EFFECT OF GERMINATION ON THE KINETICS OF UREASE EXTRACTED FROM EIGHT BEANS SAMPLES

BY

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MAY, 2012

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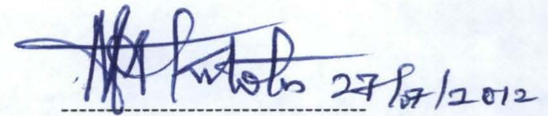
THESIS SUBMITTED TO POSTGRADUATE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTER OF TECHNOLOGY (M.TECH) IN BIOCHEMISTRY

MAY, 2012

DECLARATION

I hereby declare that this thesis titled: **Effect of Germination on the Kinetics of Urease Extracted from Eight Beans Samples** is a collection of my original research work and it has not been presented for any other qualification anywhere. Information from other sources (published or unpublished) has been duly acknowledged.



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SIGNATURE & DATE

CERTIFICATION

The thesis titled: **Effect of Germination on the Kinetics of Urease Extracted from Eight Beans Samples** by: OGUNMOLASUYI Adewoyin Martin, M.Tech/SSSE/2009/2197, meets the regulations governing the award of the degree of Master of Technology in Biochemistry of Federal University of Technology, Minna and it is approved for its contribution to scientific knowledge and literary presentation.

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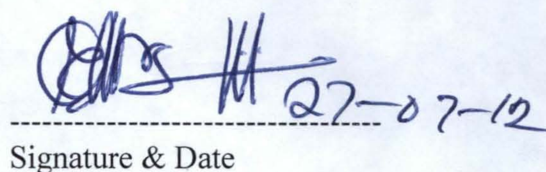

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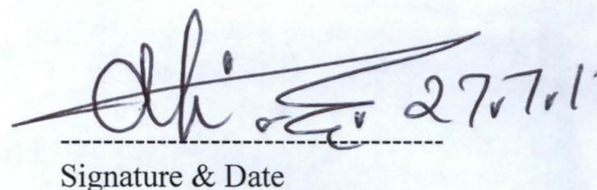

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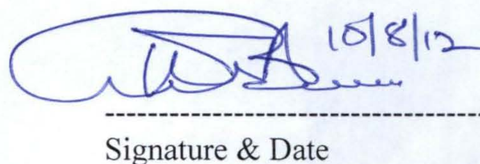

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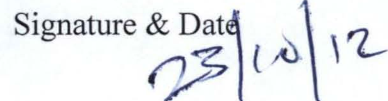

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DEDICATION

With full gratitude to Almighty God, I dedicate this thesis to the family of my beloved Brother Godwin Osanebi.

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ABSTRACT

This research work was designed to determine the activity of urease isolated from eight different species of local beans (*Phaseolus lunatus, Parchyrhizus tuberrosus, Sphenostylis stenocarpa, Glycine max, Cajanus cajan, Mucuna pruriens, Kerstings geocarpa* and *Vigna mungo*) germinated and ungerminated. The protein level in germinated and ungerminated beans species as well as specific activity, optimum pH, optimum temperature, substrate concentration and kinetic parameters, V_{max} and K_m, of urease were determined. The pH, temperature and substrate concentration ranged from 5.50-8.0, 30-80^{0}C and 0.1-0.6M respectively. The crude urease from ungerminated *Mucuna pruriens* was partially purified and immobilized on preformed chotisan beads. The free and immobilized urease was characterized for temperature, substrate concentration, storage ability and reusability. From the results, the protein level in germinated beans samples reduced significantly ($p<0.05$) compared to ungerminated beans samples. However, protein level in *Mucuna pruriens* was significantly ($p<0.05$) higher than other beans samples, germinated and ungerminated. This showed that, *Mucuna pruriens* can be a good and cheap source of protein for animal feed and other industrial processes requiring protein. The result of effect of germination on urease specific activity showed that, activity was significantly ($p<0.05$) increase in germinated than ungerminated beans samples. Although, *Glycine max* and *Cajanus cajan* have been extensively studied for urease activity, among other beans samples *Mucuna pruriens* and *Kerstings geocarpa* showed significant ($p<0.05$) urease activity. The optimum pH and temperature ranged from 6.5-7.0 and 60-70°C respectively for both germinated and ungerminated beans sample. Therefore, germination did affect the pH and temperature stability of urease compared to ungerminated. The results showed that germination significantly ($p<0.05$) increased V_{max} and there was no significant ($p<0.05$) difference in K_m of urease in germinated and ungerminated beans sample studied. However, the catalytic efficiency of urease in germinated beans was significantly ($p<0.05$) higher than ungerminated. This implies that, with germination urease activity can be activated to meet both clinical (analysis of urea in biological fluid and artificial kidney development) agricultural (plant urea metabolism) and industrial demand (water and environmental remediation). Partially purified crude urease from *Mucuna pruriens* showed one peak of protein and urease specific activity. The optimum temperature for purified urease (free and immobilized) was 40°C whereas the K_m *and* V_{max} were 0.02mM and 0.04mM and 38.46Umg^{-1} and 19.23Umg^{-1} respectiviely. From the result purification increased the catalytic efficiency of urease from *Mucuna pruriens* significantly. Urease immobilization on chitosan showed low reusability and storage ability. The free and immobilized urease retained 50% and 25% of their activity respectively, after one week (seven days). However, immobilization technology is a major way of maximizing enzyme activity, reducing cost of production and stabilizing enzyme in the process of application.

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CHAPTER ONE

1.0 INTRODUCTION

Urea is hydrolyzed with urease (E.C 3.5.15) a nickel dependent enzyme, to form ammonia and cabarmate (unstable intermediate) and the cabarmate concomitantly decomposes to yield a second molecule of ammonia and carbon (IV) oxide (Andrew *et al.*, 1986 and Pearson *et al.*, 1997), at a rate 10^5 times the rate of uncatalysed reaction (Mobley and Hausinger, 1989 and Callahan *et al.*, 2005).

The enzyme urease was first isolated and crystallized by James Summer in 1926 from jack beans (*canavalia ensiformis*) as a pure crystalline enzyme (Summer, 1926). Crystalline form of urease was the first obtained for a known enzyme, and this played a key role in establishing the fact that enzymes are majorly protein in nature. After about 50 years later, it was discovered that urease was a nickel containing enzyme (Dixon *et al.*, 1975).

For reasons not clear, some leguminous seeds are particularly rich in urease (Polacco and Haviv, 1979; Prakash and Bhushan, 1997), though it was proposed that plants which are sources of urease, utilize the ezyme for the assimilation of urea which is normally formed in plant as a result of the hydrolysis of arginine to ornithine, a reaction catalyzed by the enzyme arginase (Polacco and Holland, 1993 and Lea, 1997).

Besides the fact that most leguminous plant seeds contain urease, the enzyme has also been isolated and characterized from leaves, roots and bark of plant with actively growing tissues possessing higher activity than senescing ones (Thompson, 1980; Horgan *et al.*, 1983). The activity of the enzyme in the leaf is increased if urea is used as a fertilizer during germination. It was proposed that foliar treatment with urea heightened total leaf urease yield in mulberry plants,

improved leaf nutritional quality, and also gave rise to an increased yield of cocoon (Fotedar and Chakra, 1998; Sarker and Absar, 1995). Notwithstanding, the information available on properties of plant leaf urease is quite inadequate. Genetic and chemical blocking of leaf urease activity caused necrotic leaftip, associated with urea accumulation (Eskew *et al.*, 1983; Stebbins *et al.*, 1991 and Krogmeirer *et al.*, 1989). This information shows that urease is of great importance in the metabolism of plant urea.

However, there are several microbial sources of urease which include bacterial such as *Lactobacillus ruminis*, *Lactobacillus fermntum*, *Sporosacina pasteurii*, *Helicobacter pylori*, *ureplasma* (mocillicutes) and *Porteus vulgaris* (Kakimoto and Suzuki, 1992) and fungi such as *Rhizopus oryzea* (Farley and Santosa, 2002). Filamentus fungi serve as sources of about 40% of all available urease enzymes (Archer and Peberdy, 1997). Most industries prefer to use filamentous fungi, as a source of enzyme owing to the fact that, fermentation industries are well acquainted with the condition needed for maximum production of the homologous protein by them (Wubbolt *et al.*, 2000).

Urease possesses several clinical and industrial applications. In medical laboratories, it is used for diagnostic purposes, in the determination of urea in biological fluids such as urine and blood (Chellapandian and Krishnam, 1998), and also in drug uses of urea in topical dermatological products, for nonsurgical debridement of nails and as a diuretic (Wikipedia, 2006). Additionally, there are many industrial applications of urease. It is used as reducing agent in alcoholic beverages (Fujinawa and Dela, 1990; Fumuyiwa and Ouch, 1991). Urease is also found useful since urea has commercial importance in the manufacture of hair conditioner, glues, fertilizer, plastic, animal feed and as a browning agent in factory-produced pretzels. Urease has been

extensively used as a model enzyme to elucidate the applicability of inhibitory assay for mercury (II) (Tsai and Doong, 2005; Preininger, 1999 and Mahmod, 2001).

It is noteworthy that, the industrial application of enzymes is very limited, due to the fact that, they are expensive, available in inadequate amounts, unstable and cannot be recovered in most cases, from spent reaction, which makes them uneconomical biocatalysts. However, recent biotechnological advancement of immobilizing these enzymes has the potential to subdue these associated difficulties to enzyme industrial application (Guisan, 2006; Illanes *et al.*, 2008; Reddy *et al.*, 2004; and Yong *et al.*, 2004). With enzyme immobilization technology, there is substrate specificity, thermal and pH stability, longer time of storage, reusability, and ease of recovery from spent reaction (Yong *et al.*, 2004; Savangiker and Joshi, 1978; Hearn and Neufeld, 2000 and Won *et al.*, 2005). Immobilization of enzymes had been performed on several different carriers such as chitosan, carboxymethylcellulose, chitin, parafin e.t.c and is well documented in the literature. Among many methods for enzyme immobilization are physical adsorption on polysaccharide materials (Chitosan) (Ray *et al.,* 1994), entrapment in alginate, for example calcium alginate (Kokufuta, *et al.*, 1988), immobilization on ion-exchanger (Roy and Hedge, 1987; Deleyn and Stouffs, 1990), silica beads (German and Crichton, 1980) and agarose (Viera, *et al.*, 1988; Sheffield *et al.*, 1995).

1.1 Justification

The extraction and characterization of urease from plants and microbial sources are well established in the literature. Most of the previously published works have focused on extracting urease from bean seeds (Summer, 1926), leaves (Hirayama *et al.*, 2000), bacterial (Mobley and Hausinger, 1989), and few fungi (Mobley *et al.*, 1995; Lubbers *et al.*, 1996). Those that have considered germinated seeds only looked at the effect of germination on urease activity and the

role of assessory protein in urease activation (Freyermuth *et al.*, 2000). To date, no published work has described the the kinetic characteristics of urease extracted from germinated seeds particularly those that have not been studied. It is clear that, in order to achieve higher degree of urea hydrolysis, the assembly of nickel at the active site will be necessary. In the same vein, urease with remarkable kinetic behavior will be appropriate for successful industrial application, for instance; in the area of water remediation, blood urea analysis, determination of heavy metal in the course of environmental impact assessment e.t.c. However, immobilization of this enzyme makes it cost effective and reusable. Therefore, the objective of this work is to activate urease activity in plant with the corresponding kinetics (V_{max} and K_m) by extracting the enzyme from eight germinated beans samples.

1.2 Aim

This project aims to evaluate the effect of germination on urease kinetics with the following objectives

1.3 Specific Objectives

- Determination of protein level in germinated and ungerminated beans
- Characterization of urease enzyme for optimum pH, temperature and substrate concentration
- Determination of kinetic characteristics of the urease
- Partial purification of urease by gel filtration and characterization of immobilized urease.

CHAPTER TWO

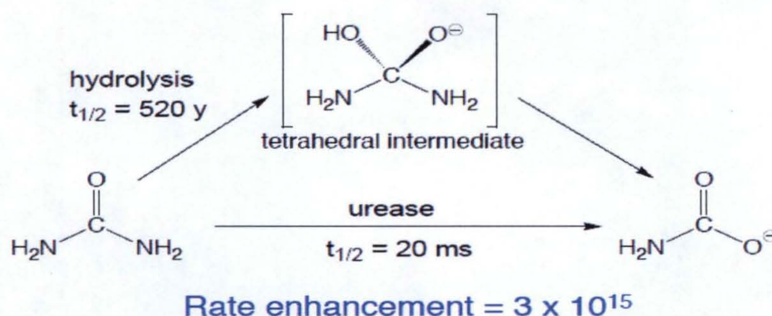
2.0 LITERATURE REVIEW

2.1 Plant ureases: Roles and Regulation

2.1.1 Enzymatic Activity of Urease

Urease (EC 3.5.1.5, urea amidohydrolase), a nickel-dependent metalloenzyme, catalyzes the hydrolysis of urea to form ammonia and carbon (IV) oxide. The hydrolysis of one molecule of urea results into the release of two molecules of ammonia and one molecule of carbon (IV) oxide (Figure 2.1). Several different assays, based mainly on the measurement of the amounts of products released during the reaction, are available for quantifying urease activity.

Urease: the most efficient enzyme



Rate enhancement = 3×10^{15}

"Urease appears to be **unique among hydrolases in containing two nickel atoms**, which presumably assist this enzyme in grappling effectively with this unusually simple substrate [urea]."

Callahan, Yuan, Wolfenden *J. Am. Chem. Soc.* **2005**, *127*, 10828

Figure 2.1: Urease the most Efficient Enzyme

Ammonia can be detected by several methods including: ion-selective electrodes, reaction with phenol-hypochlorite or with Nessler's reagent. There is possibility of including pH-sensitive

dyes in the assays or simply observe changes with pH electrodes since release of ammonia usually gives rise to increase in pH. The amount of ammonia can also be monitored spectrophotometrically using a coupled system with NADH-dependent glutamate dehydrogenase (ammonia is a substrate for this enzyme). The other product of the reaction, carbon (IV) oxide, can be trapped and monitored by radiological methods with ^{14}C-labeled urea as a substrate (Mobley and Hausinger, 1989, and Mobley *et al.*, 1995).

2.1.2 Occurrence of Urease in Organisms

There are many organisms, including plants, some bacteria, fungi and invertebrates, which are able to synthesize urease. Biochemically, the best-characterized urease is that from jack bean (*Canavalia ensiformis*) (Hirai *et al.*, 1993; Karmali and Domingos, 1993; Riddles *et al.*, 1991; Takishima *et al.*, 1988). Recently, urease from mulberry (*Morus alba*) leaves has also been purified and characterized (Hirayama *et al.*, 2000). The best genetic data concerning plant ureases are available for soybean (*Glycine max*) (Polacco and Holland, 1993; 1994). Separate genes encoding two urease isoenzymes, a tissue-ubiquitous and embryo-specific, as well as the unlinked genes encoding regulatory proteins, were identified in soybean (Meyer-Bothling and Polacco, 1987; Torisky *et al.*, 1994) and mutants are available. The embryo-specific urease is an abundant seed protein in many plant species, including soybean, jack bean (Polacco and Holland, 1994) and *Arabidopsis* (Zonia *et al.*, 1995), while the other type of urease (called ubiquitous) is found in lower amounts in vegetative tissues of most plants (Hogan *et al.*, 1983). Bacterial ureases play an important role in the pathogenesis of a number of bacterial species including *Proteus mirabilis*, *Staphylococcus saprophiticus*, *Yersinia enterocolitica*, *Ureaplasma urealiticum* and others (Mobley *et al.*, 1995). Owing to urease activity, bacteria (e.g. *Klebsiella*

aerogenes) are able to use urea as a sole nitrogen source (Mulrooney *et al.*, 1989). In the case of *Vibrio parahaemolyticus*, the ability to hydrolyze urea was proposed as a simple screening test to predict which strains are potentially pathogenic (Kaysner *et al.*, 1994). One of the most frequently mentioned examples in the recent literature is the urease from *Helicobacter pylori* because of its essential role in the pathogenesis of this microorganism and the high prevalence of this human pathogen (Eaton *et al.*, 1991). However, the best structural data are available for the urease from *K. aerogenes* (Jabri *et al.*, 1995). Urease activity was found in several species of fungi; however, the nucleotide sequences of the genes encoding urease were reported for only a few of them, including a fungal respiratory pathogen of human *Coccidioides immitis* (Yu *et al.*, 1997) and *Schizosaccharomyces pombe* (Tange and Niwa, 1997). In the invertebrate *Aplysia californica*, urease, together with carbonic anhydrase, is required for the formation and homeostasis of statoconia, calcium carbonate inclusions in the lumen of the gravity-sensing organ, the statocyst (Pedrozo *et al.*, 1996a; 1996b). Urease is a cytosolic enzyme. In most of the studied cases the majority of its activity is associated with the soluble fractions of the cells (Mobley *et al.*, 1995).

2.1.3 Protein Structure of Ureases

The plant and fungal ureases are homo-oligomeric proteins (consist of identical subunits), while the bacterial ureases are multimers formed from a complex of two or three subunits (Mobley *et al.*, 1995; Tange and Niwa, 1997). Significant amino-acid similarities were observed between all known ureases. Amino-terminal residues of the monomers of plant and fungal enzymes are similar to the small subunits of bacterial enzymes (e.g. UreA of *H. pylori*). The large subunits of bacterial ureases (e.g. UreB of *H. pylori*) resemble the carboxy-terminal portions of plant and

fungal subunits (Figure 2.2). The high sequence similarity indicates that all ureases are variants of the same enzyme and are likely to possess similar tertiary structures and catalytic mechanisms (Mobley *et al.*, 1995). This conclusion is supported by the available biochemical and structural data obtained for the best-characterized ureases, e.g. from jack bean (Hirai *et al.*, 1993) and *K. aerogenes* (Jabri *et al.*, 1995). Jack bean urease exists as a homotrimer able to aggregate to a homohexamer (Hirai *et al.*, 1993). Bacterial ureases possess structures similar to the jack bean urease. They are either trimers or hexamers of subunit complexes (Figure 2.2). They can also exist in aggregated forms. The stoichiometry of subunits (1:1 for ureases from *Helicobacter* sp. or 1:1:1 for most bacterial ureases, including the urease from *K. aerogenes*) is always maintained.

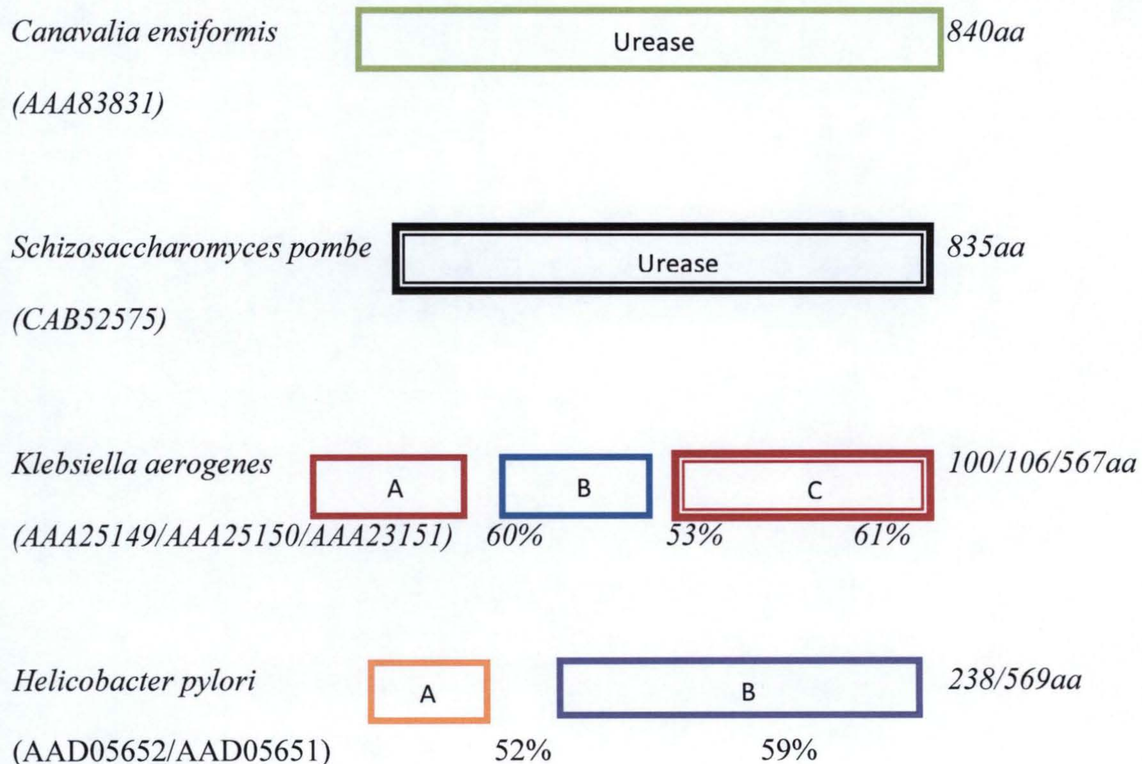


Figure 2.2: Schematic comparison of the structural subunits of ureases from selected organisms.

The GenBank accession numbers (in brackets) and the numbers of amino acids (on the right) are shown for each protein. The percent values below boxes indicate the degree of identity to urease from jack bean (*C. ensiformis*).

The catalytic site is located in subunit UreC of the *K. aerogenes* enzyme and in the respective regions (Figure 2.2) of ureases from other organisms. Each catalytic subunit contains the active site with two nickel ions that, in the case of crystallized *K. aerogenes* urease, were shown to be 3.5 Å aparts (Jabri *et al.*, 1995). In this enzyme, one nickel atom is bound to two histidine residues (His-246 and His-272), while the second nickel atom is bound to three residues: two histidines (His-134 and His-136) and aspartic acid (Asp-360). Additionally, a carbamate ligand derived from Lys-217 bridges the two nickel ions (Mobley *et al.*, 1995). The process of nickel incorporation requires participation of many accessory (or activatory) proteins, which appear to act as urease-specific chaperones (Mobley *et al.*, 1995). These proteins are required for assembling an active urease in both bacteria and plants (Polacco and Holland, 1994; Mobley *et al.*, 1995).

2.1.4 Role of Urease in Plants Urea Metabolism

There are at least three key enzymes involved in urea metabolism in plants: arginase, urease and glutamine synthetase (Figure 2.3). The primary role of ureases is to allow the organism to use external or internally generated urea as a nitrogen source (Mobley and Hausinger, 1989; Mobley *et al.*, 1995). Significant amounts of plant nitrogen flow through urea. This compound is derived from arginine (Figure 2.3) and possibly from degradation of purines and ureides (Polacco and Holland, 1994). The nitrogen present in urea is unavailable to the plant unless hydrolyzed by

urease. The product of urease activity — ammonia — is incorporated into organic compounds mainly by glutamine synthetase (Figure 2.3, 2.4). It has been reported that over-expression of the pine glutamine synthetase in transgenic poplar improves the growth of the plants, probably by increasing the efficiency of nitrogen utilization (Gallardo *et al.*, 1999). On the other hand, it has been shown that increased activity of this enzyme in the roots of a legume plant, *Lotus japonicus*, leads to decreased plant biomass production, possibly due to limited nitrate uptake (Limami *et al.*, 1999).

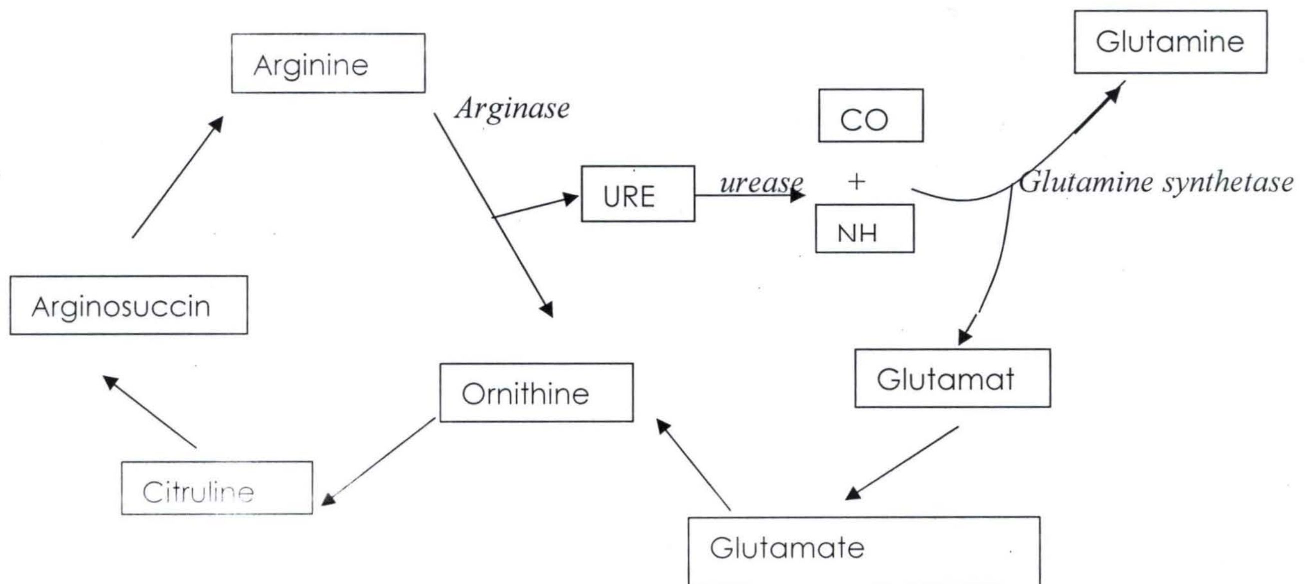


Figure 2.3: Urea metabolism in plants (Modified from Gerendas *et al.* (1998)

The names of the key enzymes involved in the production and conversion ofurea are indicated in italics.

