and the value was found to be 15 days. It may be due to the fact that continuous high temperature exposer causes the denaturation of enzyme. The storage stability of α-amylase from different sources has been reported at different assay conditions and different temperature (Al-Qodah, 2007). Malhotra, R., Noorwez, S. M., & Satyanarayana, T. (2000), who reported production of α-amylase by *B. thermooleovorans* at 70°C and half-life of 3h at 100°C. The operational stability of enzyme preparation was ascertained by quantifying their activity in five consecutive cycles. It was observed that after five reuses, α-amylase enzyme still retained 75% of enzyme activity. There have been other reports also where other enzymes have been reused many times and still retained a good amount of activity (Krishna *et al.*, 2011). Increase in protein concentration in the α-amylase also accounts for more stability due to an increase in protein-protein interaction (Cheung and Truskett *et al.*, 2005). There are numerous reports where storage stability has enhanced upon α-amylase (Sharma *et al.*, 2003; Dwevedi and Kayastha *et al.*, 2009). Retained activities of α-amylase on various polymeric supports were previously reported as 75–90% after 25 days of storage (Tumturk *et al.*, 2000; Lim *et al.*, 2003; Kahraman *et al.*, 2006).

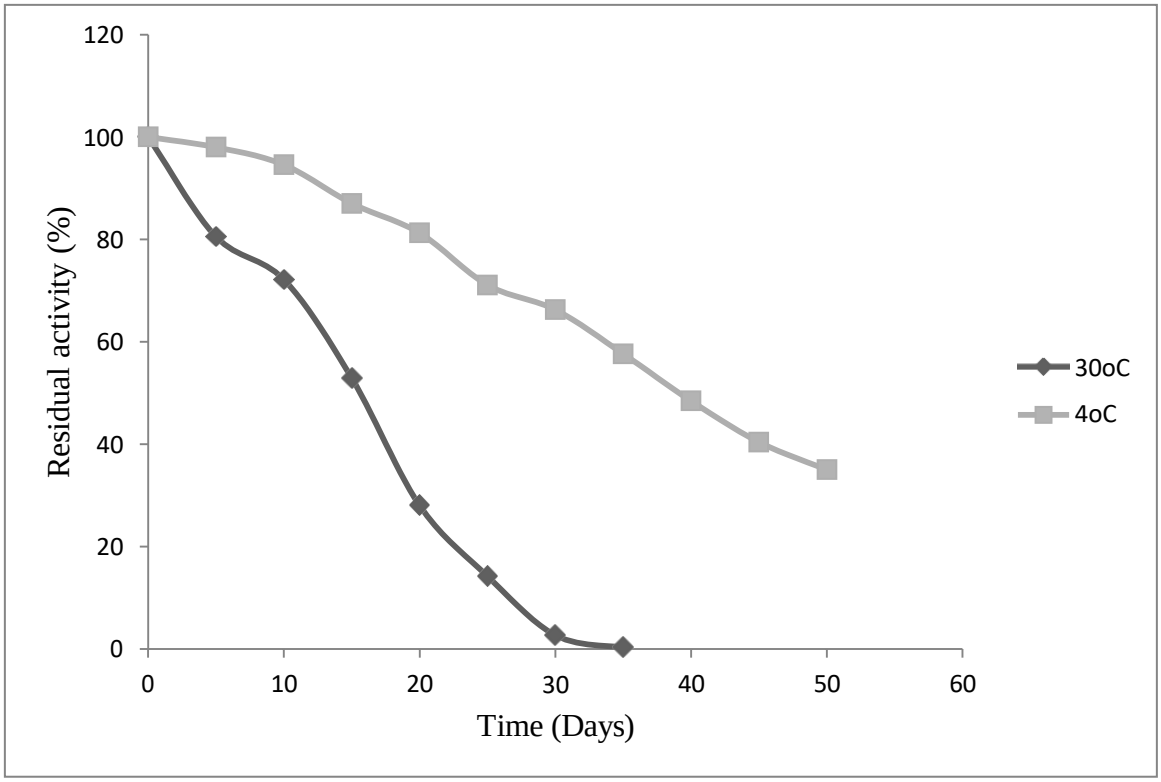


Fig.4.2. Comparison of stability of mung bean α-amylase when stored 4ºC and 37ºC. Each experimental point represents the mean of three determinations.

Steady state kinetics

Determination of optimum temperature:

The experiment was carried out to determine the effect of varying temperature on the activity of α-amylase from mung bean. The optimum temperature can be determined by measuring the amount of substrate transformed to product by an enzyme in a given time at different temperatures. The various temperatures used were 20, 30, 40, 50, 60, 70, 80 and 90^{o}C at constant assay conditions. The graph was drawn by plotting percent relative activity versus temperature. A bell-shaped curve (Fig.4.3.1.) was obtained when α-amylase was assayed at different temperatures. The optimum value of temperature was observed at 40^{o}C, where the α-amylase displayed 100% activity.

The temperature dependence of the activity of enzymes resembles in some respect the pH dependence, increasing first with rising temperature, than passing a maximum, followed by a decrease. Therefore, this behavior is frequently described as optimum temperature. At 20^{o}C, the relative activity was 27.35% whereas at 30^{o}C, the activity was 55.84%. At low temperature, the observed α-amylase activity is low because the enzyme works slowly. The substrate molecules have less energy and move slowly into the active sites. The low temperature does not denature the protein structure of the enzyme but rather slows down the enzyme and substrate collisions, thereby exhibiting low enzyme activity. At 40^{o}C, the α-amylase shows increase in activity (100%). The velocity of any chemical reaction increases 2-3 times with every 10^{o}C rise in temperature. This rule holds also for enzymatic reactions. When temperature was further decreased to 70^{o}C, the α-amylase activity declined to 38.88%. At 80^{o}C, the mung bean α-amylase showed the relative activity of 16.09%. This may be due to the fact that the 3-D structure of enzyme is thermo-sensitive and becomes destabilized at the high temperature causing the denaturation. This process opposes the acceleration of the reaction velocity and is responsible for its decline at high temperature. The progression of denaturation depends both on the actual temperature and on time, the higher the temperature, the faster denaturation. When enzyme is exposed to very high temperature (90^{o}C), this could lead to a complete thermal denaturation of the enzyme and may result in decreased enzyme activity. α-amylase from different source has been reported to be most efficient in the temperature range between 30^{o}C to 70^{o}C.

