Studies on Isolation, Purification, Kinetic Characterisation and Immobilisation of α-amylase from some legumes of Garhwal Himalayan Region.

THESIS

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Certificate

This is to certify that the thesis entitled "**Studies on Isolation, Purification, Kinetic Characterisation, and Immobilisation of α-amylase from some legumes of Garhwal Himalayan Region**" submitted by Maryada Goyal Reg. No. HNBGU/Res/ to the school of Life sciences HNB Garhwal Central University ,Srinagar (Garhwal), Uttarakhand for the award of degree of Doctor of Philosophy in Biotechnology, carries out a record of original research by her and has not been submitted in full or in part for any diploma, degree or associate ship in this or any other University. It is also certified that she has put in more than 200 days work in biotechnology laboratory, Department of Zoology and Biotechnology, HNB Garhwal Central University, Campus Pauri, Pauri Garhwal (Uttarakhand), India. I wish her every success in life.

(Prof. A.K. Dobriyal)

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ABBREVIATIONS

- α : alpha
- A° : Angstrom
- $CaCl_2$: Calcium chloride
- CCM : Cellulose coated magnetite
- CNBr : Cyanogen bromide
- CTMAB : Cetyl- tri-methyl ammonium bromide
- DEAE : Di-ethyl-amino-ethyl
- DNS : di-nitro salicylic acid
- HFD : High fat diet
- Hrs. : Hours
- I.U. : International Unit
- K_m : Michaelis-Menton's constant
- KDa : Kilodaltons.
- mM : Mili molar
- Min : Minute
- Nm : nano meter.
- O.D. : Optical Density
- Ppm : parts per million
- Rpm : Revolutions per minute.
- Pp : pages
- Sec : Second
- Spp : species
- S_o : Initial starch substrate concentration
- UV-Vis : Ultra violet-visible
- V_o : Initial velocity
- V_{max} : Maximum velocity

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Chapter I
Introduction

Origin of the current problem:

The Garhwal Himalayan Region of Uttarakhand has been largely known for its genetic and ecological diversity since times immemorial. Ever since, the state of Uttarakhand came into being on 9th November 2000 as the 27th state of Indian Republic, the rich angiospermic flora of this state became a topic of investigation for large number of biotechnologists. This state has 4700 species under 1503 genera and 213 families, thus accounting roughly for 27% of total Indian angiospermic flora. Interestingly the state hosts approximately 50% of genera of Indian flowering plants.

The Dicots dominate with 3493 species under 1163 genera and 182 families. Among the dicots, family Asteraceae has the largest number of genera, i.e., 125, followed by Fabaceae which has 76 genera. It is interesting to note that the family Fabaceae has the second largest number of species i.e., 327. The flowering plants provide wide range of useful products ranging from timber, medicine, food, vegetable oils, gums, resins and spices etc. Among the edible plants, pulses, also known as grain legumes, are second only to cereals as a source of human food. These account for nearly 20 species all belonging to the family Fabaceae. Major pulse crops are *Cajanus cajan, Cicer arietinum, Dolichos*

*spp., Phaseolus spp., Vigna spp., Glycine max, Lens culinaris, Pisum sativum and Vicia faba (*Uniyal *et.al,* 2007*).*

Biotechnological practices in this area are not new to people. Biotechnologists of this area are working largely on subjects related to biotechnology like molecular biology, enzymology and tissue culture, etc. It is interesting to note that despite the large number of products; timber, medicine, food, vegetable oils, gums, resins and spices; exploited from these multi-faceted pulse crops, the enzymatic stamina of these crops has not been explored yet. Despite many biotechnological advances in this area, the scientists were least interested regarding the idea of enzyme extraction from pulse crops. Therefore the present research on **"Studies on isolation, purification, kinetic characterization and immobilization of α-amylase from some legumes of Garhwal Himalayan Region"** has been carried out.

Brief introduction about the enzyme:

Alpha-amylase, (α-1,4 Glucan-glucanhydrolase, E.C. 3.2.1.1) an extracellular enzyme degrades α-1,4 Glucosidic linkages of starch and related substrates in an endo fashion producing oligosaccharides including maltose, glucose and alpha limit dextrin (Leach and Schoch, 1961; Chengyi *et.al.,* 1999; Vidyalaxmi *et.al.,* 1999). This breakdown reaction is an important step in many industrial processes like brewing, baking, starch liquefaction, pharmaceutical and enzyme production etc.

Starch--------→ oligosaccharides------→ Glucose

Therefore, this enzyme is extensively used in industries like starch liquefaction, brewing, food, paper, textile and pharmaceuticals (Haq *et.al.,* 2010).

According to a survey, the European food enzyme market was worth $ 200 millions in 2004. Although it was contributed by starch processing, sugar processing, bakery and dairies, but the highest growth rate was witnessed in the nutrition and dietary supplements market. Here, amylases are said to contribute 25% of the enzymes. These uses have placed greater stress on increasing indigenous α-amylase production and search for more efficient sources of the enzyme.

Among various extracellular enzymes, it ranks first in terms of commercial exploitation. Spectrum of application of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, it was also found applicable in baking, brewing, detergent, textile, paper and distilling industry.

Sources:

Large numbers of sources have been recognized by scientists and researches all around the world for extraction of α-amylase. These sources include micro-organisms, plants and animals. The enzyme can be isolated from micro-organisms (Morgan et.al., 1981; Farez-Vidal et.al., 1992; Abe et.al., 1994; Aguloglu et.al., 2000; Primarini and Ohta, 2000 and Lin et.al., 2002), human saliva (Shainkin and Birk, 1966; Mayo and Carlson, 1974 and Goyal, 2005); and porcine pancreas (Strumeyer et.al., 1988) etc.

A close investigation of various enzyme firms shows that most of the α-amylase production is done from genetically modified micro-organisms. Most of the enzyme firms are dependent on genetically modified sources of bacteria like *Bacillus*

amyloliquefaciens, Bacillus subtilis and Bacillus caldolyticus. Among plants, work has been done on sorghum, broad beans, coconut oil cake, cereals and mung beans.

From literature survey it was revealed that leguminous plants also contain α-amylase (Beers and Duke, 1988; Greenwood *et.al.,* 1965a and b; Tripathi *et.al.,* 2007; Kumari *et.al.,* 2010). The Garhwal Himalayan region is a rich source of various economic important crops. This region also exclusively produces some legumes such as kulath, tohar, rajma, mung etc. which can be a great source of α-amylase in future. No work has been reported regarding α-amylase of legumes of this region. Therefore, it was found useful to investigate the legumes found in Garhwal Himalayan region for α-amylase.

In the present research work, α-amylase has been extracted from two legume sources: *Dolichos biflorus,* commonly known as Kulath and *Phaseolus vulgaris HUR 15,* commonly known as white Rajma. The selection of these two legumes has been done on the basis of the following characteristics:

1. These two legumes are found exclusively in Garhwal Himalayan Region, since there advent into this region.
2. No literature regarding enzyme extraction from these two legumes has been found till date.
3. Enzyme assay via DNS method reveals maximum amount of enzyme, α-amylase in these legumes, amongst all other Himalayan legumes.

Dolichos biflorus (kulath):

Synonyms: Horse gram, Kulthee, Gahat

Taxonomic lineage:

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Dolichos*

Species: *biflorus*

It is a branched or trailing annual with small trifoliate leaves, and very wide climbing slender stem. On maturity, it gives narrow, flat, curved pods. The pods contain 5-7 flattened ellipsoid seeds, 1/8" to ¼" long.

Archaeological investigations have revealed the use of Kulath as food around 2000 BC. Commonly known as Horse Gram, this twining herb of old world tropics is cultivated for its seed in tropical and sub-tropical regions of the world. In India, it is found up to 1500 MSL (mean sea level). Interestingly, the entire Garhwal himalayan region is well suited for the production of this legume crop.

In experimental studies, the seeds of *Dolichos biflorus* have shown to be effective in preventing the deposition of stone material in urinary bladder of rats (Kumar *et.al.*, 1981). The extracts of the seeds of *Dolichos biflorus* have shown inhibiting effect on the formation of phosphate precipitate and has shown better potential in preventing the formation of calcium precipitate (Garimella et.al., 2001). It has also been reported to

lower lipids contents in rats and high fat diet (HFD)-induced oxidative stress in rabbits. Singh and Kumar (1973) have opined that the seeds of *Dolichos biflorus* are effective in dissolving kidney stones in human patients. Alpha-amylase extraction from this pulse crop revealed 27.7IU/ml of the enzyme (Garg and Dobriyal, 2011).

Figure 1: Plant of *Dolichos biflorus* showing pods.

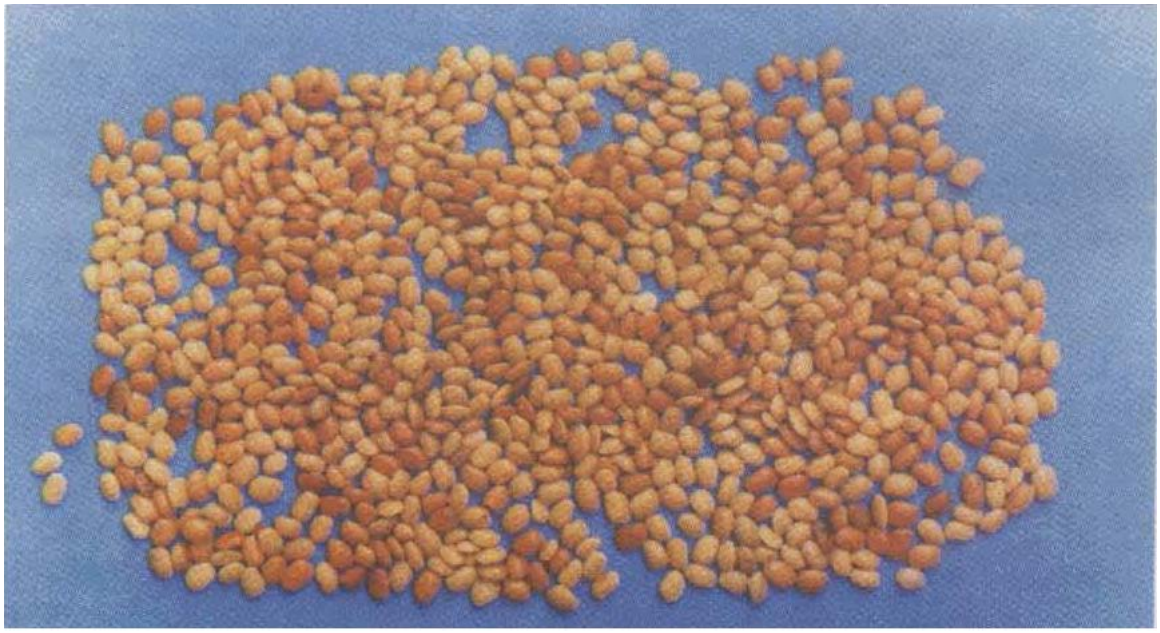

Figure 2: Seeds of Dolichos biflorus.

PLATE-I

Phaseolus vulgaris var HUR15 (white rajma):

Synonyms: Kidney bean, French bean

Taxonomic lineage:

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Papilionaceae

Genus: *Phaseolus*

Species: *vulgaris*

First developed at BHU, Varanasi, this Rabi crop was first identified in 1984. It has been cultivated for its seeds in UP, Uttarakhand, Bihar, MP and Maharashtra. Four varieties of this crop are identified: out of which white Rajma i.e., the HUR15 variety is mainly grown in tropical hilly regions of Uttarakhand as a Rabi crop. Rajma fits well in existing cropping system. It can be successfully grown after all kharif crops maturing by middle of October and spring crops may follow thereafter.

The seeds are creamy white in texture, and require 115-120 days for maturity. Being such short duration crop of 115-120 days, Rajma paves way for high intensity cropping (up to 300%). Thus spring cultivation of other pulse crops like mung bean, urad bean etc. is assured after rajma which is not possible after wheat. The cost of production of Rajma is comparable with wheat.

Unlike other pulse crops, Rajma is inefficient in symbiotic nitrogen fixation and therefore need heavy dose of nitrogen fertilizer. Seeds should be treated with fungicides to control seed borne diseases. The recommended fungicides are bavistin, thiram, and captan. Rajma needs fine seed bed and adequate moisture for proper germination of seeds. It has been estimated that under good management conditions the Rajma production is 25 to 30 quintals /ha (Ali and Lal, 1991).

In the present study α-amylase extraction from the grains has been done and the results obtained were remarkable.

(a) Seeds of *Phaseolus vulgaris* HUR15

(b) Plant of *Phaseolus vulgaris* HUR 15 showing flowers and pods.

PLATE-2

Brief introduction about the methodology used:

Isolation of enzyme from legume source is accompanied by initial crushing of the presoaked, imbibed seed using mortar-pestle. The crushed seed is then passed through four layers of cheese cloth. Then filtration is done in refrigerated centrifuge at 13000 rpm for 15 min. The supernatant is collected and pellet is discarded. This supernatant is considered as crude enzyme extract and can be stored at 4˚C.

Purification of the enzyme is then done from the stored supernatant. Two-fold purification is done: Ultrafiltration and ion-exchange chromatography. Although ultrafiltration is generally employed for concentration of very dilute enzymes, but in the present study it has been utilized for purification of low molecular weight amylases (Garg and Dobriyal, 2011).

The crude extract of the enzyme is passed through ultrafiltration membrane of 10KDa and 30KDa sizes. Then enzyme activity is measured by Miller's DNS method (Miller, 1959) in both the ultrafiltrate and the concentrated enzyme fraction. Further purification of the ultrafiltrate is performed by using ion exchange chromatography. And DEAE cellulose was used for column packing.

Kinetic characterization on the basis of following parameters; optimum pH, optimum temperature, substrate concentration, thermal stability, time, Michaelis Menton's constant (K_m) and Maximum velocity (V_{max}) is then performed. These kinetic characteristics are calculated via enzyme assays using DNS method in the ion exchange fractions of both the ultrafiltrate; for low molecular weight amylases; and dialysed sample; for large molecular weight amylases.

After kinetic characterization, the immobilization of this purified enzyme is also done and the enhanced characteristics are studied. A close comparison is made between the characteristics of immobilized and free enzyme.

It was interesting to note that the enzyme extracted from these pulse crops was as better as the traditional enzyme extracted from bacterial sources. Results prove that plant enzymes showed nearly same characteristics just like bacterial enzymes. Thus, besides finding out new, low cost sources of α-amylase, we could also deduce that these new sources can be made usable on industrial scale.

Variation of this work from earlier work done:

Earlier researchers have extracted α-amylase from all sources including plants, bacteria, fungi, and animals. But only genetically modified bacteria have been applied to commercial usage. The present research focuses on extraction of enzyme from leguminous source, which is a totally new concept.

Importance to science and society:

The increasing demand of α-amylase in food, pharmaceutical and starch industry have led researchers to think about some cheap as well as newer sources for extraction of the enzyme. In this context, this research would prove to be stepping stone towards this approach.

CHAPTER-2

REVIEW OF LITERATURE

Alpha- amylase is ubiquitous in bacteria, fungi, plants and animals. Literature survey reveals that work on α-amylase started in the mid of 20th century. In this context, work of Shainkin and Birk (1966) is worth mentioning, who isolated pure α-amylase from human saliva. After that Mayo and Carlson (1974) isolated four α-amylase isozymes from human sub-mandibular saliva by gel-filtration and iso-electric focusing and studied there different properties. This indicates that human beings and their digestive process was the main source of study of this enzyme. Now it is a well known fact that the enzyme α-amylase is present in human saliva and is responsible for degradation of starch into glucose. This glucose is further utilized in the human body for the life controlling cycle, glycolysis (discovered by Embden, Meyerhoff and Parnas in 1940) which further ends up into Kreb's cycle. These two cycles are responsible for formation of ATP (Adenosine tri-phosphate) in body which is utilized for providing energy to all other cells and cycles of the human body.

Further studies on human amylases included human pancreas. In this context, Ferey-Roux *et.al.*, (1998) studied human pancreatic α-amylase isoforms. Hokari *et.al.*,

(2002) performed a restriction endonuclease assay for expression of human α-amylase isozymes.

As it is known that all animal cells are same, and glycolysis and Kreb's cycle occurs in all animal cells, therefore it became necessary to investigate the presence of α-amylase enzyme in other animal cells also. In the meanwhile, various researchers isolated and characterized the enzyme from other animal sources as well. In this context, Strumeyer *et.al.,* (1988) studied the population distribution of isozymes of α-amylase in porcine pancreas. Perera and Hoover (1998) studied the reactivity of porcine pancreatic α-amylase towards native, defatted and heat-moisture treated potato starches before and after hydroxypropylation. It was found that among animals, α-amylase has been studied mostly in case of porcine, almost till the beginning of 21st century.

Further, among animals Oosthuizen *et.al.,* (1992) performed the isolation and partial characterization of α-amylase from pancreas of Ostrich (*Struthio camelus*). Oosthuizen *et.al.,* (1994) also performed purification and characterization of pancreatic isozymes of Ostrich α-amylase. They discovered four isozymes. Nakatani and Kobayashi (1996) studied the enzymatic properties of α-amylase from digestive tube of sea urchin, *Strongylocentrotus nudas*. Benkel *et.al.,* (1997) perfomed cloning and expression of an α-amylase gene in chicken. Strobl *et.al.,* (1998) studied crystal structure of yellow meal worm α-amylase at 1.64 A° resolution. Moreau *et.al.,* (2001) studied isolation, structural studies and inhibition kinetics of α-amylases from two tilapias *Oreochromis niloticus* and *Sarotherodon melanotheron.* They used ammonium sulfate precipitation, affinity chromatography and chromato-focusing procedures for purification of the enzyme from the intestinal cavity of these two fishes. Mohamed (2004) studied purification and

characterization of α-amylase from the infective juveniles of the nematode *Heterorhabditis bacteriophora*. Lombrana *et.al.,* (2005) discovered two forms of α-amylase in mantle tissue of *Mytilus galloprovincialis*. This was the first case when α-amylase activity was discovered in a non-digestive tissue. Wu *et.al.,* (2006) discovered amylase gene from *Ophiostoma floccosum*. Similarly α-amylase has also been discovered in other mammals and insects by Silva *et.al.,* (2009). Silva *et.al.,* (2009) studied inhibitory action of Cerrado plants against α-amylase of mammals and insects. The insect species studied were *Zabrotes subfasciatus* and *Acanthoscelides obtectus* while the mammalian sample tested was human saliva. Thus it can be said that till date there are more than fifty animals in which the enzyme has been discovered, purified and studied kinetically as well as biochemically, including human beings. Further, it is interesting to note that there has been no animal species till date whose α-amylase has been immobilized.