Nickel in urease - The mechanism

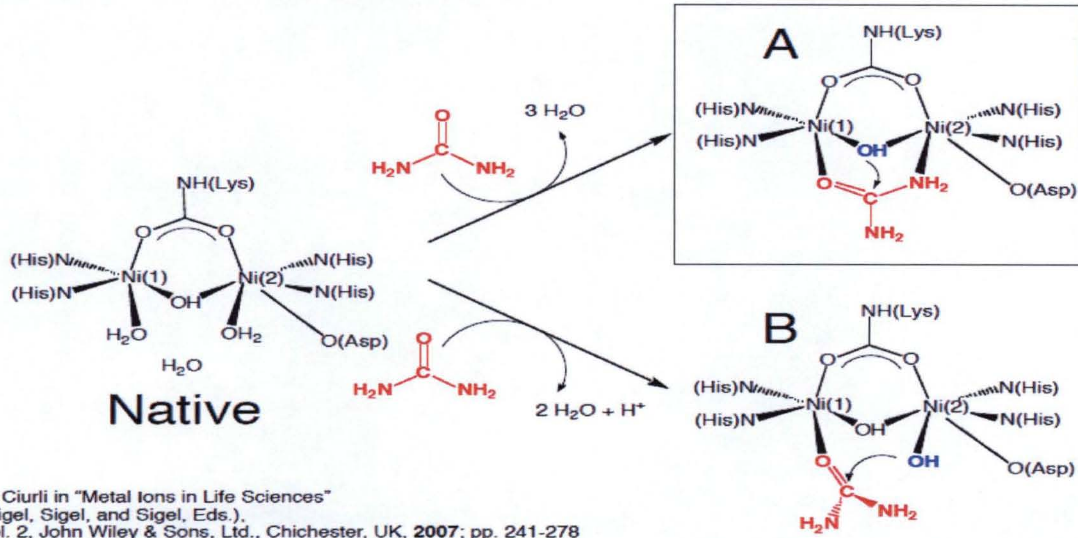


Figure 2.4: The mechanism of Nickel in Urease

These results, which seem contradictory at first glance, might reflect the different roles of glutamine synthetase isoenzymes in different plants and/or different plant organs. They indicate the significance of this enzyme for plant nitrogen metabolism. The activity of glutamine synthetase, the amount of its product, glutamine, and possibly the availability of its substrate, ammonia, seem to be important factors controlling nitrogen metabolism and affecting plant growth (Stitt, 1999; Wiren *et al.*, 2000). The ubiquitous urease (found in all plant tissues) is responsible for recycling metabolically derived urea (Polacco and Holland, 1994). In soybean, the ubiquitous urease is found at the level of 1/1000 or 1/100 that of the embryo-specific urease. The latter does not seem to have any assimilatory function. Its physiological role is unknown; however, the involvement in protection against plant pathogens (due to production of toxic ammonia) has been proposed (Polacco and Holland, 1994).

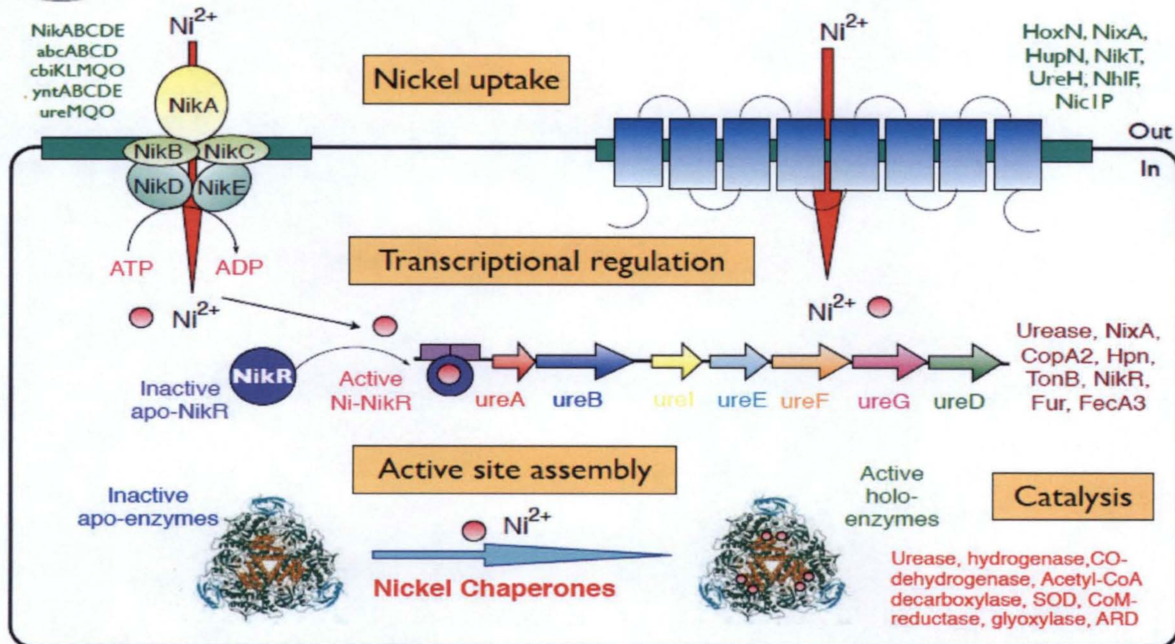


Figure 2.5: Biological Handling of Nickel

As mentioned above, urea can be assimilated exclusively by urease in higher plants. Moreover, urease is the only nickel-containing metalloenzyme (Figure 2.5) yet identified in plants (Polacco and Holland, 1993; 1994). The importance of nickel for urease activity was demonstrated by the observation that urea-grown nickel-deprived rice (*Oriza sativa*) plants showed reduced growth and accumulated large amounts of urea due to reduced urease activity (Gerendas *et al.*, 1998). Urease-negative mutant plants and nickel- deprived wild type plants have the same phenotype.

They accumulate urea and exhibit necrotic leaf tips, apparently due to urea "burn" (Polacco and Holland, 1993). It has been demonstrated that similar leaf-tip necrosis observed after fertilization with urea result from the accumulation of toxic amounts of urea rather than from the formation of a toxic amount of ammonia (a product of urease action) since addition of a urease inhibitor

increased the leaf-tip necrosis (Krogmeier *et al.*, 1989). Urease plays an important role in germination and in seedlings' nitrogen metabolism. It may function coordinately with arginase in the utilization of seed protein reserves during germination (Polacco and Holland, 1993). Imbibition of *Arabidopsis* seeds in water containing urease inhibitor delayed germination by 36hours and completely blocked germination of aged seeds. This inhibition could be abolished by supplying nitrogenous compounds into the imbibition medium (Zonia *et al.*, 1995).

2.1.5 Plants Accessory Proteins involved in Urease Maturation

Genetic analysis of urease expression in soybean revealed several genes whose inactivation leads to the lack of urease activity (Polacco and Holland, 1993; 1994). Two of these genes, *Eu1* and *Eu4*, encode embryo-specific and tissue-ubiquitous ureases, respectively. Two other genes, *Eu2* and *Eu3*, are analogous to bacterial urease accessory genes that are involved in urease maturation i.e. the placing of essential nickel in the urease active site. Mutation in either of the two latter genes eliminates the activity of the ubiquitous and embryo ureases but has little effect on the amounts of these proteins (Meyer-Bothling and Polacco, 1987; Meyer- Bothling *et al.*, 1987). It was recently shown that *Eu3* gene encodes the 32-kDa protein (Freyermuth *et al.*, 2000). Its homologues have also been found in *Arabidopsis* (Freyermuth *et al.*, 1999) and in *Medicago truncatula* (accession AA660998). Eu3 protein interaction with the product of *Eu2* in the activation of embryo-specific urease was demonstrated (Freyermuth *et al.*, 2000) but sequence data are not yet available for *Eu2*. Freyermuth and coworkers have shown that the Eu3 protein is developmentally controlled and accumulates in developing embryos. They have proved the direct involvement of this protein in urease activation since anti-*Eu3* antibodies blocked this activation *in vitro* (Freyermuth *et al.*, 2000). Lack of *Eu3* protein prevented accumulation of embryo

specific urease since the enzyme is most probably unstable without nickel insertion. Although the authors could not prove the direct involvement of *Eu3* in the activation of tissue-specific urease (encoded by *Eu4*) because of the low sensitivity of the method (Freyermuth *et al.*, 2000), such an involvement is rather expected, since both functional *Eu3* and functional *Eu2* are necessary for the activation of both types of ureases (Polacco and Holland, 1994). Urease activation in *K. aerogenes* involves the action of four accessory proteins (UreD, UreE, UreF and UreG) (Figure 2.6) (Moncrief and Hausinger, 1997). These proteins are well conserved in bacteria that possess urease activity (Mobley *et al.*, 1995). The *Eu3* protein shows 55–60% identity in its C-terminal part to bacterial UreG proteins, including a nucleotide- binding P-loop that is essential for *in vivo* activation (Moncrief and Hausinger, 1997).

Urease active site assembly

UreF
Nickel cannot bind before lysine carbamylation
UreD
UreG
GTP hydrolysis, lysine carbamylation
UreF
UreG
CO_2
Pi + GDP
GTP
UreD
Conformational change of apo-urease
Carboxyphosphate
Nickel delivery
UreE
UreE
Apo-(UreABC)$_3$
Holo-(UreABC)$_3$

Hausinger, R.P. - *JBIC* **1997**

M. Stola, F. Musiani, S. Mangani, P. Turano, N. Safarov, B. Zambelli, S. Ciurli - *Biochemistry* **2006**

Figure 2.6: Urease Active Site Assembly

The N-terminal part of *Eu3* is rich in histidines and in this regard is similar to the C-terminus of *K. aerogenes* UreE (Freyermuth *et al.*, 2000). No plant homologues of bacterial UreD, UreE or UreF have been yet identified.

In summary, several pieces of evidence suggest that both urease enzymes and the machinery required for their activation are evolutionarily conserved. First, there is a significant sequence homology not only between urease enzymes (Figure 2.2) but also between activatory factors (Freyermuth *et al.*, 2000), although the latter is much less striking. Second, it was postulated that urease of soybean-commensal bacteria (*Methylobacter*) required some plant factors for activation (Polacco and Holland, 1994). Third, an increase of urease activity was observed in plants transformed with the *ureA* and *ureB* genes of *H. pylori* encoding subunits of urease (Brodzik *et al.*, 2000). This result suggests that the plant accessory factors were able to incorporate nickel into a bacterial apoenzyme. The same enzyme produced in *E. coli* was inactive unless co-expressed with the *H. pylori* genes encoding accessory proteins (Hu and Mobley, 1993).

2.2 Application of Urease

2.2.1 Agriculture

Urea fertilizer is the predominant solid nitrogen (N) fertilizer in Chinese agriculture, and constitutes > 50% national nitrogenous fertilizer consumption. Nitrogen, after carbon, is the most limiting element in plant nutrition. Efficient recycling of reduced nitrogen present in the form of urea is important for plant growth since urea contains a significant amount of this element (Polacco and Holland, 1993). In addition to internally generated urea, externally applied urea can also be utilized by plants. Urea is a widely used fertilizer because of its low costs, ease in

handling and high nitrogen content (Mobley and Hausinger, 1989). In plants, urease is the only enzyme that is able to recapture nitrogen from urea (Polacco and Holland, 1993). Fertilization with urea through leaves could be an efficient method of plant feeding and any modifications leading to increased urease activity in leaves could result in more effective assimilation of this fertilizer. Such an increase might have a positive impact on the nitrogen metabolism in plants since more ammonia would be available for assimilation *via* glutamine into a variety of nitrogenous compounds.

2.2.2 Environmental Monitoring

Mercury (II) is a highly toxic element that is found both naturally and as an introduced contaminant in the environment. Mercury (II) is widely distributed in the Earth's crust; sea water, ground and rain water, and its toxic effects on biological systems through direct uptake as well as by accumulation in food chain are well known (Bradstreet *et al.*, 2003). Average mercury (II) levels in the atmosphere are 3–6fold higher than the pre-industrial estimates (Selid *et al.*, 2009). Mercury (II) pollution has always been the area of concern. History records several major cases of mercury (II) poisoning. Among them was the infamous Mina Mata tragedy in 1956 where mercury (II) was dumped into the sea and residents of the Mina Mata bay, Japan area began coming down with a strange nervous disorder (Ninomiya e*t al.*, 1995). The persistent nature of mercury (II) and its ill effect for years is supported by recent report on high concentration of mercury (II) in the water of Bhopal, India (CSE Study, 2009). Atmospheric deposition of mercury (II) contains three principal forms, although the major component is inorganic mercury as mercury (II). Due to its high toxicity World Health Organization has set a limit for mercury (II) in drinking water which is 0.001 µg.mL−1 for water quality monitoring

purposes (GDQ, 2009). This necessitates extensive study of existing analytical methods, identifying the gap between them and developing alternative methods for simple and quick determination of mercury (II) in the environment at very low concentrations.

Several analytical methods such as atomic absorption spectrometry, liquid chromatography with inductively coupled plasma mass spectrometry and others have been developed for mercury (II) analysis (Tuzen *et al.*, 2009). These techniques, although very sensitive for mercury (II) analysis, require extensive sample pretreatment, they use large amounts of organic solvent and do not give toxicological information. Biosensors provide a good alternative to all these problems as a simple, rapid and cost effective tool for the analysis of mercury (II) in the environment (Arduini *et al.*, 2009). The remarkable affinity of mercury (II) for amino acids and proteins can cause structural and functional changes which can be utilized in the development of bio-analytical techniques. For mercury (II) compounds the primary route responsible for their toxicity is depletion of glutathione and bonding to the sulfhydryl (-SH) groups of proteins (Chapleau *et al.*, 2008). Reported inhibition based mercury biosensors mainly exploit the properties of enzyme inhibition or micro-organism toxicity for mercury determination. Mercury ions are known as effective inhibitors of the catalytic activities of various enzymes such as alcohol oxidase (AlOx) (Pirvutiou *et al.*, 2002), butyrylcholinesterase (BuChE) (Mahmod, 2001), glucose oxidase (GOD) (Guascito *et al.*, 2008), peroxidase (HRP) (Han *et al.*, 2001 and Shekhovtsova and Chernetskaya, 1994), invertase (Kestwal *et al.*, 2008 and Mohammadi *et al.*, 2005), glycerol 3-phosphate oxidase (Ciucu *et al.*, 2001) and urease (Kuralay *et al.*, 2007; Krawczyk *et al.*, 2000; Preininger *et al.*, 1999; Tsai and Doong, 2005 and Rodriguez *et al.*, 2004). Recently array based enzyme biosensors have been reported for screening various environmental pollutants, mainly heavy metals and pesticides. The urease enzyme has been extensively used as a model enzyme to

elucidate the applicability of inhibition assays for mercury (II), where reported work has focused on assays with immobilized urease (Tsai and Doong, 2005). The detection limit of optical determination of mercury (II) by urease is reported to be as low as 1ng·mL−1 (Preininger *et al.*, 1999), whereas for free BuChE it is reported up to 6 μM (Mahmod, 2001). Successful mercury (II) analysis using AlOx using flow injection analysis by a thermal technique is reported to have a detection limit as low as 5 ng·mL−1 (Preininger *et al.*, 1999).

2.2.3 Diagnosis

Urea has considerable significance in clinical chemistry in kidney disease and renal failure. Removal of excess urea has been a major problem for patients suffering from renal failure (Abdel and Guilbault, 1990). Urease is utilized for diagnostic purposes, in the determination of urea in biological fluids. Approximately half a million patients', world-wide, are being supported by heamodialysis (Nose, 1990). The conventional artificial kidney is bulky, heavy, expensive, complex, and difficult to handle, limiting the mobility of the patients. The use of microencapsulated urease is being developed as a useful system in kidney machines to maintain the urea level in the blood of patients suffering alterations in kidney function and in an attempt to construct a portable/wearable artificial kidney (Nose, 1990 and Kayastha *et al.*, 1999). Urease has been used in immobilized form in kidney machines for blood urea detoxication (Lee *et al.*, 1995).

2.3 Enzyme Immobilization Technology

2.3.1 History

Enzymes have been used for several years to modify the structure and composition of foods but they have only recently become available for large-scale use in industry, mainly because of the

high cost of enzymes. However, progress in genetic engineering and in process technology may now enable the enzyme industry to offer products with improved properties and at reduced costs. Economical usage of ureases in hospitals and industry requires enzyme immobilization, which enables enzyme reuse and facilitation of the continuous process. Immobilized enzyme was defined by Katchalski-Katzir at the first Enzyme Engineering Conference, held at Henniker, NH, USA, in 1971, as the confinement or localization of enzyme physically in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Katchalski-Katzir and Kraemer, 2000).

The concept of immobilizing proteins and enzymes to insoluble supports has been the subject of considerable research for over 30 years and consequently, many different methodologies and a vast range of applications have been suggested. Aims often include such factors as the reuse or better use of enzymes, especially if they are scarce or expensive, better quality products as there should be little enzyme in the product requiring inactivation or downstream purification, the production of biosensors, flow-through analytical devices or the development of continuous manufacturing processes. Although large tonnages of immobilized enzymes are used industrially, for example in the production of various syrups from starch, and there are several smaller-scale industrial applications, the introduction of such biocatalysts has been disappointingly slow. With many current manufacturing applications, the cost of the enzyme itself is not a large proportion of overall production costs but the trend toward more complex processing operations and more sophisticated products in the pharmaceutical, food, chemical and other bio-processing industries will require the use of a wider range of enzymes of greater

purity and specificity and hence, higher value. Optimizing use and reuse of enzymes will be increasingly required.

Nelson and Griffin carried out the first investigation on immobilization in 1916. In their work, invertase was adsorbed both on charcoal and aluminum hydroxide (Pitcher, 1980). Meanwhile, the first lipase immobilization study was carried out in 1956 by Brandenberger H. who covalently linked the lipase on ion-exchange resin. However, the first attempts for the usage of immobilized lipase for hydrolysis or ester synthesis reactions were done by Iwai et al. in 1964 (Akşamoğlu, 1997). The first industrial application of immobilized enzymes was carried out first at the Tanabe Seiyaku in Japan who developed columns of immobilized *Aspergillus oryzae,* aminoacylase for the resolution of synthetic, racemic-amino acids into the corresponding, optically active enantiomers. Around 1970, two other immobilized systems were launched on a pilot-plant scale. In England, immobilized penicillin acylase, also referred to as penicillin amidase, was used to prepare 6-amino penicillanic acid (6-APA) from penicillin G or V, and in the USA, immobilized glucose isomerase was employed to convert glucose into fructose. These successful industrial applications prompted extensive research in enzyme technology, leading to a steady increase in the number of industrial processes based on sophisticated, immobilized- enzyme reactors (Katchalski-Katzir and Kraemer, 2000).

2.3.2 Advantages of Enzyme Immobilization

Immobilization process seems to offer mainly the economical advantages. However, there are a number of advantages to attaching enzymes to a solid support and a few of the major reasons are; enzymes can be reused, processes can be operated continuously and can be readily controlled,

products are easily separated, effluent problems and materials handling are minimized, in some cases, enzyme properties (activity and stability) can be altered favorably by immobilization, provides higher purity and product yields, product inhibition is less apparent, greater, pH and thermal stability, no contamination due to added enzyme, continuous operation and greater, flexibility in reactor design (Banu, 2001).

Despite these advantages, industrial application is still limited by; the comparatively low cost of soluble enzymes, traditional attitudes, the investment needed for introducing new equipment to already implanted processes, the nature and cost of the immobilizing support and the immobilizing process (including losses of activity) and the performance of the system (Banu, 2001).

2.3.3 Factors Affecting Immobilization Performance

There are many factors that influence the performance of an immobilized enzyme preparation. Some of the most important factors are the choice of a carrier and the selection of an immobilization strategy.

2.3.4 Support Materials

An enormous number of different types of matrices have been used in laboratory immobilization studies. However, selection of the optimum support is the major parameter that affects the immobilization performance. The properties that an enzyme carrier should have are; large surface area, permeability, insolubility, chemical, mechanical and thermal stability, high rigidity, suitable shape and particle size, resistance to microbial attach and regenerability (Banu, 2001).

However, the exact nature of the process design, the physical properties of the feedstock and the product, the reaction conditions and many other factors will place constraints on the type of matrix, which will be most suitable. In an industrial operation, maximized enzyme-matrix life span is vital component. In Table 2.1, factors that affect the matrix selection can be seen.

Table 2.1: Factors to be considered in the selection of an immobilization matrix

Factors under consideration	Example
Cost of matrix	Beaded dextrans and controlled-pore glass are expensive compared with ceramics and celluloses.
Chemical resistance of the matrix	Silica-based matrices are significantly soluble at pH values above 8.0. Cellulose and dextran-based matrices can be degraded enzymically. Some organic polymers change shape in organic solvents.
Physical properties of the matrix	Ceramic matrices have a high resistance to pressure compared with beaded dextrans.
Complexity of the immobilization	Ion exchange adsorption interactions can be generated rapidly and minimize the inactivation of unstable enzymes. Covalent attachment can be laborious, expensive and destructive.
Stability of the enzyme-matrix association	Enzymes immobilized by physical means undergo slow leakage compared with covalently attached enzymes.
Special requirements of the process	Pressure sensitive matrices (e.g. Agarose) are unsuitable for plug-flow reactors. Magnetic matrices are useful for the recovery of enzyme in CSTR* systems.

*continuous stirred tank reactor (Banu, 2001)

2.3.5 Enzyme Immobilization Methods

The selection of an immobilization technique is based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of the urease utilization, deactivation and regeneration characteristics, cost of immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized urease. Chemical methods feature the formation of covalent bonds between the urease and the modifier, while physical methods are characterized by weaker interactions of the enzyme with the support material, or mechanical containment of the urease within the support. Methods for enzyme immobilization can be classified into three main categories: entrapment, carrier binding and cross linking (Banu, 2001).

2.3.5.1 Carrier Binding

Carrier-Binding method is the oldest immobilization method for enzymes and is defined as the binding of enzymes to water-insoluble carriers. In this method, enzymes bound to the carrier with their amino acid residues containing chemically reactive groups, ionic groups, and/or hydrophobic groups as well as hydrophobic domains. These amino acid residues and the hydrophobic domains can participate in the immobilization of the enzymes through covalent linkage and physical adsorption, though immobilization also depends on the nature of the carrier (Wolfgang, 2007). The following picture shows how the enzyme is bonded to the carrier in this method:

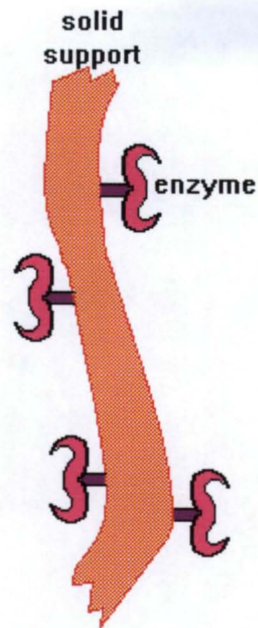
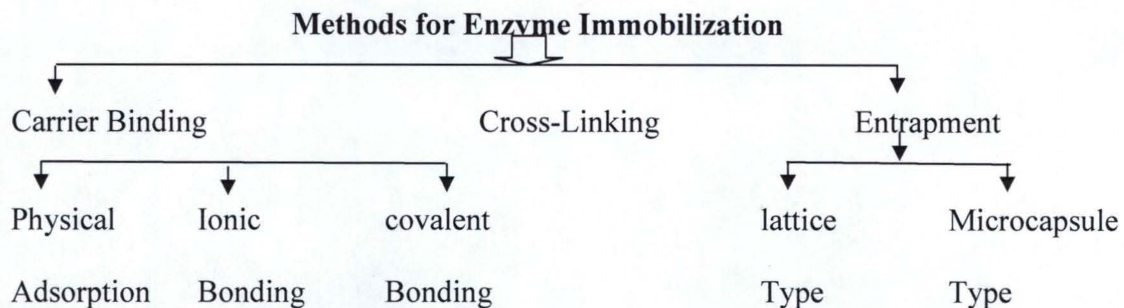


Figure 2.7: Schematic illustration of carrier-binding method (http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).



The selection of the carrier depends on the nature of the enzyme itself, as well as the particle size, surface area, molar ratio of hydrophilic to hydrophobic groups and chemical compositions. In general, an increase in the ratio of hydrophilic groups and in the concentration of bounded enzyme results in a higher activity of the immobilized enzymes. The most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel. According to the binding mode of the enzyme, carrier-binding

method can further be sub-classified into: physical adsorption, ionic binding and covalent binding (Banu, 2001).