The optimum temperature of α-amylase from different sources has been reported viz. (Tapan Kumar *et al.*, 2005). Effect of temperature on partially purified enzyme activity was measured at pH 6.0 over a temperature range of 5-80ºC. The optimum temperature of the enzyme was 30ºC. Fifty percent of hydrolyzing activity occurred between 10-55ºC. At 70ºC, activity was 15% and at 80ºC, activity was virtually undetectable. The enzymes obtained from *B. licheniformis* generally were stable than those from other *bacillus species* (Fossi *et al.,* 2005). In such studies the optimum temperature was recorded at 50-60ºC (Asghar *et al.,* 2005; De-Souza and Martins, *et al.*, 2000). The effect of pH on enzyme stability and activity is also dependent on time and temperature. The maximum, enzyme activity was observed at 45ºC. Singh and coworker (2010) ave revealed that the highest enzyme activity was observed at 45ºC. The optimal temperature (60°C) of *A. terreus* α-amylase is significantly higher than that of most fungal α-amylases (Li *et al.,* 2011; Michelin *et al.,* 2010). Moreover, α-amylase from *A. terreus* showed excellent thermostability, whereas most fungal α-amylases were stable up to 50°C (Hostinova, Janecek, & Gasperik *et al.*, 2010).

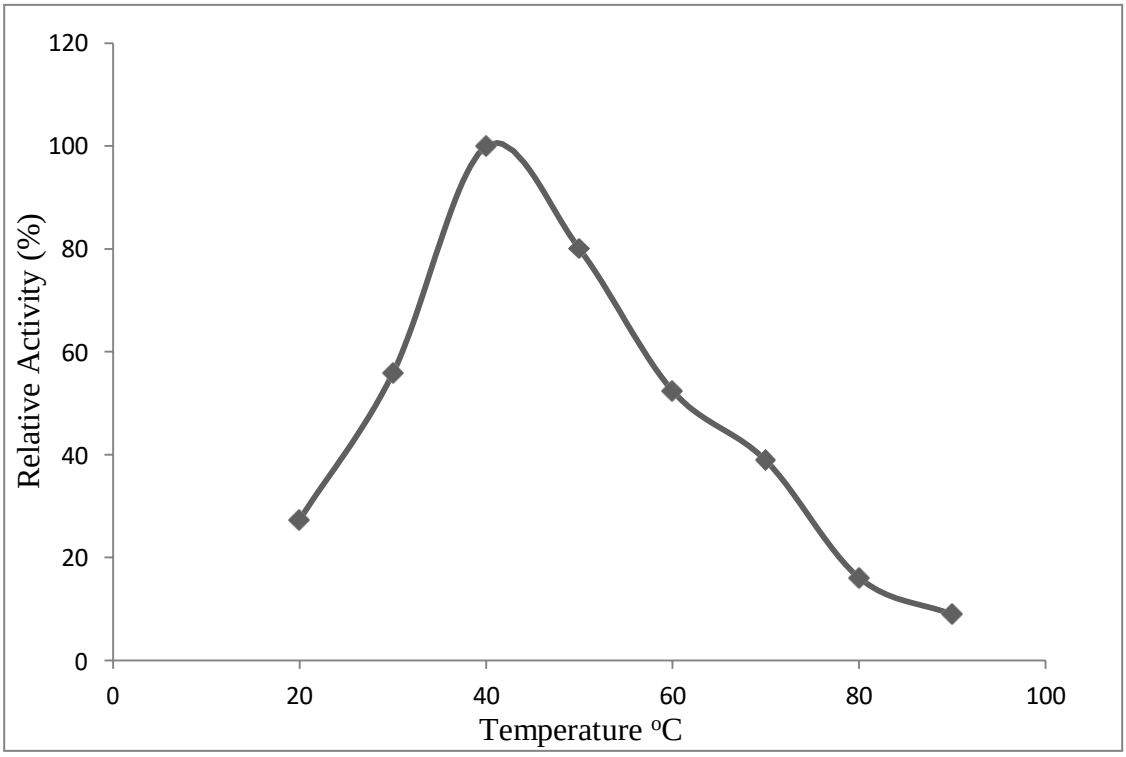


Fig.4.3.1. Effect of temperature on the activity of mung bean α-amylase. Each experimental point represents the mean of the three determinations.

Determination of optimum pH:

The activity of enzymes depends strictly on the pH of the assay mixture. Activities of most enzymes follow a bell shaped curve, increasing from zero in the strong acid region up to maximum value, and decreasing to zero to the strong alkaline region. Two different effects are responsible for this behavior: First, the state of protonation of functional group of amino acids and cofactor involved in the catalytic reaction. Secondly, the native three dimensional protein structure of enzyme. While protonation is a reversible process, damaging of the protein structure is mostly irreversible. In the simplest case protonation of one functional group promotes the catalytic activity, while protonation of another essential group break it down. The pH-value of the maximum of the pH-activity curve is the pH optimum. Since here the enzyme exhibits its highest activity, it is usually chosen as standard pH for the assay of the enzyme. The pH optimum of many enzymes is within the physiological range (about 6.8), not in any case accurately at this pH, but frequently between 6 to 8 (Bisswanger *et al*., 2014).