For industrial purposes most of the workers have proposed to isolate the enzyme α-amylase from micro-organisms like bacteria, fungi and also the genetically modified micro-organisms. Amongst micro-organisms, most of the work regarding isolation, purification, kinetic characterization and immobilization of the enzyme α-amylase has been done in bacteria. Various properties like size, thermal stability, optimum pH, K_m, V_{max} etc. have been studied by different workers. Research on bacterial α-amylase started very early in the mid of 20^{th} century, and by the mid of 20^{th} century it was realized that on an industrial scale, strains of *Bacillus* are best suited for the production of enzyme. This is so because the enzyme α-amylase exists in both membrane associated and extracellular forms in *Bacillus* sp. and in other bacteria (Farez-Vidal *et.al.,* 1992). The α-

amylase synthesized by *Bacillus subtilis* (pAMY430) which is a wild type gene is almost completely secreted from the cell by the aid of various amino acids in media. Cysteine in media shows highest secretion of α-amylase (Aguloglu *et.al.*, 2000).

A close investigation of bacterial amylases indicated that more than 20 species of the genus *Bacillus* have been investigated for the enzyme α-amylase on an industrial scale. These include wild type as well as genetically modified bacteria. Among these the work of Welker and Campbell (1967) is quite interesting. They performed experiments on comparison of α-amylase of *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Both these strains have been considered to be commercial α-amylase concentrates. Yutani (1973) studied molecular weight of a thermostable α-amylase from *Bacillus stearothermophilus*. Heinen and Lauwers (1975) worked on molecular size of extracellular amylase produced from intact cells and protoplasts of *Bacillus caldolyticus*. This enzyme had a typical property of disintegrating into smaller units of less than 10,000 when subjected to ultrafiltration. Ivanova and Dobriva (1994) studied catalytic properties of immobilized purified thermostable α-amylase from *Bacillus licheniformis* (44MB82-A). Raabe and Knorr (1996) studied the kinetics of starch hydrolysis in *Bacillus amyloliquefaciens* α-amylase under high hydrostatic pressure.

With the beginning of 21st century, research work on bacterial strains was further strengthened with increase in demand of enzyme. These strains mostly included **genetically modified strains** of the genus *Bacillus*. In 2001, Hagihara *et.al.*, produced a novel α-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic isolate, *Bacillus* KSM-K38. Further, Jin *et.al.*, (2001) studied and modeled extracellular thermostable α-amylase from aerobic *Bacillus sp*. JF strain. Lin

et.al., (2002) performed the isolation of a recombinant *Bacillus sp.* TS-23 α-amylase by adsorption elution on raw starch. Das *et.al.,* 2004 performed purification and biochemical characterization of a thermostable, alkaliphilic, extracellular α-amylase from *Bacillus subtilis* DM-03, a strain isolated from traditional fermented food of India. This traditional fermented food was a starter culture used for the production of alcohol by local Assam tribes. The enzyme was purified by ion-exchange, gel filtration and reverse phase HPLC. Bernhardsdotter *et.al.,* (2005) studied the enzymatic properties of an alkaline chelator resistant α-amylase from an alkaliphilic *Bacillus sp.* isolate L1711. This *Bacillus* isolate was selected from 13 soda lakes isolates. Anto *et.al.,* (2006) performed α-amylase production by *Bacillus cereus* MTCC 1305 using solid state fermentation, while Murakami *et.al.,* (2008) performed purification and characterization of five alkaline thermotolerant, and maltotetraose producing α-amylases from *Bacillus halodurans* MS-2-5. Gangadharan *et.al.,* (2009) used submerged fermentation for isolation of the enzyme from *Bacillus amyloliquefaciens*. They further utilized ion-exchange chromatography for purification of raw-starch digesting α-amylase. Riaz *et.al.,* (2009) performed immobilization of a thermostable α-amylase on Calcium alginate beads from *Bacillus subtilis* KIBGE-HAR. Haq *et.al.,* (2010) performed production of α-amylase from a randomly induced mutant strain of *Bacillus amyloliquefaciens*. Femi-Ola and Olowe (2011) performed characterization of α-amylase from *Bacillus subtilis* BS5 isolated from hindgut of wood eating termite *Amitermes evuncifer Silvestri*.

Today it is the *Bacillus spp*. whose strain is being used world wide by industry for production of this industrially important enzyme. It is interesting to note that various researchers have used various methods for purification of the enzyme from time to time.

Brena *et.al.*, (1996) studied a range of chromatographic methods for separation of amylases from complex extracts, including separation of isozymes. Till date, almost all types of chromatographic methods have been utilized for purification of extracellular/ membrane bound α-amylase from various bacterial isolates.

Besides *Bacillus*, work on other bacterial genus has also been done from time to time. In this regard, Freer (1993) performed purification and characterization of extracellular α-amylase from *Streptococcus bovis* JB1. Abe *et.al.*, (1994) performed purification and characterization of periplasmic α-amylase from *Xanthomonas campestris* K-11151. This α-amylase isolated by Abe *et.al.*, was of new type as it showed the activities of cyclodextrinase and neopullanase also along with α-amylase. In 1977, Glymph and Stutzenberger performed production, purification and characterization of α-amylase from *Thermomonospora curvata*. Further, Primarini and Ohta (2000) performed purification and characterization of some raw starch digesting amylases of *Streptomyces praecox* NA- 273 and *Streptomyces aureofaciens*. Talamond *et.al.*, (2002) studied α-amylases of *Lactobacillus species*. They performed isolation, characterization and inhibition by acarbose of α-amylase from *Lb. fermentum*. They also compared the α-amylase of *Lb. fermentum* with *Lb. manihotivorans* and *Lb. plantarum*. Ezeji and Bahl (2006) studied purification, characterization and synergistic action of phytate-resistant α-amylase and α-glucosidase from *Geobacillus thermodenitrificans* HRO10. Shafiei *et.al.*, (2010) performed purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic α-amylase from a moderately halophilic bacterium, *Nesterenkonia sp*. strain F. Mollania *et.al.*, (2010) performed

purification and characterization of a thermostable phytate- resistant α-amylase from *Geobacillus sp.* LH8.

Work on fungal strains regarding isolation of α-amylase enzyme started a bit later than bacteria. A significant contribution in this field is made by Ramachandran *et.al.*, (2004) who discovered that coconut oil cake is a potent raw material for production of α-amylase. They used solid state fermentation for production of α amylase using fungal culture of *Aspergillus oryzae.*

Some workers have isolated and characterized α-amylase from plant sources as well. Among plants also, isolation, purification, kinetic characterization and immobilization has been done from a variety of species. The earliest known research among plant amylases is from the cereal family, done by Dube and Nordin (1961). They purified α-amylase in good yield from Sorghum (also known as jowar) malt by procedures based on ultracentrifugation, salt fractionation and adsorption on starch granules and studied their properties as well. Greenwood *et.al.,* (1965a) studied the properties of purified enzyme from broad beans *Vicia faba* belonging to the family Fabaceae/leguminoceae. As a result of this study, characteristic α-amylolytic degradation patterns for the action of the enzyme on various substrates were obtained. In the same year, Greenwood *et.al.,* (1965b) performed studies on purification and properties of starch-degrading enzymes of one more leguminoseae member, soyabean (*Glycine max*). Beers and Duke (1988) observed that the enzyme α-amylase is present in apoplast of pea (*Pisum sativum L*) stems. They found that virtually all α-amylase activity of pea is located in stems. Kumari *et.al.,* (2010) worked on α-amylase from germinating soybean (*Glycine max*) seeds. Along with purification and characterization, they also studied the

sequence similarity of conserved and catalytic amino acid residues in soybean. Thus we can say that the earliest research in plant amylases was from the grass family, Poaceae and legume family, Leguminoseae also known as Fabaceae.

With the classification and evolution of α-amylase genes of plants (Huang *et.al.*, 1992), the studies on isolation and characterization of α-amylase from plant sources speeded up. Warner and Knutson (1991) performed isolation of α-amylase and other starch-degrading enzymes from endosperm of germinating maize (family Poaceae). Their scientific work was significant improvement over use of protein precipitation and affinity chromatography for purification of α-amylase. Later, Rashad *et.al.,* (1995) experimented on localization of Radish (family, Brassicaceae) β-amylases in root. They carried out extraction, purification and characterization of these radish root β-amylases, unlike stem α-amylases of pea. Berbezy *et.al.,* (1996) performed the purification and characterization of α-amylase from vine shoot internodes (family, Vitaceae). Azad *et.al.,* (2009) studied isolation and characterization of a novel thermostable α-amylase from Korean pine seeds (family Pinaceae). These amylases also showed good prospects for industrial application.

At the start of 21st century, work on the family Poaceae (grass family) once again got momentum. Mohamed *et.al.,* (2009) studied partial purification and characterization of five α-amylase from a wheat local variety (Balady) during germination. They found that activity of α-amylase increased from day 0 to day 6 of germination, followed by decrease of activity till day 16. The highest level of activity was discovered on day 6 with 2300 units/ g seeds. Biazus *et.al.,* (2009) performed production and characterization of amylases from *Zea mays* malt. Nour and Yagoub, (2010) performed partial purification

and characterization of α and β-amylases isolated from *Sorghum bicolor* cv. (Feterita) Malt.

It is worth mentioning that certain α-amylase inhibitors have been discovered in all sorts of plants. In this context, Gibbs and Alli (1998) studied the characterization of purified α-amylase inhibitor from white kidney beans (*Phaseolus vulgaris*).

With increase in industrial applicability of the enzyme, immobilization of enzyme on various matrices started. Most immobilization processes are based on covalent immobilization. Bayramoglu *et.al.,* (1992) performed immobilization of α-amylase into photographic gelatin by chemical cross-linking. Chemical cross-linking was done with chromium (III) acetate and chromium (III) sulphate. In this context, Somers *et.al.,* (1995) performed isolation of α-amylase on cross-linked starch. This has been proved to be one of the most economically attractive purification processes because the adsorbent is cheap and easy to prepare. Tanyolac *et.al.,* (1998) performed immobilization of a thermostable α-amylase Termamyl® onto nitrocellulose membrane by Cibacron Blue F3GA dye binding. He *et.al.,* (2000) proposed a new method for immobilization of α-amylase by UV-curing coating on piezoelectric crystal. The activity of immobilized α-amylase is monitored by a technique based on bulk-acoustic wave (BAW) sensor. Experimental results show that immobilized α-amylase entrapped by this method can be reused more than 50 times under experimental conditions. Tumturk *et.al.,* (2000) performed covalent immobilization of α-amylase onto p-(HEMA), (poly(2-hydroxyethyl methacrylate)), and p(St-HEMA), (poly(styrene -2-hydroxyethyl methacrylate)), microspheres. Bryjak (2003) performed glucoamylase, α-amylase and beta amylase immobilization on acrylic carriers. Bayramoglu *et.al.,* (2004) performed immobilization of a thermostable α-amylase on

reactive membranes. They also studied kinetics, characterization and application to contimuous starch hydrolysis. Liao and Syu (2005) performed affinity binding of α-amylase on beta cyclodextrin matrix. Beta cyclodextrin was cross linked with epichlorohydrin to improve its rigidity. El-Batal *et.al.,* (2005) described the entrapment of α-amylase into butyl acrylate-acrylic acid copolymer using γ-radiation. The effect of an anionic surfactant (AOT), the reuse efficiency, and kinetic behaviour of immobilized α-amylase were studied. Kara *et.al.,* (2005) performed immobilization of α-amylase on Cu^{+2} chelated poly (ethylene glycol dimethacrylate –n-vinyl imidazole) matrix via adsorption. Kahraman *et.al.,* (2007) performed immobilization of α-amylase on functional glass-beads by covalent attachment. This covalently bound α-amylase lost all its activity within 25 days unlike the free enzyme which was stable upto 15 days. Konieczna-Molenda *et.al.,* (2009) performed immmobilisation of α-amylase on six poly(vinylamines) and three poly(vinylformamides) hydrogels. Here also the enzyme was covalently bound to the supports using glutaraldehyde as a spacer. Turunc *et.al.,* (2009) performed immobilization of α-amylase onto cyclic carbonate bearing hybrid material. Hybrid matrix was prepared using sol-gel method and α-amylase was covalently bounded onto matrix via cyclic carbonate functionality. Sedaghat *et.al.,* (2009) studied immobilization of α-amylase onto sodium bentonite and modified bentonite. Bentonite was modified with cetyl trimethyl ammonium bromide (CTMAB).

Among fungi, immobilization of α-amylase of *Aspergillus niger* has been performed. Pascoal *et.al.,* (2010) immobilized this fungal α-amylase onto polyaniline.

Most studies on α-amylase indicate that the enzyme is calcium-dependent. In this context Sajedi *et.al.,* (2005) discovered a calcium independent α-amylase from *Bacillus species* KR-8104. It is interesting to note that this α-amylase was active and stable at low pH.

In India, some scanty references are available on α-amylase sources. Shah *et.al.*, (1989) isolated a high yielding stable mutant of *Bacillus subtilis*, which yielded 5-fold more α-amylase activity by subjecting the strain to irradiation. Lonsane and Ramesh (1990) performed production of bacterial thermostable α-amylase by solid state fermentation; this process is highly economic in enzyme production and starch hydrolysis Dubey *et.al.,* (2000) discovered α-amylase in *Aspergillus niger*, and proved that this α-amylase is a proteolytically processed product of a precursor enzyme. Gupta *et.al.,* (2003) studied microbial α-amylases and their application with a biotechnological perspective. Gopal et.al., (2008) studied porcine pancreatic α-amylase and its isoforms- effect of deglycosylation by peptide-N-glycosidase F. Prakash *et.al.*, (2009) studied production, purification and characterization of two extremely halotolerant, thermostable and alkali stable α-amylases from *Chromahalobacter sp.* TVSP 101. These two α-amylases were named as amylase I and amylase II. These enzymes efficiently hydrolysed carbohydrates to yield maltotetraose, maltotriose, maltose and glucose as end products.

In this context of plant α-amylases the work performed by Nirmala and Muralikrishna since the year 2000 is worth mentioning. They mostly studied the properties of α-amylases of cereals. First of all, Nirmala *et.al.*, (2000) studied carbohydrate and their degrading enzymes from malted finger millet (Ragi, *Eleusine coracana*, Indaf-15). Indaf -15 is a hybrid ragi crop developed at CFTRI, Mysore. They observed that Indaf-15 is a potential variety for malting purposes and it develops high

levels of amylases during germination. They also found that its malt form is a rich source of reducing sugar. Nirmala and Muralikrishna (2003a) studied purification and partial characterization of three α-amylases from this same malted finger millet (Ragi, *Eleusine coracana* Indaf-15). These three α-amylases were designated as α-1, α-2 and α-3 respectively. In the same year, they performed in-vitro digestibility studies of cereal flours and starches using these three purified finger millet amylases. In these studies α3 was found to be the most efficient followed by α1 and α2 in their hydrolyzing capacity (Nirmala and Muralikrishna, 2003b). Later on Nirmala and Muralikrishna (2003c) together studied the properties of these three purified α-amylases from this same malted finger millet once again. Muralikrishna and Nirmala (2005), studied various facets of cereal α-amylases regarding definition, history, types, sources, classification, assay methods, molecular basis of α-amylase during malting, isolation, fractionation, purification etc. Emphasis was also given to recently characterized finger millet α-amylases.

Immobilization from α-amylases has also been done in India to a certain extent. Sahukhan *et.al.,* (1993) performed immobilization of α-amylase from *Myceliophthora thermophila* D-14 (ATCC 48104). In this research, three immobilization methods were involved; covalently bound to CNBr-activated Sepharose, and entrapped within crosslinked poly-acrylamide gels and Calcium alginate beads. Of the three methods, Calcium alginate beads proved to be the best carrier for immobilization. Reshmi *et.al.*, (2007) performed immobilization of α-amylase from zirconia. Zirconia is a heterogenous biocatalyst for starch hydrolysis. Tripathi *et.al.,* (2007) performed immobilization of α-amylase from mung beans (*Vigna radiata*). They performed the immobilization on

Amberlite MB-150 and Chitosan beads. Gangadharan *et.al.*, (2009) immobilized bacterial α-amylase for effective hydrolysis of raw and soluble starch. The α-amylase produced by *Bacillus amyloliquefaciens* ATCC 23842 was immobilized in calcium –alginate beads and used for effective hydrolysis of soluble and raw potato starch which was comparable to the free enzyme. Namdeo and Bajpai (2009) performed immobilization of α-amylase onto cellulose-coated magnetite (CCM) nanoparticles. CCM nanoparticles were obtained by coagulation of aqueous solution of cellulose containing magnetite nanoparticles. Kumari and Kayastha (2011) performed immobilization of soyabean α-amylase onto Chitosan and Amberlite MB-150 beads. Optimisation and characterization studies were also performed on the immobilized enzyme. Singh and Kumar (2011) used a very different approach for immobilization of α-amylase. They used carboxymethyl tamarind gum-silica nanohybrids for effective immobilization of the enzyme.