Physical Adsorption

The earliest example of enzyme immobilization using this method is the adsorption of β-D-fructo-furanosidase onto aluminium hydroxide. This method for the immobilization of enzyme is based on the physical adsorption of enzyme amino acid residue as a result of the physical interaction such as hydrogen bonding, hydrophobic interaction and Van der Waal's forces that exist between enzyme amino acid residues and the functional groups of the polysaccharide (chitosan). Although enzyme is immobilized without any modification, interaction between enzyme and support is generally weak and affected by environmental factors such as temperature or concentration of reactants (Wolfgang, 2007). Hence, the method causes little or no conformational change of the enzyme, or destruction of its active center. If a suitable carrier is found, this method can be both simple and cheap. Also, they lend themselves a minimal resistance in the reaction mixtures, and possible supports for physical adsorption are mechanically durable and re-useable (Oliveira *et al.*, 2000). Thus, this method is found to be the most suitable for large-scale immobilization.

However, it has the disadvantage that the adsorbed enzyme may leak from the carrier during utilization, because the binding force between the enzyme and the carrier is weak. The enzyme immobilized onto a solid support by low energy binding forces, e.g., Van der Waal's interactions, hydrophobic interactions, hydrogen bonds, ionic bonds. Many carrier materials exist, the choice of one often depending on properties that are important for potential industrial

applications: mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme load capacity and cost. Initially, mineral supports such as porous glass beads, diatomaceous earth, silica and alumina were used. More recently, the most used supports are ion exchange resins, celite and biopolymers (Villeneuve *et al.*, 2000).

The success and efficiency of the physical adsorption of the enzyme on a solid support is dependent of several parameters. The size of the protein to be adsorbed, the specific area of the carrier and the nature of its surface (porosity, pore size) are crucial. Typically, the use of a porous support is advantageous since the enzyme will be adsorbed at the outer surface of the material and within the pores as well. An efficient immobilization is also dependent on the enzyme concentration. The amount of adsorbed enzyme per amount of support increases with the enzyme concentration reaching a plateau at the saturation of the carrier. This operation is usually carried out at constant temperature, and, consequently, adsorption isotherms are obtained which follow the Langmuir or Freundlich equations. The pH at which the adsorption is conducted is equally important since ionic interactions are crucial in such an immobilization. Usually, the maximum adsorption is observed for pH values close to the isoelectric point of the enzyme. Finally, addition of water miscible solvents during the immobilization process favors the adsorption by reducing the solubility of the enzyme in the aqueous phase (Villeneuve *et al.*, 2000).

Urease immobilization by physical adsorption finds applications in urea bioconversion. Their unique specificities allow the design of synthetic routes that predetermine product structure and distribution whereas chemical catalysts generally lead to random reaction product mixtures.

Thus, the use of urease makes it feasible to obtain new products with predetermined physical and chemical properties (Villeneuve *et al.*, 2000).

During the immobilization of urease by physical adsorption, driving force is mainly hydrogen bonding because of the structural properties of the enzyme. Hydrogen bonding arises from the interaction hydroxyl groups of the polysaccharide (chitosan) and enzyme molecules (Wolfgang, 2007).

Ionic Binding

The ionic binding method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues. Polysaccharides and synthetic polymers having ion-exchange centers are usually used for carriers. The binding of enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme, and so yields immobilized enzymes with high activity in most cases. As the binding forces between enzyme protein and carriers are less strong than in covalent binding; leakage of enzyme from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH. The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages is much stronger for ionic binding although less strong than in covalent binding.

Covalent Binding

The covalent binding method is based on the binding of enzymes and water insoluble carriers by covalent bonds. The functional groups that take part in this binding of enzyme to carrier can be

amino, carboxyl, sulfhydryl, hydroxyl, imidazole or phenolic groups which are not essential for the catalytic activity. In order to protect the active site, immobilization can be carried out in the presence of its substrate or a competitive inhibitor. Activity of the covalent bonded enzyme depends on the size and shape of carrier material, nature of the coupling method, composition of the carrier material and specific conditions during coupling.

The main advantage of the covalent attachment is that such an immobilization is very solid. Unlike physical adsorption, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength. Moreover, the obtained immobilized enzymes are usually very stable and resistant to extreme conditions (pH range, temperature). Finally, a large number of different supports and methods to activate them are available. However, experimental procedures are obviously more difficult to carry out than for physical adsorption. The 3-D structure of the protein is considerably modified after the attachment to the support. This modification generally leads to a significant loss of the initial activity of the biocatalyst (Villeneuve *et al.*, 2000).

2.3.5.2 Cross-Linking

This can be defined as the intermolecular cross-linking of enzymes by bifunctional or multifunctional reagents and it is based on the formation of chemical bonds, as in the covalent binding method, but water-insoluble carriers are not used. The immobilization is performed by the formation of intermolecular cross-linkages between the enzyme molecules by means of bi or multifunctional reagents. The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions

can change the conformation of active center of the enzyme; and so may lead to significant loss of activity. This method can be applied in three different manners: mixing the prepolymers with a photosensitizer (e.g., benzoin ethyl ether), melting, mixing with an enzyme solution and gelling by exposure to near ultraviolet radiation, freezing a monomer solution containing the enzyme in the form of small beads, polymerization is then started using gamma radiation an mixing the enzyme in a buffered aqueous solution of acrylamide monomer and a cross-linking agent. Polymerization can be initiated by the addition of some chemicals (Villeneuve *et al.*, 2000).

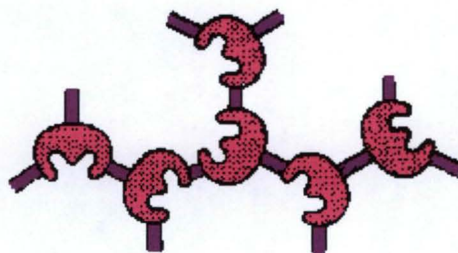


Figure 2.8: Schematic illustration of cross-linking method (http://www.eng.rpi.edu/ dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

2.3.5.3 Entrapment

This method of immobilization involves incorporating enzymes into the lattices of a semi permeable gel or enclosing the enzymes in a semi permeable polymer membrane. The entrapment for immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane in such a way as to retain protein while allowing penetration of substrate. This method differs from the covalent binding and cross-linking in that enzyme itself does not bind to the gel matrix or membrane; and thus, has a wide applicability. The conditions, used in the chemical polymerization reaction, are relatively severe, resulting in the loss of enzyme activity. Therefore, careful selection of the most suitable conditions for the immobilization of various enzymes is required (Wolfgang, 2007).

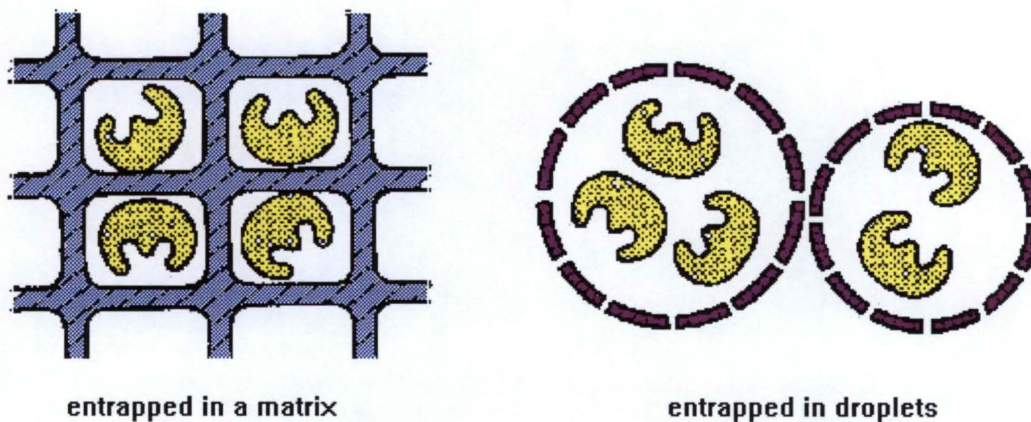


Figure 2.9: Schematic illustration of entrapment methods (http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

Entrapment can be classified into lattice and microcapsule types.

1. Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as polyacrylamide, polyvinylalcohol, etc. and natural polymer (starch) have been used to immobilize enzymes using this technique.

2. Microcapsule-Type entrapping involves enclosing the enzymes within semi permeable polymer membranes. This is probably the less developed immobilization technique, is very similar to entrapment, although in this case, it is the enzyme and its whole environment that are immobilized. Microencapsulation creates artificial cells delimited by a membrane. Large molecules such as enzymes are not able to diffuse across the synthetic membrane whereas small molecules, e.g., substrates and products, can pass through it (Villeneuve *et al.*, 2000). The preparation of enzyme microcapsules requires extremely well controlled conditions; and the procedures for microencapsulation of enzymes are liquid drying, phase separation and interfacial polymerization method.

The advantage of such an immobilization technique is that the enzyme does not chemically interact with the polymer; therefore, denaturation is usually avoided. However, mass transfer phenomena around the membrane are problematic. The diffusion rate across the membrane of the substrate and the product is often the limiting parameter. Generally, high substrate concentrations are necessary in order to limit its influence. Finally, entrapped enzymes are better used with small substrates since larger ones may not be able to pass the membrane and reach the active site of the biocatalyst (Villeneuve *et al.*, 2000).

Table 2.2: Comparison of immobilization methods (Akşamoğlu, 1997)

Characteristic	Cross Linking	Physical Adsorption	Ionic Binding	Covalent Binding	Entrapment
Preparation	Intermediate	Easy	Easy	Difficult	Difficult
Binding force	High	Low	Medium	High	Medium
Enzyme activity	Low	Medium	High	High	Low
Reusability	Impossible	Possible	Possible	Rare	Impossible
Cost	Intermediate	Low	Low	High	Intermediate
Stability	High	Low	Intermediate	High	High
Applicability	No	Yes	Yes	No	Yes

2.3.6 Enzyme Immobilization Technology and its Application

Enzymes are the ubiquitous magicians of the biological world, catalyzing one substance into a material that is substantially different. Agriculture, manufacturing, pharmaceuticals, energy generation, all aspects of industry and human endeavor rely in some ways on enzyme reactions. However, enzymes are fragile and operate within very specific temperatures and environments that reflect their cellular origins. This fragility has, until now, limited researchers' ability to precisely control enzyme reactions or to reuse the enzymes."An enzyme is like a very efficient, environmentally friendly chemical factory that doesn't require extreme conditions to operate. Cells have thousands of enzymes carrying out the chemical reactions that sustain life, and many of these enzymes can be tapped for useful applications" (Doaa and Wafaa, 2009).

The possibilities for using immobilized enzymes to carry out desirable targeted chemical reactions are endless. New and highly diverse areas of research such as generating energy more efficiently in hydrogen fuel cells, purifying chemical and biological materials for prescription drug use, and detecting and neutralizing dangerous chemical and biological agents are just a few of the possible applications of targeted enzyme reaction (Doaa and Wafaa, 2009).

For several years, scientists have been working to immobilize enzymes for the purpose of carrying out targeted chemical reactions. However, the work has not been very successful due to enzyme fragility that is, its inability to retain activity in an incompatible environment. Immobilizing an enzyme in an optimal framework with a compatible environment could prevent its deterioration (Doaa and Wafaa, 2009).

2.3.6.1 Enzymes for Kidney Disorders: Uricase: Gout, an inflammation of joints occurring frequently in the age group of 30-50 years is due to the accumulation of uric acid in the joints. Uric acid is the final product of purine catabolism in humans. It has poor solubility (0.42mmol/lit.). When its blood level is increased it will precipitate mainly in terminal joints. This painful pathological condition can be due to the over production of urate by an excessive purine diet (excess meat) an accelerated ATP degradation or an enzymic problem (i.e. deficiency of hypoxanthine-guanine phosphoribosyl transferase) or hyper activity of phosphoribosyl pyrophosphatase synthetase. It may also be due to renal under excretion of urate due to a kidney dysfunction, an inhibition of urate secretion by tubules due to competitive anions or an enhanced tubular reabsorption of urate. Presently patients with acute gout are treated with anti inflammatory agents to ease pain followed by uricosuric agents to alter renal excretion to cure hyper uricaemia. An alternative approach is the utilization of uricase (urate oxidase) which catalyses the oxidation of urate to allantoin to decrease the urate blood level. Allantoin is approximately 100 times more soluble than urate (Doaa and Wafaa, 2009)

2.3.6.2 Diabetics: Glucose Oxidase: The immobilized glucose oxidase on a polycarbonate membrane modified by a urethane coupling with a poly-(L-lysine) activated with glutaraldehyde has been described (Shin-ichiro *et al.*, 1998). The enzymic properties of immobilized enzyme were investigated and compared with those of native glucose oxidase. The thermal stability and pH stability of the immobilized glucose oxidase were greater than native enzyme. The molecular mass of poly- (L-lysine) was investigated as a possible influencing agent on immobilization of glucose oxidase on porous polycarbonate membrane. They used standard immobilization procedure except that the molecular mass of poly-(L-lysine) was varied in the range 5-300Kda.

The effect of molecular mass on the immobilized glucose oxidase activity showed that 50 Kda or above was required for optimum immobilization of glucose oxidase. The comparison of enzyme activity with the method of immobilization showed a quantity of glucose oxidase adsorbed on ordinary poly carbonate membrane was negligible, while covalent binding with aldehyde groups in the derivatives membrane was string and no leakage was observed. The membrane was applied as glucose sensor.

2.3.7 Application of Immobilized Enzyme as Biosensors

Phosphate detection Biosensors (Alkaline phospatase immobilized on glass fiber for detection of phosphate in water samples, milk and shrimp). Attachment of enzymes to the surface of glass fiber is a technique used for developing biosensors. This attachment occurs by the covalent bond or physical adsorption. It has been used widely as one of the traditional enzyme immobilization technologies. Although glass provides desirable mechanical strength and thermo chemical stability, it is difficult to achieve high enzyme loading as required for efficient bioprocess application. The development could potentially enhance the area of rapid detection using biosensor Procedure, amide bonds were formed between carboxyl groups on the protein and amino group on the glass surface was attempted to overcome the problem (Hobson *et al.*, 1996).

2.3.8 Application in Cleaning up Pesticide

In many circumstances pesticides are the only effective means of controlling weeds, insect, and fungus, parasitic and rodent pests. Application of pesticide in agriculture serves to lower the cost of production, increase crop yields, provide better quality produce and also reduce soil erosion. Due to the toxic nature of pesticide, application of these pesticides also has the potential of

adverse effects on human health and the environment. Treatment of pesticide-contaminated waste is an approach that could assist to reduce the impact of pesticides on the environment. One of the most important technologies to be applied for this approach is immobilized enzyme. The immobilized enzyme is capable of breaking down a range of organophosphate insecticides (Sharmin *et al.*, 2007 and Horne *et al.*, 2002).

2.3.9 Application in Textile Industry

Textile industry is a conventional industry in many countries, which possesses a considerable proportion of the economy .In recent years, special attention has been paid to the application of biotechnology in textile industries. One of these technologies is enzyme immobilization, including bio-stone washing of denim with cellulases and desizing of cotton fabrics with amylases (Chen *et al.*, 2007).

2.4 Lima Beans (*Phaseolus lunatus*)

In Delaware, lima beans are considered the cornerstone crop of the vegetable-processing industry. Lima beans are double-cropped on as much as three-fourths of the acreage, thus offering producers maximum utilization of the land. Limas are often planted in June or July after a pea or small grain crop.

The nutritional status of lima beans is presented below.

Table 2.3: Nutritional Value of Lima Beans

Serving size: 1/2 cup, boiled		Primary Nutrients		%RDA(m)	%RDA(f)
Calories	109	Folic acid	78 mcg	39	43
Fat	0.35	Iron	2.25 mg	22.5	15
Calories from fat	3	Magnesium	41 mg	12	15
Cholesterol	0	Thiamine	0.15 mg	10	14
Sodium	2 mg	Zinc	0.9 mg	6	7.5
Protein	7.3 g				
Carbohydrate	19.7 g			% Mini. Requirement	
Dietary fiber	6.8 g	Potassium	478 mg	24	

Source: UC Davis, Vegetable Research and Information Center (2002).

2.5.0 The Mexican Yam Bean (*Pachyrhizus Tuberosus* (L.) Urban)

This cultivated yam bean species is found in Central America as well as South East Asia. The species is named Jicama in Mexico and Bang Kuang in Indonesia. It is a herbaceous vine with great variation in the outline of the leaflets, from dentate to palmate (Sørensen, 1996). Moreover the species is defined by the lack of hairs on the petals, the number of flowers (4-11) per lateral inflorescence axis by complex racemes. Morphological characters of the pods are also used to distinguish the species. A number of seed characters are also specific (Sørensen, 1996).

2.5.1 Chemical Composition and Nutritional Value

The characterization and quantification of the amino acids composition of *P. tuberosus* seeds compared with soya bean seeds were reported by Sales *et al.* (1990). The seeds are rich in both proteins and lipids/oil (Grüneberg *et al.*, 1999). Yam bean seeds are characterized by high oil

(about 20 to 28 %) and protein (about 23 to 34 %) contents. Seed oil contains high concentrations of palmitic (about 25-30 % of the total fatty acids), oleic (21-29 %) and linoleic (35-40 %) acids. The levels of linolenic acid are very low, from 1.0 to 2.5 %. Total tocopherol content of the seeds was relatively low in *P. erosus* (from 249 to 585 mg kg-1 oil) and *P. tuberosus* (from 260 to 312 mg kg-1 oil) compared with the levels found in *P. ahipa* under identical conditions (508 to 858 mg kg-1 oil) (Grüneberg *et al.*, 1999). Other studies also showed the chemical composition and quality of the *P. erosus* oil (Cruz, 1950; Broadbent and Shone, 1963; Jimenez B., 1994; Santos *et al.*, 1996). These studies agree that if the insecticidal compounds are removed, the oil has a composition comparable with that of groundnut and cottonseeds oil.

2.6.0 Pigeonpea

The high nutritive value of pigeonpea is perhaps the most important reason why it should find an important place among the smallholder poor farmers in Africa. Pigeonpea is wonderfully abundant in protein, making it an ideal supplement to traditional cereal-, banana- or tuber-based diets of most Africans which are generally protein-deficient. The protein content of commonly grown pigeonpea has been reported to range between 18–26% (Swaminathan and Jain, 1973) while up to 30% has been reported in other closely related *Cajanus* spp. (Reddy *et al.*, 1979). Researchers at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India have developed high protein lines (HPL) with up to 32.5% protein content and significantly higher sulphur-containing amino acids (cysteine and methionine) (Singh *et al.*, 1990; Saxena *et al.*, 2002). Pigeonpea is therefore a good source of amino acids (Elegbede, 1998).

Anti-nutritional factors such as protease (trypsin and chymotrypsin) inhibitors, amylase inhibitors and polyphenols, which are a known problem in most legumes, are less problematic in pigeonpea than soybean, peas (*Pisum sativum*) and field beans (Singh and Eggum, 1984; Singh, 1988; Faris and Singh, 1990). Within pigeonpea cultivars, anti-nutritional factors are mainly found among darkseeded genotypes (Faris and Singh, 1990) that are typically grown in Asia. The native African pigeonpea types are largely cream or white seeded with relatively less antinutritional factors.

The supplementation of cereals with protein rich legumes is considered as one of the best solutions to protein-calorie malnutrition in the developing world (Chitra *et al.*, 1996). Pigeonpea flour has been tested and found to be suitable as a protein source for supplementing baked products such as bread, cookies and *chapattis* due to its high level of protein, iron (Fe) and P (Harinder *et al.*, 1999). It has therefore been recommended in school feeding programs and vulnerable sections of the populations in developing nations. The protein-rich seeds have also been incorporated into cassava flour to produce acceptable extruded products (Rampersad *et al.*, 2003).

Pigeonpea is a rich source of carbohydrates, minerals and vitamins. The seeds contain a range of 51.4–58.8% carbohydrates (Faris and Singh, 1990), 1.2–8.1% crude fibre and 0.6–3.8% lipids (Sinha, 1977). It is a good source of dietary minerals such as calcium (Ca), P, magnesium (Mg), Fe, sulphur (S) and potassium (K) (Sinha, 1977), and water soluble vitamins especially thiamine, riboflavin and niacin (Salunkhe *et al.*, 1986). Pigeonpea contains more minerals, ten times more fat, five times more vitamin A and three times more vitamin C than ordinary peas (Foodnet, 2002).

In Africa, pigeonpea seeds are mainly eaten green unlike in India, where dry dehulled split-pea (*dhal*) is most popular. Such green seeds are a richer source of Fe, Cu and Zn than the mature seed (Singh *et al.*, 1984) and have a greater edible portion (72% vs 53%), more protein, carbohydrates, fibre, fat, minerals and vitamins than *dhal* (Faris *et al.*, 1987). An estimated 30% of children under the age of five in sub-Saharan Africa are reportedly underweight due to deficiencies in energy and nutrients. Wide adoption of pigeonpea in Africa thus stands to play an important role in food security, balanced diet and alleviation of poverty.

2.6.1 Nutritional Evaluation of pigeon pea Chemical analysis

Proximate analysis of pigeon pea (both raw and processed seeds) was carried out using the methods outlined by the Association of Official Analytical Chemists (AOAC, 1990). The proximate compositions of the pigeon pea and details of quality characters and nutritional values of pigeon pea are presented in the tables 6 and 7 below.

Table 2.4: Proximate composition (%) of raw and roasted pigeon pea seeds

Contents	Raw seed	Roasted seed
Dry matter	95.89	96.34
Crude protein	21.03	21.07
Crude fat	4.43	5.96
Crude fibre	7.16	7.52
Ash	3.76	4.02
Nitrogen free extract	59.51	57.77

Akande *et al.*, (2010).

Amino acid analysis

Table 2.5: Amino acid profiles of raw and roasted pigeon pea seeds (g/16 gN)

Amino acids	Raw pigeon pea	Roasted pigeon pea
Lysine	7.79	7.55
Histidine	3.66	2.88
Arginine	5.86	6.18
Aspartic acid	11.56	12.20
Threonine	3.12	3.28
Serine	3.59	3.84
Glutamic acid	9.23	14.21
Proline	3.17	3.16
Glycine	3.07	3.36
Alanine	3.79	4.06
Cystine	1.19	0.69
Valine	5.85	4.27
Methionine	1.19	0.89
Isoleucine	3.47	2.73
Leucine	6.78	7.23
Tyrosine	2.63	2.86
Phenylalanine	6.15	5.54
Tryptophan	ND	ND

ND = Not Determined. (Akande, *et al.*, 2010).

The amino acid profile of pigeon pea is comparable with the conventional plant protein sources. Roasted pigeon pea seeds are recommended as valuable feed ingredient at both domestic and industrial levels for monogastric animals. Production of pigeon pea of high-protein genotype should be encouraged.

2.7 African Yam Beans (*Sphenostylis stenocarpa*)

African yam bean (*Sphenostylis stenocarpa* Ex. A. Rich, Harms) is an under-utilized food legume crop in the tropics that is not popular as other major food legumes (Azeke *et al.*, 2005; Moyib *et al.*, 2008). It produces nutritious pods, highly proteinous seeds and capable of growth in marginal areas where other pulses fail to thrive (Okpara and Omaliko, 1997). The crop thus has the potential to meet the ever increasing protein demands of the people in the sub-Sahara Africa if grown on a large scale. Presently, low quantities are offered for sale in the markets compared to other pulses. It is a good source of high plant protein (21% by wt) and calcium (61 mg/100 g) which compares well to that of Soybean (Baudoin and Mergeai, 2001). African yam bean (AYB) contributes to sustainable agriculture, survives well in weathered soils where rainfall could be extremely high, tolerates acidic, leached and infertile soils (NRC, 2007). Asare, *et al.*, 1984 and DSC, 2006 observed that African yam bean is a good source of fodder for ruminant animals and performed better in seed yield when intercropped with maize, yam and okra. It is resistant to crop pests such as *Clavigralla tomentosicollis*; *Maruca vitrara* than other food legumes like cowpea [*Vigna unguiculata* (L) Walp] (Okigbo, 1973; Evans and Boulter, 1974; IITA, 1985; Nwokolo, 1987). However, with some of these attributes, very little efforts have been invested to improve the crop in terms of yield, disease resistance, reduced cooking time and other desirable qualities, when compared to other major legumes (Potter, 1991).