To determine the effect of pH on mung bean α-amylase, the experiment was conducted where the activity assay was performed at different pH value (4, 5, 6, 7, 8, 9, 10 and 11) at constant assay conditions. The graph was drawn by plotting the percent relative activity versus pH to give a bell-shaped curve (Fig. 4.3.2.). The rate of hydrolysis by mung bean α-amylase at 37^{o}C showed the optimum pH at 7.0 in pH range of (4 to 11). From the graph it is clear that at acidic pH of 4.0, mung bean α-amylase is only 14.50% efficient as compare to 100% activity at pH 7.0. When pH was increased from 4 to 5, then there was enhancement of α-amylase activity from 14.50% to 39.36%. The rate of hydrolysis of starch by the mung bean α-amylase showed the maximum activity of 100% at pH 7.0, that illustrate the neutral nature of mung bean α-amylase with that of other α-amylase viz., (Tapan Kumar *et al*., 2005), optimum pH of the enzyme was 6.0. The α-amylase retained more than 50% of its original activity between pH 4.6 and 6.8. The enzyme was stable over a wide pH range. More than 50% residual activity was obtained between pH 4.7 and 9.0. The enzyme was not stable below pH 3.5 or above pH 10.0. Boucher and Samain (1974) found pH 6.8 to be optimal for copepods and Mayzaud (1985) found pH 6.4 for Acartia clausi as optimal.

Van Wormhoudt *et al.,* (1983), however, reported an alkaline condition, 7.2 pH.). The optimum pH of the purified enzyme was 6.0, but the enzyme can work in the pH range of 5.0 – 9.0 (Sundarram and Murthy *et al*., 2014). It is evident that the optimum activity was obtained at pH 7.0 and it retained relatively high activity in the pH range 7 - 10. For instance, at pH 10 the enzyme retained about 80% of its maximal relative activity. However, steep reduction in α-amylase activity in acidic media. This activity is 30% at pH 5.5 and complete deactivation occurred at pH 4.5. This behavior is attributed to the structure of functional groups in the active site which seem to be basic and react with the hydrogen ion and the result was deformation of the enzyme active site. These results are in agreement with those of (Srivastava and Baruah *et al*., 1986) for the growth of *G. sterothermophilus* where complete deactivation occurred at pH 4.0. However, there are other α-amylases that have optimum pH in the acidic range. Stamford et al. (2001) reported that the optimum pH for the α-amylase produced by *Nocardiopsis sp.* at 70^{o}C was 5.0.

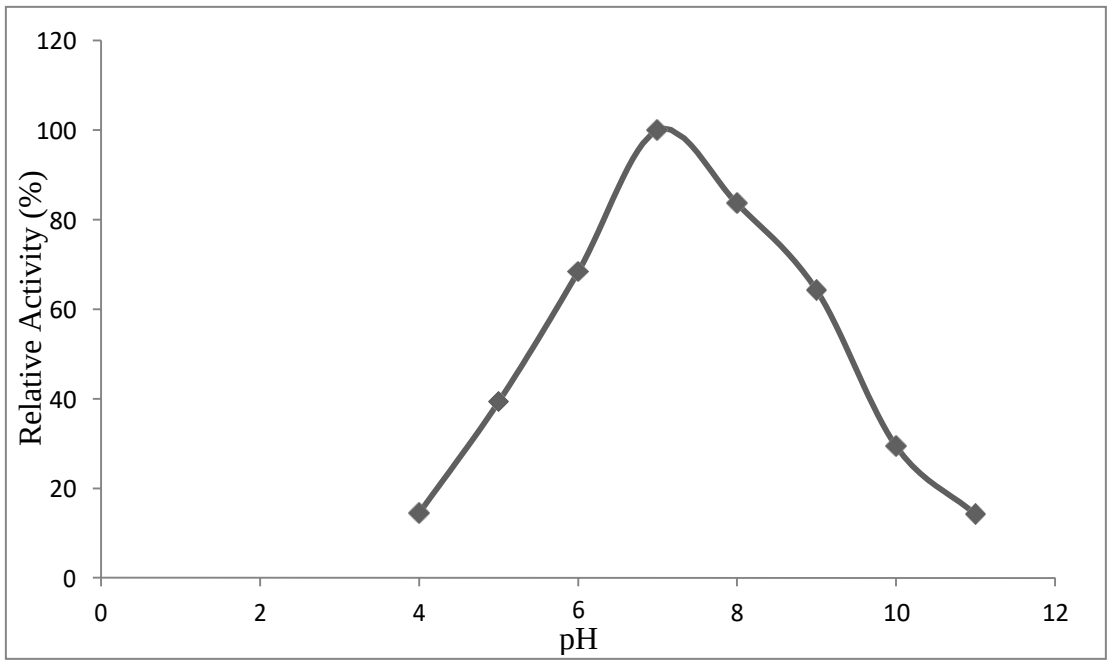


Fig.4.3.2. Effect of pH on the activity of mung bean α-amylase. Each experimental point represents the mean of three determinations.

Effect of substrate concentration on α-amylase activity:

The Mung bean α-amylase was assayed in the presence of varying concentration of starch (100-1000mg) and at standard conditions as described under materials and methods. It is clear from the Fig. (4.3.3), that the α-amylase follows Michaelis-Menton kinetics. As the concentration of the substrate increases, there is a corresponding increase in the *Vo*. However, beyond a particular substrate concentration, the velocity remains almost constant without any further increase. This maximum velocity achieved under substrate saturating conditions is called the *Vmax*. The Line weaver Burk Plot (Fig. 4.3.4) was used to calculate the *Km* of α-amylase and value was 483.09mg.ml^{-1}. The value of *Vmax* was also calculated and was found to be 50.5µmole/min. A small *Km* indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations. A large *Km* indicates the need for high substrate concentrations to achieve maximum reaction velocity. At low concentration of substrate, there is a steep increase in the rate of reaction with increasing substrate concentration. The catalytic site of the enzyme is empty, waiting for substrate to bind, for much of the time, and the rate at which product can be formed is limited by the concentration of substrate which is available.