All the above work studied shows that a lot of work on α-amylase enzyme has been done all around the world. Till date, around 25,000 research papers based on the enzyme α-amylase have been published in various journals, both India and abroad. Among these, around 500 research papers, reviews, short communications etc. have been published regarding isolation, purification, kinetic characterization, and immobilization of α-amylase. Still, the above literature clearly reveals complete lacunae of literature on legume α-amylase in India. Therefore, the present study entitled "Studies on Isolation, purification, kinetic characterization and Immobilisation of α-amylase from some legumes of Garhwal Himalayan Region" has been undertaken.

CHAPTER-3

MATERIALS AND METHODS

Sampling material and sampling sites:

The legumes were collected from local markets of Garhwal Himalayan Region and were considered as source of α-amylase enzyme. Various legumes including *Dolichos biflorus* (kulath), *Phaseolus vulgaris* (Rajma), *Vigna ungiculata* (lobia), *Cajanus cajan* (Tohar), *Pisum sativum* (pea), *Glycine max* (soyabean) and mung etc., were collected initially. These legumes were studied based on their different varieties obtained exclusivity in Garhwal Himalayan Region. We found four varieties of Rajma, one of kulath, two of lobia, and one variety each of tohar, pea, soyabean and mung.

Selection of material:

In the present research work, it was decided to extract α-amylase from two legume sources: *Dolichos biflorus*, commonly known as Kulath and *Phaseolus vulgaris* HUR 15, commonly known as white Rajma. The selection of these two legumes has been done due to following reasons:

1. These two legumes are found exclusively and extensively in Garhwal Himalaya, since their advent into this region.

2. No literature regarding enzyme extraction from these two legumes has been found till date.

3. Enzyme assay via DNS method reveals maximum amount of enzyme, α-amylase in these legumes amongst all other himalayan legumes.

Initial treatment of legumes:

All these legumes were first of all washed with tap water followed by a wash with distilled water. Then chemical treatments were given to the legumes:

First, these were thoroughly washed with mild non-ionic detergent Tween-20 **(**Molecular formula= $(C_{58}H_{114}O_{27})20$**)** for 20 minutes. Tween-20, also known as polysorbate-20 is a polysorbate surfactant whose stability and relative non- toxicity allows it to be used as detergent and emulsifier in a number of domestic, scientific and pharmacological applications. Secondly, these were treated with an ionic surfactant, 0.01% mercuric chloride. At last, both the legume samples were thoroughly washed with distilled water before proceeding to the next step.

Sprouting of seedlings:

As well known, the α-amylase enzyme shows maximum activity in germinating seedlings, therefore all the seedlings were imbibed overnight (Warner and Knutson, 1991). Then these were kept in incubator at 37C for 24 hrs. to initiate germination. The

seedlings were kept in a petri-plate on the top of wet cotton to retain moisture during germination.

Adequately long sprouts were obtained for *Dolichos biflorus* within 24 hrs. For *Phaseolus vulgaris* HUR15 adequate sprouting was attained in 48 hours. These results defied earlier results of Warner and Knutson (1991) who stated adequate time for sprouting of seedlings to be 6 days in case of cereals. It was further witnessed that sprouting time can be shortened by use of incubator for germination. The seeds were further induced to higher sprouting for a maximum of 6 days. Higher the sprouting, larger will be the production of enzyme. After 6 days, further sprouting could not be witnessed due to appearance of visible moulds. It was further observed that within 5-6 days visible secondary roots could be seen in the sprouts

Initial studies of sprouted seedlings:

Germination percentage and seedling length were calculated for both the legumes after five days.

- **Germination percentage**: 100 seeds each of *Dolichos biflorus* and *Phaseolus vulgaris* were kept in three Petri plates. These were made to sprout for 5 days. Then total germinated seeds were calculated.
- **Seedling length**: Seeds of each of the legumes were kept by side of ruler and measured for the length of their axis. Maximum length was calculated for up to three seedlings.

Enzyme Extraction/Isolation:

All germinated seeds were first of all homogenized in phosphate buffer (pH=7) using mortar-pestle. Homogenate was filtered through four layers of cheese cloth. Filtrate was centrifuged at 13,000 rpm for 15 minutes in cooling centrifuges (manufactured by Remi) at 4C. The supernatant was taken as crude extract and stored at 4˚C in Refrigerator (Figure 8). This crude extract was utilized for further enzyme assays, kinetic characterization and immobilization studies.

Enzyme assay:

The α-amylase activity was assayed in crude extract by DNS method given by Miller (1959). The α-amylase catalyses the hydrolysis of α-1, 4 linkages in starch with the production of reducing sugars like glucose and maltose. As the hydrolysis reaction proceeds, there is an increase in the number of reducing end groups. To determine the increase in number of these reducing end groups, DNS solution, i.e. 3, 5 di-nitrosalicylic acid is added to the reaction mixture. DNS is an oxidizing agent, which on reaction with reducing end groups converts to 3-amino, 5-nitro salicylic acid. DNS is a yellow coloured reagent. It serves two purposes in the reaction-

- First, it stops the reaction between starch and α-amylase immediately. Thus, the amount of reducing sugars obtained within the given period of time under given conditions can be determined.
- Second, it imparts colour to the reaction mixture which further enables us to detect the reagent in visible spectrum range.

Since, the reagent is solely responsible for the entire reaction to take place, therefore the name of method is DNS method. Substrate utilized for DNS method was D-glucose (Dextrose Fisher Scientific, Product No. 15405, Molecular weight = 180.16) and standard graph was plotted (Figure 10).

Preparation of Standard graph:

Concentrations of glucose starting from 50 ppm, 100 ppm ($mg.l^{-1}$), 200 ppm, 300 ppm, 400 ppm, 500 ppm, 600 ppm, 700 ppm, 800 ppm, 900 ppm, and 1000 ppm were prepared first of all. For the same, first of all a stock of 10,000 ppm glucose was prepared. 1 ml of each glucose concentration sample was added in separate test tube. One test tube was kept aside as blank. In this test tube 1ml water was added. Then, in all the 12 test tubes, 3ml DNS solution (prepared 1% in 1% NaOH) was added. Then all these test tubes were kept in water bath (manufactured by Remi Equipments Ltd.), heated up to a temperature of 45°C. After 10 minutes, 1 -1 ml of Sodium potassium tartarate (40%) was added to each of the test tubes. Then, 15 ml of distilled water was added to all the tubes. All these tubes were kept in boiling water bath at 80°C for 15 minutes until colour (orange-yellow) change was observed. O.D was recorded in UV-Visible spectrophotometer at 620 nm. A graph was plotted taking glucose concentration (in ppm) on X-axis and O.D. on Y-axis.

Enzyme assay

(a) For crude extract of *Dolichos biflorus*:

Step 1: A set of test tubes were taken. One tube was marked as main "**M**" and the other tube was marked as reference "**R**".

Step 2: To each of the test tubes 1ml of 1% starch solution was added.

Step 3: Then, 2.5 ml buffer solution, of pH 7 was added to both the tubes. The tubes were kept in hot water bath at 45°C. The enzyme was also kept in hot water bath at the same temperature for equilibration.

Step 4: After 10minutes, 0.1 ml of crude enzyme, was added to the test tube marked as **"M"** and equal amount of distilled water was added to the test tube marked as **"R"**. The reaction was allowed for 10min

Step 5: Then 3 ml DNS solution was added to both the test tubes. These tubes were put to boiling for 15min.

Step 6: Then, 1ml of sodium potassium tartarate and 15ml distilled water was added to both the tubes.

Step 7: The absorbance was recorded at 620nm in UV-Visible spectrophotometer.

One unit of enzyme was calculated as micromoles of glucose formed per ml of enzyme per unit time (in min) (Palmer, 2004), from standard graph. Enzyme activity is based on conversion of DNS to 3-amino 5-nitro salicylic acid.

(b) For crude extract of *Phaseolus vulgaris* HUR 15:

Similarly enzyme assay was performed with the crude extract of *Phaseolus vulgaris* HUR 15.

Purification:

Basically two methods were employed for purification of enzyme from the crude extract:-ultrafiltration and column chromatography.

Ultrafiltration:

The supernatant was made to pass through 30kDa and 10kDa ultra filtration membrane (Millipore) respectively at 55psi pressure and ˚C temperature. Enzyme assays were performed with the ultra filtered extracts, for calculating the percent recovery of α-amylase in these extracts. This is accompanied with DNS method for quantitative estimation of enzymes.

Column chromatography:

Ion-exchange chromatography was performed for further purification of the ultrafiltered extract.

* **Column packing**: DEAE cellulose was used for column packing (Welker and Campbell, 1967). The DEAE cellulose was washed with buffer of pH 8 and packed to a height of 5cm in chromatographic column, 2cm in diameter.

* **Equilibration of column:** Then the column was washed exhaustively with equilibration buffer, pH 8, twice.

* **Sample addition**: Then α-amylase solution was applied to the column. Care should be taken that no air-bubbles enter the column during packing of column, addition of equilibration buffer, and addition of sample. The column is covered on the top each

time to avoid any evaporation losses and contamination of sample with micro-organisms from the air.

* **Elution**: The α-amylase was eluted by stepwise elution with increasing molarities of sodium-phosphate buffer (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.5M) pH 7.2. The fractionation was carried out at 4°C and the eluate was collected in 2 -2ml fractions. Each fraction was assayed for enzyme activity. The fraction showing maximum amylase activity (via DNS method) was further subjected to kinetic characterization.

Kinetic Characterisation:

Following biochemical properties were determined to characterize the purified enzyme in both the samples. All assays were done via DNS method.

Optimum pH:

There are a number of distinct effects that a change in pH can have on an enzyme catalyzed reaction. Outside a certain pH range,

* inactivation of enzyme can occur.
* Also there can be a change in ionization state of a substrate
* There could be a change in equilibrium position and changes in the equilibrium position might displace the equilibrium of the reaction in favor of the product.
* The most important effect of pH is that it results in changes in the ionization state of the amino acid side chains that are essential for catalysis of the enzyme.

For determining optimum pH, phosphate buffer (pH 6.2-8.2), tris buffer (pH>8), and acetate buffer (pH 3-8) were prepared. From these prepared buffers, 8 pH solutions were taken. A separate set of test tubes were taken, for each of the 8 pH solution taken

Test tube of each set was marked as Reference **"R"** and Main **"M"** respectively along with the pH . Then, enzyme assay was done at a range of pH concentrations from pH 3-9, from step 1-7 as mentioned in section Enzyme assays (discussed ahead). The only difference was in Step 3, where the buffer of requisite pH value was added to each set. That pH was noted down at which maximum O.D was obtained. From the standard graph, enzyme activity corresponding to these O.D values was determined.

A graph is plotted with pH on X-axis and O.D (620nm) on the Y-axis. From this graph, that pH was determined at which maximum enzyme activity was obtained. This pH value is known as optimum pH for the given enzyme.

Optimum Temperature:

Every enzyme has a particular temperature at which its enzyme activity is maximal. This optimum temperature is calculated by checking the enzyme activity of α-amylase at different temperatures. The temperature at which maximum enzyme activity is obtained is said to be optimum temperature of the enzyme. While performing experiment with a particular enzyme knowledge of this temperature is necessary. Heating above the optimum temperature disrupts the folded structure of the enzyme by increasing the vibrational and rotational motions of atoms.

Enzyme assay is done as mentioned earlier in section 'Enzyme assays'. Optimum temperature was investigated by incubating the enzyme extracts at 0˚C, 10˚C, 20˚C, 30˚C, 40˚C, 50˚C, 60˚C, 70˚C, and 80˚C respectively in Step-3.

Thermal Stability:

Checking the thermal stability means finding that maximum temperature up till which an enzyme is stable. It is also calculated that for how much time, an enzyme remains stable at maximum temperature. For this first of all, the optimum temperature is found. Then O.D. of enzyme is calculated 10 °C above optimum temperature for 10, 15, 20, 25 and 30 minutes respectively. That temperature above the optimum temperature, was obtained at which the enzyme remains stable. The difference was in Step-4 where the reaction was carried out with five sets of test tubes for 10, 15, 20 and 25 min respectively for each temperature above the optimum temperature. O.D. was calculated up till that time till which it became almost nullified.

Substrate Concentration:

The difference was in Step-1 where enzyme reaction was performed at various substrate concentrations of starch ranging from 200ppm, 300ppm----- 5000ppm. Optimum substrate concentration was that concentration at which maximum O.D. was obtained.

Time:

Optimum time is calculated as that time duration in which best reaction was obtained. For this, enzyme assay was done as usual using DNS method as mentioned earlier. The only difference was that the reaction time was varied in Step-4. The enzyme reaction was performed at various time ranges:- 2, 4, 6, 8, 10, 12, and 14 minutes.

V_{max} and K_m:

After a particular substrate concentration, the enzyme activity stops increasing. The enzyme activity obtained at this maximum substrate concentration is known as V_{max}. For this, the enzyme activity of the enzyme was checked at various substrate concentrations of starch. The substrate concentration, at which maximum enzyme activity (V_{max}) became half, was calculated as Michaelis- Menton's constant (K_m). A graph was plotted between substrate concentration (S_o) and enzyme activity (V_o) and K_m and V_{max} marked accordingly.

Immobilisation:

The major concern in an enzymatic process is the instability of enzyme under repetitive or prolonged use and inhibition by high substrate and product concentration. Immobilization is a very effective alternative in overcoming problems of instability and repetitive use of enzymes. Entrapment method of immobilization is advantageous over other methods as they do not involve chemical modification of the enzyme (Gangadharan *et.al.*, 2009).

Both the purified enzyme extracts were subjected to entrapment method for immobilization. In this approach, enzyme molecules are held or entrapped within Calcium alginate gels. This is one of the most widely used application.

Preparation of Sodium Alginate:

100ml water was kept on stirrer. In stirring conditions, 3% sodium alginate was added to the water. Stirring was allowed for 10-15 minutes. Then the solution was left for ½ hour so that any bubbles present in the solution disappear. Then at room temperature, 2ml enzyme extract was added to the sodium alginate solution.

Calcium Chloride solution:

0.2M $CaCl_2$ solution was prepared. This solution was kept in freezer for ½ hour to maintain chilled conditions.

Preparation of beads:

The sodium alginate- enzyme solution was filled in a 10ml syringe. This solution was added drop wise to chilled $CaCl_2$ solution. Small rounded beads about 2cm in diameter were obtained. These beads are the immobilized α-amylase enzyme beads. Kinetic characterization of the immobilized enzymes was performed using these beads. In the subsequent experiments, these beads will be added to DNS extract instead of free enzyme. These beads can be recovered after each experiment. And the subsequent experiment can be performed using the same beads.

Kinetic Characterization of the immobilized enzyme:

The kinetic characteristics were calculated using the immobilized enzyme. To find out these kinetic characteristics, DNS method was applied as mentioned earlier in text. The only difference was that in place of free enzyme, the immobilized enzyme beads were added to the mixture. Enhanced characteristics were determined.

Comparison:

A close comparison between the kinetic characteristics of immobilized as well as free enzyme was done. Also the kinetic characteristics of the free enzyme were compared with the kinetic characteristics of α-amylase of other plant and bacterial species discovered so far.

Chapter 4
Results and Discussion

(All the experiments were performed in triplicates, and the mean values were taken for results.)

Initial studies

Initially the legume samples of Kulath and white Rajma procured from local markets of Garhwal Himalayan Region were soaked and made to germinate for a maximum of 6 days. These 6 day old legumes were used for further studies.

Germination percentage:

- Germination percentage of seedlings for white Rajma :

  Total seeds taken = 41

  Ungerminated seeds= 6

  Germinated seeds= 36

  Germination percentage= (36 / 41) x 100

  = 87.8%

- **Germination percentage of seedlings for Kulath:**

  Total seeds taken = 279

  Ungerminated seeds= 80

  Germinated seeds= 199

  Germination percentage= (199 / 279) x 100

  =71.3%

Figure 5: 5 day old sprout of White Rajma with secondary roots (*Phaseolus vulgaris* HUR 15)

Figure 6: 1-5 day seedling length of White Rajma (*Phaseolus vulgaris HUR 15*)

PLATE-3

Figure 7:
5 day Seedling length of kulath

Figure 8:
Crude extract of kulath

Figure 9:
2 day old sprouts of kulath

PLATE-4

Seedling length:

From these germinated seedlings, **seedling length** was calculated. For Rajma, highest seedling length was 6.5cm, 6.1cm and 5.9cm (Figure 6). In case of kulath, highest seedling length was 3.6cm, 1.7cm and 1.6cm respectively (Figure 7).

Preparation of standard graph:

A range of glucose concentrations starting from 50 ppm to 7000ppm were taken. At every glucose concentration the optical density was calculated and a table was prepared (Table-1). This table was plotted on graph by taking glucose concentration on X-axis and corresponding O.D. on Y-axis. A straight line graph obeying Beer-Lambert's law was obtained. This straight line graph is known as the standard graph (Figure 10). And this graph is used throughout the experiments for calculating enzyme activities under varying conditions.