2.7.1 Proximate analysis of the seed and tuber of *S.* stenocarpa

The compositional analysis of the seed of five accessions of *S. stenocarpa* is shown in Table 2.6. The crude protein fraction of the seed was highest in accession 3 (23.7%), closely followed by accession 5 (23.0%) while the lowest crude protein component of the seed was recorded in accession 4 (9.9%). There were highly significant differences between the crude protein fractions in the five accessions of African yam bean seed. The ash fraction of the seed was more in accession 1 (2.8%), followed by accession 2 (2.5%) while accession 4 showed the lowest ash fraction (1.3%). The percentage ash fractions showed highly significant differences between the five accessions of *S. stenocarpa* seed. Accession 4 showed the highest nitrogen-free extract fraction (65.9%), followed by accession 2 (65.7%), while the least nitrogen-free extract percentage was shown in accession 3 (53.6%). There were highly significant differences between the nitrogen-free extract fractions in the five accessions of African yam bean seed. The ether extract component of the seed was highest in accession 1 (1.2%), followed by accession 3 (1.1%) while accession 5 recorded the lowest ether extract fraction (0.8%). The various fractions of ether extract did not differ significantly between the five accessions. The moisture content of the seed was highest in accession 4 (9.6%), followed by accession 1(9.1%), while the lowest percentage moisture content was shown in accession 2 (8.0%). There were however no significant differences between the percentage moisture content in the five accessions of African yam bean seed. The crude fibre fraction of the seed in the five accessions of *S. stenocarpa* was highest in accession 4 (12.4%), followed by accession 2 (12.2%) while accession 5 showed the least (7.4%) crude fibre fraction. There were highly significant differences between the crude fibre components in the five accessions of African yam bean seed. The results in Table 2.6 indicate that the seed of accession 4 had the highest carbohydrate content (78.3%), followed by

accession 2 (77.9%) while accession 3 showed the lowest (65.0%) carbohydrate content. There were highly significant differences between the carbohydrate contents in the five accessions of *S. stenocarpa*. It could be seen from the results above that the seed of the five accessions of African yam bean have high percentage carbohydrate content than crude protein. The proximate composition of African yam bean tuber is shown in Table 2.6. The results showed a crude protein and carbohydrate composition of 8.32% and 79.24%, respectively. The results equally presented the tuber of *S. stenocarpa* as being composed mainly of carbohydrate. The other constituents showed low percentages except nitrogen-free extract (71.18%) that was more than those found in the seed of all the accessions. However, the crude protein level in the tuber was found to be smaller than those recorded in the five accessions of African yam bean seeds. Both the seed and tuber of *S. stenocarpa* therefore possess higher levels of carbohydrate than crude protein or other constituents.

2.7.2 Mineral content of African yam bean seed and tuber

The mineral composition of African yam bean seed and tuber is shown in Table 2.7. Comparison of the minerals found in both seed and tuber indicate that magnesium; phosphorus, potassium, iron and manganese were more in the tuber than seed. However the quantity of sodium, calcium, zinc and copper in the tuber were less than those found in the seed. The use of paired t-test showed that both the seed and tuber did not differ significantly in the composition of their mineral contents (Table 2.7). NS = Not significant

Table 2.6: Proximate analysis of the seeds of five accessions of *S. stenocarpa*

Crude Protein (%)	Ash (%)	Nitrogen free extract	Ether extract (%)	Moisture (%)	Crude fiber (%)	Carbohydrat (%)
21.0	2.8	56.8	1.2	9.1	9.1	65.9
10.7	2.5	65.7	1.0	8.0	12.2	77.9
23.7	1.6	53.6	1.10	8.7	11.4	65.0
9.9	1.3	65.9	1.0	9.6	12.4	78.3
23.0	2.4	57.7	0.8	8.6	7.4	65.2

Ameh, (2007).

Table 2.7: Mineral composition of African yam bean seed and tuber

Minerals	Seed	Tuber
Mg (mg/100g)	43.2	46.7
P (mg/100g)	27.4	660.1
K (mg/100g)	116.4	487.9
Na (mg/100g)	421.3	214.4
Ca (mg/100g)	43.6	26.8
Zn (μg/100g)	50.0	37.5
Cu (μg/100g)	23.0	16.0
Fe (mg/100g)	12.6	31.6
Mn (μg/100g)	14.0	18.0
Mean	83.5	171
CV (%)	156.3	140.6

t (0.05) for comparing the two means = 0.96. Ameh, (2007).

The proximate composition of African yam bean seed and tuber is comparable to those of other legumes. The highest percentage crude protein of 23.7 % was obtained in the seed of three accessions of African yam bean.

2.8 Soya Beans (*Glycine max*)

The soybeans plant (*Glycine max*) was cultivated in China 3000BC, and was classified as one of the five sacred crops. The fisrt written record is a 2200BC farming manually advising, Chinesse farmer on how to get the best crops. Soya was introduced into USA in the early 19th century (originally arriving as ballast abroad returning clipper ships) but soay farming in the USA only expanded dramatically after world war II, when production in china was devastated (insttute of food research website). Since 1945, it has become the most important world's main protein and oil crop, being widely used for both human and animal food stuffs (FDA UN 2004). Soya beans contain approximately 38% protein and 18% oil. Japanese soya bean is reported to have a high fat content, while China and India soybean are known for thiere high protein content. A lot of genetic variation exists with the soybean crop as there are thousand of varieties available, including red, black and green. The most popular variety is the yellow soya bean (*Glycine max*) (Burke *et al.*, 1995).

2.8.1 Nutritional Value of Soya Beans

The details of quality characters and nutritional value of soya beans are presented in the table below

Table 2.8: Soya Bean Seed Composition

Character	Fresh seed (g/100g)	Dried yellow seed (g/100g)
K calories	139	400
Moisture content (%)	68.2	10.2
Protein	13.0	35.10
Fat	5.7	17.7
Carbohydrate	11.4	32.0
Fiber	1.9	4.2
Ash	1.7	5.0
Calcium	78mg	226mg
Phosphorus	158mg	546mg
Iron	3.8mg	
Sodium	15.0mg	
Potassium	607mg	
B-carotene equivalent	360μg	
Thiamine	0.4mg	
Riboflavin	0.17mg	8.5mg
Niacine	1.5mg	2.2mg
Ascorbic acid	27mg	

Source: Anderson, *et al.*, (1995).

2.8.2 Protein Content of Soya beans

Soya bean has been identified as a complete source of protein (http://wwwtruesterhealth.com/members/cniarchives12m13pia8.html). A complete protein is one that contain significant amount of all the essential amino acids that must be provided to the human body because of the body's inability to synthesize them. In this reason, soy protein is similar to that of other legume seeds, but has the highest yield per square meter growing area and is the least expensive source of dietary protein.

The original protein efficiency ratio (PER) method of measuring soya protein quality was found to be flawed for humans because the young rats used in the study have higher relative requirements for sulphur containing amino acids. As such, the analytical method that is universally recognized by the FAO/WHO, (1990) as well as the FDA, USDA, United Nations University (UNU) and national academy of science when judging the quality of protein digestibility corrected amino acid score, as it is viewed as accurately measuring the corrected relative nutritional value of animals and vegetable sources of protein in the diet (FAO/WHO, 1991; Schaifema, 2000).

A globular protein, glycine, accounts for 20-90% of the total protein content of the seed and in turn is made up of the total following amino acids.

Table 2.9: Amino acid content of soya beans protein

Amino acid	Amount (%)
Glutamine	1.9
Aspartic acid	5.7
Threonine	2.1
Proline	4.3
Alanine	1.7
Glycine	0.7
Valine	1.6
Cysteine	1.1
Methionine	1.8
Isoleucine	2.4
Leucine	9.2
Tyrosine	3.9
Phenylalanine	4.3
Lysine	5.4
Histidine	2.2
Tryptophan	1.7
Arginine	8.3
Proline	4.3

Source: FAO/WHO (1990)

Soya bean protein is comparable to meat and egg. The vegetable oil is made up of polyunsaturated fatty acid which little or no tendency to generate cholesterol. Soy protein is said to have the effect of reducing cholesterol level in hypercholesterolemia people (Anderson, 1995)

2.9 Velvet Bean (*Mucuna pruriens*)

Mucuna pruriens is a tropical legume known as **velvet bean** or **cowitch** and by other common names (see below), found in Africa, India and the Caribbean. The plant is infamous for its extreme itchiness produced on contact, particularly with the young foliage and the seed pods. It has value in agricultural and horticultural use and has a range of medicinal properties.

2.9.1 Nutritional and Antinutritional Assessment of *Mucuna pruriens*

The crude protein content of all the three accessions of *Mucuna pruriens* var. *pruriens* show little variation and contain higher crude protein when compared with commonly consumed pulse crops such as black gram, green gram, pigeon pea, chick pea and cow pea (Gupta and Wagle, 1978; Jambunathan and Singh, 1980; Nwokolo and Oji, 1985; Nwokolo, 1987) and other *Mucuna* species which have been reported earlier (Janardhanan and Lakshmanan, 1985; Arulmozhi and Janardhanan , 1992; Mohan and Janardhanan, 1995; Arinathan *et al.*, 2009; Vadivel and Janardhanan, 2000). The crude lipid content of all the accessions of *M. pruriens* var. *pruriens* seems to be higher than the previously studied common/tribal pulses such as *Vigna radiata* (Khan *et al.*, 1979); *Cicer arietinum* (Jambunathan and Singh, 1980), *Cajanus cajan* (Nwokolo, 1987), *Vigna capensis* and *V. sinensis* (Mohan and Janardhanan, 1993), *V. trilobata* (Siddhuraju, 1992), *Atylosia scarabaeoides*, *Neonotonia wighii* var. *coimbatorensis*, *Rhynchosia filipes* and *Vigna unguiculata* subsp. *unguiculata* (Arinathan *et al.*, 2003). All the currently

investigated samples contain high levels of total dietary fibre when compared with the other tribal pulses reported earlier *Canavalia gladiata*, *Lablab purpureus* var. *lignosus* and *Vigna unguiculuta* subsp. *unguiculata* (Arinathan *et al.*, 2003). The ash content of the investigated tribal pulse (Table 2.10) would be important to the extent that it contains the nutritionally important mineral elements, which are presented in Table 2.11. The range in calorific values exceeds the energetic values of cowpea, green gram, horse gram, moth bean and peas (Narasinga Rao, *et al.*, 1989), which are in the range of 1318 – 1394 kJ100g-1 DM.

Table 2.10: Proximate composition of the seeds of three accessions of *Mucuna pruriens* var. *pruriens* (g 100g-1) a.

Components	Saduragiri	Siruvani	Thallaianai
Moisture	10.21 ± 0.01	9.48 ± 0.23	9.78 ± 0.23
Crude protein (Kjeldahl Nx6.25)	32.48 ± 0.47	28.80 ± 0.46	29.40 ± 2.54
Crude lipid	8.50 ± 0.41	7.66 ± 0.47	8.94 ± 0.24
TDF (Total Dietary Fibre)	7.41 ± 0.01	6.20 ± 0.56	6.78 ± 0.01
Ash	4.10 ± 0.01	4.30 ± 0.01	4.52 ± 0.01
Nitrogen Free Extractives (NFE)	47.51	53.04	50.36
Calorific value (kJ100g-1DM)	1656.28	1655.51	1669.03

a All values are of means of triplicate determination expressed on dry weight basis. ± denotes standard error.

Table 2.11: Mineral composition and vitamins (niacin and ascorbic acid) of the seeds of three accessions of *Mucuna pruriens* var. *pruriens* (mg 100g-1) a

Components	Saduragiri	Siruvani	Thallaianai
Sodium	54.12 ± 0.42	88.20 ± 0.46	69.21 ± 0.08
Potassium	1527.94 ± 0.04	1638.40 ± 0.48	1421.35 ± 0.44
Calcium	562.51 ± 0.23	710.42 ± 0.46	630.20 ± 0.38
Magnesium	410.10 ± 0.47	512.42 ± 0.25	478.82 ± 0.46
Phosphorus	408.52 ± 0.46	530.82 ± 0.46	448.12 ± 0.47
Iron	8.16 ± 0.47	7.15 ± 0.94	6.84 ± 0.23
Zinc	1.98 ± 0.01	3.34 ± 0.01	3.60 ± 0.19
Copper	0.66 ± 0.02	0.54 ± 0.01	0.72 ± 0.09
Manganese	7.10 ± 0.27	5.28 ± 0.04	6.48 ± 0.09
Niacin	34.20 ± 0.46	48.10 ± 0.50	42.64 ± 0.61
Ascorbic acid	45.70 ± 0.01	52.66 ± 0.55	38.12 ± 0.48

aAll values are of means of triplicate determination expressed on dry weight basis. ± denotes standard error (Fathima *et al*., 2010)

Table 2.11 shows the elemental composition of the sample. In the investigation, the Sivagiri accession registered a higher level of potassium than the other two accession and their levels of potassium seem to be higher compared to that of *Mucuna monosperma* (Arulmozhi and Janardhanan, 1992), *M. pruriens* (Siddhuraju *et al*., 1996) and *M. utilis* (Mohan and Janardhanan, 1995). Among the three accessions, Sivagiri accession registers the highest levels of calcium, magnesium and phosphorus and its calcium level is found to be higher than that of the

recommended dietary allowances of calcium (400g) for children by the Indian Council of Medical Research (ICMR. 1992), and its magnesium and phosphorus levels seem to be higher compared to that of *M. atropurpurea* and *M. utilis* (Mohan and Janardhanan, 1995). The contents of iron, copper and zinc reported in all the three accessions are higher than that of the previous study in the *M. utilis* (Mohan and Janardhanan, 1995). Similarly, the manganese content of all the three accessions seems to be low compared to that of *M. utilis* (Mohan and Janardhanan, 1995). The variability in the content of minerals for the same species may be related to genetic origin, geographical source and the levels of soil fertility. The low sodium range makes the legume a good food source for people on low sodium diets (Vadivel and Janardhanan, 2000).

The investigated tribal pulse, itching bean exhibits the highest level of niacin content (Table 2.11). According to the author, this was found to be higher than that of earlier reports in *Cajanus cajan, Dolichos lablab, D. biflorus, Mucuna pruriens, Phaseolus mungo, Vigna catjang* and *Vigna* sp (Rajyalaksmi and Geervani, 1994) *Rhyncosia filipes, R. suaveolens, Vigna unguiculata* subsp. *unguiculata, V. unguiculata* subsp. *Cylindrical* (Arinathan *et al.*, 2009; Arinathan *et al.*, 2003). The investigated tribal pulse also registers higher level of ascorbic acid content than *Cicer arietnum* (Fernandez and Berry, 1988), *Atylosia scarabaeoides, Lablab purpureus* var. *lignosus, Dolichos trilobus* and *Teramnus labialis* (Arinathan *et al.*, 2009; Arinathan *et al.*, 2003).

The amino acid profiles of the purified seed proteins and the essential amino acid score are presented in Table 2.12. The essential amino acid profiles of total seed proteins compared favourably with the FAO/WHO (1991) requirement pattern, except that there are deficiencies of sulphur containing amino acid in all the three accessions and also in the leucine in Thallaianani

accession, lysine in Siruvani accession and tryptophan contents in all the accessions. All the three accessions of seed materials of itching beans, the Thallaianai accession registers the highest level of *in vitro* protein digestibility (75.16%) compared to other accessions and its protein digestibility is found to be higher than that of an earlier study in the other species of *Mucuna* (Ravindran and Ravindran, 1988) and Soya beans (Ekfenyong and Brochers , 1979). In the present study, the high level of trypsin inhibitor activity in the Saduragiri accession (48.30 TIU mg-1 proteins) might be attributed for low protein digestibility. The presence of antinutritional factors is one of the major drawbacks limiting the nutritional and food qualities of the legumes (Salunkhe *et al.*, 1982). For this reason, a preliminary evaluation of some of these factors in raw itching bean is made. Total free phenolics occurred within the range of 4.38 – 5.02% and tannins ranged from 0.24 to 0.30%. Tannins have been claimed to affect adversely protein digestibility (1988). In *Mucuna* beans, Ravindran and Ravindran and Mary Josephine and Janardhanan (Mary Josephine and Janardhanan, 1992; Ravindran and Ravindran, 1988) reported that most of the tannins are located in the seed coat with only traces in the cotyledons.

Table 2.12: Amino acid profiles of acid- hydrolysed, purified total seed proteins of the seeds of three accessions of *Mucuna pruriens* var. *pruriens* (g 100g-1)a.

Amino acid	Saduragiri	EAAS	Siruvani	EAAS	Thallaianai	EAAS
Glutamic acid	14.11		13.50		10.38	
Aspartic acid	12.98		12.44		13.11	
Serine	4.40		3.50		4.24	
Threonine	3.78	111.67	4.12	121.17	3.56	104.70
Proline	2.80		4.04		2.44	
Alanine	4.24		4.94		5.12	
Glycine	5.95		4.44		4.90	
Vaaline	3.90	111.42	4.06	116.00	3.56	101.71
Cystine	0.54		0.73	1.01		
Methionine	1.24	71.20	0.68	56.40	0.74	70.00
Isoleucine	5.94	212.14	6.94	247.85	6.24	222.85
Leucine	7.24	109.69	6.30	95.45	5.94	90.00
Tyrosine	4.94		5.01		4.24	
Phenylalanine	3.98	141.58	3.44	134.12	4.01	130.95
Lysine	6.01	103.62	5.68	97.93	6.61	113.96
Histidine	4.44	233.68	3.60	189.47	2.90	152.63
Tryptophan	0.88	80.00	0.92	83.63	0.56	50.90
Arginine	5.06		5.94		6.66	

EAAS-Essential amino acid core (Fathima *et al.*, 2010).

Since the seed coats are usually removed by soaking prior to consumption, the tannins in *Mucuna* beans are of little significance from the nutritional point of view. Besides, Siddhuraju *et al* and Vijayakumari *et al* (Vijayakumari *et al.*, 1996; Siddhuraju *et al.*, 1996) reported that in *M. pruriens*, the levels of phenolics and tannins are reduced significantly during dry and wet heat treatments and their reduction improves the protein digestibility.

2.10 Ground Beans-Kersting's *(Kerstingiella geocarpa)*

Groundbean (*Kerstingiella geocarpa harm*) is a lesser known and under-exploited grain legume crop. It is said to have originated in the savanna areas of West Africa and has a very restricted range of cultivation being confined to tropical Africa, particularly Nigeria, Mali, Burkina-Faso, Upper Volta, Niger, Benin and Togo (Kay, 1979; Obasi and Agbatse, 2003) where it is grown at subsistence level.

Germination is one of the methods used in elimination of various anti-nutritional factors present in foods. It is a natural process in which dormant but viable seeds are induced to start growing into seedlings. This is the process by which amylase degrade starches into dextrin and maltose. Germination of seed is a simple process that does not require sunlight or soil and requires only short sprouting time. However, its yield is high (Chen, 1970). It is characterized by a changing array of enzymatic activities. Some of these activities increase dramatically from an initially low or even undetected state to a moderate level. Later these activities then decline to an activity lower than initially present. Another group of enzyme activities are found in dry seed at an intermediate level which remain constant throughout germination e.g, amylase, amyloglucosidase and gluthamine. The enzymes convert the stored foods such as insoluble carbohydrates and proteins to soluble components (Enwere, 1998). Nout and Ngoddy (1997)

reported that germination of seeds triggers the enzyme systems that cause breakdown of complex macromolecules of proteins, carbohydrates and lipids into simpler forms that are much easily assimilated. Proteins are broken down to peptides and amino acids by protease activity. Nitrogen is transferred to developing axis, carbohydrate to simpler sugars by amylase, phytic acid to inositol and phosphoric by phytase and breakdown of tannin-proteinenzyme- mineral complexes. Sprouting seeds of most cereals and legumes have shown improvements in nutrients in human diet and compare well with their fresh counterparts if not better (Kakade and Evans, 1966; Kylen and McCready, 1975).

Studies have shown that although legumes are known for their high protein content, their utility is limited because of the low protein digestibility. A combination of sprouting and cooking resulted in an excellent digestibility coefficient (El-Hag *et al.*, 1978; Ologhobo and Fetuga, 1986). Germination resulted in greater retention of all minerals and B-complex vitamins compared to cooking treatment in chickpeas (El-Adawy, 2002). Germination increased amount of thiamin, riboflavin, niacin and ascorbic acid in both soybean and mung bean (Abdullah and Baldwin, 1984). Mineral values in sprouted beans increased with germination except with iron. Iron values decreased in sprouted seeds but its availability increased due to an increase in phytase activity during seed germination (Bates *et al.*, 1977; Walker and Kochhar, 1982). As germination takes place, the anti-nutritional factors are greatly decreased to insignificant levels or to nothing (El-Adawy, 2002; Ugwu and Oranye, 2006).

Table 2.13: Proximate composition of ungerminated and germinated groundbean

Composition (%)	GGB0	GGB24	GGB48	GGB72	GGB96	±SEM
Moisture	8.75	8.25	8.25	8.75	8.55	0.022
Crude protein	22.19	22.00	22.19	22.29	22.61	0.060
True protein	20.86	20.68	20.86	21.26	21.25	0.040
True nitrogen	3.36	3.31	3.34	3.40	3.40	0.015
Non protein nitrogen	0.21	0.21	0.21	0.22	0.22	0.000
Ash	2.65	3.45	3.81	3.95	3.59	0.015
Fat	1.05	1.23	1.20	1.11	1.22	0.013
Fiber	2.92	3.48	3.25	3.36	3.62	0.017
Carbohydrate	67.40	61.59	61.29	60.14	60.41	0.064

Values are means of triplicate samples ±SEM; Means bearing different superscripts in the same column differed ($p<0.05$) GGB0 = 0h Germinated Groundbean GGB24 = 24h Germinated Groundbean GGB48 = 48h Germinated Groundbean GGB72 = 72h Germinated Groundbean GGB96 = 96h Germinated Groundbean (Echendu, *et al.*, 2009).

2.11 Black gram (*Vigna mungo*)

Black gram or urid is one of the important pulse crops in India. Black gram (*Vigna mungo* L) reported to be originated in India. Its references have also been found in Vedic texts such as Kautilya's 'Arthasasthra' and in 'Charak Samhita' lends support to the presumption of its origin in India. India is the largest producer and consumer of Black gram in the world.

Black gram is a rich protein food. It contains about 26% protein, which is almost three times that of cereals. Black gram supplies a major share of protein requirement of vegetarian population of Indian. It is consumed in the form of split pulse as well as whole pulse, which is an essential supplement of cereal based diet. The combination of dal-chawal (pulse-rice) or dal-roti (pulse-wheat bread) is an important ingredient in the average Indian diet. The biological value improves greatly, when wheat or rice is combined with Black gram because of the complementary relationship of the essential amino acids such as arginine, leucine, lysine, isoleucine, valine and phenylalanine etc.

In addition, being an important source of human food and animal feed, it also plays an important role in sustaining soil fertility by improving soil physical properties and fixing atmospheric nitrogen. Being a drought resistant crop, it is suitable for dryland farming and predominantly used as an intercrop with other crops. The chemical composition of Black gram is given as under:

Table 2.14: Nutritional composition of Black gram

Calorie	Crude protein	Fat	CHO	Ca	Fe	P	Vitamin (mg/ 100g)		
(cal./100g)	(%)	(%)	(%)	(mg/100g)			B1	B2	Niacin
350	26.2	1.2	56.6	185	8.7	345	0.42	0.37	2.0

Source: Pulse Crops, IARI, New Delhi, (2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials-Beans

Eight different local beans species were screened for their urease activities in this experiment. The beans samples were identified at International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The common and botanical names of the beans samples are shown in table 3.10. Six of the samples Lima beans-*Phaseolus lunatus*, Mexican yam beans-*Parchyrhizus tuberrosus*, African yam beans-*Sphenostylis stenocarpa*, Soya bean-*glycine max*, Pigeon pea-*Cajanus cajan*, Black gram-*Vigna mungo*, were procured from Sabo market in Kaduna, Kaduna State-Northern Nigeria and the remaining two; *Mucuna prureins* and Kerstingella-*Kerstings geocarpa* were got from Owo in Ondo State-Western Nigeria, in the month of December, 2010.

Table 3.1: Taxonomic Identification of Beans

Common name	Botanical name	Identity abbreviation
Lima beans	*Phaseolus lunatus*	Tpl
Mexican yam beans	*Parchyrhizus tuberrosus*	Tptu
African yam beans	*Sphenostylis stenocarpa*	Tss
Soya beans	*Glycine max*	---
Pigeon pea	*Cajanus cajan*	Tcc
Mucuna spp	*Mucuna pruriens*	---
Kerstingiella	*Kerstings geocarpa*	Tkg
Black gram	*Vigna mungo*	---

3.2 Equipment and Laboratory Apparatus

- RC-16M refrigerated centrifuge Sherwood medicals England.
- Biochrom (Biochrom Ltd) UV 2800 Double beam; UV/VIS scanning spectrophotometer.
- DNP-982 Laboratory incubator; Gulflex Medicals and Scientific England.
- Thermo cool refrigerator
- pH meter- Crison micropH 2000
- Weighing balance – Scout pro OHAUS-200g and Brain weigh B-300 OHAUS.
- General glasswares

3.3 Reagents and their Preparation

3.3.1 Reagents

All the reagents used were of analytical grades

- Tris buffer (Laboratory Technology Chemicals, pH=7.5-10.0)
- Acetic acid (BDH chemicals)
- Nessler's reagent (Sodium Hydroxide, potassium Iodide and Mercuric chloride- BDH chemicals)
- Acetone (BDH chemicals)
- Chitosan
- Distilled water
- Deionized water

3.3.2 Preparation of Extraction and Assay Buffers

The buffering system used for the extraction of urease enzyme was Tris-acetate buffer as described by Kayastha and Nilanjana (1999). The extraction buffer contained 25mM, pH 6.5 Tris-acetate. Where the Acid is acetic acid and the Base is Tris.

Acetic acid (0.14ml) was measured into 100ml distilled water and 0.3g of Tris into another 100ml of distilled water. The two solution were titrated (Acid-Base) and the extraction buffer was graduated to pH 6.5 while the assay buffer was graduated to pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 with the pH meter.