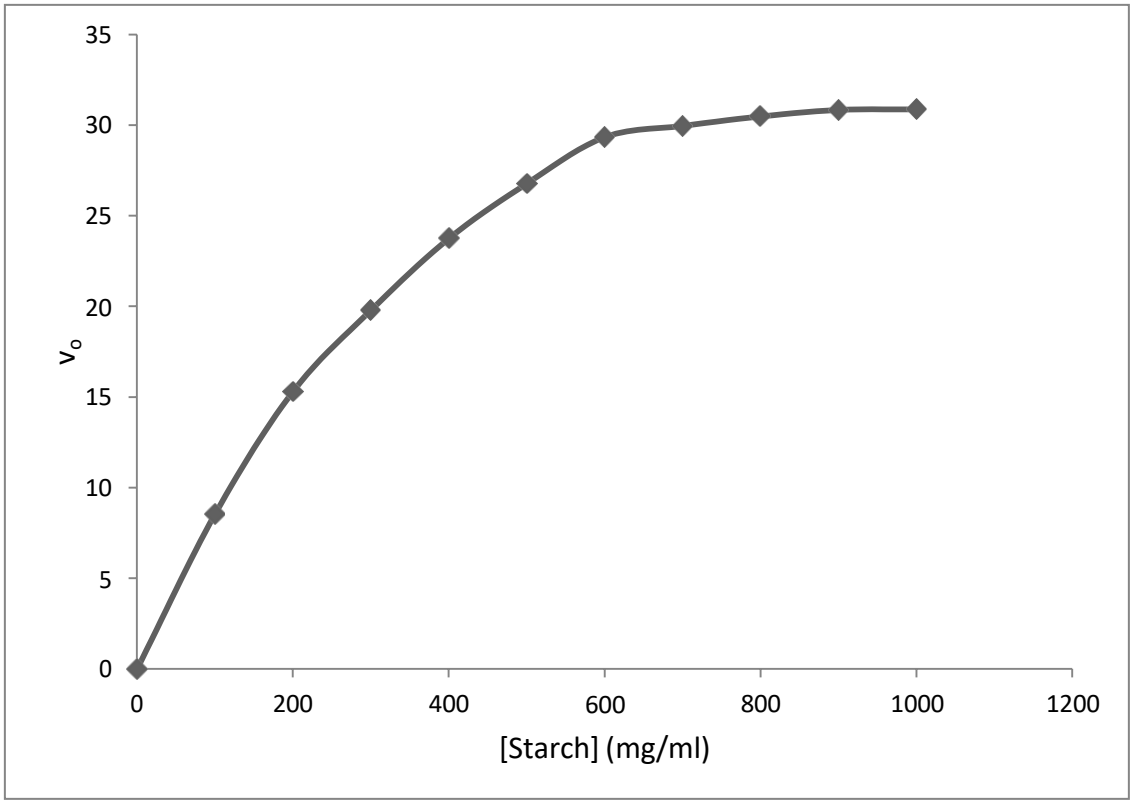


Fig.4.3.3. Effect of substrate concentration on the activity of mung bean α-amylase. Each experimental point represents the mean of the three determinations.

From similar studies, it has been reported that the specificity appears to be restricted to α (1-4)-linked glucans. The reaction velocity followed Michaelis-Menten type kinetics. The lowest *Km* values, i.e. the highest substrate affinity, was for amylose (*Km* = 1.82mg/ml). his was followed by starch (*Km* = 1.96mg/ml), amylopectin (*Km* = 2.86mg/ml), and glycogen (*Km* = 7.69mg/ml), in relation to their corresponding *Vmax* of 1420.45 µg ml-1 min^{-1}, 1162.79 µg ml-1 min-1, 980.4 µg ml-1 min-1, and 12.5 µg ml-1 min-1, respectively (Tapan Kumar *et al*., 2005). Mayzaud and Mayzaud (1981) found the *Km* value of α-amylase for unsorted neritic copepods to be 1.77mg/ml, which is quite close to the *Km* value for *H. viduus* (1.82 mg/ml), but it is less than the value obtained by Mayzaud (1985) for Acartia clausi (4.5 mg/ml). The *Km* value is higher with respect to the soluble α-amylase (0.5 mg/mL) determined at pH 7.0. Increase in *Km* value has been observed in results obtained with other immobilized enzymes (Kerdalin Kharkrang *et al*., 2019). The velocity of the enzyme reaction with the tested substrates was linear up to 0.2 mg/ml. A reciprocal plot of the enzyme velocity against substrate showed that the enzyme had *Km* values of 0.71 mg/ml, 0.83 mg/ml, 0.83 mg/ml, 1.67 mg/ml and 10.0 mg/ml for glycogen, soluble starch, amylopectin, pullulan and amylose, respectively (Opurum C. *et al*., 2019).