Table 1: Standard graph (via DNS method)

Glucose concentration (In ppm)	O.D. (620 nm)
50	0.04
100	0.06
200	0.07
300	0.1
400	0.12
500	0.13
600	0.16
700	0.19
800	0.2
1000	0.24
2000	0.44
3000	0.62
4000	0.73
5000	0.95
6000	1.1
7000	1.25

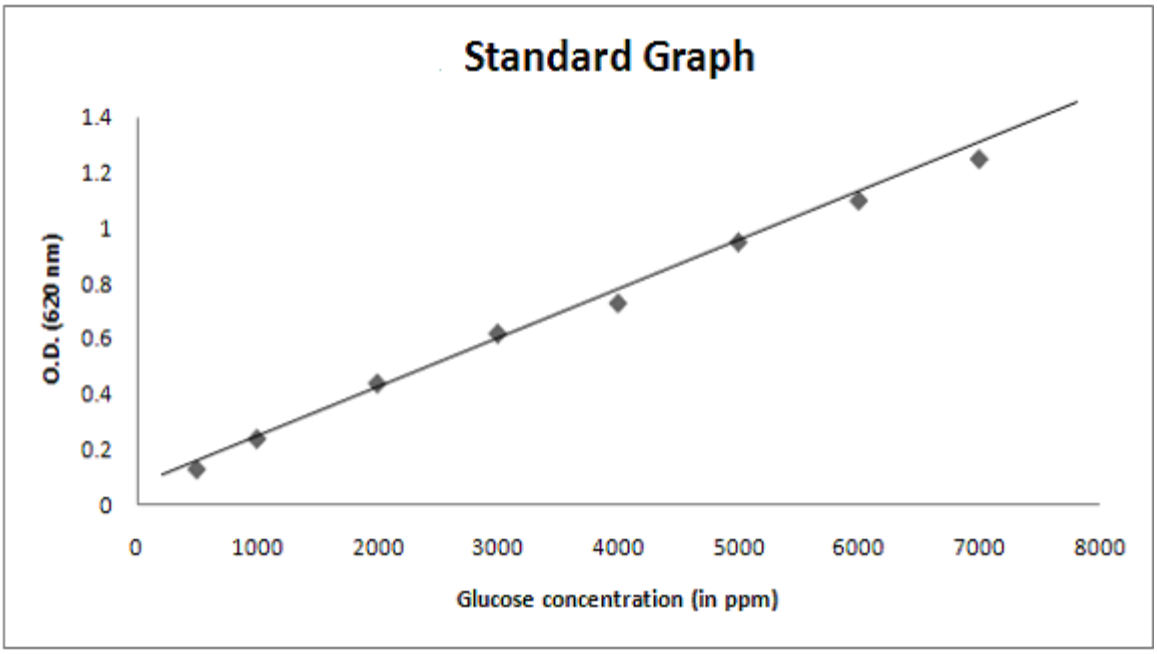

Figure 10: Graph showing O.D. values against varying glucose concentrations.

Isolation:

The isolated enzyme was found in the supernatant. O.D. of crude *Dolichos biflorus* extract was found to be 0.17 while that of crude *Phaseolus vulgaris* HUR15 extract was found to be 0.09.

From the standard graph (Figure 11), the corresponding glucose concentration against these O.D values came out to be **680ppm** for *Dolichos biflorus* and **290ppm** for *Phaseolus vulgaris* HUR 15.

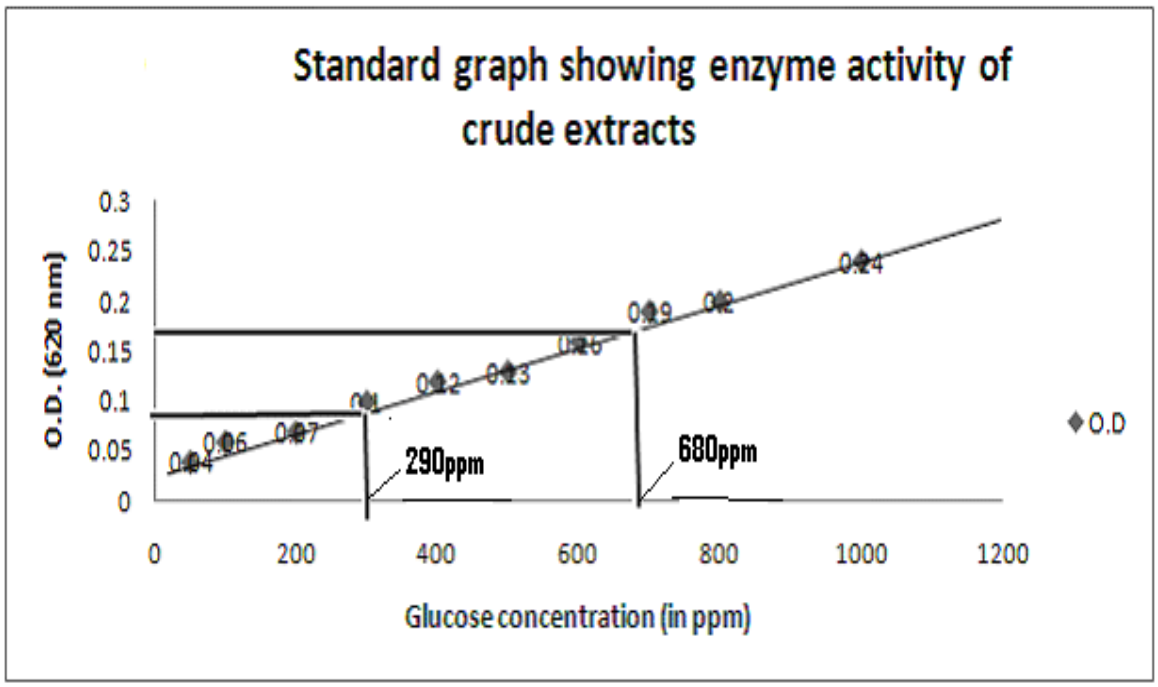

Figure 11: Standard graph showing enzyme activity of crude extracts of kulath and white Rajma.

Enzyme activity of α-amylase in crude extract was calculated as micromoles of product formed per ml of enzyme per unit time (in min). Thus, the corresponding value will be calculated as follows:-

$$\frac{\text{Glucose concentration (in ppm)}}{180.16 * \text{time (in minutes)} * \text{qty. of enzyme (in ml)}}$$

Enzyme activity of crude extract of *Dolichos biflorus*:

= 680/ (180.16 *10*0.1)

= 3.77 IU/ml

Enzyme activity of crude extract of *Phaseolus vulgaris* HUR 15:

= 290/ (180.16*10*0.1)

= 1.61 IU/ml

Further, these isolated crude enzyme extracts were subjected to purification.

Purification of Crude Extracts:

Two fold purification was carried out first by ultra filtration and then by ion exchange chromatography.

Ultra filtration:

a) *Dolichos biflorus*:

The ultra filtered extract from 10kDa extract showed an O.D of 0.01, of which glucose concentration could not be determined from standard graph. Thus, 10kDa extract does not have any α-amylase.

The ultra filtered extract from 30kDa extract showed an O.D of 0.13. From standard graph, the corresponding glucose concentration came out to be 500 ppm. Thus, amount of reducing sugars were estimated to be (as per formula)

= 500/ (180.16*10*0.1)

= 2.77 IU/ml

As the crude extract contained 3.78 IU/ml enzyme, therefore it was deduced that nearly 2/3 of alpha amylase of this plant is below 30kDa size. This 2/3 enzyme was further purified by ion exchange chromatography. Further 1/3 of the enzyme which is above 30kDa was not investigated.

b) *Phaseolus vulgaris* HUR15

In case of *Phaseolus vulgaris* HUR15 (white Rajma),10 kDa extract showed an O.D. of 0.01 while 30kDa extract showed an O.D. of 0.08. From standard graph, the corresponding glucose concentration came out to be 275 ppm. Thus, amount of reducing sugars were estimated to be (as per formula):

= 275/ (180.16*10*0.1)

= 1.52 IU/ml.

Thus, 94% of enzyme has been found to be below 30KDa size. Therefore, this 94% enzyme was used for kinetic characterization.

It was finally concluded that there are two types of α-amylases, viz., Small and large. Small α-amylase is that whose size ranges upto<= 30kDa while large alpha amylase is that whose size is above > 30kDa.

In the present research, α-amylase of Kulath as well as white Rajma comes under small alpha amylases. α-amylases of bacteria generally come under large amylases (discussed later).

It is also important to note that the recent research focuses basically on the kinetic characteristics of purified alpha amylases of these two plants, therefore the exact size of alpha amylases was not required, thus not calculated.

Ion exchange chromatography

Ion exchange chromatography of 30 kDa extract was done.

In case of *Dolichos biflorus*, highly purified α-amylase was obtained at 250 mM concentration (Table 2).

Table 2: Ion exchange chromatography results for *Dolichos biflorus* extract.

Molar Concentration (In mM)	O.D. (620 nm)
50	**0.06**
100	**0.07**
150	**0.07**
200	**0.10**
250	**0.14**
300	**0.13**
350	**0.13**
400	**0.08**
500	**0.07**

At this concentration; O.D. was 0.14. From standard graph, glucose concentration was deduced to be 520 ppm. Thus enzyme activity was calculated as

= 520/180.16x10x0.1

= 2.89 IU/ml

Thus, 2.89 micromoles of glucose product are formed by 1ml of purified α-amylase of *Dolichos biflorus* in one min.

In case of *Phaseolus vulgaris* HUR15, ion exchange chromatography of crude extract was done. Purified alpha amylase was eluted at 150mM concentration (Table 3).

Table 3: Ion exchange chromatography results for *Phaseolus vulgaris* HUR 15 extract.

Molar Concentration (In mM)	O.D. (620 nm)
50	**0.04**
100	**0.06**
150	**0.11**
200	**0.08**
250	**0.07**
300	**0.07**
350	**0.06**
400	**0.05**
500	**0.04**

Maximum O.D. was obtained at 150 mM concentration was 0.11. From standard graph glucose concentration was deduced to be 360 ppm. Enzyme activity was calculated as

= 360/180.16x10x0.1

= 1.998 IU/ml

~2IU/ml.

Thus, 2 micromoles of glucose product are formed by 1ml of purified α-amylase of *Phaseolus vulgaris* HUR 15 in one minute.

To increase this enzyme activity of the purified enzyme, further kinetic characterization is done. As it is a well known fact the under optimum reaction conditions best enzyme activity will be reached.

Kinetic Characterisation

Study of kinetic characteristics of enzymes is important because only after studying the kinetic characteristics, a particular enzyme can be used on industrial scale. More the stability of enzyme in a wide range of conditions more is the usefulness of enzyme on industrial scale.

We calculated the kinetic characteristics of pure enzyme as well as the crude extracts of the enzyme. And we found out that the characteristics of both the extracts were almost the same. This is so because reference on standard graph is glucose.

- **Optimum pH**

pH optima of kulath extract was 6.1. From table 4, O.D. values against each pH concentration starting from pH 3.6 to 7 could be tabulated. The table simply shows maximum O.D. value of 0.94 at pH 6.1. Therefore, pH 6.1 was considered as optimum pH.

Table 4: Table showing optimum pH of crude kulath extract.

pH	O.D. (620 nm)
3.6	**0.24**
4	**0.27**
5	**0.67**
6.1	**0.94**
6.3	**0.91**
6.7	**0.67**
7	**0.17**

On plotting a graph, a bell shaped graph was obtained (Figure 12). Figure 12 shows that the enzyme showed maximum activity between pH 5.5 to 7. But the activity reduced in alkaline range i.e.>7. The drop in activity was more in phosphate buffer than in Tris-HCl buffer, indicating the stability of enzyme in Tris-HCl buffer. After pH 7.5 the

enzyme activity almost nullified. These results correspond with previous results of Nirmala and Muralikrishna (2002).

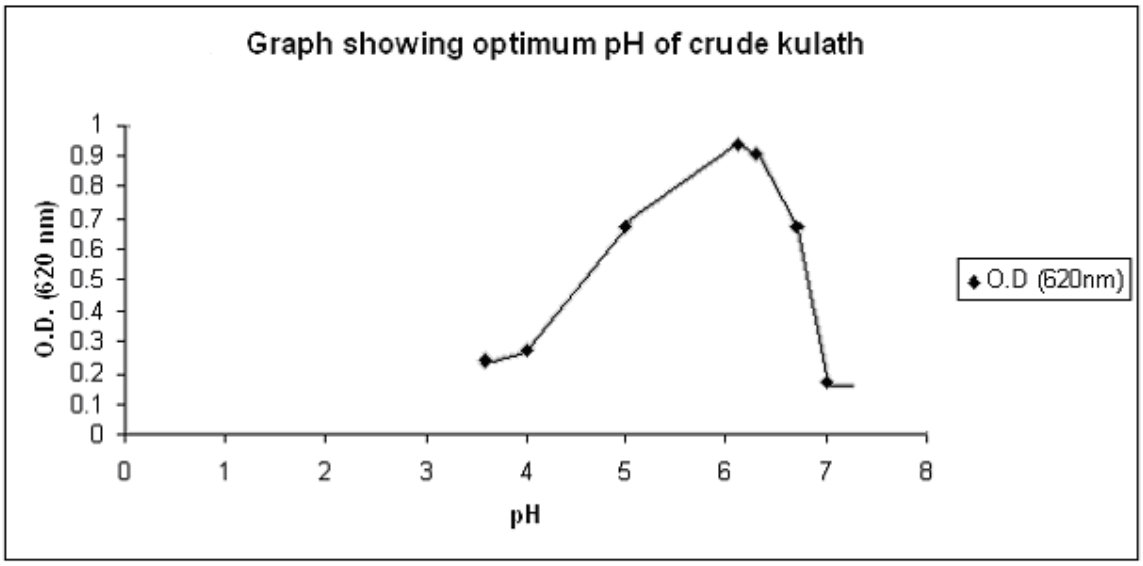

Figure 12: Optimum pH of crude kulath extract.

pH optima of purified kulath enzyme obtained via ion-exchange chromatography was also calculated. Maximum O.D. was obtained to be 1 at pH 6.1. Thus, pH 6.1 was considered to be optimum pH of the purified enzyme (Table 5).

Table 5: Table showing optimum pH of purified kulath extract.

pH	O.D. (620 nm)
3.6	**0.33**
4	**0.46**
5	**0.53**
6.1	**1**
6.3	**0.94**
6.7	**0.93**
7	**0.14**

A graph was also plotted taking pH on X-axis and corresponding O.D. values on the Y-axis. Again a bell shaped graph was obtained showing pH optima of purified enzyme to be 6.1 pH. Purified enzyme also showed best stability in acidic pH range, i.e. 5.5 to 6 (Figure 13).

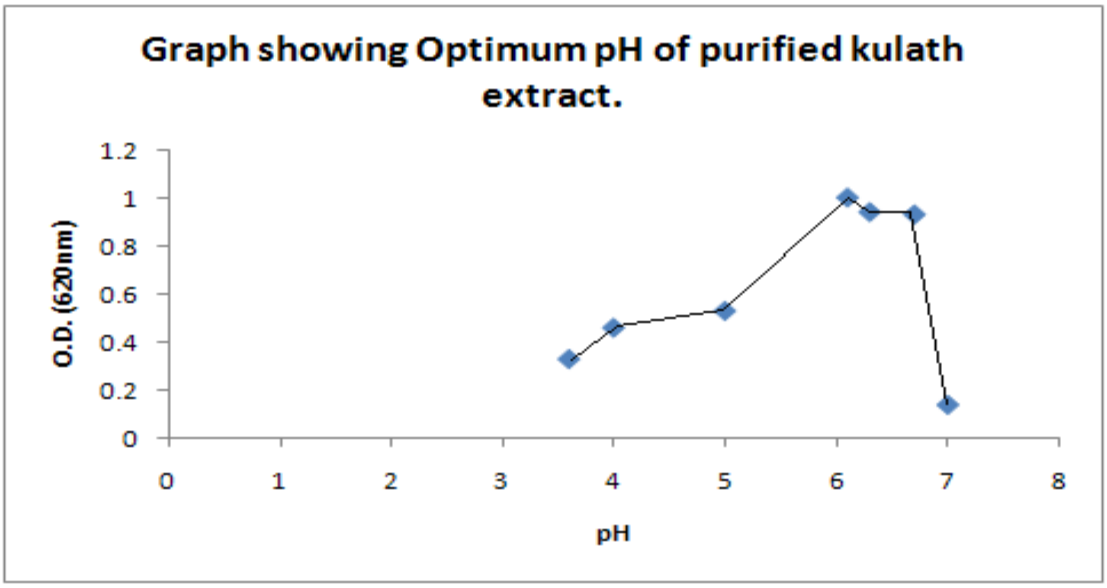

Figure 13: Optimum pH of purified kulath extract.

pH optima of white rajma extract was calculated similarly. Maximum O.D. was obtained to be 0.14 at pH 6.5 (Table 6).

Table 6: Table showing pH optima of white Rajma extract (crude).

pH	O.D. (620 nm)
4	**0.11**
5	**0.12**
6.1	**0.13**
6.3	**0.13**
6.5	**0.14**
6.7	**0.11**
7	**0.09**
7.5	**0.07**
8	**0.06**
8.6	**0.01**

On plotting a graph of pH (X-axis) against O.D values (Y-axis), a bell shaped graph was obtained. The enzyme showed best stability in 4 to 6.5 pH range. Enzyme activity totally nullified after pH range 8 (Figure 14).