3.4 Extraction and Characterization of Urease

Urease from Ungerminated Beans

Urease was extracted from different beans samples according to the method described by Kayastha and Nilanjana (1999). The beans samples were blended coarsely with laboratory mortar and pestle and 50g of the beans meal was soaked overnight in 100ml extraction buffer (0.025m Tris-acetate buffer, pH 6.5) at 4^0C (Refrigeration temperature). The soaked beans meals were swirled for 2 minutes and then sieved with four layers of pre-washed and dried white muslin cloth, the filtrate was centrifuged at 15000rev/min for 15mins under refrigeration condition 4^0C. The clear supematant was collected while the sediment was washed and discarded. The filtrate from the protein was added to the general pool.

3.5 Precipitation with Acetone

Procedure

To the clear suprantant was added a cold acctone in a range of one-third the volume of the crude extract at 0^0C (ice-chest with constant swirling). After 15mins in ice-chest, the suspension (precipitate) was centrifuged at 15000rev/min for 2 mins at 4^0C. Equal volume of cold acetone was added to the supernatant for further precipitation. The precipitate was dissolved in a minimum volume of 0.05M Tris-acetate buffer which represent the crude urease enzyme and was stored at frozen temperature for further analysis.

Germination

The various beans samples were layered on woolen material and also covered with the same material, and water was applied to moisturize the beans. Under this humid and warm condition, the bean seeds initially swelled then began to germinate (develop radicles) after 72hour.
The germination process was stopped by freezing the beans for 12 hours followed by 2days sun drying. The enzyme process was repeated for germinated beans.

3.6 Determination of Protein in Crude Extract

The determination of the protein content of the crude extract is essential for the calculation of the specific activity of the urease enzyme (Bergunger and Bergmeyr, 1986).

3.6.1 Spectrophotometric Determination of Protein

Procedure

To 0.lml of beans crude protein in a test tube was added 9.9ml of 0.05M Tris-acetate buffer (pH6.5) solution. The content was stirred with a glass rod and lml of the diluted crude protien was transferred into a clean test tube with syringe and was made up to 10ml. The absorbance of the sample solution was taken against the Tris-acetate buffer on a UV/VIS spectrophotometer (Biochrom UV 2800 Double beam; UV/VIS scanning spectrophotometer) at two wavelengths 260 and 280nm.

Calculation:

To calculate the protein concetration calcar's emperical formula was used:

$$X = 1.45\ E_{280} - 0.74E_{260}$$

Where x is the protein concentration in solution g/litre and E is the absorbance. E_{280} and E_{260} are absorbance at 280 and 260nm respectively.

3.7 Urease Enzyme Assay

Nesslers' Reagent Preparation

Fifty gram of potassium iodide was dissolved in 50ml of cold distilled water. Then a saturated solution of mercuric chloride (22g of HgCl2 in 350ml of water was added) until an excess was indicated by the formation of a precipitate. Then 200ml of 5N NaOH solution was added. The reaction mixture was stirred and diluted to one liter. The mixture was allowed to settle and filtered. The residue was discarded and the clear supernatant was used. This procedure was

adapted from 'SALT LAKE METALS (Rocky Patterson) COMPANY, 115 EAST BRIGADOON, SALTLAKE CITY UT84117'.

Assay Procedure

To 0.9ml of assay buffer (0.05M Tris-acetate, pH 7.0) was added 0.1ml of appropriately diluted enzyme (20-fold dilution in same buffer) and incubated at 60°C in 1ml of 0.2M urea. After 30mins, the reaction was stopped by reducing temperature to 10°C (stopping an enzyme catalysed reaction is concerned with changing the environmental condition such as the pH and temperature of the enzyme). An aliquot (1ml) of the reaction mixture was transferred to a 50ml beaker and 1ml Nesslers' reagent was added with constant swirling and the volume made up with distilled water after 1min of reaction. The yellow colour produced was measured at 405nm with UV/VIS spectrophotometer (Biochrom UV 2800 Double beam; UV/VIS scanning spectrophotometer). A blank was run without the enzyme and urea. An enzyme Unit (U) in this present work is defined as the amount of enzyme required to liberate 1μmol ammonia in 1min under the reaction conditions (0.2M urea, 0.05M Tris-acetate buffer, pH 7 at 60°C). The experiments were conducted in triplicate.

The urease from both germinated and ungerminated beans were charactirzed for optimum pH, temperature and kinectic parameters under standard assay condition.

3.7.1 Optimum pH

In order to determine the optimum pH for urease activity from germinated and ungerminated beans samples, enzyme activity were assayed in pH ranging from 5.5 to 8.0 at constant temperature 60^0C and 0.2M urea for 30mins.

3.7.2 Optimum Temperature

To determine the optimal temperature up to which the urease enzyme from both germinated and ungerminated beans can withstand temperature urease enzyme was suspended in Tris-acetate buffer (0.05 M) and optimum pH above, and incubated at different temperatures (30 to 80 °C) for 30 min before the activity was measured.

3.7.3 Determination of the Kinetic Parameters (*Vmax* and *Km*)

To determine the kinetic parameters (*Km* and *Vmax*) the substrate concentration was varied (0.1-0.6M), at optimum pH and temperature of urease from germinated and ungerminated beans. The values of *K*m and *V*max were determined using Lineweaver–Burke plots.

The catalytic efficiency was determined for urease from germinated and ungerminated beans samples: V_{max}/K_m according to the method of Juan *et al.*, (2010).

3.8 Partial Purification of Urease

Urease would be further purified from any samples that showed higher activity compared to soya beans and pigeon pea for immobilization. These samples (soya beans and pigeon pea) have been shown in literature to have high urease activity (Kayastha, 1999 and Polacco & Holland, 1993; 1994). The protein so precipitated was redissolved in Tris buffer (10ml) and loaded (2ml) onto a sephadex G-100 column (3.0x15cm), previously equilibrated with 0.05M Tris-acetate buffer, pH 7.0 (15ml/g bed volume). The adsorbed protein was eluted using 0.1 M Tris-acetate buffers, pH7.0. Eluents were collected at 20ml per tube. After collecting the pass through, the column was washed with two-bed volumes of the same buffer.

3.9 Urease Ezyme Assay

The protein content of the fractions (20ml eluent) were determined spectrophotometrically following the procedure discussed earlier

3.10 Preparation of Immobilization Buffer

The preparation of immobilization buffer followed the same procedure as the extraction buffer. The buffering system contained 100mM Tris-acetate (pH 7.0).

The enzyme assay was performed in each eluent collected (20ml). The reaction mixture contained 0.1M Tris-accetate buffer, pH 7.0, 0.02M urea and the enzyme was incubated for 30min at 37°C, and the reaction was stopped reducing the temperature to 10^0C. Urease activity was determined by measuring the amount of ammonia released from urea using the Nessler's reagent. The reaction was monitored spectrophotometrically at 405nm. The protein level of the active fractions (100-140ml) was determined before and after immobilization.

3.10.1 Formation of Chitosan Beads

Chitosan beads were prepared according to the method Nastratum *et al.*, (2009). The chitosan powder (3g) was first dissolved in 1% acetic acid. The solution of the chitosan powder was then introduced into a 20ml syringe and extruded through the syringe needle into a solution of 1M NaOH containing 26% (v/v) ethanol under constant stirring. The spherical beads (5mm in diameter) were formed as a result of coagulating effect of NaOH and ethanol. The mixture was allowed to remain overnight at 4°C refrigeration temperature. The spherical beads were harvested by filtration and washed with deionized water until neutrality and were stored in deionized water at 4^0C until needed.

3.10.2 Immobilization Urease Enzyme

Procedure

The immobilization was performed according to the method of Zu pei LiANG, *et al.*, (2005). The chitosan beads were immersed in urease solution with a given enzyme/beads ratio (2ml/gbeads). The mixture was gently stirred for 10mins and then placed in refrigerator at 4^0C for 24hours. The supernatant was removed and the beads washed three times with deionized water. The immobilized urease was recoverved from spent solution and stored at 4^0C for reuse.

3.10.3 Assay for the Activity of Free and Immobilized Urease

The urease enzyme activities were determined in both free and immobilized enzyme, according to the reaction condition stated in section 3.9. There after the free and immobilized urease were characterized for optimum temperature, substrate concentration, storage ability and reusability.

3.10.4 Optimum Temperature

To determine the optimal temperature at what limit the immobilized enzyme can withstand thermal stress, free and immobilized enzyme were suspended in Tris-acetate buffer (0.10 M, pH 7) and incubated at different temperatures (30 to 80 °C) for 30 min before the activity was measured as described in section 3.9.

3.10.5 Determination of the Kinetic Parameters (*Vmax* and *Km*)

To determine the kinetic parameter of the partially purified urease, the substrate concentration was varied (0.01-0.1M) for both free and immobilized enzyme at optimum temperature, 40^0C and 0.1M Tris-acetate buffer pH7.0. The rate of reaction was measured at 405nm. The values of *Km* and *Vmax* were determined by using Lineweaver-Burke double reciprocal plots.

3.10.6 Storage Ability and Reusability

To determine the storage ability of free and immobilized urease, the activity was monitored weekly. However, the reusability of the immobilized enzyme was determined by using it repeatedly at 40^0C, 0.02M urea and 0.1M Tris-acetate buffer (pH 7.0).

3.11 Statistical Analysis

All data obtained in this work were statistically analyzed on the basis of germinated and ungerminated beans samples, using descriptive, analysis of variance and Students T-test with SPSS 15.0, Graphpad and SAS system for windows 9.0, at $p < 0.05$ level of significance.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Protien Level in Germinated and Ungerminated Beans

The results of protein level in germinated and ungerminated beans are presented in Table 4.1 respectively. The result showed that, germination significantly ($p<0.05$) reduced the protein level in the beans when compared to the ungerminated beans samples. However, effect of germination on protein level of *Cajanus cajan* was significantly ($p<0.05$) higher than other beans samples with 71.36% and 3.49fold decrease in protein level, whereas, the effect of germination on *Parchyrhizus tuberrosus* was least significant ($p<0.05$) with 2.89% and 1.03fold decrease in protein level when compared to others. Additionally, the protein content of both germinated and ungerminated *Mucuna pruriens* was significantly ($p<0.05$) higher compared to protein of other beans samples.

4.2 Urease Specific Activity in Germinated and Ungerminated Beans

The urease activities pattern in germinated and ungerminated beans is shown in Table 4.2. From the result, germination significantly ($p<0.05$) increased the urease activity compared to the ungerminated beans samples. The effect of germination on urease activity in *Glycine max* was significantly ($p<0.05$) higher than other beans sample with 672.20% and 7.72fold increase, while, germination effect on *Parchyrhizus tuberrosus* was least significant ($p<0.05$) with 13.70% and 1.14fold increase in urease activity when compared to others (See Appendix A). However, germination reduced the urease activity in *Sphenostylis stenocarpa*.

Table 4.1: Protein Level in Germinated and Ungerminated Beans Samples

Beans samples	Germinated (mg/g)	Ungerminated(mg/g)	% Decrease	Fold decrease
Glycine max	60.13 ± 8.89^{b}	90.17 ± 2.11^{a}	33.32	1.50
Parchyrhizus tuber	$43.73 \pm 0.47^{a***}$	$455.03 \pm 7.65^{a***}$	2.89	1.03
Phaseolus lunatus	$46.93 \pm 9.25^{a***}$	51.00 ± 4.16^{ans}	7.98	1.09
Cajanus cajan	15.13 ± 2.14^{bns}	52.83 ± 2.02^{ans}	71.36	3.49
Mucuna pruriens	$91.13 \pm 5.54^{b***}$	$95.57 \pm 7.68^{a**}$	4.65	1.05
Kerstings geocarpa	$29.30 \pm 2.08^{b***}$	67.17 ± 2.14^{ans}	56.38	2.29
Vigna mungo	$22.37 \pm 6.04^{b***}$	72.90 ± 2.08^{ans}	69.31	3.26

Down columns, values with ns, *, ** and *** are statistically different from *Glycine max* at $p < 0.05$, < 0.01 and < 0.001 level of significance respectively. Across rows, values with different letters (superscripts) are statistically different at $p < 0.05$. Each data is mean±SD of three replicates. NS: not significant.

Table 4.2: Urease Specific Ativity in Germinated and Ungerminated Beans

Beans samples	Germinated (U/mg)	Ungerminated (U/mg)	% Increase	Fold increase
Glycine max	6.95 ± 0.05^{b}	0.90 ± 0.03^{a}	672.2	7.72
Parchyrhizus tuber	$1.66\pm0.07^{a***}$	$1.46\pm0.07^{a***}$	13.70	1.14
Sphenostylis stenoc	1.20 ± 0.06^{b}***	1.40±0.06	-16.67	0.86
Phaseolus lunatus	$1.51\pm0.06^{b***}$	1.04 ± 0.06^{ans}	45.19	1.45
Cajanus cajan	$5.47\pm0.19^{b***}$	$1.24\pm0.06^{a***}$	341.13	4.41
Mucuna pruriens	$3.59\pm0.06^{b***}$	$1.17\pm0.03^{a***}$	206.84	3.07
Kerstings geocarpa	$3.49\pm0.10^{b***}$	0.93 ± 0.04^{ans}	275.27	3.75
Vigna mungo	$2.99\pm0.15^{b***}$	$0.77\pm0.04^{a*}$	288.31	3.88

Down columns, values with ns, *, ** and *** are statistically different from *Glycine max* at $p< 0.05$, < 0.01 and <0.001 level of significance respectively. Across rows, values with different letters (superscripts) are statistically different at $p<0.05$. Each data is mean±SD of three replicates. NS: not significant.

4.3.1 Optimum pH

The result of optimum pH for urease extracted from germinated and ungerminated beans samples are presented in Figure 4.1 (a-c) to 4.2 (a-c) and summarized in Table 4.3. The result showed that, the optimum pH of urease from both germinated and ungerminated beans samples ranged between 6.5 and 7.0.

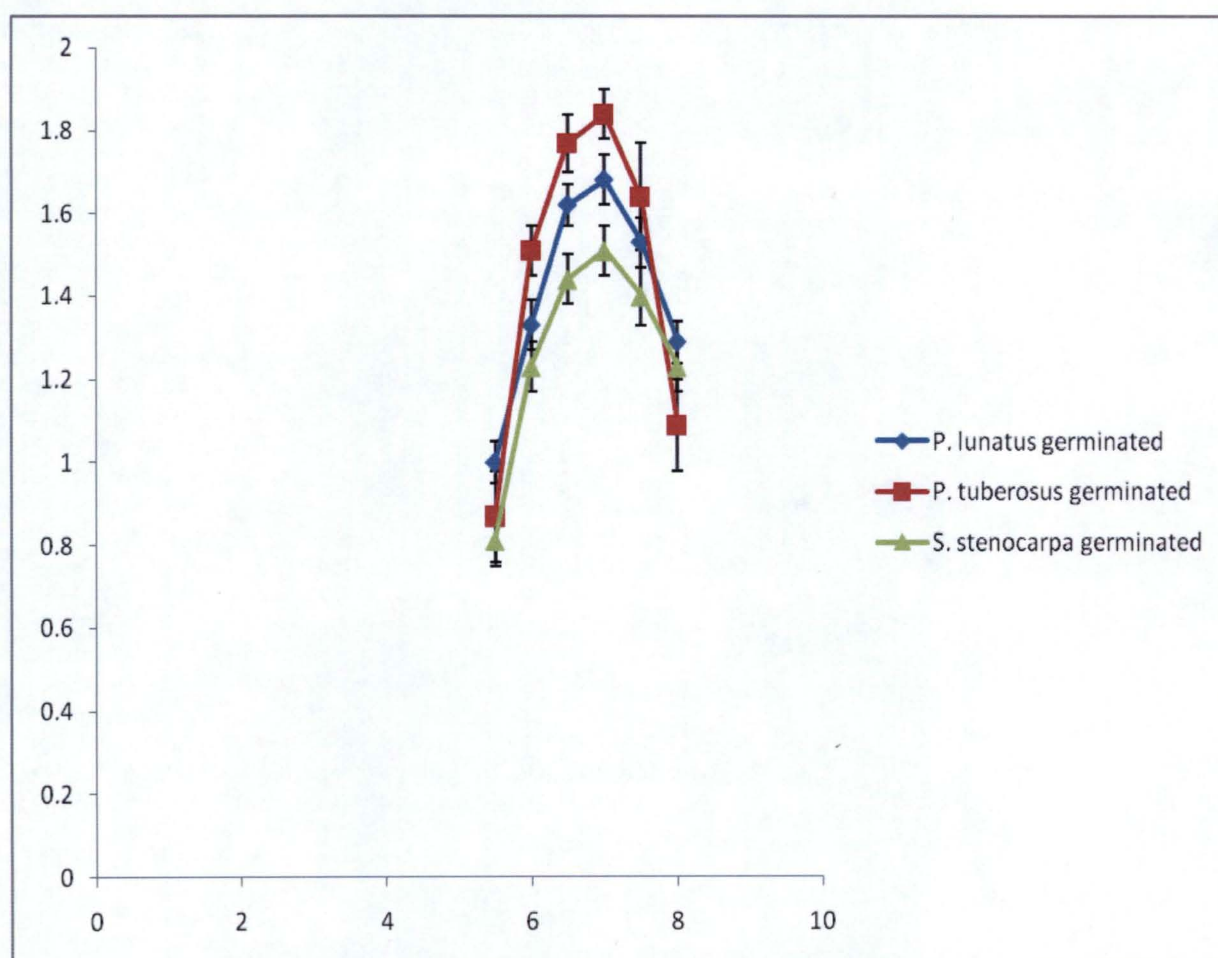


Figure 4.1a: Effect of pH on urease from germinated beans.

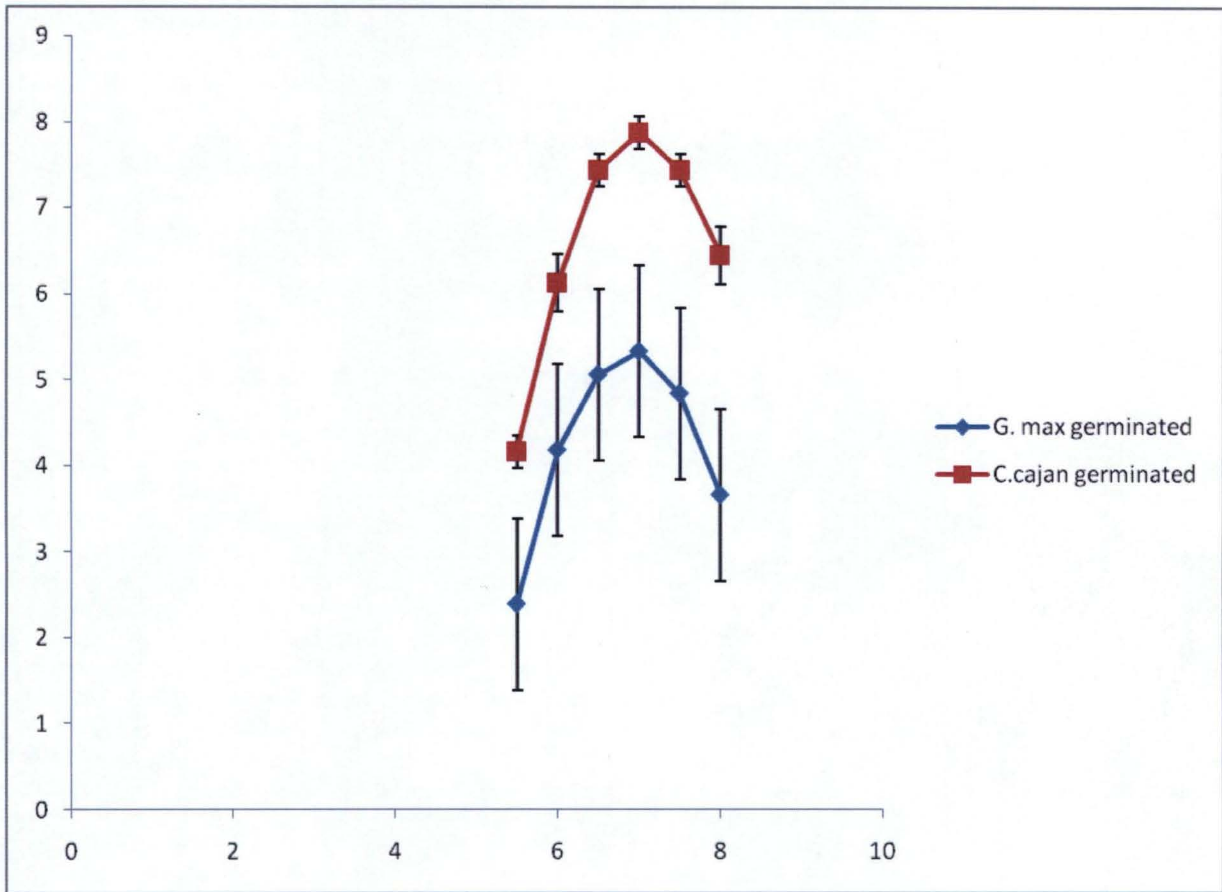


Figure 4.1b : Effect of pH on urease from germinated beans.

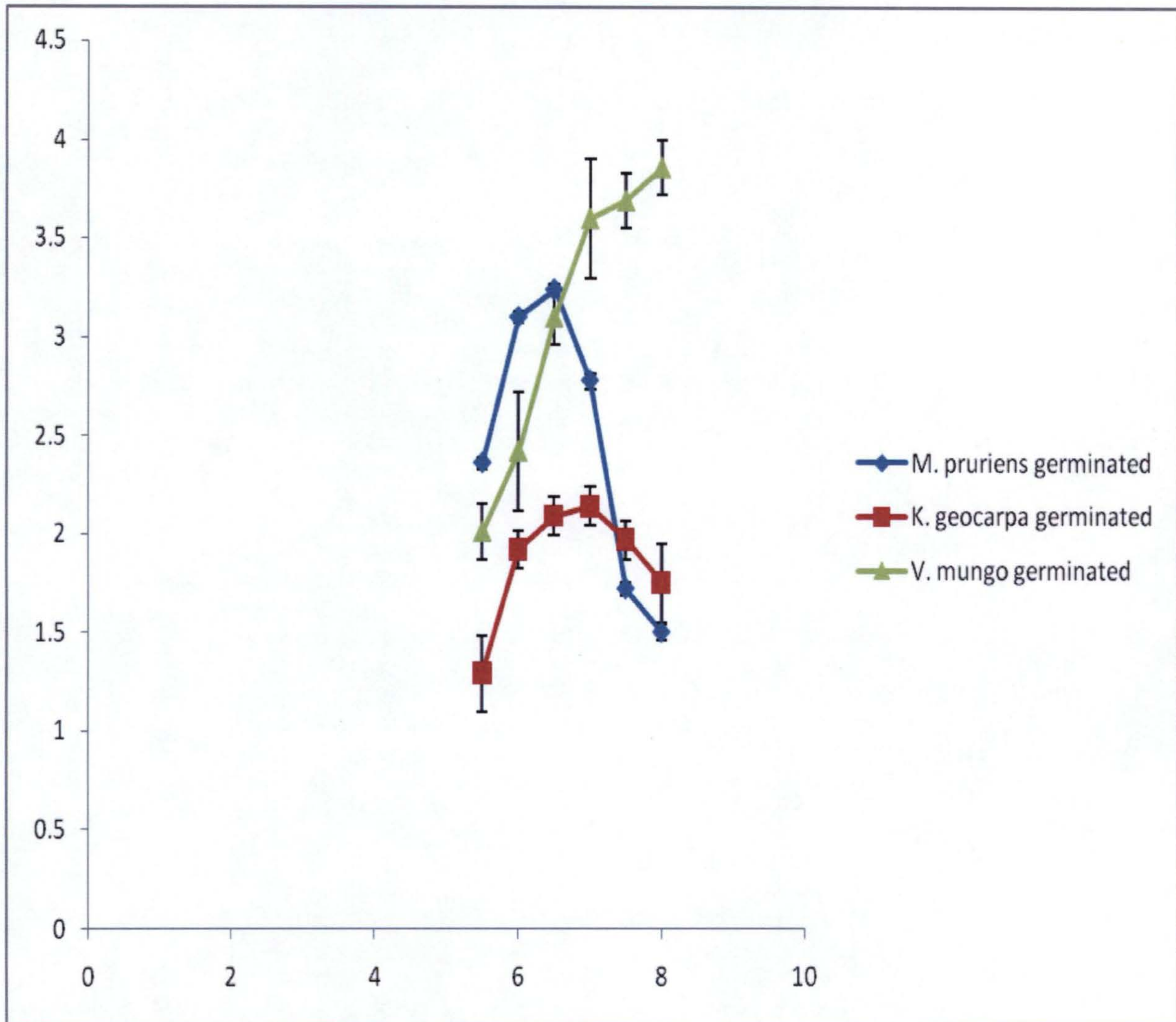


Figure 4.1c : Effect of pH on urease from germinated beans.