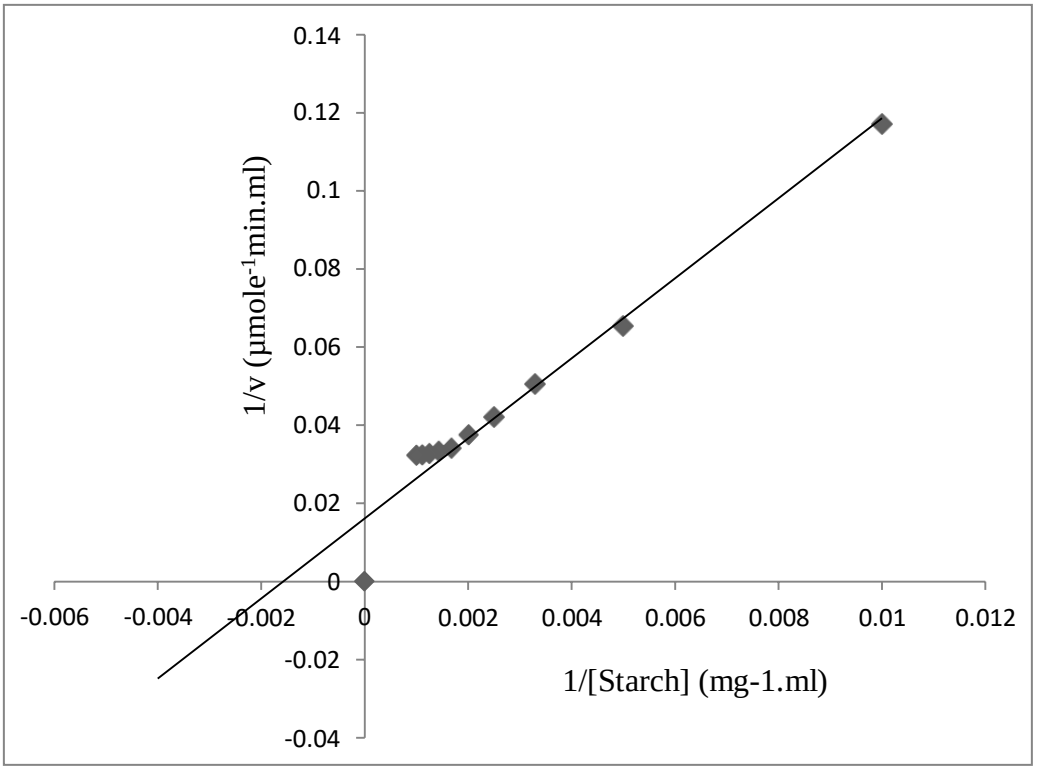


Fig.4.3.4. Line Weaver Burk Plot to determine the K_m and *Vmax* value of mung bean α-amylase. Each experimental point represents the mean of three determinations.

Inhibition studies of mung bean α-amylase:

Inhibition of α-amylase, enzyme that plays a role in digestion of starch and glycogen, is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity, as well as, dental caries and periodontal diseases. Plants are an important source of chemical constituents with potential for inhibition of α-amylase and can be used as therapeutic or functional food sources.

The use of heavy metals such as mercury, copper, lead, cadmium etc. in agriculture and industrial areas has been increased tremendously. Since different environmental pollutants are likely to affect biological systems in different ways according to their respective properties, the sum of physiological changes created by a particular pollutant is likely to be the characteristics of that pollutant. The reason for such studies is that, the heavy metals have specific binding affinity to sulfhydryl group of the enzymes. This would naturally alter the activity of the enzymes that may range from activation to total inhibition. The metal induced alteration in enzyme activity may be taken as more or less accurate indicator of metal toxicity. The change in enzyme activity further provides significant information about sub cellular biochemical adjustment and consequent ability of animals to adapt any environmental change.

The higher concentration of toxicants brings the adverse effect on aquatic organism at cellular level or molecular level. Ultimately it leads to disorder in biochemical composition, alteration in the functional efficiency of the nervous system on exposure to heavy metal causes change in the enzyme activity level of carbohydrate and protein metabolism of the organism. Concentrations of specific metal ions are very essential for microbial growth of *Bacillus subtilis* (Linden *et al.,* 2003). Previous literature indicated that most α-amylase activities were inhibited in the presence of Ni^{+2}, Cd^{+2}, $Cu^{+2,}$ Ag^{+}, Pb^{+2}, Fe^{+2} and Zn^{+2}. The α-amylases from *Bacillus spp.* were strongly inhibited by Ni^{+2}, Cd^{+2}, Zn^{+2} and Hg^{+2} (Cordeiro *et al.,* 2002). Zinc chloride has inhibitory effect on the activity of enzyme in *Aspergillus flavus* (Abou zeid *et al*., 1997). The α-amylase from *Thermus sp*. was strongly inhibited by Cu^{+2} and Fe^{+2} (Shen *et al.,* 1998) and the α-amylase from *Bacillus subtilis* was strongly inhibited by $Zn^{+2,}$ Ag^{+}, Cu^{+2} and Fe^{2+} (Elif & Velittin *et al*., 2000). However, the activity of *Nocardiopsis*

species α-amylase was not affected by $Zn^{+2,}$ Ni^{+2} and Fe^{+2} but activated by Cd^{+2} and Cu^{+2}. α-amylase was strongly inhibited by Ag^{+2} in *Bacillus species*. (Cordeiro *et al.,* 2002; Gupta *et al.,* 2003; Sun *et al.,* 2010; Elif Demirkan & Demirkan *et al*., 2011) and α-amylase was inhibited by Hg^{+2} in *Bacillus spp.* (Pandey *et al.,* 2000; Ramirez zavala *et al.,* 2004; Sindhu 2005; Sun *et al.,* 2010). The activity is inhibited in the presence of zinc ions in *Bacillus spp.* (Elif & Velittin 2000; Roheena Abdullah *et al*., 2005), *Bacillus dipsosauri* (Deutch, 2002), *Bacillus stearothermophilus* (Chakraborty *et al.,* 2000) and *Bacillus pumilus* (Ming *et al.,* 1992).