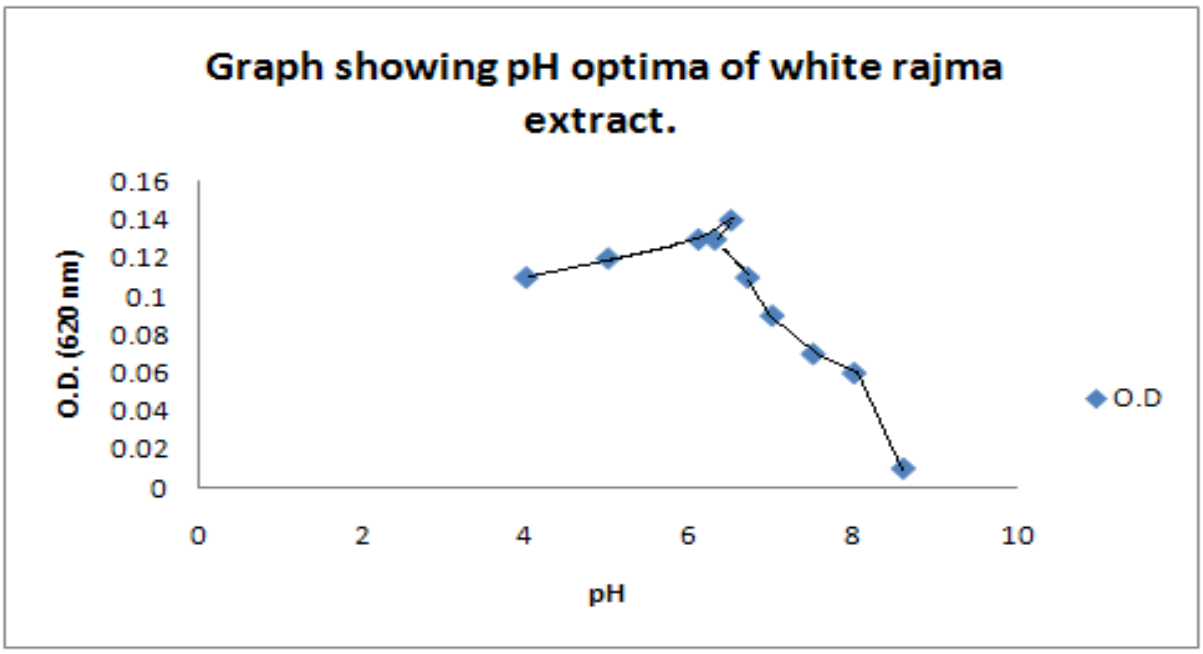

Figure 14: Optimum pH of white Rajma extract (crude).

Optimum pH of purified white rajma extract (obtained via ion-exchange chromatography) was also determined and it also came out to be 6.5. At this pH, the O.D. value was found out to be 0.46 (Table 7).

Table 7: Table showing optimum pH of purified white rajma extract.

pH	O.D. (620 nm)
4	**0.21**
5	**0.22**
6.1	**0.25**
6.3	**0.35**
6.5	**0.46**
6.7	**0.33**
7	**0.21**
7.5	**0.11**
8	**0.03**
8.6	**0.02**

On plotting a graph, a bell shaped graph was obtained (Figure 15), showing best stability of enzyme between pH 6 to 7, i.e acidic range.

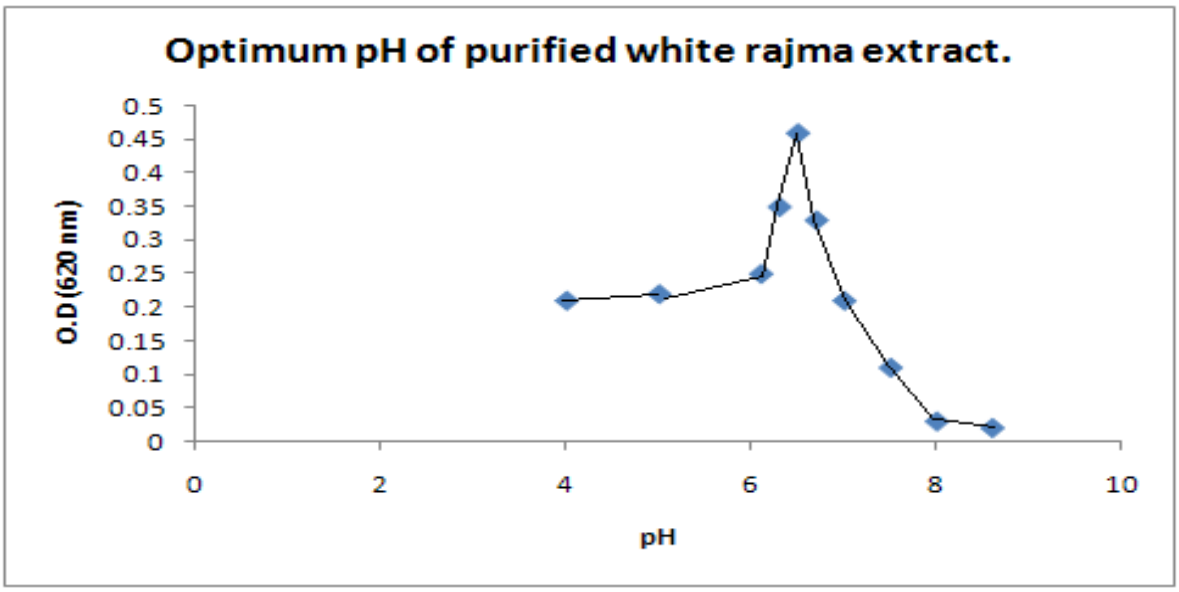

Figure 15: Optimum pH of purified white rajma extract.

We can finally deduce that α- amylase of these two legumes remains stable in acidic pH range and is an acidic amylase.

(Further kinetic characteristics were determined only with the purified enzyme.)

Optimum temperature and thermal stability

The optimum temperature of kulath α-amylase was found out to be 45°C. At this temperature, a maximum O.D. of 0.88 was obtained (Table 8).

Table- 8: Optimum temperature of purified enzyme in kulath.

Temperature (°C)	O.D. (620 nm)
0	**0.02**
4	**0.05**
10	**0.22**
20	**0.44**
30	**0.57**
40	**0.78**
45	**0.88**
50	**0.76**
60	**0.77**
70	**0.21**
80	**0.06**

A graph was plotted taking temperature in (°C) on X-axis and temperature on Y-axis. A slightly distorted bell shaped graph was obtained. The enzyme showed best stability in temperature range 42°C to 48°C (Figure 16).

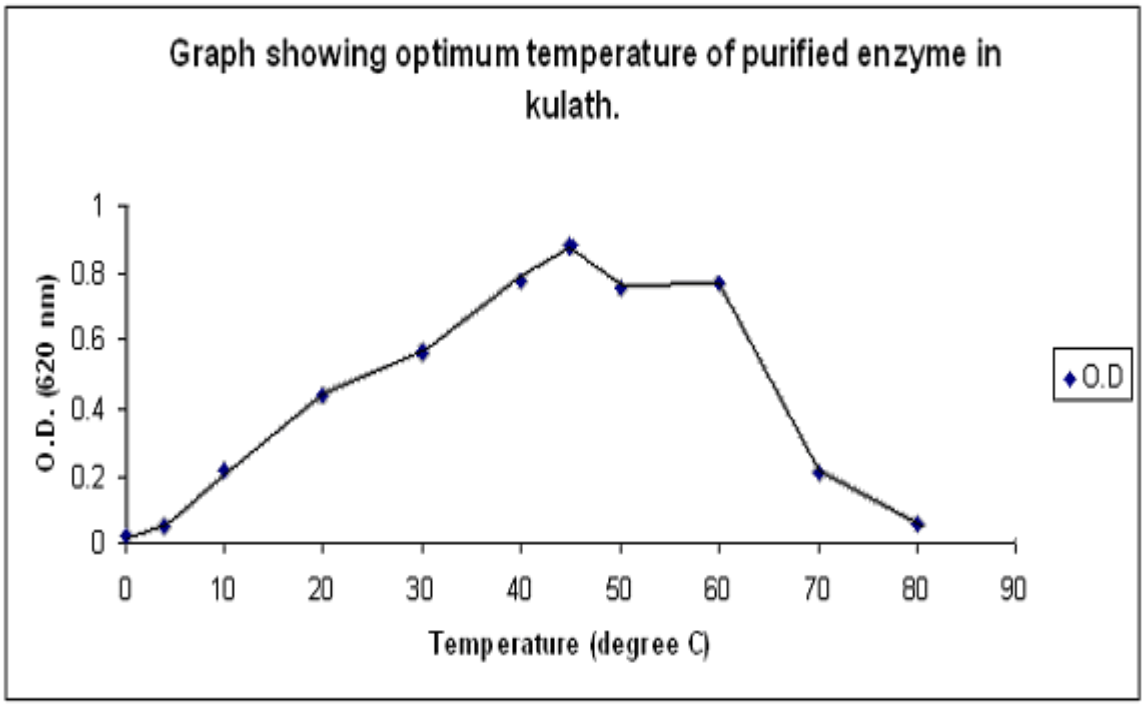

Figure 16: Optimum temperature of purified kulath enzyme.

Since optimum temperature is found to be 45°C, therefore determination of thermal stability was started at 55°C (i.e. 10°C above the optimum temperature). Enzyme assay was done at 55°C, 65°C and 75°C for 10, 15, 20, 25 and 30 minutes respectively. It was observed that O.D keeps on decreasing with increasing temperature. A very slight O.D. change was observed at 55°C; the O.D change increased extensively at 65°C; and at 75°C, after 20minutes, the enzyme was almost totally unstable (Table-9). This enzyme was considered to be highly thermostable as it shows stability upto 75°C. Table 9 shows exact values of changing O.D. with increasing temperature and time.

The optimum temperature of white rajma α-amylase was found out to be 50°C. (Table-10). At this temperature, the maximum O.D. value obtained was 0.2

Table- 9: Table showing thermal stability of purified kulath enzyme.

At 55°C temperature and Time (in minutes)	**O.D.**
10	0.77
15	0.76
20	0.65
25	0.62
30	0.61
At 65°C temperature and Time (in minutes)	**O.D.**
10	0.48
15	0.31
20	0.22
25	0.21
30	0.20
At 75°C temperature and Time (in minutes)	**O.D.**
10	0.08
15	0.08
20	0.07
25	0.06
30	0.05

Table -10: Table showing optimum temperature of white rajma purified enzyme.

Temperature (°C)	**O.D. (620 nm)**
30	**0.09**
40	**0.13**
42	**0.13**
44	**0.15**
46	**0.14**
48	**0.19**
50	**0.2**
60	**0.12**
70	**0.05**
80	**0.02**

On plotting a graph, a slightly distorted bell shaped graph was obtained. Best stability of enzyme was observed in temperature range 45°C to 55°C (Figure 17).

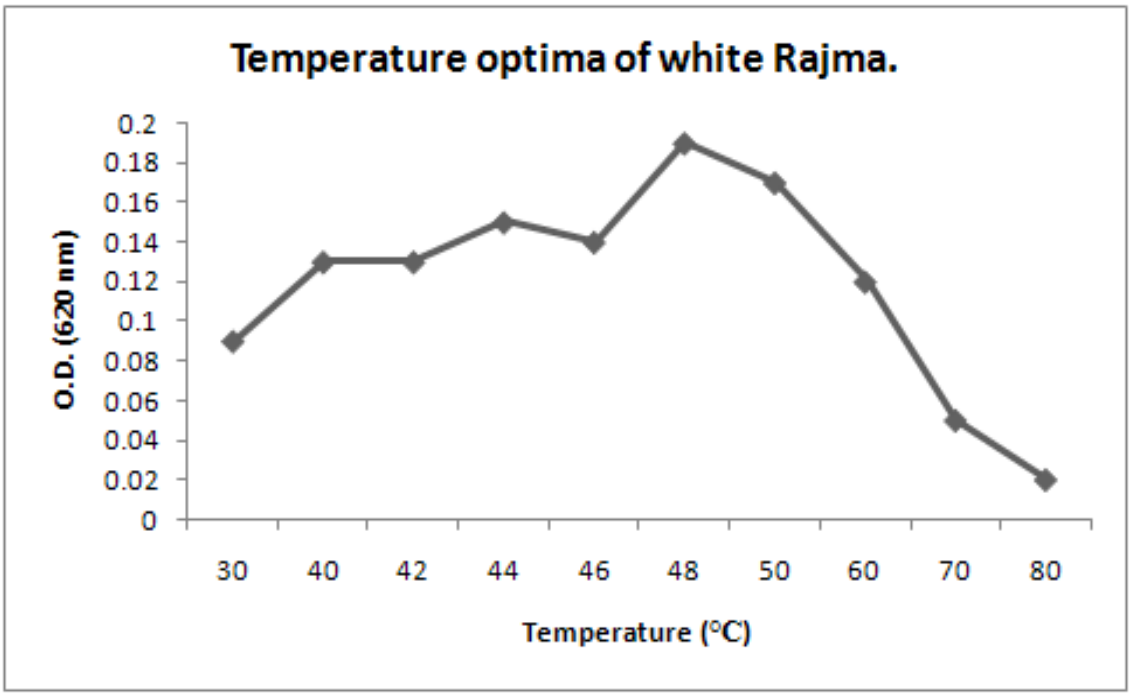

Figure 17: Graph showing temperature optima of white Rajma enzyme.

For thermal stability of white rajma, enzyme assay was done at 60°C and 70°C for 10, 15, 20, 25 and 30 minutes respectively. The O.D. started reducing with increasing temperature and time. Drastic O.D. change was observed between 60°C and 70°C (from 0.12 to 0.05 for 10 minutes). After 20minutes at 70°C, the enzyme activity almost nullified (Table-11). Graph-7 also showed that after 80°C, the activity finished almost completely showing complete instability of enzyme in temperature ranges higher than this, thus accounting to high thermo stability (Figure 15).

Table -11: Table showing thermal stability of white Rajma enzyme.

At 60°C temperature and Time (in minutes)	O.D.
10	0.12
15	0.11
20	0.09
25	0.08
30	0.08
At 70°C temperature and Time (in minutes)	**O.D.**
10	0.05
15	0.05
20	0.04
25	0.02
30	0.02

Km and Vmax

For calculating K_m and V_{max} values, O.D. of the enzyme at various substrate concentrations was calculated first of all. Then that substrate concentration was determined at which maximum O.D. was obtained. In case of kulath, optimum substrate concentration was found to be 2.4mg/ml. At this substrate concentration, O.D. was found to be 0.90. This value is known as the optimum substrate concentration (Table 12).

Table 12: Table showing S_o against V_o (kulath).

S_o= starch concentration in mg/ml	O.D. (620 nm)	V_o = ppm value/ (180.16 X time (in min) X qty. of enzyme (in ml))
0.4	0.07	1.11
0.8	0.1	1.66
1.2	0.16	3.33
1.6	0.21	4.83
2	0.58	16.5
2.4	0.90	27.11
2.8	0.88	26.6
3.2	0.87	26.5
3.6	0.83	25.5
4	0.88	26.6
5	0.88	26.6

A graph was plotted taking S_o on X-axis and V_o on Y-axis. **In** case of kulath, a slightly distorted parabolic graph obeying Michaelis Menton equation was obtained (Figure 18). The enzyme activity first increased very slowly and then after increasing starch substrate concentration to 2mg/ml, it suddenly increased four times. V_{max} was obtained at $28IUml^{-1.}$ From the graph K_m was calculated as 1.95mg/ml.

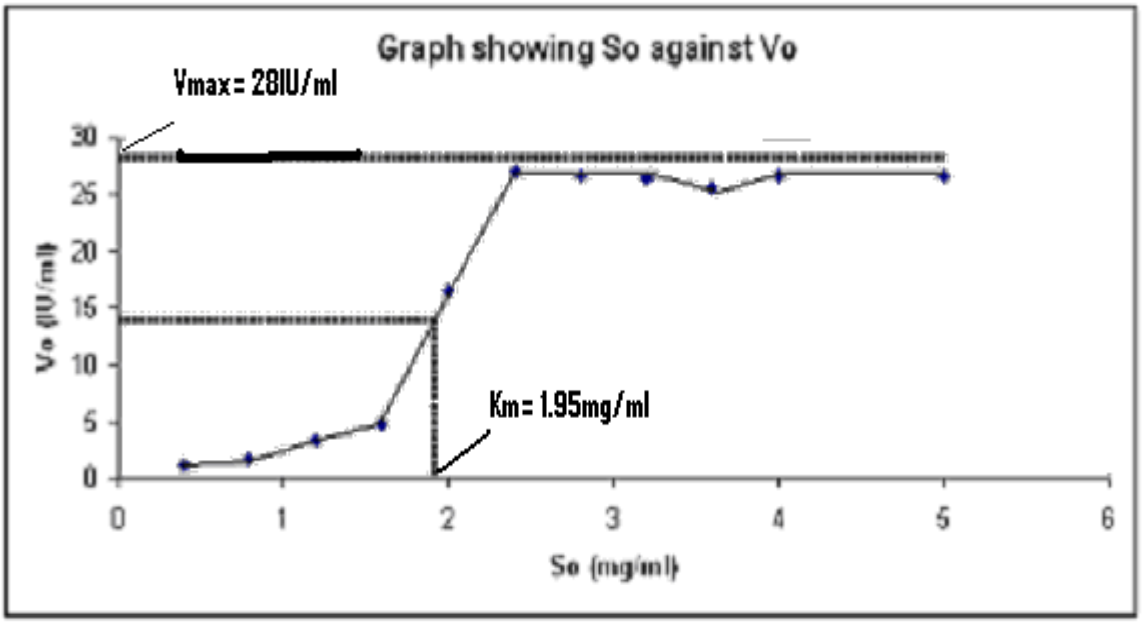

Figure 18: K_m and V_{max} of α-amylase of Kulath.

In case of white Rajma also, first of all optimum substrate concentration was determined. Optimum substrate concentration was found out to be 2mg/ml. At this substrate concentration, maximum O.D. was obtained, i.e. 0.15 (Table 13).

Table 13: Table showing S_o against V_o (white rajma).

So= starch concentration in mg/ml	**O.D. (620 nm)**	**Vo = ppm value/ (180.16 X time (in min) X qty. of enzyme (in ml))**
0.2	**0.09**	**1.55**
0.4	**0.08**	**1.33**
0.6	**0.08**	**1.33**
0.8	**0.09**	**1.61**
1	**0.07**	**1.11**
2	**0.15**	**3.27**
3	**0.15**	**3.16**
4	**0.13**	**2.7**
5	**0.15**	**3.27**
10	**0.14**	**2.88**

On plotting a graph of S_o against V_o, a parabolic graph obeying Michaelis Menton was obtained (Figure 19), but the parabola was observed after 1mg/ml S_o value. V_{max} was obtained at 3.4 IU/ml. Initially V_o values swirled between 1.5 to 1.6IU/ml. After 1mg/ml So value, the V_o value increased to 3.27IU/ml. the values again started rotating between 2.8 to 3.2 IU/ml. thus, we could say that the graph became parabolic after S_o values came up to 1mg/ml.