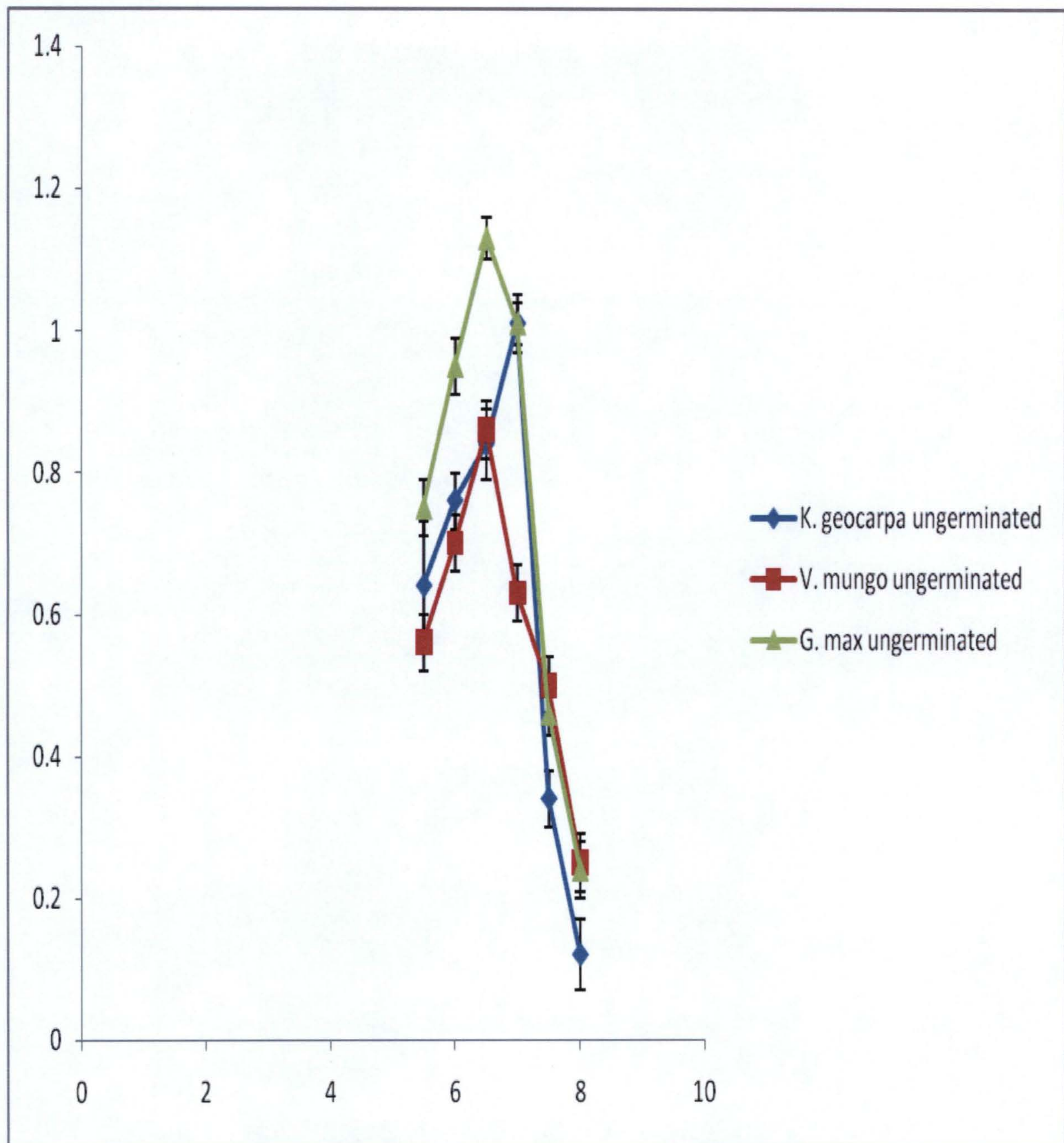


Figure 4.2a: Effect of pH on urease from ungerminated beans.

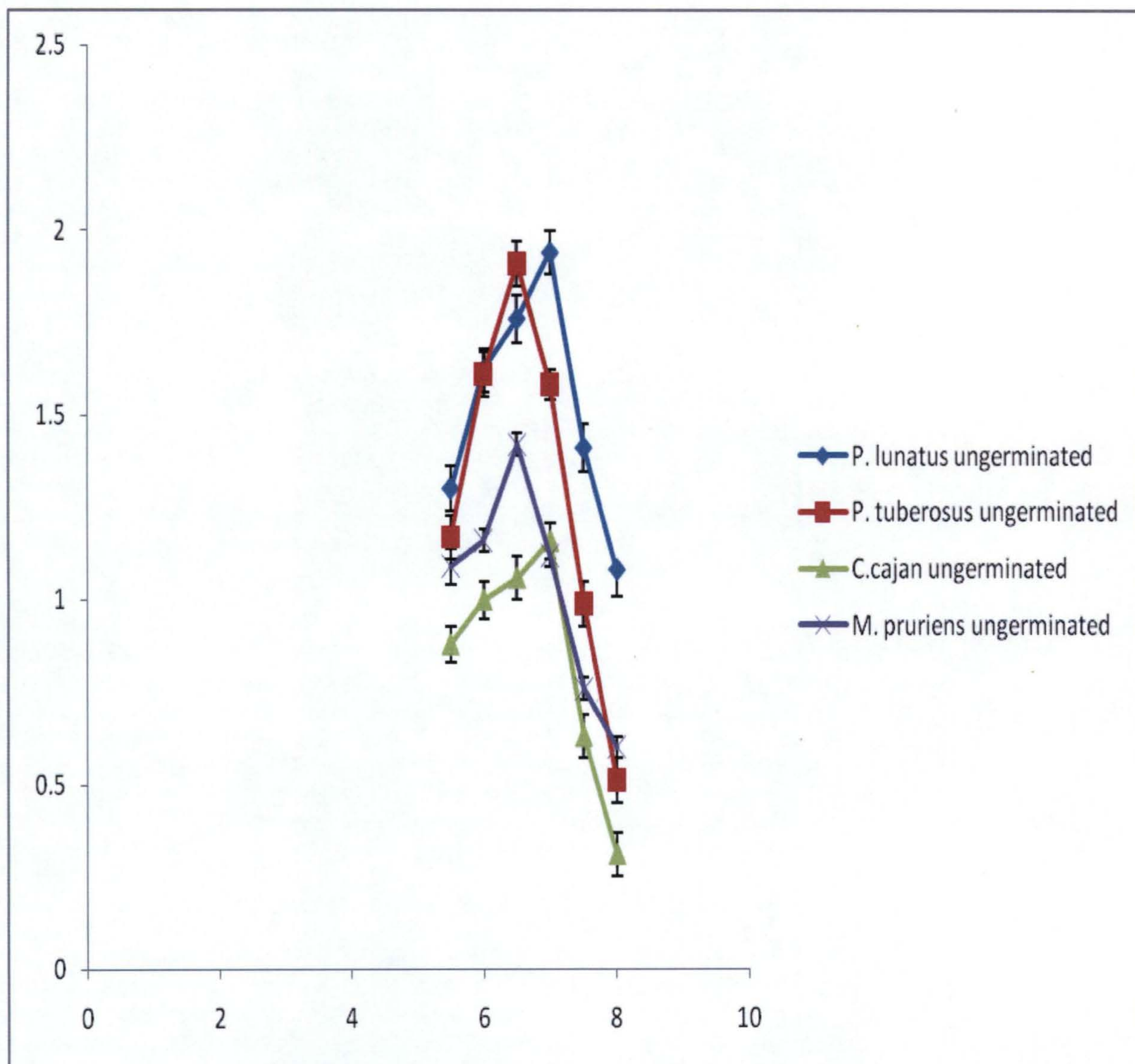


Figure 4.2b: Effect of pH on urease from ungerminated beans.

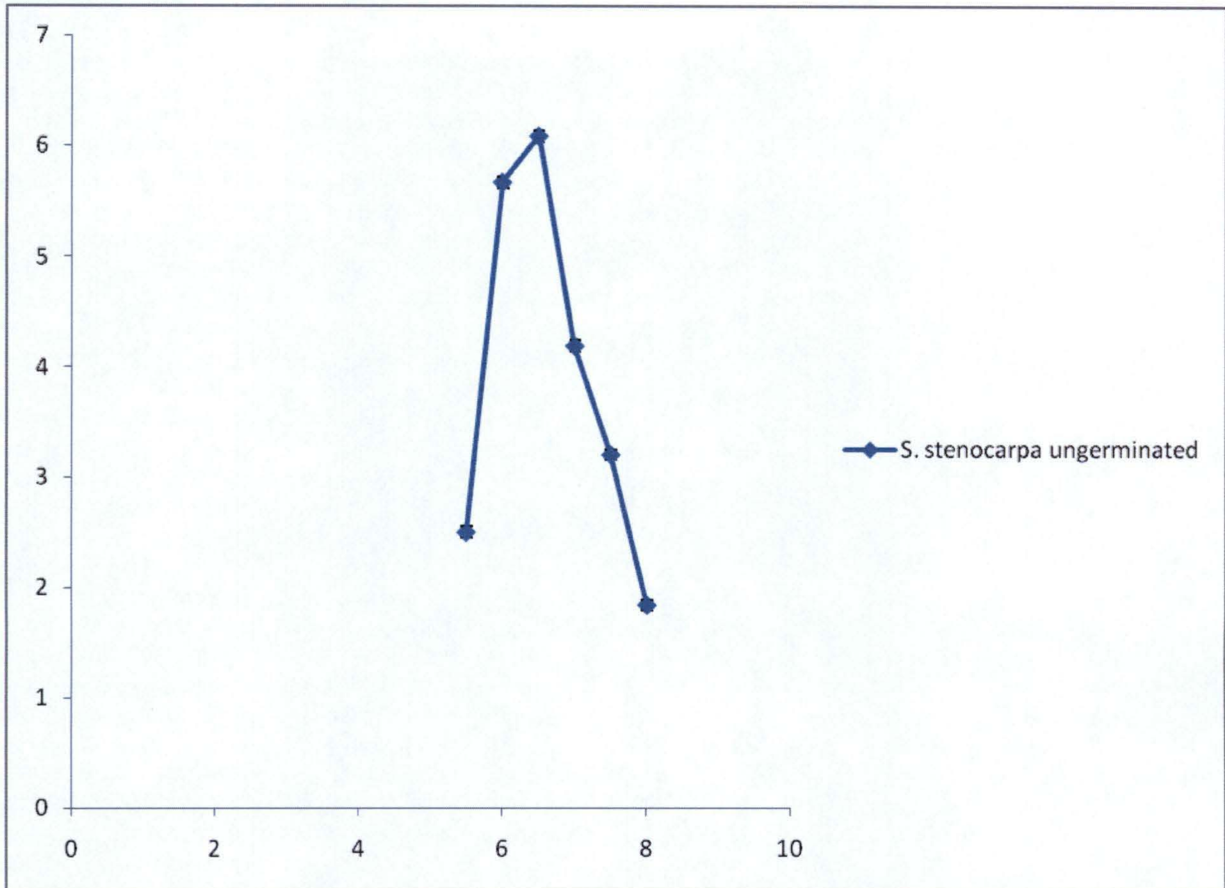


Figure 4.2c: Effect of pH on urease from ungerminated beans.

4.3.2 Optimum Temperature

The results of optimum temperature for urease from germinated and ungerminated beans are shown in Figure 4.3 (a-c) to 4.4 (a-c) and summarized in Table 4.4. Results showed that, the optimum temperature for urease from both germinated and ungerminated beans samples ranged between 65 and 70^0C.

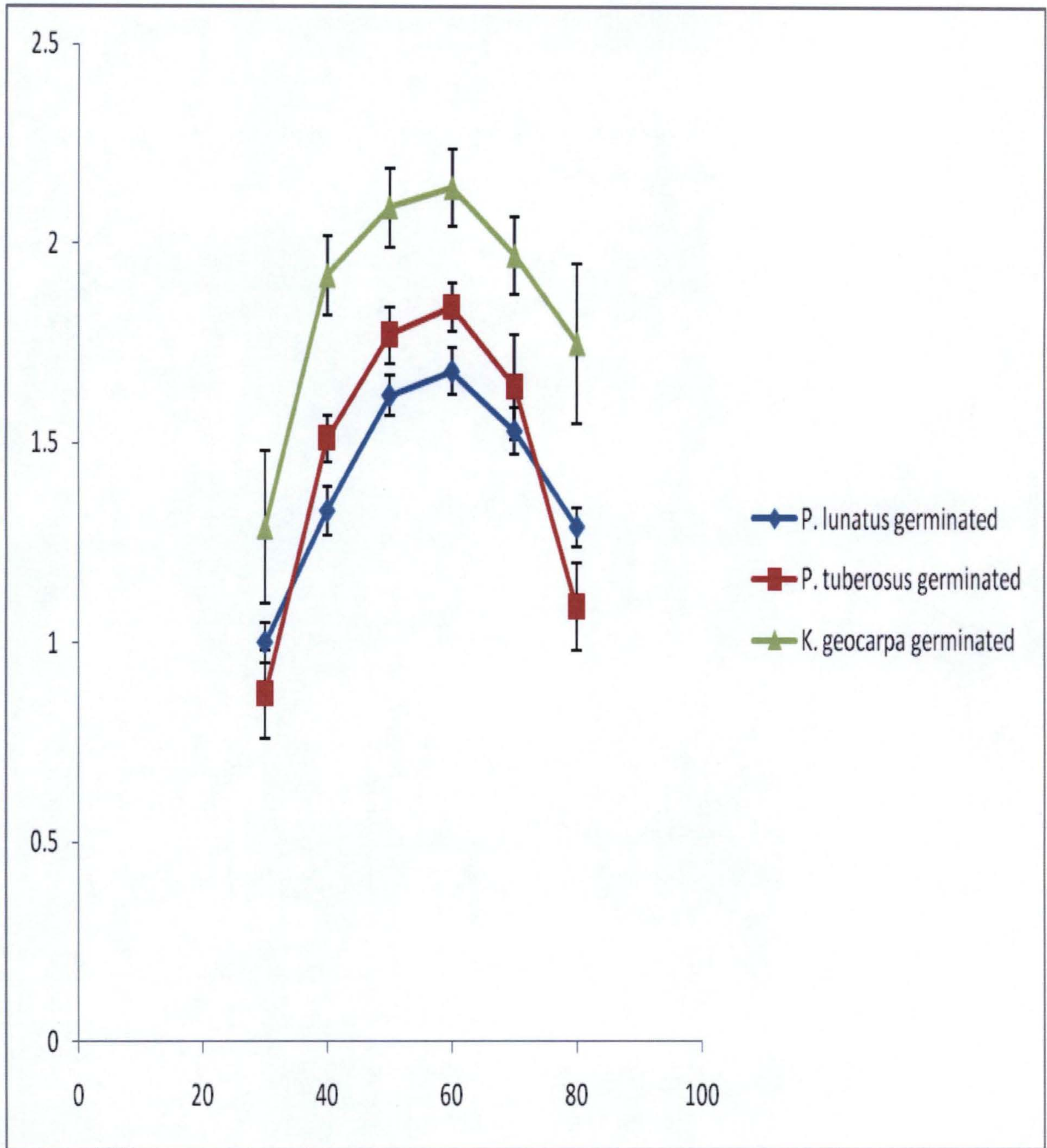


Figure 4.3a: Effect of temperature on urease from germinated beans

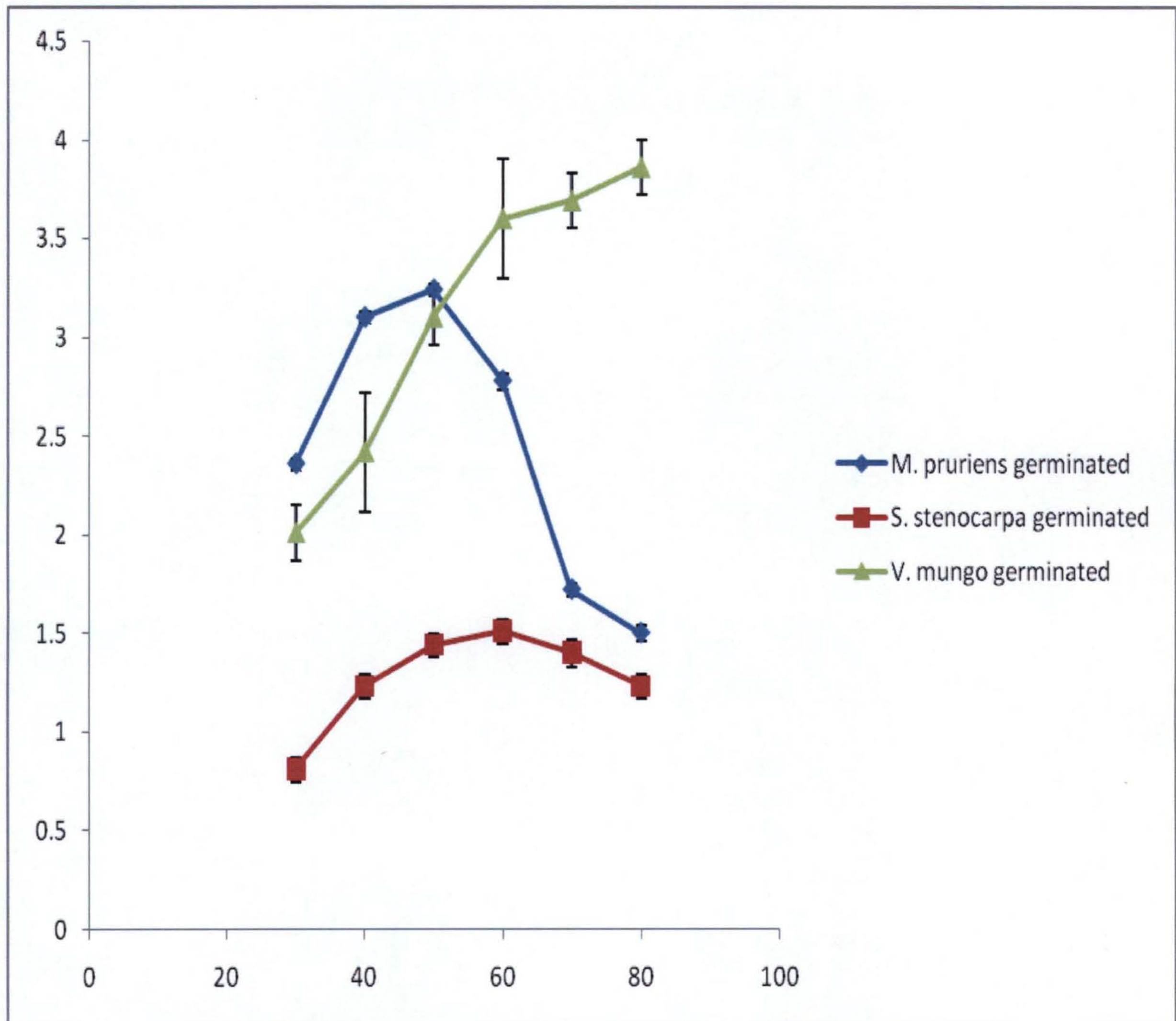


Figure 4.3b: Effect of temperature on urease from germinated beans.

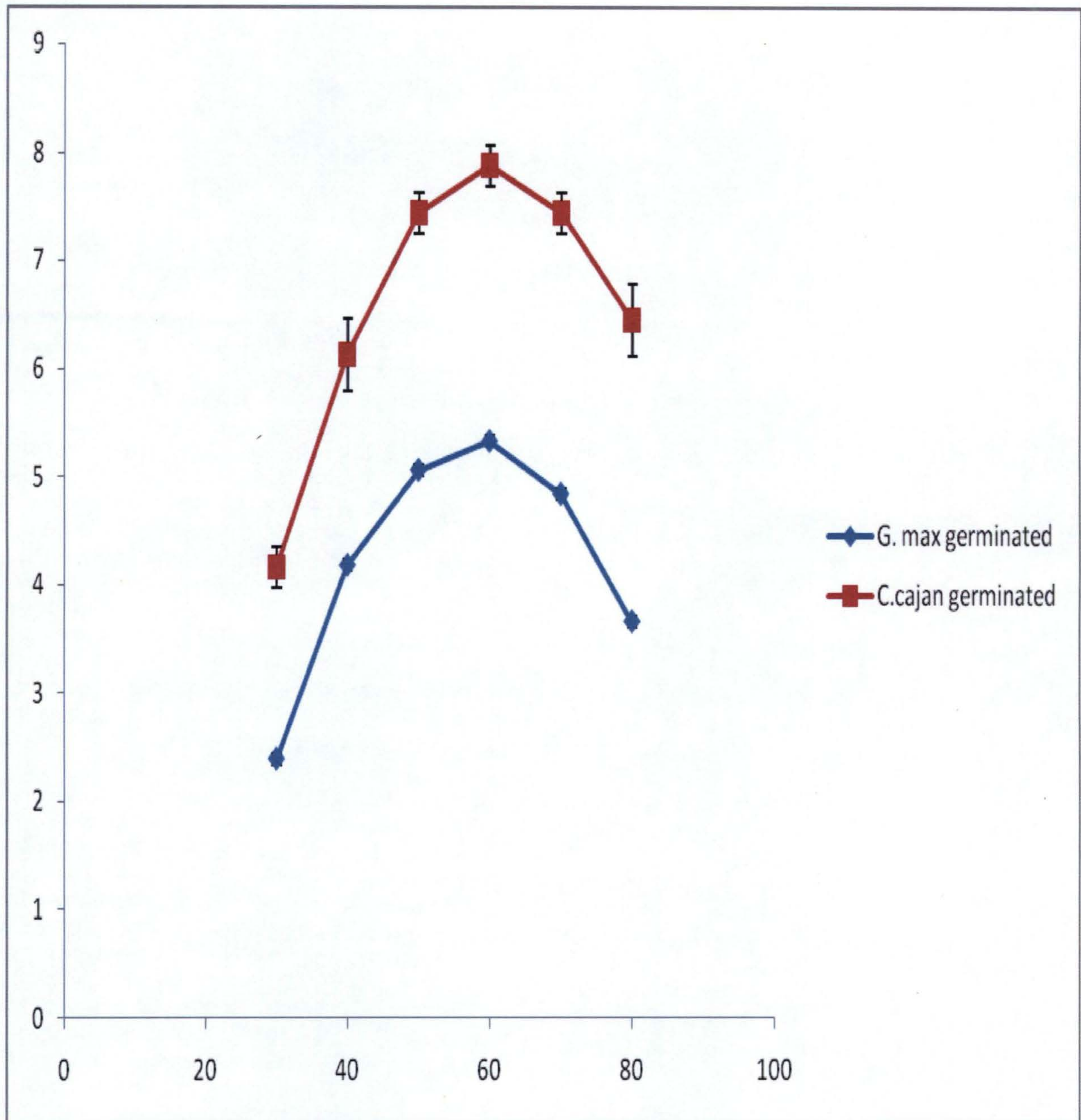


Figure 4.3c: Effect of temperature on urease from germinated beans.

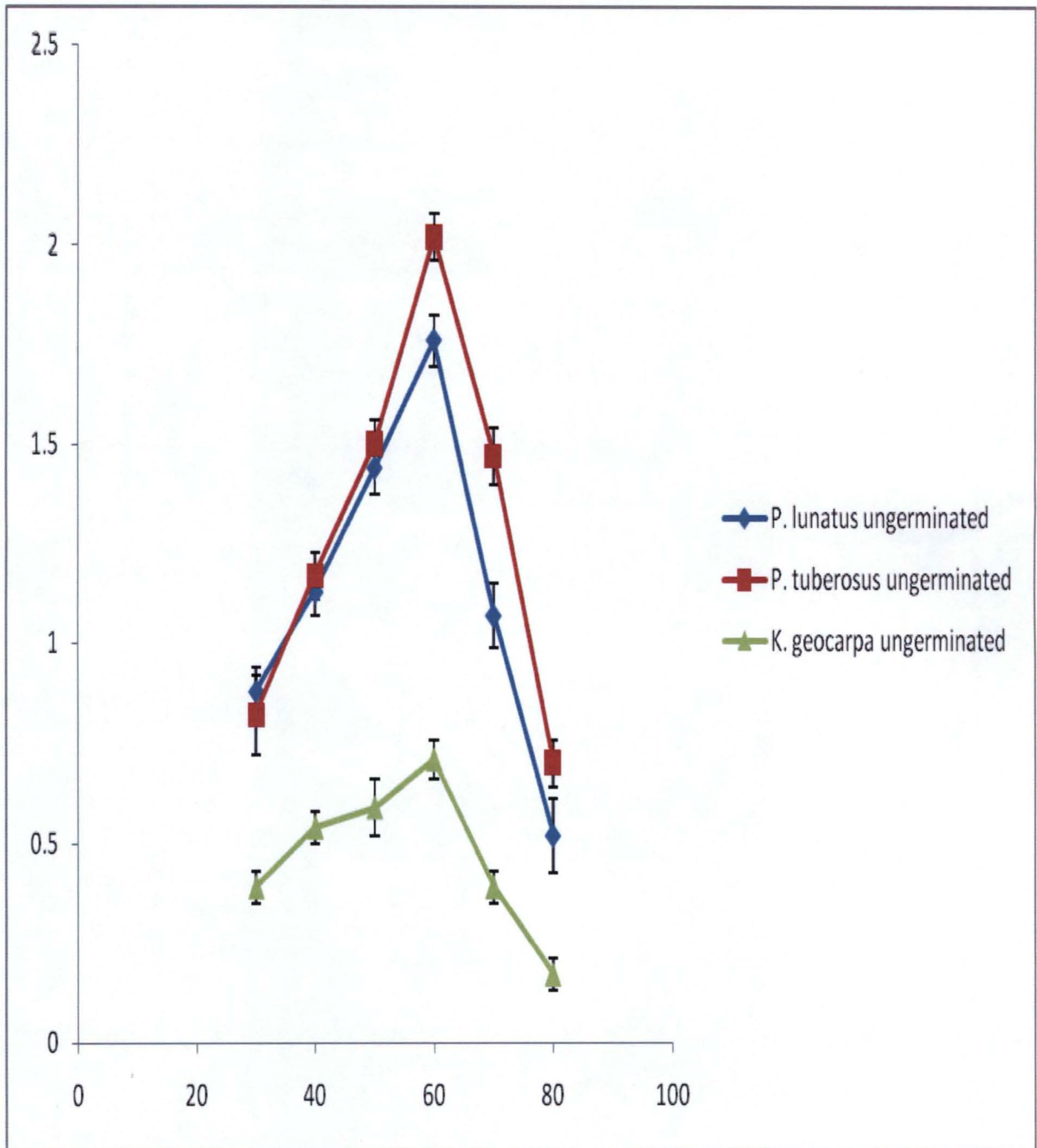


Figure 4.4a: Effect of temperature on urease from ungerminated beans.