Inhibition with $HgCl_2$:

Mercury is a heavy metal which at the molecular level, form bonds with sulfhydryl group on an enzyme. The active site of enzymes consists of amino acid cysteine in which sulfhydryl group in present in the side chain. It is well known from the literature that some heavy metal ions are strong inhibitors of α-amylase (Shaw, 1995; Zaborska *et al.,* 2004). Therefore, heavy metal ions Ag^{+} was investigated for its inhibitory effect on mung bean α-amylase for the better understanding of α-amylase action and also for inhibition-based metal detection that could be exploited in the construction of biosensor and biosensing system. The I_{50} value for Hg^{+2} ions was determined. The I_{50} value for the Hg^{+2} ion found to be 46.41mg/ml. Due to quit low value of I_{50} as compare to other inhibitors mercuric chloride has been found to be a strong inhibitor of α-amylase. α-amylase from different sources has been found to be inhibited by heavy metal ions. Kumar and kayastha (2014) have been investigated the inhibitory effect of heavy metal ion such as Ag^{+}, Hg^{+2} and Cu^{+2} and have shown a strong inhibition of soybean α-amylase viz. I_{50} = 2.3×10^{-8} Mm, 7.1×10^{-5} Mm, and 3.3×10^{-3} Mm, respectively with the silver ion being a potent inhibitor. The relative effectiveness of the heavy metal ions as inhibitors of jack been α-amylase has been reported to decrease in the following approximate order: $Hg^{+2} > Ag^{+} > Cu^{+2} > Ni^{+2} > Cd^{+2} > Zn^{+2} > Co^{+2} > Fe^{+3} > Pb^{+2} > Mn^{+2}$ (Krajewska, 1991; Zaborska *et al.,* 2004), with Hg^{+2}, Ag^{+} and Cu^{+2} ions nearly always listed as the most effective inhibitors (Shaw 1954; Krajewska, 1991). This inhibition has been habitually as described to the reaction of the ions with the thiol groups of cysteine residues of the enzyme, resulting in the formation of mercaptides (Hellerman *et al.,* 1943; Shaw 1954; Krajewska *et al.,* 1991).

This was supported by a conclusion of Shaw (Shaw 1954; Shaw and Raval, 1961) that the order of the effectiveness of the heavy metal ions as α-amylase inhibitors correlated with the solubility product constants of corresponding metal sulfides. However, very importantly, heavy metal ions can also bind to functions in the proteins other than thiols. These mainly include nitrogen (histidine) and oxygen (aspartic and glutamic acids) containing functional groups (Ruliek and Vondraek *et al.*, 1998), and in fact, the relative frequency of sites reported as utilized by metals in metallo-proteins follows the order: His > Cys > Asp > Glu.

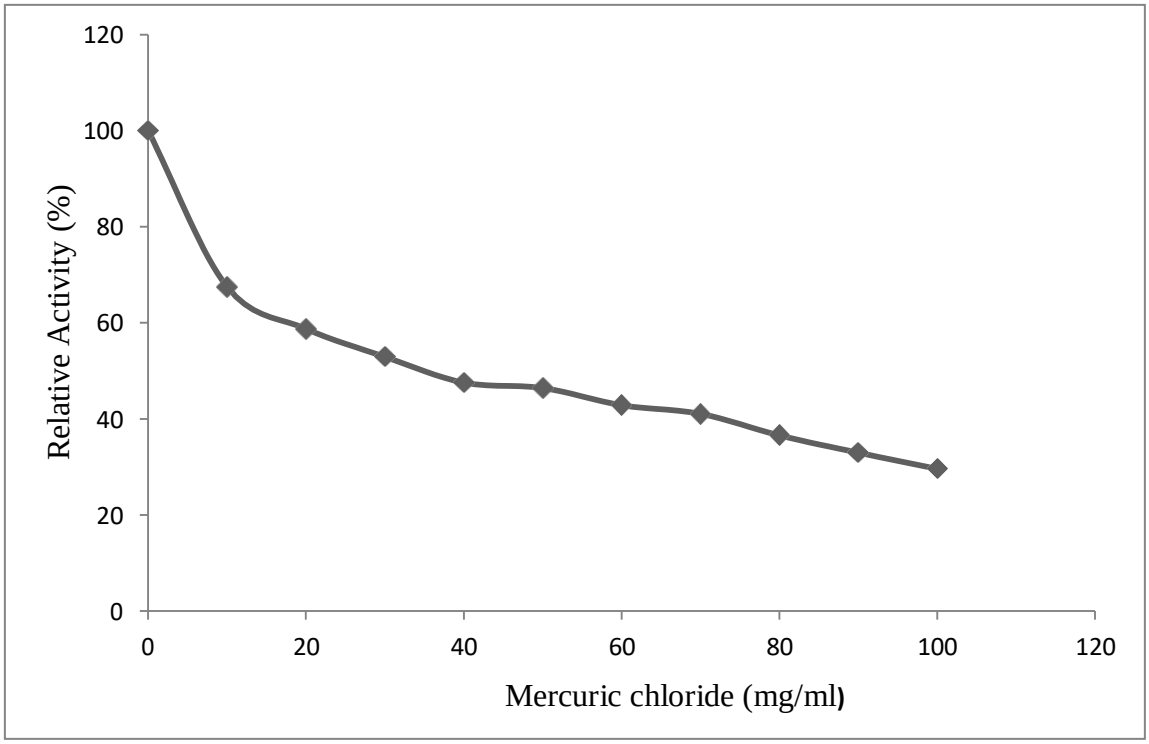


Fig.4.4.1. Inhibition studies of $HgCl_2$ on the activity of the mung bean α-amylase. Each experiment point represents the mean of three determinations.

Inhibition with $CuSO_4$:

Copper, an essential element for normal plant growth and metabolism plays a significant role in a number of physiological processes such as the photosynthetic and respiratory electron transport chains, nitrogen fixation, protein metabolism, antioxidant activity, cell wall metabolism, and hormone perception (R. K. Sharma and M. Agrawal *et al*., 2005). As a structural and catalytic component of proteins and enzymes, it is also well documented and has been reported to be among the most toxic heavy metals. However, when absorbed in excess quantities, Copper is highly toxic to plant growth potentially leading to physiological disorders that inhibit plant growth. The I_{50} value for Cu^{+2} ions was determined. The I_{50} value for the Cu^{+2} ions found to be 46.94mg/ml. It has been reported that excess Copper, at the cellular level, causes molecular damage to plants via the generation of reactive oxygen species (ROS) and free radicals (J. Liu, Z. Xiong, T. Li, and H. Huang *et al*., 2004).