It is important to note that further readings (after 10mg/ml concentration of starch) could not be calculated using DNS method because of very intense dark colour.

Thus, finally V_{max} was estimated to be 3.4 IU/ml. From the graph K_m was found out to be 1.3mg/ml.

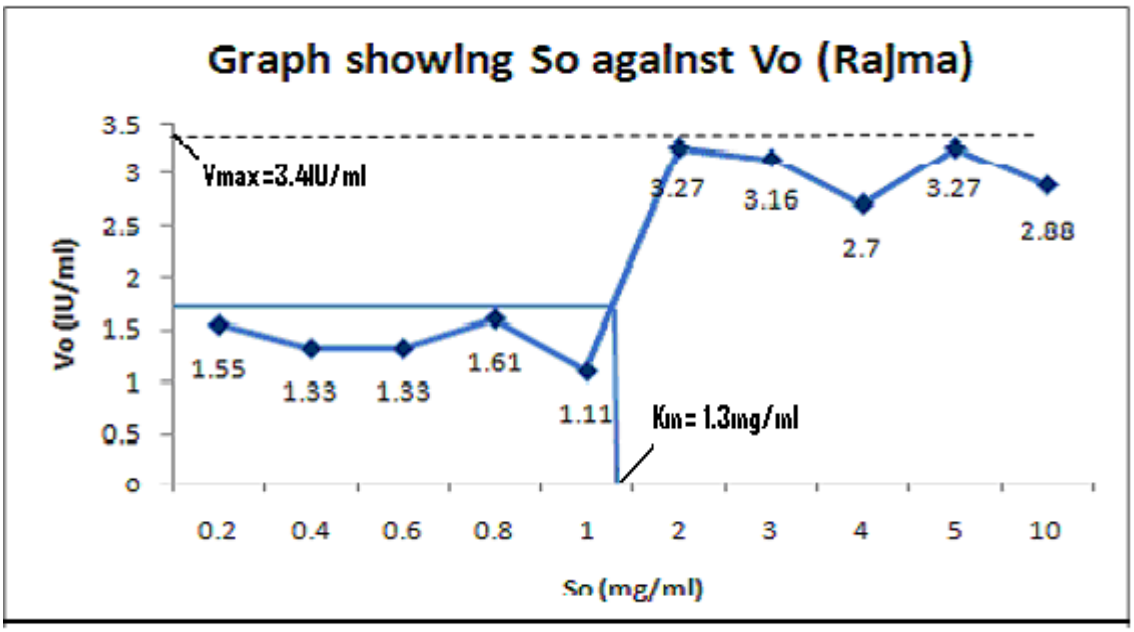

Figure 19: K_m and V_{max} of alpha- amylase of white Rajma.

Time

In case of kulath, maximum enzyme activity was observed in 10 minutes in this time maximum O.D. = 0.18 was obtained (Table 14).

Table 14: Table showing optimum time of α-amylase of kulath.

Time (in minutes)	O.D. (620 nm)
2	**0.04**
4	**0.06**
6	**0.09**
8	**0.14**
10	**0.18**
12	**0.11**
14	**0.09**

A graph was plotted with time on X-axis and corresponding O.D. on Y-axis. Again a bell shaped graph was obtained (Figure 20)

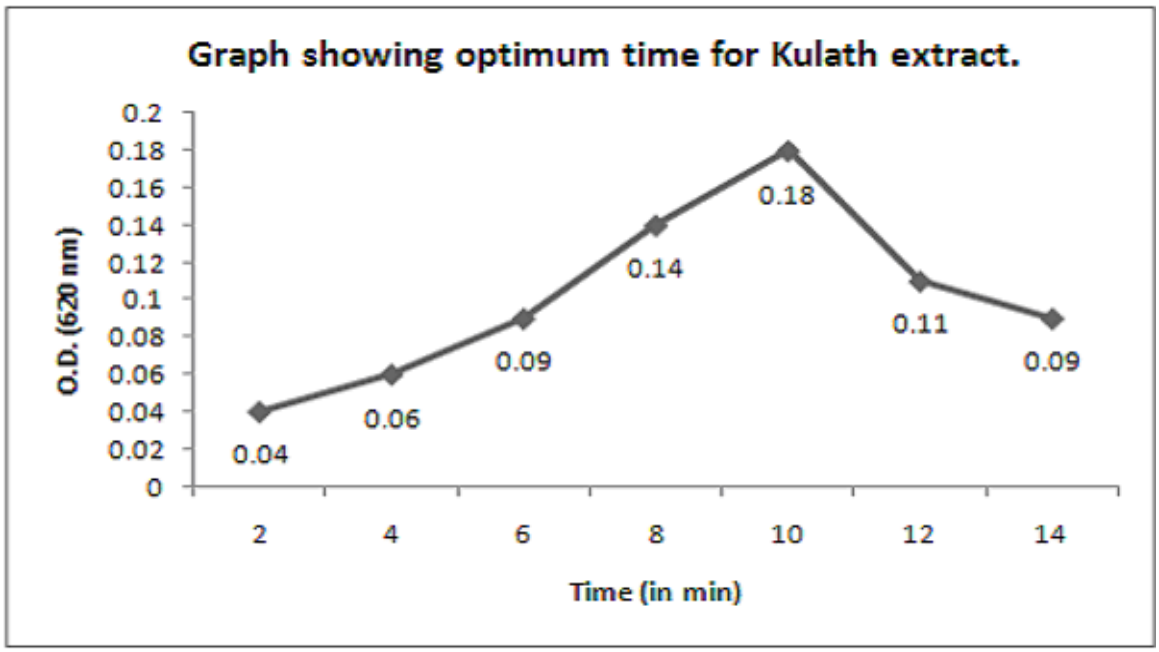

Figure 20: Graph showing optimum time of α-amylase of kulath.

In case of white rajma optimum time was observed to be 14 minutes in which maximum O.D. was obtained that is 1.19 (Table-15).

Table 15: Table showing optimum time of α-amylase of white rajma.

Time (in minutes)	O.D. (620 nm)
2	**0.03**
4	**0.04**
6	**0.07**
8	**0.89**
10	**0.94**
12	**1.03**
14	**1.19**
16	**0.72**

A graph was plotted with time on X-axis and corresponding O.D. on Y-axis. The graph was distorted bell shaped type in case of this sample (Figure 21).

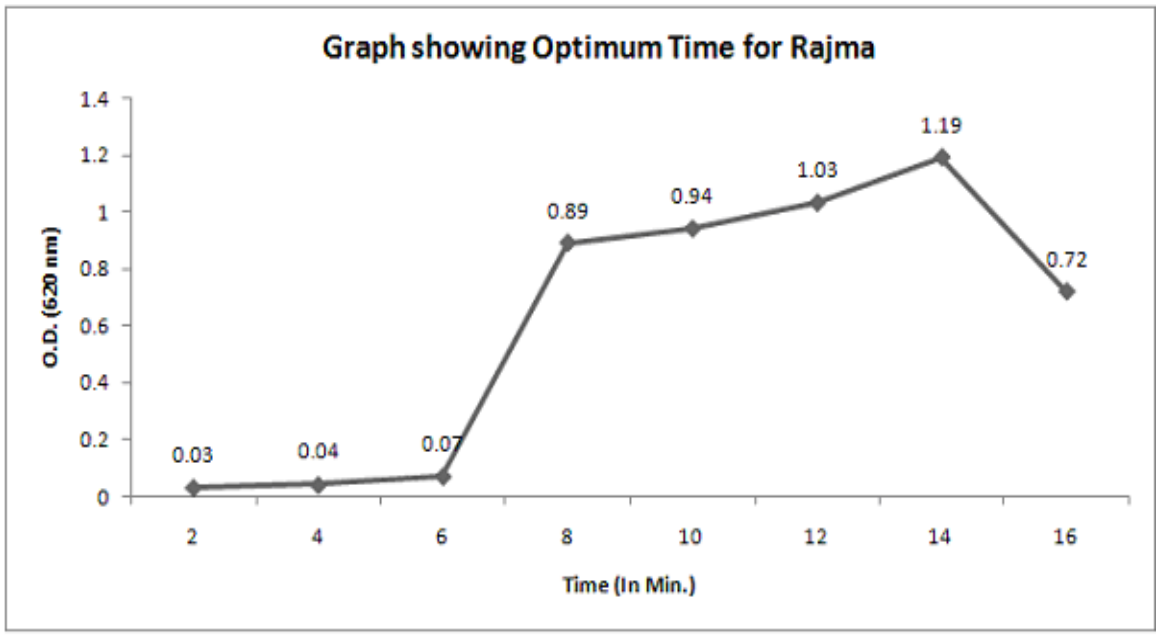

Figure 21: Graph showing optimum time of α-amylase of white Rajma.

In both the cases, it was observed that in the initial four minutes hardly any reaction could take place. It was thus deduced that 4 min is the time for optimization of the overall process so that reaction can take place speedily.

Yield

Best yield is always obtained at optimum kinetic conditions. Therefore, in case of *Dolichos biflorus*, Yield of enzyme was best at pH 6.1, temperature 45ºC, time 10 minutes, and starch concentration 2.8 mg/ml. Under these conditions, enzyme activity came out to be was quite promising accounting to 27.7IU/ml Yield in case of *Phaseolus vulgaris HUR 15* was best at pH 6.5, temperature 44ºC, time 14 minutes, and starch concentration 2mg/ml. Under these conditions the enzyme activity came out to be 35 IU/ml. Thus, we could conclude that alpha amylase of these two legumes is a new, low cost source of this multi faceted enzyme.

Immobilisation

Immobilization of both enzymes was done on calcium alginate matrix. Shiny transparent beads of two centimeter diameter with slightly yellowish brown coloration were obtained in case of kulath. Beads of ultra filtered kulath extract (~30kDa) were also prepared which were shiny yellow in colouration (Plate 5).

In case of rajma opaque white color beads of two centimeter diameter were obtained.

Kinetic characteristics of immobilized enzyme:

Kinetic characteristics of these immobilised enzymes beads were calculated and it was found out that the characteristics were highly enhanced. Although much change in optimum pH was not observed, still we observed a remarkable increase in enzyme activity. Optimum pH of free enzyme came out to be 6.1 and that of immobilized enzyme came out to be 6.3 (Table 16).

Table -16: Comparison between enzyme activities of crude and immobilized kulath enzyme at different pH concentrations.

pH	Enzyme activity (IU/ml) of crude enzyme.	Enzyme activity (IU/ml) of immobilized enzyme.
3.6	**5.55**	**4.44**
4	**6.11**	**6.67**
5	**18.87**	**18.05**
6.1	**27.22**	**22.78**
6.3	**26.11**	**37.22**
6.7	**18.87**	**44.44**
7	**3.39**	**38.89**
8	**<0.20**	**16.96**
8.6	**<0.20**	**17.19**
9	**<0.20**	**2.08**

Figure 22:
Shiny yellow brown beads of crude extract of *Dolichos biflorus*

Figure 23:
Shiny beads of Ultrafiltered extract of *Dolichos biflorus*.

Figure 24:
White beads of *Phaseolus vulgaris* HUR 15
(white rajma)

Plate 3- Immobilised beads of Kulath and Rajma.

Figure 25 shows a close comparison between optimum pH of free and immobilized enzyme. Most enzymes are stable in pH range 6 to 8 (Welker and Campbell, 1967). This table further puts light on this statement. Uptil pH 6.1, we could not observe much increase in enzyme activity. Drastic increase in enzyme activity was observed after pH 6.3. We observed that at pH 7 enzyme activity increased more than 11 times (~11.5 times). Although we could not observe much increase in optimum pH; Free enzyme showed an optimum pH of 6.1 (Table 5) while the immobilized enzyme showed an optimum pH of 6.7 (Table 16). We also observed that the immobilized enzyme was stable at much higher pH values than the free enzyme. While the free enzyme showed no activity at pH 8, the immobilized enzyme showed an activity of 16.96 IU/ml at pH 8.

An important point of consideration was that at pH 3.6, the enzyme activity of immobilized enzyme was slight lower than that of free enzyme. Free enzyme showed enzyme activity of 5.55 IU/ml while immobilized enzyme showed enzyme activity of 4.44 IU/ml. This might be so because immobilized enzyme takes higher time for optimization than the free enzyme.

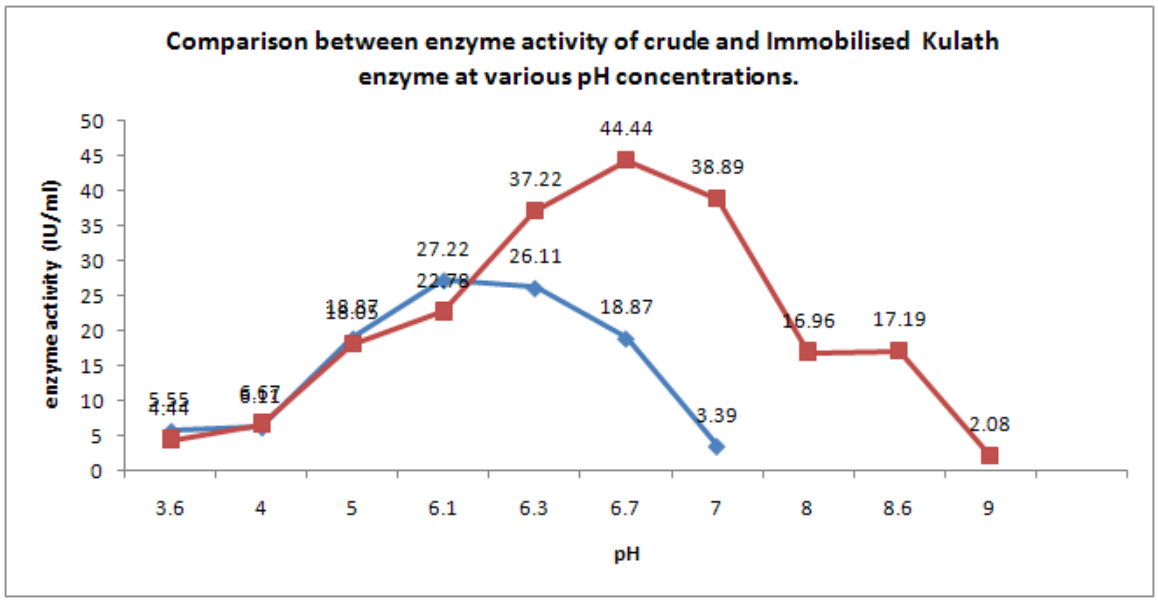

Figure 25: Graph showing comparison between enzyme activities of free and immobilized kulath at various pH concentrations.

Certain similar results were seen in case of optimum temperature. Optimum temperature of free enzyme was 45°C (Table 8) while optimum temperature of immobilized enzyme was 50°C (Table 17). This simply proves the increase in thermal stability. Around 9 times increase in enzyme activity was observed at 70° C (Figure 26). While the free enzyme showed very less activity at 80°C, the immobilized enzyme showed a high activity of 10.74IU/ml at 80°C.

Table -17: Comparison between enzyme activities of crude and immobilized kulath enzyme at various temperatures.

Temperature (°C)	Enzyme activity (IU/ml) of crude enzyme.	Enzyme activity (IU/ml) of immobilized enzyme.
0	**<0.20**	**0.28**
4	**0.42**	**0.55**
10	**5**	**5.55**
20	**11.11**	**14.44**
30	**15.55**	**17.22**
40	**22.22**	**22.78**
45	**25.55**	**28.94**
50	**21.67**	**>44.4**
60	**22.19**	**>44.4**
70	**4.44**	**38.89**
80	**0.55**	**24.8**
85	**--**	**10.42**
90	**--**	**1.11**
95	**--**	**0.55**

Kinetic characterization of immobilized α-amylase of white rajma also revealed similar results. Optimum pH of immobilized white rajma extract came out to be 6.5 . This was same as that of free enzyme (Table 7). Enzyme activity of immobilized enzyme increased 20 times at pH 8. While the free enzyme showed no activity at pH 8.6, the immobilized enzyme showed an activity of 8.89 IU/ml at pH 8.6. Thus, it was finally proved that characteristics are enhanced in immobilized enzyme. While the pH stability range for free enzyme is 4 to 7, the pH stability range for immobilized enzyme is observed to be 4 to 9 (Table 18; Figure 27).