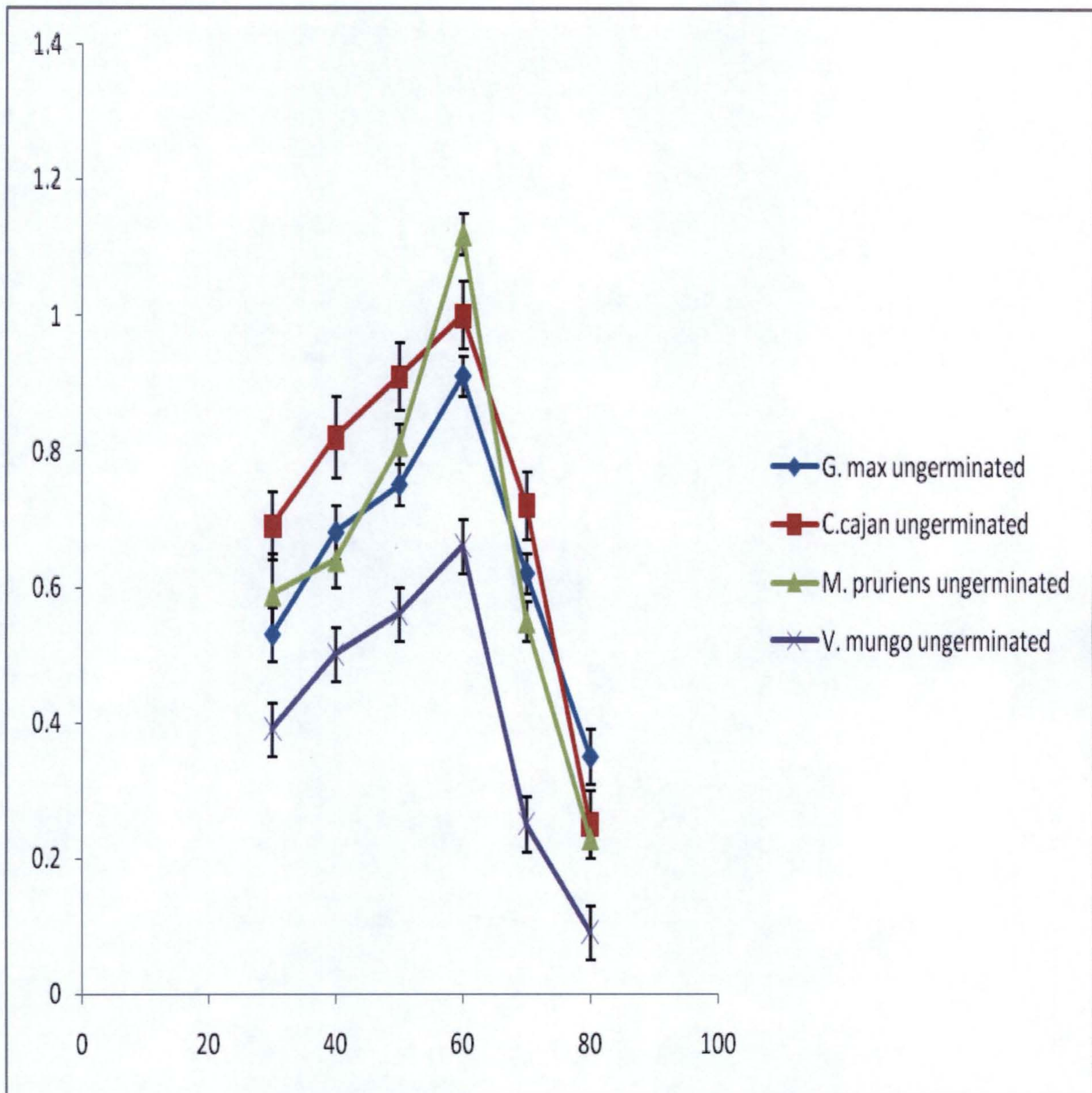


Figure 4.4b : Effect of temperature on urease from ungerminated beans.

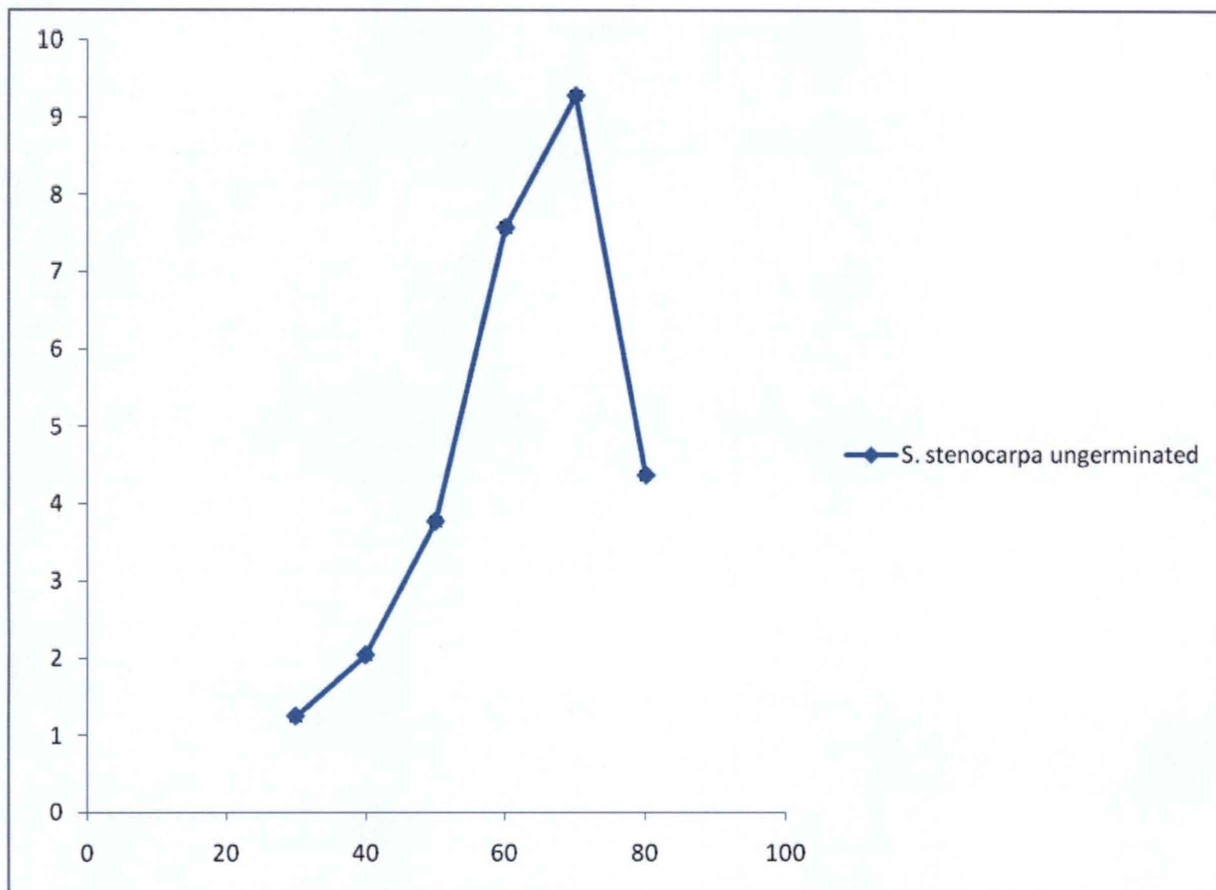


Figure 4.4c: Effect of temperature on urease from ungerminated beans

4.3.3 Substrate Effect and Enzyme Kinetics (V_{max} and K_m)

The results of substrate effect on uresae enzyme activity are presented in Figures 4.5 (a-c) and 4.6 (a-c). From the results, urease activity follows the expected Michelis-Menten curve. The results of reaction velocity (V_{max}) and the Michelis-Menten's constant (K_m) are presented in Figures 4.7 (a,b) and 4.8 (a,b) and the summary of the kinetic parameters in Table 4.3. The V_{max} of urease from germinated beans samples was significantly ($p<0.05$) higher than the ungerminated beans, while the *Km* showed a corresponding Michelis-Menten pattern