It is a fact that copper containing materials, capable of entering the body via the food chain, are harmful to the ecosystem. A way of analyzing these illnesses is to highlight the effects of these harmful chemicals on the enzymes. The changes in aspirate amino transferases (ASAT) and alanine amino transferases (ALAT), catalase and protein levels in gill, liver and kidney and following different exposure periods of sub-lethal copper concentration suggested that Channa punctatus showed adaptive elevation in the activity of enzymes in these tissues. These enzymes have a very important role in the metabolically process since they are biological catalysts. The enzymes could be successfully used as potential biomarker of fish health. Their deficiency or surplus indicates damage of body organs in fish. Intake of such type of food materials is very harmful to human beings.

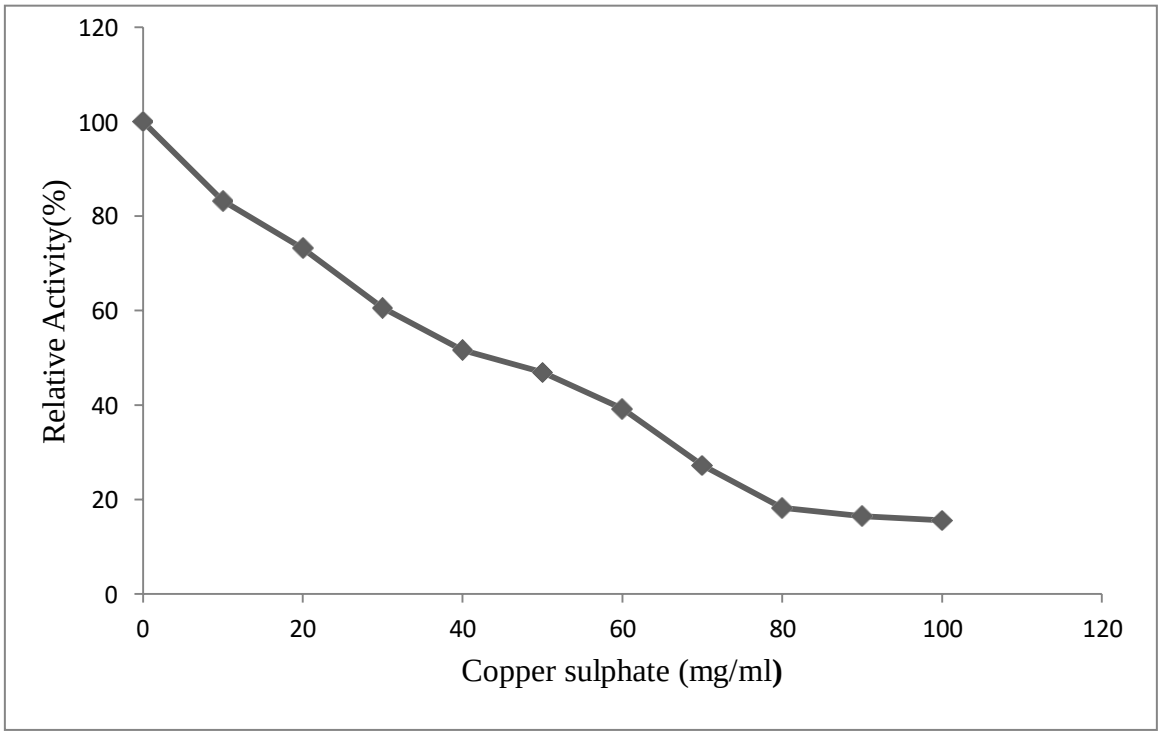


Fig.4.4.2. Inhibition studies of $CuSO_4$on the activity of the mung bean α-amylase. Each experiment point represents the mean of three determinations.

Chapter 5

SUMMARY & CONCLUSION

α-amylase are ubiquitous in nature and have been isolated, purified and characterized from a number of animal, plant, fungus as well as bacterial sources. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes and are of great significance in biotechnological applications ranging from food, fermentation, detergent, pharmaceutical, brewing and textile to paper industries due to their inherent properties, such as stability, reusability and specificity. The history of α-amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff. Ohlsson suggested the classification of starch digestive enzymes in malt as α- and β-amylases according to the anomeric type of sugars produced by the enzyme reaction. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases. Endoamylases: cleave internal α-1, 4 bonds resulting in an anomeric products, exoamylases: cleave α-1, 4 or α--1, 6 bonds of the external glucose residues resulting in alpha or beta anomeric products. α-amylase have received a great deal of attention because of their significance especially in biotechnology. α-amylase constitutes a class of industrial enzymes having approximately 25% of the enzyme market world-wide.

Enzymes are capable to act as biocatalyst for a wide variety of chemical reactions. α-amylase is one of the most important enzymes in various point of view especially in industries, that catalyses the breakdown of starch into sugar. α-amylase is produced by a variety of living organisms, ranging from bacteria to plants & humans. Bacteria & fungi secrete α-amylases to the outside and inside of their cell to carry out extracellular and intracellular enzyme. When they have broken down the insoluble starch, the soluble end products are produced such as glucose or maltose. This α-amylase producing microbes are generally present in degraded atmosphere. Micro-organisms perform their metabolic processes rapidly and with remarkable specificity under ambient conditions. Catalyzed by their diverse enzyme alternatives to harsh chemical technologies has led to extensive exploration of natural microbial diversity to discover enzyme which could function effectively and generate pollution free “Dream technologies” in the immediate future. Due to the similar catalytic mechanism exhibited by all amylase, the inhibition studies

can be successfully used in conjugation with amylases of any origin for controlling/ inhibiting amylase activity in plant and pathogenic microbes to solve the various problems.