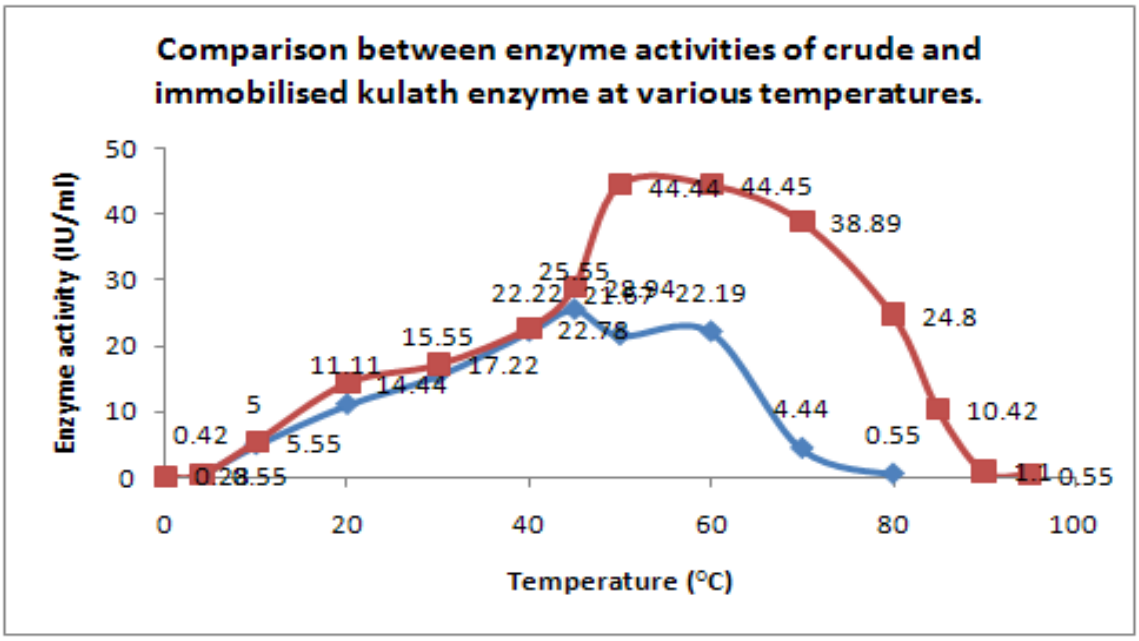

Figure 26: Graph showing comparison between enzyme activities of free and immobilized kulath enzyme at various temperatures.

Table 18: Comparison between enzyme activities of crude and immobilized white rajma enzyme at various pH concentrations.

pH	Enzyme activity (IU/ml) of crude enzyme.	Enzyme activity (IU/ml) of immobilized enzyme.
4	2	1.67
5	2.22	2.22
6.1	2.78	6.78
6.3	2.78	10.5
6.5	2.89	35.55
6.7	2	30.55
7	1.55	29.72
7.5	1.11	21.39
8	0.55	10.89
8.6	<0.20	8.89

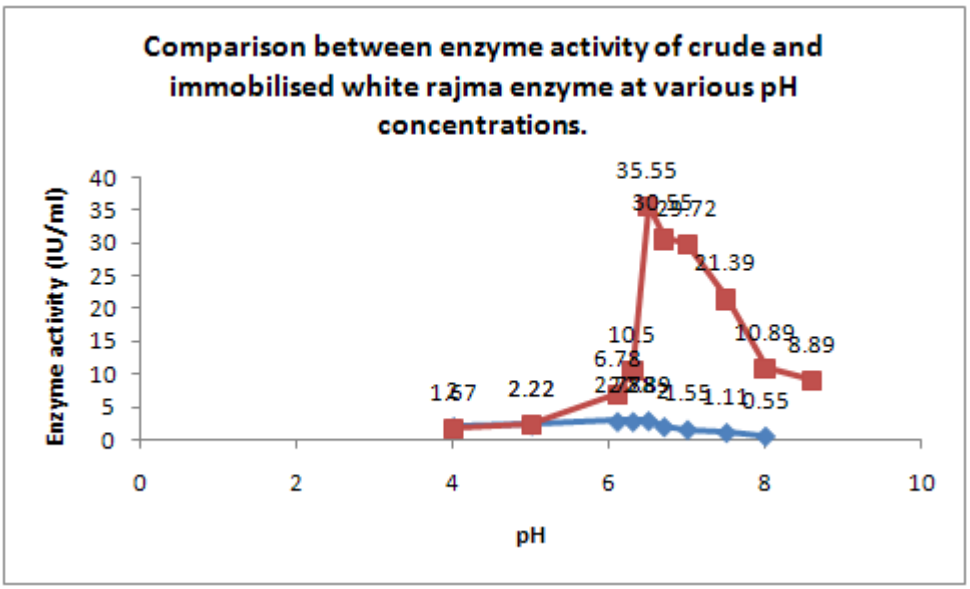

Figure 27: Graph showing comparison between enzyme activities of free and immobilized white rajma enzyme at various pH concentrations.

Coming to temperature, optimum temperature of immobilized white rajma α-amylase enzyme came out to be 60°C, against temperature optimum of 48°C for free enzyme (Table 10). Enzyme activity increased 24 times at 70°C temperature. The immobilized enzyme was found to be stable at 80° C; enzyme activity corresponding to 8.42 IU/ml. Thus, high thermostability of immobilized enzyme was proved.

Table 19: Comparison between enzyme activities of crude and immobilized rajma enzyme at various temperatures.

Temperature (°C)	Enzyme activity (IU/ml) of crude enzyme.	Enzyme activity (IU/ml) of immobilized enzyme.
30	1.55	1.67
40	2.78	2.78
42	2.78	2.22
44	2.69	5.83
46	2.89	9.72
48	3.89	14.44
50	4.44	19.17
60	2.22	22.5
70	0.42	10.14
80	<0.20	8.42

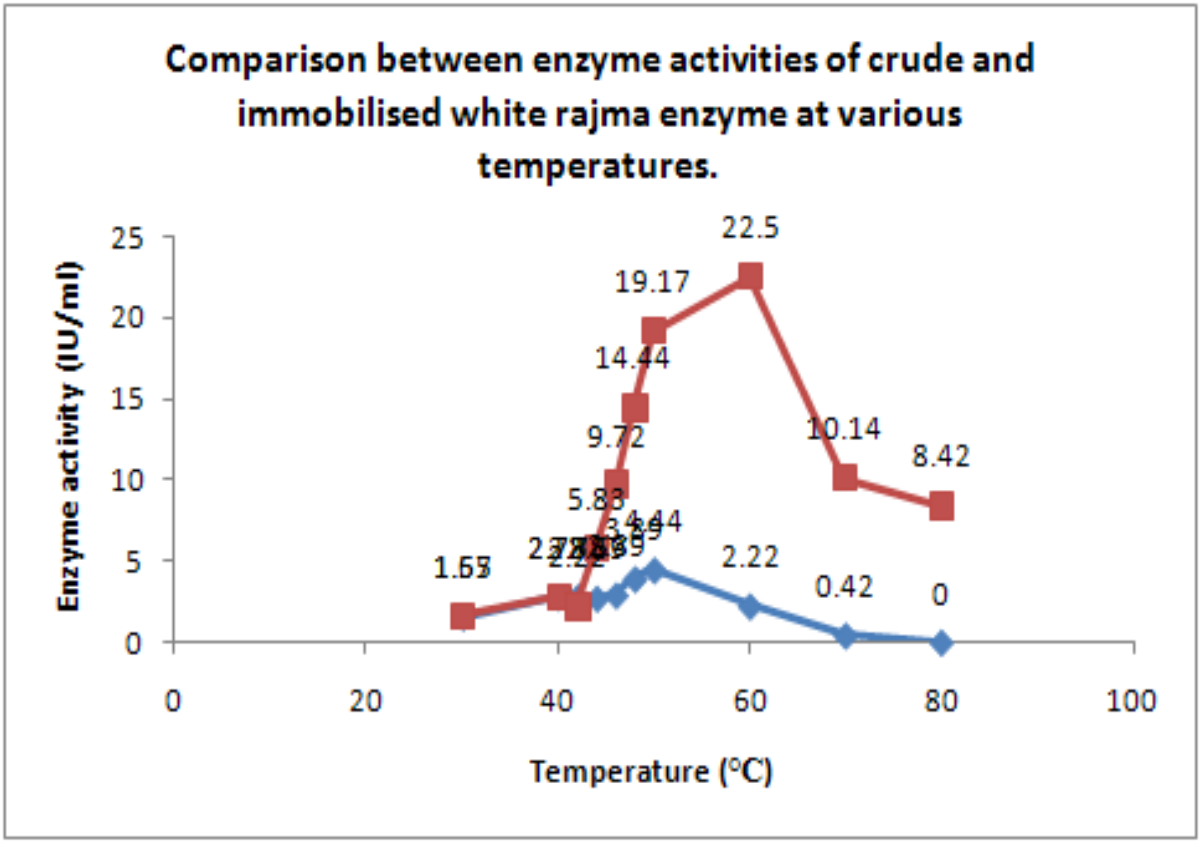

Figure 25: Graph showing comparison between enzyme activities of free and immobilized white rajma enzyme at various temperatures.

Re-utilisation of immobilized beads was not possible more than twice as during enzyme assay, when the beads came to boiling (Refer Step 5 of enzyme assay, Chapter-3); they almost dissolved, thus leaving only the contaminated solution for taking out the O.D values. But since, immobilized beads could be reused 2 times, therefore usage of immobilized enzyme in comparison to free enzyme is easy and user friendly.

Further preparation of immobilized beads which could be used more than twice remains a topic of further research.

Comparison:

We also performed a small comparison between 14 plant and bacterial species for α-amylase enzyme present in them (Garg and Dobriyal, 2010). Comparison was made on the basis of optimum pH, optimum temperature, K_m, V_{max}, pH stability range and thermal stability of enzyme. We found out that the plant enzyme is equally good than the bacterial enzyme in terms of characteristics.

α-amylase of *Dolichos biflorus* and *Phaseolus vulgaris* (family Fabaceae) showed complete instability in highly alkaline pH range. Optimum pH of white Rajma came out to be 6.5 while that of Kulath came out to be 6.1. These were completely unstable above pH 7.5. However, these were compared with α-amylase of the family Poaceae, which were stable in acidic pH range only, and become completely unstable above pH 7. *Eleusine coracana* Indaf-15, *Sorghum bicolor*, *Zea mays* and *Triticum aestivum* were studied. All these have pH stability range and optimum pH below 7 (Table 20).

Table -20: Comparison between temperature and pH for plant α-amylase.

S.No.	α-amylase source	Common name	pH stability range	Optimum pH	Thermal stability (°C)	Optimum temperature (°C)
1	*Dolichos biflorus*	Kulath	5.5-7	6.1	40-75	45
2	*Phaseolus vulgaris* HUR 15	White rajma	4-7.5	6.5	30-80	50
3	*Glycine max*	Soyabean	4-6	5.5	25-85	50
4	*Eleusine coracana* Indaf-15	Ragi or finger millet	5-5.5	5.3	45-50	47
5	*Sorghum bicolor*	Jowar	4.5-6	5.5	30-70	70
6	*Trticum aestivum (5 isozymes)*	Wheat	5.5-7	5.5-7 (for all five isozymes)	40-50	50
7	*Zea mays*	Corn	4-6.5	5.5	50-90	55

Among bacteria, 6 bacterial strains were studied, of which 3 belonged to the genus *Bacillus.* All *Bacillus* species have an optimum pH below 6.5, and are unstable in alkaline range. *Thermomonospora curvata* and *Streptococcus* had showed slight stability in alkaline range, only up to pH 8.5 (Table 21).

It was thus concluded that that optimum pH of almost all bacterial and plant amylases range between pH 4 to 8.

The thermal stability of all amylases is not the same. It was deduced that the legume family has a high thermal stability, and the α-amylase is stable almost up to 80˚C. However, the grass family has a relatively low thermal stability with amylase stability up to a temperature of 50˚C. Among bacteria, the thermal stability is varying. Strains of *Bacillus* spp. are highly thermo stable, while *Streptococcus* and *Xanthomonas* have low thermal stability. Generally, the optimum temperature ranges between 40-55˚C (Table 20 and 21).

Table - 21: Comparison between temperature and pH for bacterial α- amylase

S.No.	α- amylase source	pH stability range	Optimum pH	Thermal stability (°C)	Optimum temperature (°C)
1	***Bacillus amyloliquefaciens strain F***	5.5-6	5.9	0-7-	65
2	***Bacillus subtilis strains W23 and BS5***	5.7-6	6.3	0-70	65
3	***Bacillus cereus***	4-6	5	35-75	55
4	***Thermomonospora curvata***	6-8	7.5	40-70	53
5	***Xanthomonas campestris***	4.5-5	4.5	45-55	45
6	***Streptococcus bovis JB1***	5.5-8.5	5-6	Below 50	Below 50

Further comparison was made on the basis of size. Among plants, it was found that the plant amylases are small in size in comparison to bacterial amylases. While the size of bacterial enzymes was found to be as high as 149KDa for *Bacillus subtilis* AX 20,

the size of plant enzyme was as low as 22KDa for *Eleusine coracana* Indaf 15. Such small sized proteins are beneficial in food industry. Recent researches conducted by food industries prove that small protein molecules are easily digestible in the small intestine, and provide very high surface area for reaction (Table 22 and 23).

Table -22: Molecular weight of α-amylase from bacterial sources.

S.No.	Bacterial source	Molecular weight (KDa)	Reference
1	***Bacillus stearothermophilus***	43 to 46	Yutani, K (1973)
2	***Thermomonospora curvata***	62	Glymph, J. L. and Stutzenberger, F., (2005)
3	***Bacillus subtilis*** AX20	149	Najafi et.al., (2005)
4	***Bacillus subtilis*** BS5	63	
5	***Xanthomonas campestris*** K-11151	55	Abe et. al., (1994)
6	***Streptococcus bovis*** JB1	77	Freer, 2010

Table 23: Molecular weight of α-amylase from plant sources.

S.No.	Plant source	Molecular weight (KDa)	Reference
1	***Dolichos biflorus***	Below 30	Goyal and Dobriyal, 2011
2	***Phaseolus vulgaris*** HUR 15	Below 30	Goyal and Dobriyal, 2010
3	***Eleusine coracana I*ndaf 15**	22	Nirmala, M., and Muralikrishna, G., (2003)

Low K_m values of plant α- amylases studied indicate high affinity of the enzyme for starch substrates, like some bacterial strains (Table 24).

Not all workers have stated the V_{max} values in their work owing to the fact that, V_{max} values of different enzymes are difficult to compare as they depend on the substrate used and the reaction conditions.

Table 24: Comparison of K_m values of α-amylase from plant and bacterial sources.

S. No.	Source of α-amylase	Km value (mg/ml)
1	*Dolichos biflorus*	1.95
2	*Triticum aestivum* (5 isozymes)	1.42-1.7
3	*Bacillus amyloliquefaciens* strain F	3.076
4	*Bacillus subtilis* BS5	16.67
5	*Streptococcus bovis* JB1	0.88
6	*Thermomonospora curvata*	0.39

The investigation simply proves that the kinetic characteristics of plant and bacterial amylases are similar.

It was finally concluded that the plant enzyme is as good as bacterial enzyme in terms of characteristics. The study also proves that the enzyme can be better produced commercially by legume sources.

Conclusion:

Some reports are already available on extraction of alpha-amylase from pulse crops like mung beans, soyabean and pea etc. (Greenwood *et.al.*, 1965; Beers and Duke, 1988; Tripathi *et.al.*, 2007). Kapoor (2005) has shown the presence of urease enzyme in *Dolichos biflorus.* The present report is first report on extraction of alpha amylase from both these plants, *Dolichos biflorus* and *Phaseolus vulgaris* HUR 15. While on one hand, we studied *Dolichos biflorus*, whose seeds have high medicinal importance as astringent to bowels, antipyretic, diuretic, tonic, antihelminthic, appetizing and lithontriptic. It is useful in piles, tumours, bronchitis, heart trouble, kidney stone, enlargement of spleen, hiccough, asthma, leucoderma, and in abdominal complaints. On the other hand we studied *Phaseolus vulgaris* HUR 15, whose seeds have not been found to have any medicinal properties, nor have any enzymes been extracted from white rajma.

Both the enzymes were found to be highly thermostable, active over a range of pH values and had low K_m values. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications (Haki and Rakshit, 2003).

Activeness in a range of pH values also increases the industrial applicability of the enzyme. Although low Km values, did not prove to be much effective because substrate was not limiting, still we could say that this characteristic is important when the enzyme will be produced on laboratory scale; and further industrial scale.

Till date, very less literature could be cited regarding immobilization of plant α-amylase. No work regarding immobilization of plant amylase on Calcium alginate could be found. This research proved to be a very new decent approach on immobilization of plant α-amylase on Calcium alginate. We used Calcium alginate because Calcium alginate has been found to be the best matrix material for immobilization of bacterial α-amylases (Refer Chapter-2).

Previously researches avoided working on plant amylases because of the tediousness witnessed during extraction of the enzyme and further purification. A large amount of spent material is to be removed during enzyme extraction. In the present work, we also proved that the kinetic characteristics of crude as well as purified enzyme are almost the same. Thus, even the crude enzyme can be used for calculating the kinetic characteristics. This approach can reduce not only the tediousness of the work, but also made the work cost- effective.

The very basic thought behind starting this research work was to find out whether the α-amylase enzyme present in these two legumes is of industrial importance or not. We could finally deduce that even this α-amylase can be used in industries like starch industry, brewing and baking industry and detergent industry.

Future perspectives of the study:

- We have proved the high enzyme potential of Kulath and White Rajma in the present study. This high enzyme potential can be further enhanced by application of biotechnology and genetic engineering in this field. Studies can be performed on the same.
- Further studies on other legume sources, (of Garhwal and outside Garhwal) can also be performed.

- We have immobilized the enzyme only on Calcium alginate matrix. Studies can be perfomed on other matrices also. Also we used beads only of 2cm diameter. Further studies can be performed with different bead sizes and different matrices.
- Research can also be performed to find out the suitable matrix whose beads could be used more than twice.

Chapter 5

Summary

The thesis entitled, “Studies on isolation, purification, kinetic characterisation and immobilisation of α-amylase from some legumes of Garhwal Himalayan Region” can be summarised as follows:

1. There is a bulk of genetic and ecological diversity in the Garhwal Himalayan region of Uttarakhand. This diversity includes 4700 species under 1503 genera and 213 families. Among all these, the dicots dominate with 3493 species under 1163 genera and 182 families. Among dicots, the family Asteraceae has the largest number of genera, i.e., 125 followed by Fabaceae which has 76 genera. The flowering plants provide wide range of useful products ranging from timber, medicine, food, vegetable oils, gums, resins and spices etc. Among the edible plants, pulses, also known as grain legumes, are second only to cereals as source of human food. These account for nearly 20 species all belonging to the family Fabaceae. Major pulse crops are *Cajanus cajan, Cicer arientum, Dolichos spp, Phaseolus spp, Vigna spp, Glycine max, Lens culinaris, Pisum sativum* and *Vicia faba*.