From the present studies, it may be concluded that:

- The α-amylase has been isolated from the germinating seeds of mung bean. The stability temperature of the mung bean α-amylase was determined from a plot of the percent residual activity versus the number of days. The value of $t_{1/2}$ was found to be 40 days for amylase stored at 4^{o}C and 15 days for amylase that was stored at 37^{o}C. Therefore α-amylase is more stable and can be stored for longer durations when stored at 4^{o}C. Therefore maximum α-amylase activity can be retained when stored in 0.05mM sodium phosphate buffer, pH-7.0.
- When activity assay was performed at various temperature, it was found that at first α-amylase activity increases with rise in temperature till it maximum value at 40^{o}C and there after it starts declining until its denaturation. The optimum temperature of mung bean α-amylase was 40^{o}C. Therefore, for activity assay, the temperature 40^{o}C is the most suitable temperature. The rate of hydrolysis of starch by mung bean α-amylase at different pH revealed at the optimum pH at 7.0. Therefore for activity assay, pH 7.0 was most suitable pH.
- The experiments were carried out to study the effect of the substrate concentration, by measuring the amount of product formed at different substrate concentration. The *Km* and *Vmax* of α-amylase was found to be 483.091mg ml^{-1} and 50.50µmole/min. The value of *Km* indicates that mung bean α-amylase has high affinity for starch as a substrate.
- Further, α-amylase was investigated for the inhibitory effect of compounds such as $HgCl_2$ and $CuSO_4$. The I_{50} values for Hg^{2+} and Cu^{4+} inhibition were found to be 46.41mg/ml and 46.94mg/ml respectively. The result clearly shows the strong inhibitory nature of Hg^{2+} and Cu^{4+} due to its high I_{50} value.

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ABSTRACT

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α-amylase, (endo-1,4-D glucose-D glucohydrolase; EC 3.2.1.1) is an enzyme that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose. It is the major form of α-amylase found in humans and other mammals. α-amylase was isolated from the germinated seeds of *vigna radiata*. At first, α-amylase was stored at two different temperatures for several days in order to check its storage stability with time. The plot of percent residual activity versus number of days gave a $t_{1/2}$ of 50 days for α-amylase stored at 4^oC and 16 days for amylase that stored at 37^oC. The optimum temperature and pH was found to be 40^oC and 7.0 respectively. To study the effect of substrate concentration, the experiment was carried out by measuring the amount of product formed at different substrate concentration. The *Km* and *Vmax* of α-amylase was found to be 483.091 mg ml^{-1} and 50.50µmole/min respectively. Further, α-amylase was investigated for the inhibitory effect of compounds such as $HgCl_2$ and $CuSO_4$. The effect of inhibitors on α-amylase activity from *vigna radiata* was studied. All the inhibitors inhibited the activity. The α-amylase activity strongly inhibited by several inhibitors since, the amino acid sequence alignment reveals that all known α-amylase, isolated from different sources, are highly similar and shares a common phylogenic relationship and proposed to have common structure and catalytic mechanism. Therefore, α-amylase from any source, may be microbes or plant, can be used as model system for studies and results would equally applicable for any system or field of application.

Keywords: α-amylase; *vigna radiata*; inhibition; activity.

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Examinations		UNIVERSITY/COLLEGE	SUBJECTS	YEAR	DIVISION
10th		UDAI PRATAP INTER COLLEGE VARANASI INDIA	HINDI ,ENGLISH, MATHS, SCIENCE, COMMERCE, SOCIAL SCI.,	2009-2010	1st
12th		INTER COLLEGE BIRAPATTI VARANASI INDIA	HINDI, ENGLISH PHYSICS, CHEMISTRY, BIOLOGY,	2012-2013	1st
B.TECH		S.V.P.U.A.T MEERUT INDIA	BIOTECHNOLOGY ***THESIS- Oyster mushroom cultivation from different substrates (Wheat, Rice, Bagasse).***	2014-2018	1st
M.TECH		S.V.P.U.A.T MEERUT INDIA	BIOTECHNOLOGY ***THESIS- Stability studies and steady state kinetics of α-amylase from Vigna radiate.***	2018-2020	1st

SKILLS AND INTRESTS:

SKILLS • LABORATORY	PCR, RT-PCR(qPCR), HPLC, ELISA, SPECTROPHOTOMETRY, CENTRIFUGATION, GEL ELECTROPHORESIS & DOCUMENTATION,
• COMPUTER	MS-WORDS, MS-EXCEL, POWER POINT

EXPOSURE:

S.No.	Event	Country	Duration
1.	2nd National conference on climate change. PSRM-at UTU campus, sudhowala, deharadune, Uttarakhand.	Uttarakhand.	16 to 17 Feb, 2019
2.	3rd International Conference on "Global Initiatives in Agricultural and Applied Sciences for Eco Friendly Environment (GIASE-2019), Organized by Agricultural Technology Development Society (ATDS) at Tribhuvan University, Kathmandu, Nepal.	Nepal	16 to 18June, 2019

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- **Poornima maurya,** Piyush katiyar, Ajit kumar, Annu verma, Ajay singh, Purushottam. Oyster mushroom cultivation from different substrates (Wheat, Rice and Bagasse), ISSN: 2456-9259.
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TRAINING:

- "One week Hand-on Industrial Skill Training Programme with State of Art Lecture on **HPLC, PCR, RT-PCR, & ELISA Reader"** organized by MRD LifeSciences, Lucknow on September 14-20, 2019

CERTIFICATE / WORKSHOP:

- Course on Computer Concepts: NIELIT June 2018.
- 3rd International conference: Global Initiative in Agriculture and Applied Sciences for Eco Friendly Environment (GIASE-2019).
- 2nd national conference on Plant Science Researchers Meet (PSRM-2019).
- Brainstorming Workshop on Elevated temperature and its impact on fragrance of Basmati rice and strategies for mitigation: Feb-2019.
- Biotech Skill Development Series (BSDS-2019).
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