2. Biotechnologists of this area are known to work on subjects related to biotechnology like molecular biology, enzymology and tissue culture, etc. It is interesting to note that despite the large number of products; timber, medicine, food, vegetable oils, gums, resins and spices; exploited from these multi-faceted pulse crops, the enzymatic stamina of these pulse crops has not been explored yet.

Despite many biotechnological advances in this area, the scientists were found to be least interested regarding the idea of enzyme extraction from pulse crops. Therefore the present research on "Studies on isolation, purification, kinetic characterisation and immobilisation of α-amylase from some legumes of Garhwal Himalayan Region" has been carried out.

3. α- amylase (α-1,4 glucan-glucanhydrolase; E.C. 3.2.1.1) is an extracellular enzyme which degrades α-1,4 glucosidic linkages of starch and related substrates in an endo fashion producing oligosaccharides including maltose, glucose and alpha limit dextrins. This breakdown reaction is an important step in many industrial processes like brewing, baking, starch liquefaction, pharmaceutical and enzyme production etc. Therefore, this enzyme is extensively used in industries like starch liquefaction, brewing, food, paper, textile and pharmaceuticals.

4. A large number of sources have been recognised by scientists and researchers all around the world for extraction of α-amylase. These sources include micro-organisms, plants and animals. A close investigation of various enzyme firms shows that most of the α-amylase production is done from genetically modified micro-organisms of *Bacillus* spp. During the research it was thoroughly investigated that even though the enzyme is present in all sorts of sources discussed above, still no plant or animal source has been utilised on large scale for production of the enzyme. Although the enzyme has been located in plant sources like sorghum, broad beans, coconut oil cake, cereals and mung beans, no researcher has tried to locate the enzyme in the flora of Garhwal Himalayan Region. In the present research, the enzyme has been extracted from two leguminous sources, *Dolichos biflorus,* commonly known as Kulathha and *Phaseolus vulgaris* HUR15, commonly known as white rajma. Enzyme assays via DNS method (given by Miller, 1959) revealed maximum amount of enzyme α-amylase in these two legumes, amongst all other Himalayan legumes.

5. Dolichos biflorus, commonly known as kulthee, gahat or horse gram is a branched or trailing annual with small trifoliate leaves, and very wide climbing slender stem. On maturity, it gives narrow, flat, curved pods containing ellipsoid seeds

1/8” to ¼” long. Interestingly, the entire Garhwal Himalayan Region is well suited for production of this crop. In experimental studies, the seeds of *Dolichos biflorus* have shown to be effective in preventing the deposition of stone material in urinary bladder of rats. In humans also, the studies have proved that the seeds of Dolichos biflorus are effective in dissolving kidney stones.

6. *Phaseolus vulgaris* HUR 15, commonly known as white kidney beans, white rajma, or French bean was first developed at BHU, Varanasi as a Rabi crop in 1984. out of the four varieties of rajma, the HUR15 variety (white rajma) fits well in the existing cropping system of tropical hilly regions of Uttarakhand. The seeds are creamy white in texture, and require about 115-120 days for maturity. The cost of production of rajma is comparable with wheat. Literature survey reveals neither any enzyme extraction from this pulse crop, nor this crop has been identified to pave any kind of industrial advantage of therapeutic or commercial value.

7. Isolation of enzyme from legume source is accompanied by initial crushing of the presoaked, imbibed seed using mortar-pestle. The crushed seed is then passed through four layers of cheese cloth. Then filtration is done in refrigerated centrifuge at 13000 rpm for 15 minutes. The supernatant is collected and pellet is discarded. This supernatant is considered as crude enzyme extract and can be stored at 4˚C.

8. Purification of the enzyme is then done from the stored supernatant. Two fold purification is done: Ultrafiltration and ion-exchange chromatography. Although ultrafiltration is generally employed for concentration of very dilute enzymes, but in the present study it has been utilized for purification of low molecular weight amylases. The crude extract of the enzyme is passed through ultrafiltration membrane of 10KDa and 30KDa sizes. Then enzyme activity is measured by Miller’s DNS method in both the ultrafiltrate and the concentrated enzyme fraction. Further purification of the ultrafiltrate is performed by using ion exchange chromatography. DEAE cellulose was used for column packing. DEAE cellulose was washed with buffer of pH 8 and packed to a height of 5 cm in

chromatographic column, 2 cm in diameter. Then the column was washed exhaustively with equilibration buffer, pH 8, twice. Then sample of α-amylase was added to the column with utmost care avoiding any air bubbles. Elution was done using stepwise elution method with increasing molarities of sodium-phosphate buffer (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.5M), pH 7.2. The fractionation of was carried out at 4° C and the eluate was collected in 2-2ml fractions. Each fraction was assayed for enzyme activity. The fraction showing maximum α-amylase activity was further subjected to kinetic characterization.

9. Kinetic characterization on the basis of following parameters; optimum pH, optimum temperature, substrate concentration, thermal stability, time, Michaelis Menton's constant (K_m) and Maximum velocity ($\mathbf{V_{max}}$) is then performed. These kinetic characteristics are calculated via enzyme assays using DNS method in the ion exchange fractions

10. After kinetic characterization, the immobilization of this purified enzyme is also done and the enhanced characteristics are studied. A close comparison is made between the characteristics of immobilized and free enzyme.It was interesting to note that the enzyme extracted from these pulse crops was as better as the traditional enzyme extracted from bacterial sources. Results prove that plant enzymes showed nearly same characteristics just like bacterial enzymes. Thus, besides finding out new, low cost sources of α-amylase, we could also deduce that these new sources can be made usable on industrial scale.

11. Initial studies were performed on the legume samples of kulath and white rajma procured from local markets of Garhwal Himalayan Region. Germination percentage of seedling for white rajma came out to be 87.8% while the germination percentage of kulath came out to be 71.3%. Seedling length for white rajma was calculated as 6.5cm, 6.1cm and 5.9cm. in case of kulath, the seedling length came out to be 3.6cm, 1.7cm and 1.6cm. Then isolation of the enzyme was done. The supernatant obtained from centrifuged filtered extract of seeds was considered as isolated crude extract of the enzyme. Optical density of crude *Dolichos biflorus* extract was found to be 0.17 while that of *Phaseolus vulgaris*

HUR 15 extract was found to be 0.09. From standard graph, corresponding glucose concentration against these O.D. values came out to be 680 ppm for *Dolichos biflorus* and 290 ppm for *Phaseolus vulgaris* HUR15. Enzyme activity of α-amylase was calculated as micromoles of product formed per ml of enzyme per unit time (in minutes).

12. Enzyme activity of crude extract of *Dolichos biflorus* came out to be 3.77 IU/ml while that of crude extract of *Phaseolus vulgaris* HUR 15 came out to be 1.61 IU/ml.Further these isolated crude enzymes were subjected to two-fold purification, first by ultrafiltration and then by ion-exchange chromatography. The ultrafiltered extract from 10kDa extract of *Dolichos biflorus* showed and O.D. of 0.01, of which glucose concentration concentration could not be determined from standard graph. Thus, 10kDa extract does not have any α-amylase. The ultra filtered extract from 30kDa extract showed an O.D of 0.13. From standard graph, the corresponding glucose concentration came out to be 500 ppm. Thus, amount of reducing sugars were estimated to be 2.77 IU/ml. As the crude extract contained 3.78 IU/ml enzyme, therefore it was deduced that nearly 2/3 of α-amylase of this plant is below 30kDa size. This 2/3 enzyme was further purified by ion exchange chromatography. Further 1/3 of the enzyme which is above 30kDa was not investigated.

13. In case of *Phaseolus vulgaris* HUR15 (white Rajma),10 kDa ultrafiltered extract showed an O.D. of 0.01 while 30kDa extract showed an O.D. of 0.08. From standard graph, the corresponding glucose concentration came out to be 275 ppm. Thus, amount of reducing sugars were estimated to be 1.52 IU/ml. Thus, 94% of enzyme has been found to be below 30KDa size. Therefore, this 94% enzyme was used for kinetic characterization.

14. It was finally concluded that there are two types of α-amylases:- Small and large. Small α-amylase is that whose size ranges upto<= 30kDa while large α-amylase is that whose size is greater than 30kDa. In the present research, alpha amylase of Kulath as well as white Rajma comes under small α-amylases.

15. Ion exchange chromatography of 30 kDa extract was done. In case of *Dolichos biflorus*, highly purified alpha amylase was obtained at 250 mM. At this concentration; O.D. was 0.14. From standard graph, glucose concentration was deduced to be 520 ppm. Thus enzyme activity was calculated as 2.89 IU/ml (as per formula). Thus, 2.89 micromoles of glucose product are formed by 1ml of purified α-amylase of *Dolichos biflorus* in one minute.Purified α-amylase of white rajma was obtained at 150mM concentration. Maximum O.D. was obtained at 150 mM concentration was 0.11. From standard graph, glucose concentration was deduced to be 360 ppm. Enzyme activity was calculated as 1.998 IU/ml i.e. ~2IU/ml. Thus, 2 micromoles of glucose product are formed by 1ml of purified α-amylase of *Phaseolus vulgaris* HUR 15 in one minute.To increase this enzyme activity of the purified enzyme, further kinetic characterization is done. As it is a well known fact that under optimum reaction conditions best enzyme activity will be reached.
16. pH optima of kulath extract was 6.1. A bell shaped graph was obtained. The enzyme showed maximum activity between pH 5.5 to 7. But the activity reduced in alkaline range i.e.>7. The drop in activity was more in phosphate buffer than in Tris-HCl buffer, indicating the stability of enzyme in Tris-HCl buffer. After pH 7.5 the enzyme activity almost nullified. These results correspond with previous results of Nirmala and Muralikrishna (2002). pH optima of purified kulath enzyme also came out to be 6.1. pH optima of white rajma extract was 6.5. In this case also, a bell shaped graph was obtained. The enzyme showed best stability in 4 to 6.5 pH range. Enzyme activity totally nullified after pH range 8. We can deduce that alpha amylase of these two legumes remains stable in acidic pH range and is an acidic amylase. Optimum pH of purified white rajma extract (obtained via ion-exchange chromatography) was also determined and it also came out to be 6.5.
17. The optimum temperature of kulath α-amylase was found out to be 45°C. A slightly distorted bell shaped graph was obtained. The enzyme showed best stability in temperature range 42°C to 48°C. Thermal stability of the enzyme was checked by calculating the enzyme activity at temperatures above 45°C. This

enzyme showed stability upto 75°C and was considered to be highly thermostable. The optimum temperature of white rajma α-amylase was found out to be 50°C. Best stability of enzyme was observed in temperature range 45°C to 55°C. For thermal stability, enzyme assay was done at various temperatures above 50°C. After 80°C, the activity finished almost completely showing complete instability of enzyme in temperature ranges higher than this, thus accounting to high thermo stability.

18. For calculation of optimum time, a graph was plotted with time on X-axis and corresponding O.D. on Y-axis. Again a bell shaped graph was obtained in both cases. In case of kulath maximum enzyme activity was observed in 10 minutes in this time maximum O.D. = 0.18 was obtained. In case of white rajma optimum time was observed to be 14 minutes in which maximum O.D. was obtained that is 1.19. The graph was distorted bell shaped type in case of this sample.
19. Coming to Km and Vmax, In case of kulath, a slightly distorted parabolic graph obeying Michaelis Menton equation was obtained. The enzyme activity first increased very slowly and then after increasing starch substrate concentration to 2mg/ml, it suddenly increased four times. Vmax was obtained at 28 $IUml^{-1.}$ From the graph Km was calculated as 1.95mg/ml. In case of white Rajma also, parabolic graph obeying Michaelis Menton equation was obtained. Vmax was obtained at 3.4 IU/ml. From the graph Km was found out to be 1.3mg/ml.
20. Immobilization of both enzymes was done on calcium alginate matrix. Shiny transparent beads of 2cm diameter with slightly yellowish brown coloration were obtained in case of kulath. Beads of ultra filtered kulath extract (~30kDa) were also prepared which were shiny yellow in colouration. In case of rajma opaque white color beads of two centimeter diameter were obtained. Kinetic characteristics of these immobilised enzymes beads were calculated and it was found out that the characteristics were highly enhanced. Although much change in optimum pH was not observed, still we observed a remarkable increase in enzyme activity. Optimum pH of free enzyme came out to be 6.1 and that of immobilized enzyme came out to be 6.3. A close comparison between optimum pH of free and immobilized enzyme was done. Drastic increase in enzyme activity was observed

after pH 6.3. It was observed that at pH 7 enzyme activity increased more than 11 times (~11.5 times). We also observed that the immobilized enzyme was stable at much higher pH values than the free enzyme. While the free enzyme showed no activity at pH 8, the immobilized enzyme showed an activity of 16.96 IU/ml at pH 8. Certain similar results were seen in case of optimum temperature. Optimum temperature of free enzyme was 45°C while optimum temperature of immobilized enzyme was 50°C. This simply proves the increase in thermal stability. Around 9 times increase in enzyme activity was observed at 70° C. while the free enzyme showed very less activity at 80°C, the immobilized enzyme showed a high activity of 10.74IU/ml at 80°C.

21. Kinetic characterization of immobilized α-amylase of white rajma also revealed similar results. Optimum pH of immobilized white rajma extract came out to be 6.5. This was same as that of free enzyme. Enzyme activity of immobilized enzyme increased 20 times at pH 8. While the free enzyme showed no activity at pH 8.6, the immobilized enzyme showed an activity of 8.89 IU/ml at pH 8.6. Thus, it was finally proved that characteristics are enhanced in immobilized enzyme. While the pH stability range for free enzyme is 4 to 7, the pH stability range for immobilized enzyme is observed to be 4 to 9. One important point of consideration is that pH stability increases only in basic range. Coming to temperature, optimum temperature of immobilized enzyme came out to be 60°C, against temperature optimum of 48°C for free enzyme. Enzyme activity increased 24 times at 70°C temperature. The immobilized enzyme was found to be stable at 80° C; enzyme activity corresponding to 8.42 IU/ml. Thus, high thermostability of immobilized enzyme was proved.
23. A small comparison between 14 plant and bacterial species for α- amylase enzyme present in them was also done. Comparison was made on the basis of optimum pH, optimum temperature, K_m, V_{max}, pH stability range and thermal stability of enzyme. We found out that the plant enzyme is equally good than the bacterial enzyme in terms of characteristics.
24. α-amylase of *Dolichos biflorus* and *Phaseolus vulgaris* HUR 15 (family Leguminoseae) shows complete instability in highly alkaline pH range. Optimum

pH of white Rajma came out to be 6.5 while that of Kulath came out to be 6.1. These were completely unstable above pH 7.5. However, these were compared with α-amylase of the family Poaceae, which were stable in acidic pH range only, and become completely unstable above pH 7. *Eleusine coracana* Indaf-15, *Sorghum bicolor, Zea mays* and *Triticum aestivum* were studied. All these have pH stability range and optimum pH below 7. Among bacteria, 6 bacterial strains were studied, of which 3 belonged to the genus *Bacillus.* All *Bacillus* species have an optimum pH below 6.5, and are unstable in alkaline range. *Thermomonospora curvata* and *Streptococcus* had showed slight stability in alkaline range, only up to pH 8.5. It was thus concluded that that optimum pH of almost all bacterial and plant amylases range between pH 4 to 8.

25. The thermal stability of all amylases is not the same. It was deduced that the legume family has a high thermal stability, and the amylase is stable almost up to 80˚C. However, the grass family has a relatively low thermal stability with amylase stability up to a temperature of 50˚C. Among bacteria, the thermal stability is varying. Strains of *Bacillus* spp. are highly thermo stable, while *Streptococcus* and *Xanthomonas* have low thermal stability. Generally, the optimum temperature ranges between 40-55˚C.
26. Further comparison was made on the basis of size. Among plants, it was found that the plant amylases are small in size in comparison to bacterial amylases. Such small sized proteins are beneficial in food industry. Recent researches conducted by food industries prove that small protein molecules are easily digestible in the small intestine, and provide very high surface area for reaction.
27. Low Km values of plant α- amylases studied indicate high affinity of the enzyme for starch substrates, like some bacterial strains.The investigation simply proves that the kinetic characteristics of plant and bacterial amylases are similar. It was finally concluded that the plant enzyme is as good as bacterial enzyme in terms of characteristics. The study also proves that the enzyme can be better produced commercially by legume sources.

Conclusion:

The present report is first report on extraction of alpha amylase from both these plants, *Dolichos biflorus* and *Phaseolus vulgaris* HUR 15. While on one hand, we studied *Dolichos biflorus*, whose seeds have high medicinal importance as astringent to bowels, antipyretic, diuretic, tonic, antihelminthic, appetizing and lithontriptic. It is useful in piles, tumours, bronchitis, heart trouble, kidney stone, enlargement of spleen, hiccough, asthma, leucoderma, and in abdominal complaints. On the other hand we studied *Phaseolus vulgaris HUR 15*, whose seeds have not been found to have any medicinal properties, nor have any enzymes been extracted from white rajma.

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Previously researches avoided working on plant amylases because of the tediousness witnessed during extraction of the enzyme and further purification. A large amount of spent material is to be removed during enzyme extraction. In the present work, we also proved that the kinetic characteristics of crude as well as purified enzyme are almost the same. Thus, even the crude enzyme can be used for calculating the kinetic characteristics. This approach can reduce not only the tediousness of the work, but also made the work cost- effective.

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Chapter-6
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