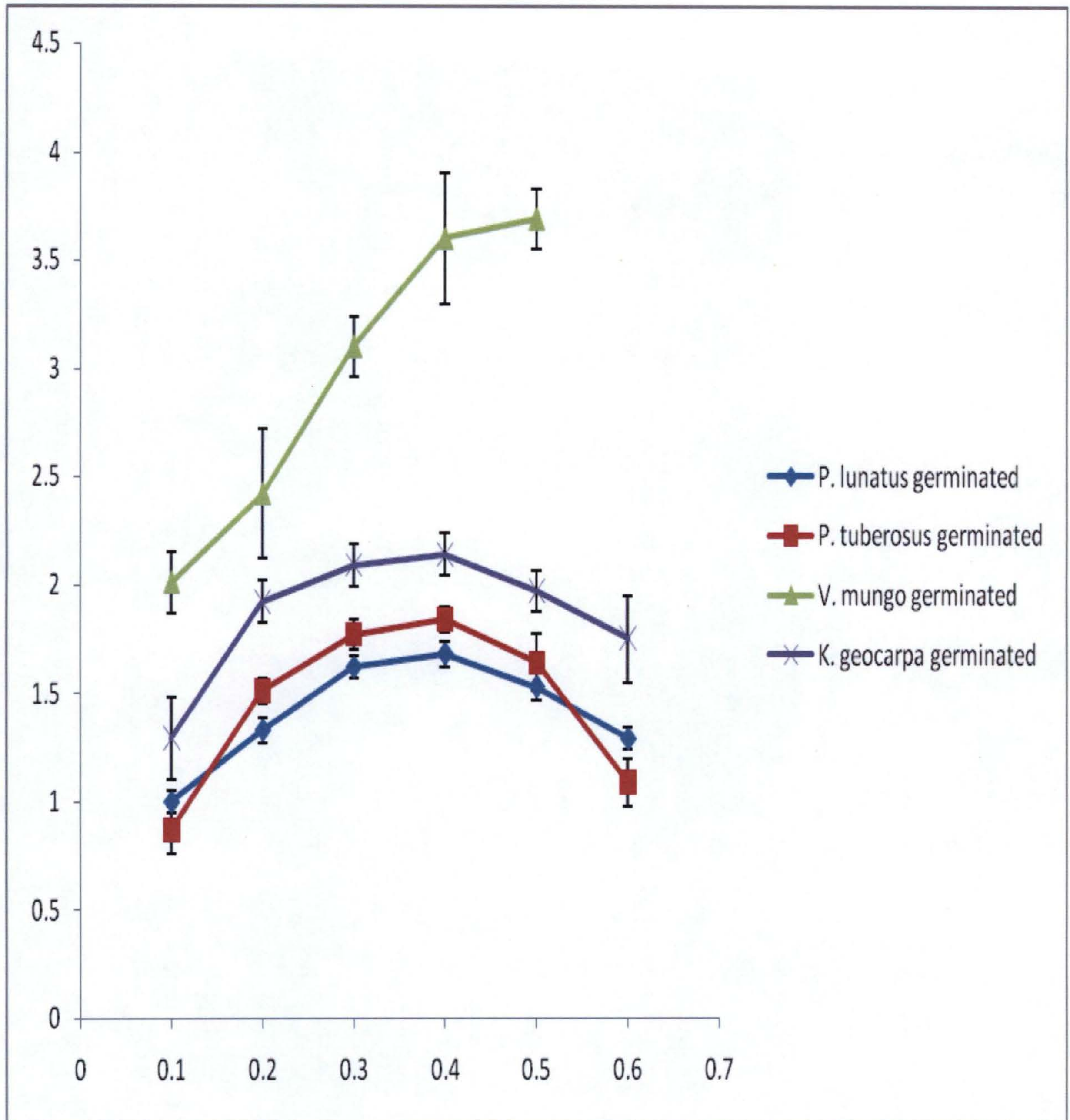


Figure 4.5a: Effect of substrate concentration on urease from germinated beans

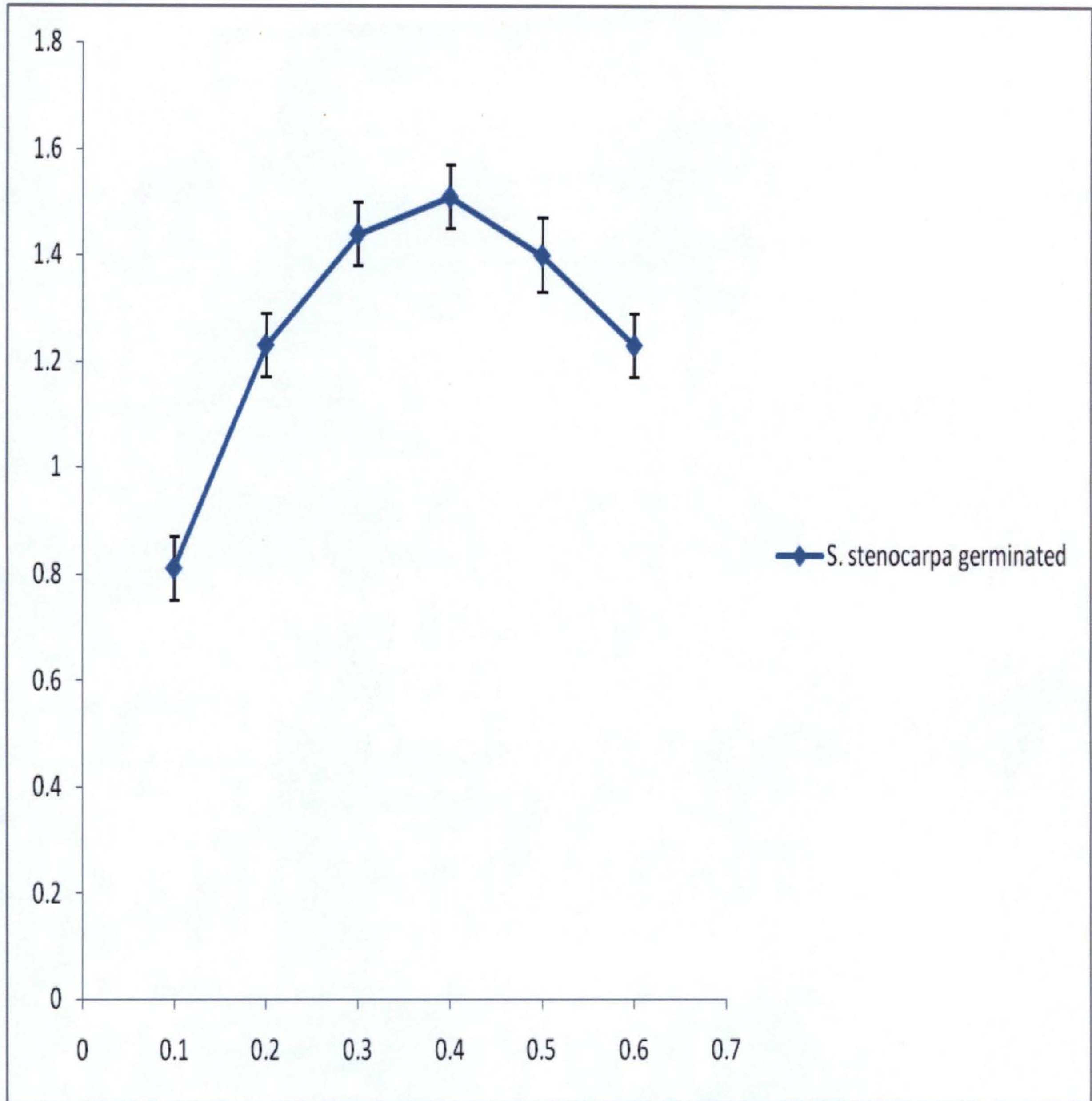


Figure 4.5b: Effect of substrate concentration on urease from germinated beans

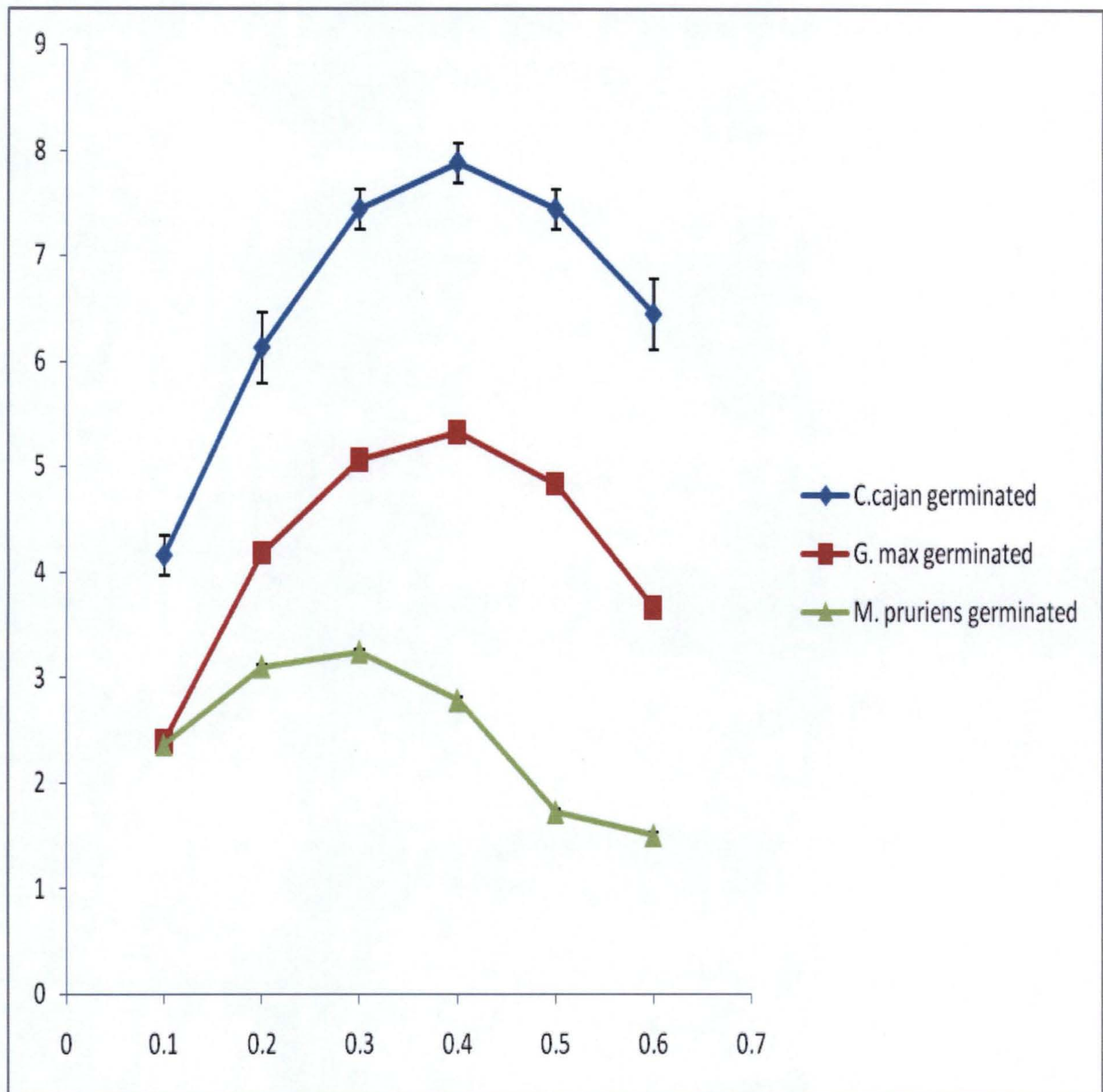


Figure 4.5c: Effect of substrate concentration on urease from germinated beans

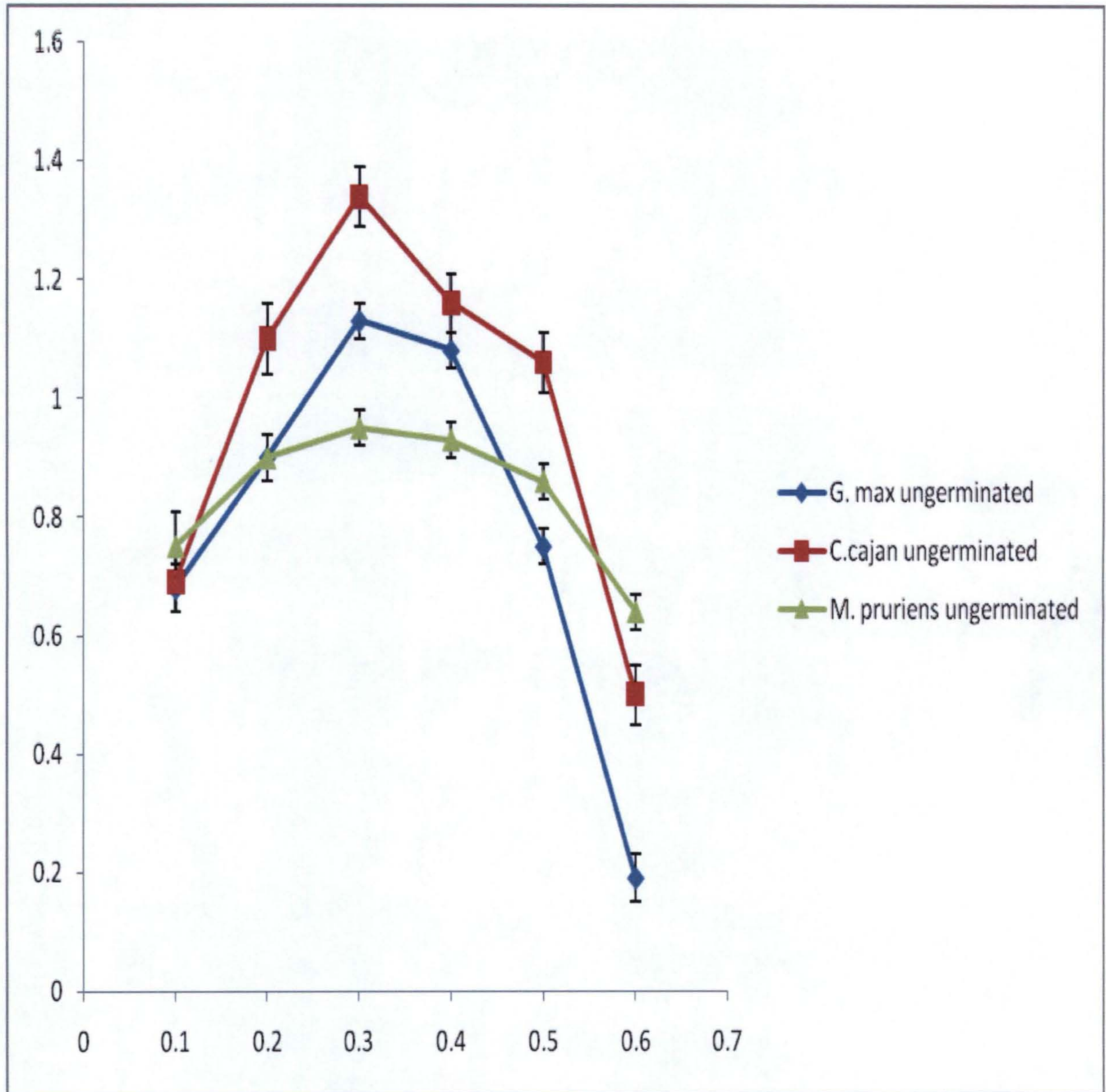


Figure 4.6a: Effect of substrate concentration on urease from ungerminated beans

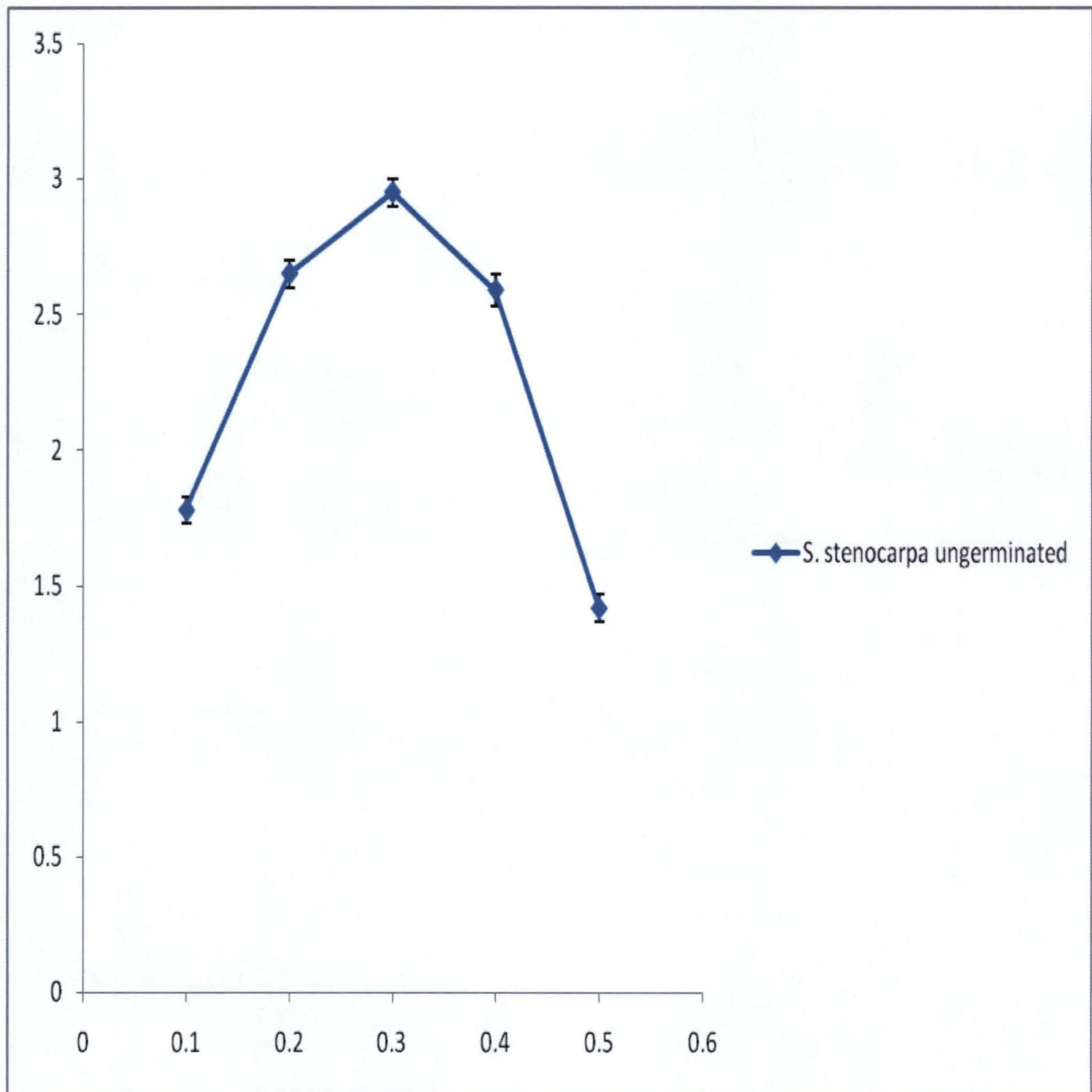


Figure 4.6b: Effect of substrate concentration on urease from ungerminated beans

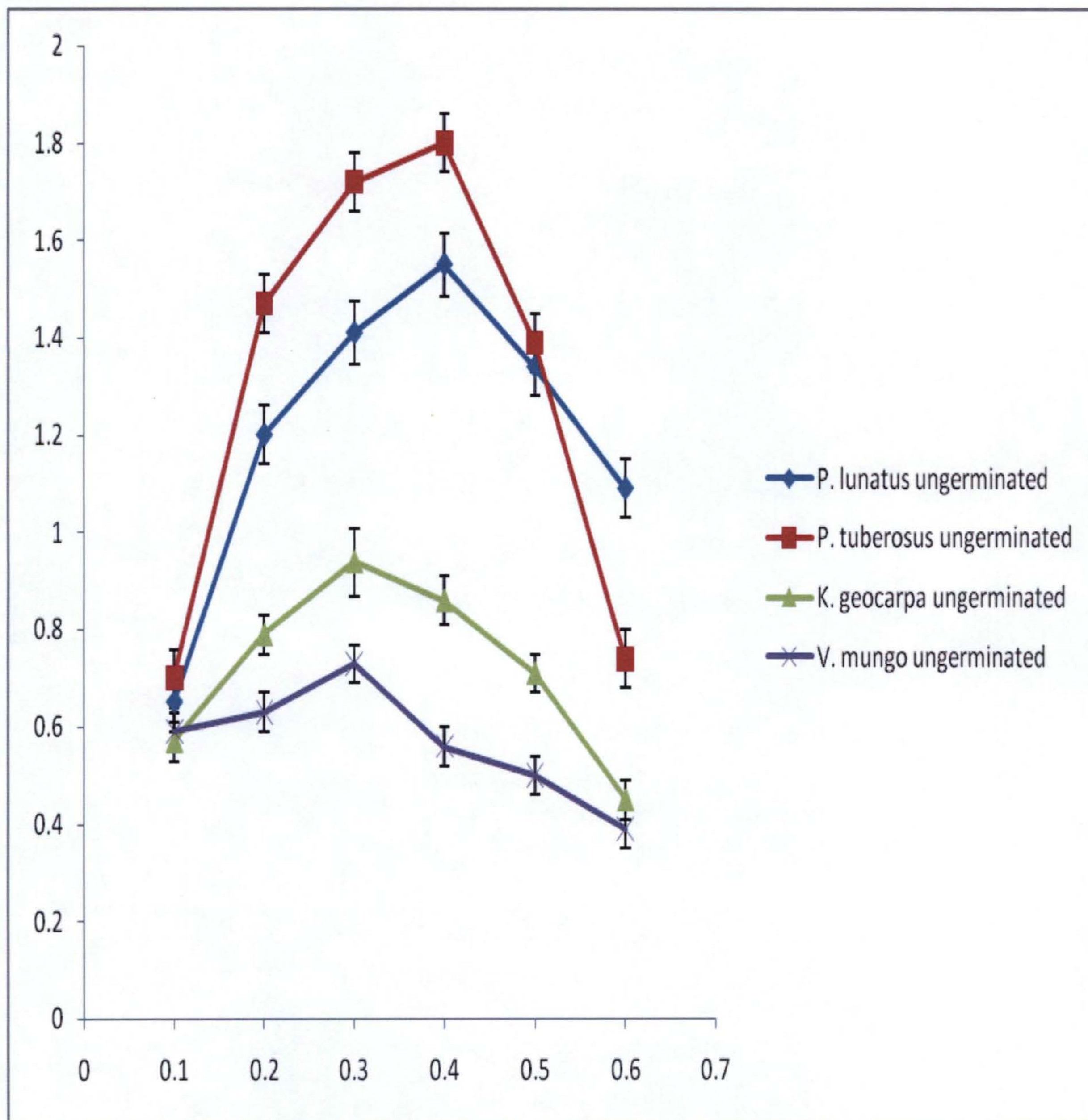


Figure 4.6c: Effect of substrate concentration on urease from ungerminated beans

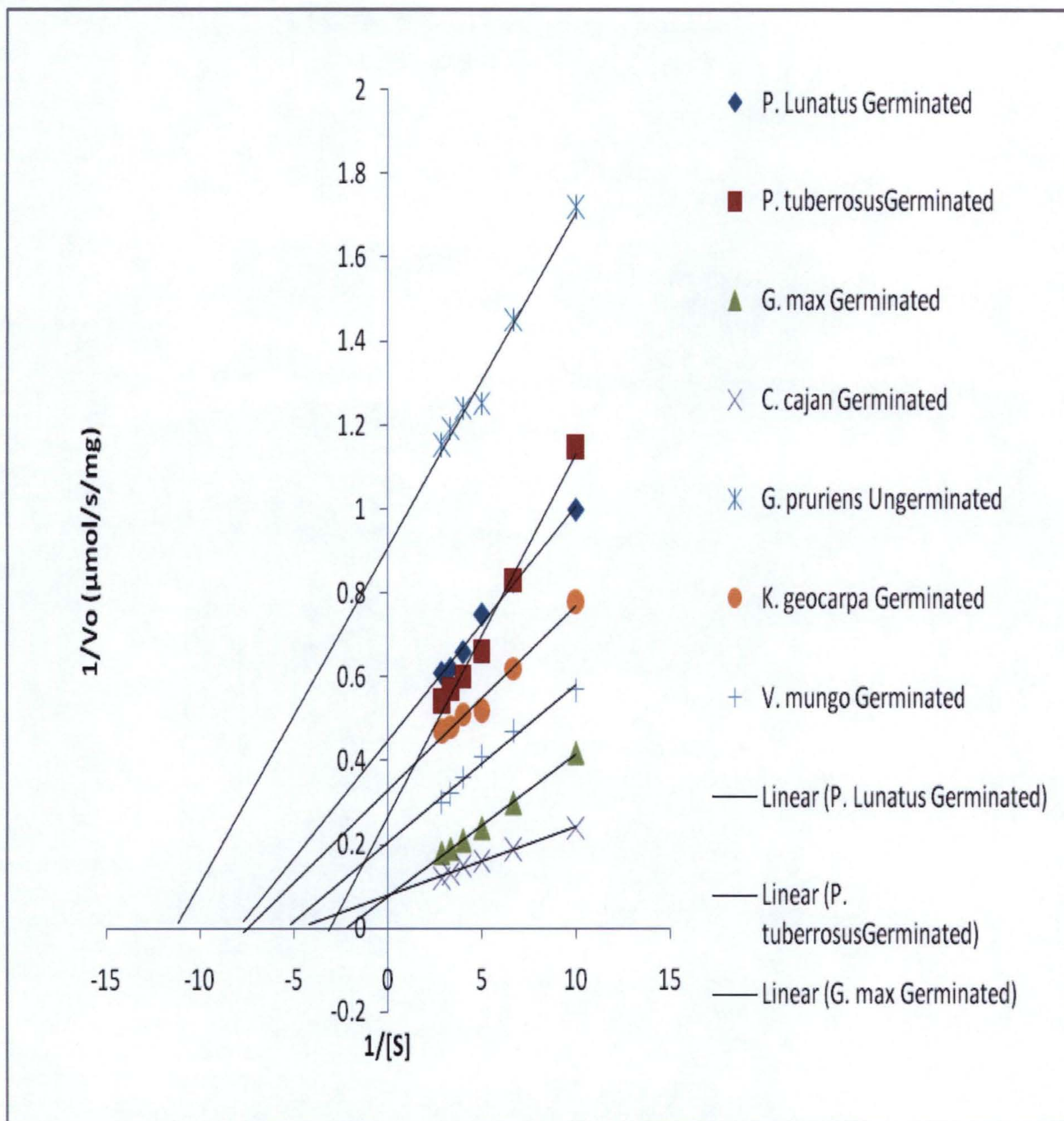


Figure 4.7a: Double reciprocal plot of rate V^{-1} *vs* $1/[S]$ for urease from germinated beans.

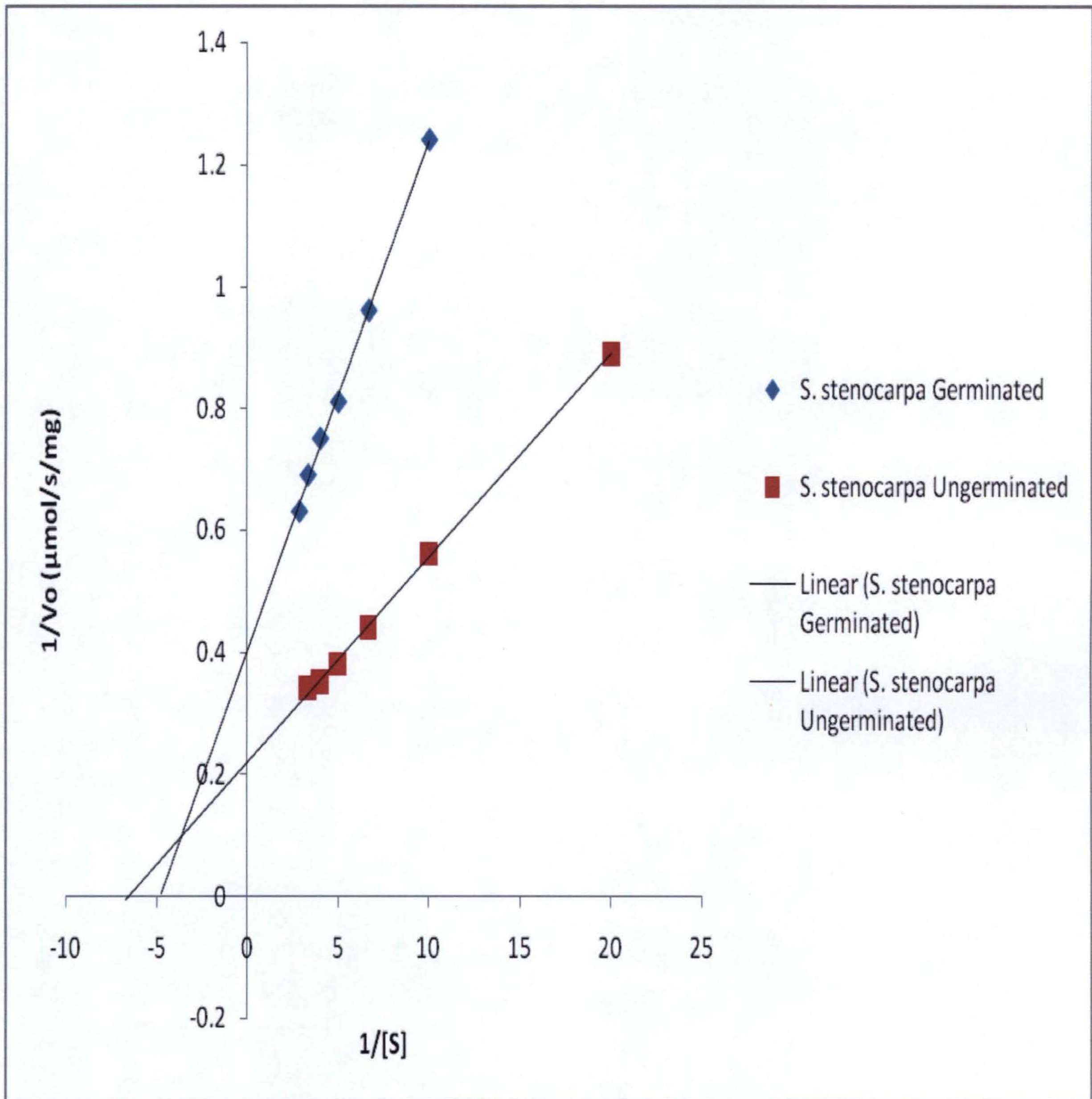


Figure 4.7b: Double reciprocal plot of rate V^{-1} *vs* *1/[S]* for urease from germinated beans.

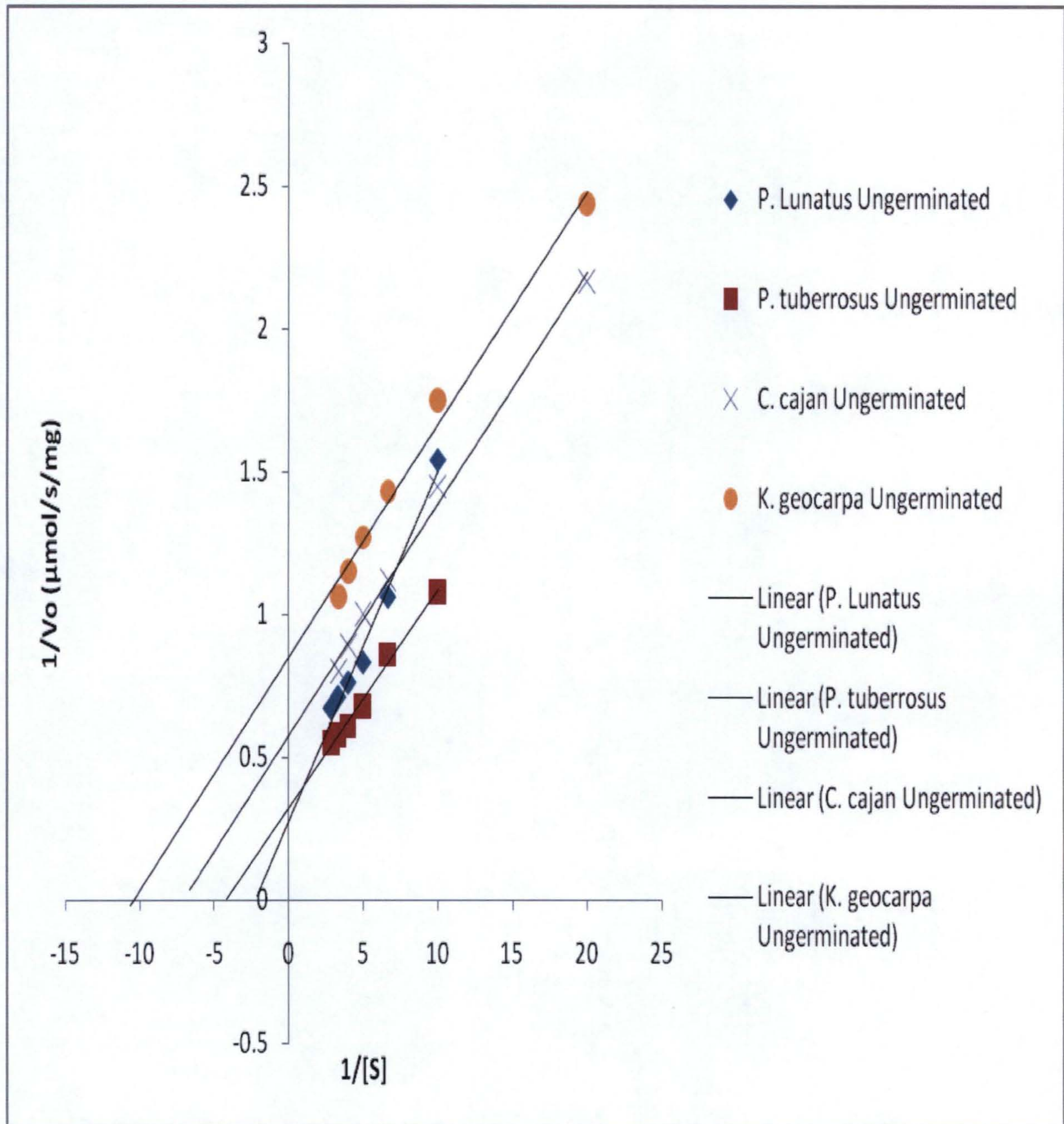


Figure 4.8a: Double reciprocal plot of rate V^{-1} *vs* $1/[S]$ for urease from ungerminated beans

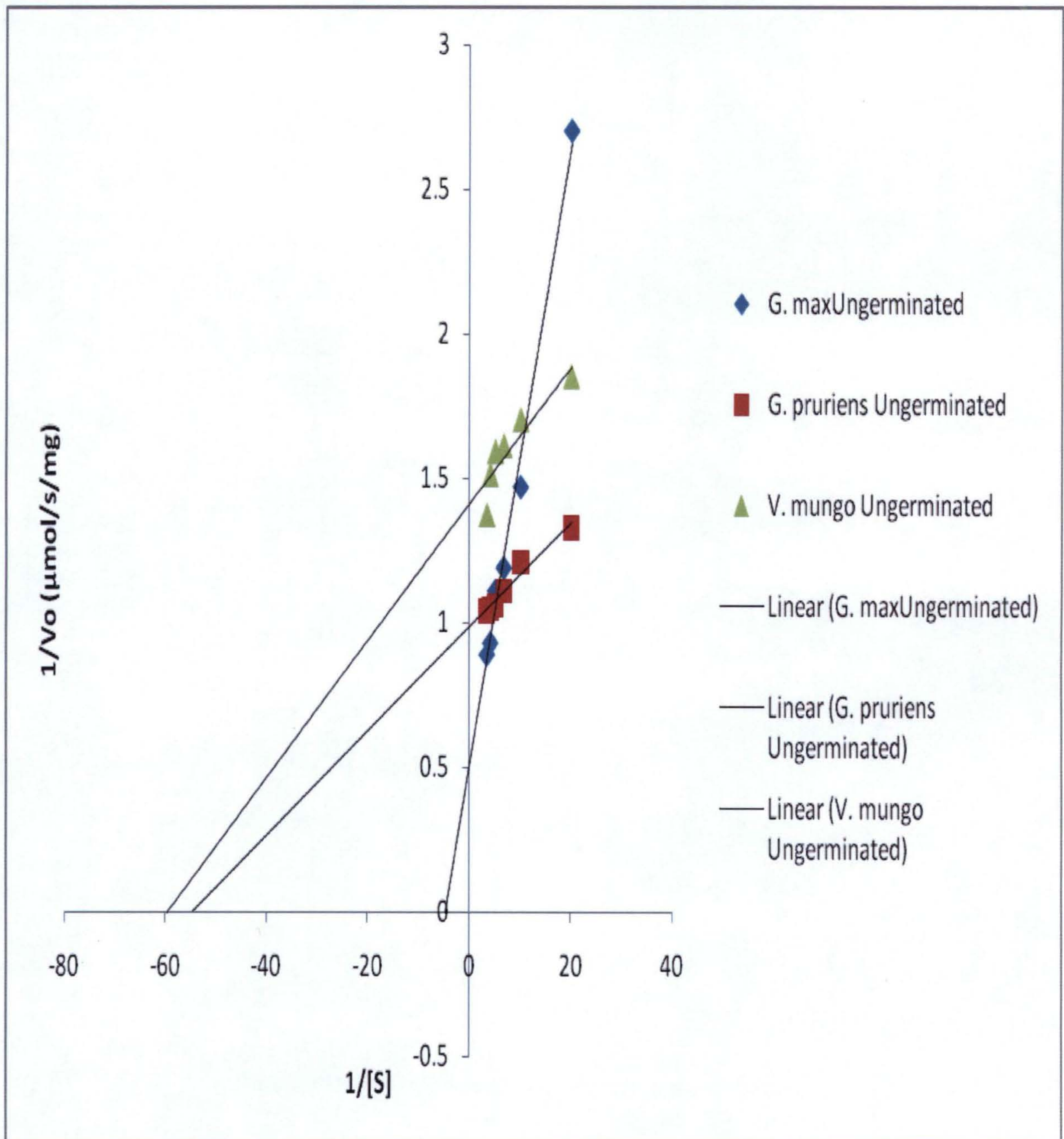


Figure 4.8b: Double reciprocal plot of rate V^{-1} *vs* $1/[S]$ for urease from ungerminated beans

Table 4.3: **Kinetic Characteristic of Urease from Germinated and Ungerminated beans**

	V_{max}(U/mg)		K_m(mM)		Catalytic efficiency	
Beans samples	**Germinated**	**Ungerminated**	**Germinated**	**Ungerminated**	**Germinated**	**Ungerminatd**
Glycine max	11.67 ± 0.73^{b}	1.64 ± 0.17^{a}	0.38 ± 0.04^{b}	0.15 ± 0.03^{a}	30.71	10.93
Parchyrhizus tuber	$4.09 \pm 0.47^{a***}$	$3.68 \pm 0.88^{a***}$	0.30 ± 0.10^{ans}	$0.35 \pm 0.15^{a**}$	13.63	10.51
Sphenostylis steno	$2.37 \pm 0.38^{b***}$	$4.71 \pm 0.23^{a***}$	$0.19 \pm 0.06^{a*}$	0.16 ± 0.02^{ans}	12.47	29.44
Phaseolus lunatus	$2.23 \pm 0.16^{a***}$	1.87 ± 1.63^{ans}	$0.15 \pm 0.05^{a***}$	$0.29 \pm 0.04^{a**}$	14.87	6.45
Cajanus cajan	12.49 ± 0.56^{bns}	2.04 ± 0.65^{ans}	$0.21 \pm 0.01^{a*}$	0.20 ± 0.11^{ans}	59.48	10.20
Mucuna pruriens	$6.68 \pm 0.81^{b***}$	$3.39 \pm 0.20^{a**}$	0.28 ± 0.05^{ans}	$0.29 \pm 0.22^{a*}$	25.69	12.11
Kerstings geocarpa	$3.06 \pm 0.26^{b***}$	1.32 ± 0.08^{ans}	$0.14 \pm 0.04^{a**}$	0.13 ± 0.01^{ans}	21.86	10.15
Vigna mungo	$4.95 \pm 0.71^{b***}$	$1.02 \pm .06^{ans}$	$0.18 \pm 0.06^{a**}$	$0.046 \pm 0.004^{b*}$	27.50	22.17

Down columns, values with ns, *, ** and *** are statistically different from *Glycine max* at $p < 0.05$, < 0.01 and < 0.001 level of significance respectively. Across rows, values with different letters (superscripts) are statistically different at $p < 0.05$. Each data is mean±SD of three replicates. NS: not significant.

4.4.1 Protein Level and Urease Enzyme Activity of Fractions

The results of protein level and urease specific activity in *Mucuna pruriens* after partial purification are presented in Figure 4.9. The protein level showed one peak at fraction 120ml. The results of urease specific activity showed that, activity was highest in fraction 120ml. The active fractions 100-140ml were pooled together for immobilization. The protein levels of the active fractions pooled was 17.30mg/g, while 10.80mg/g protein was observed in immobilized urease.

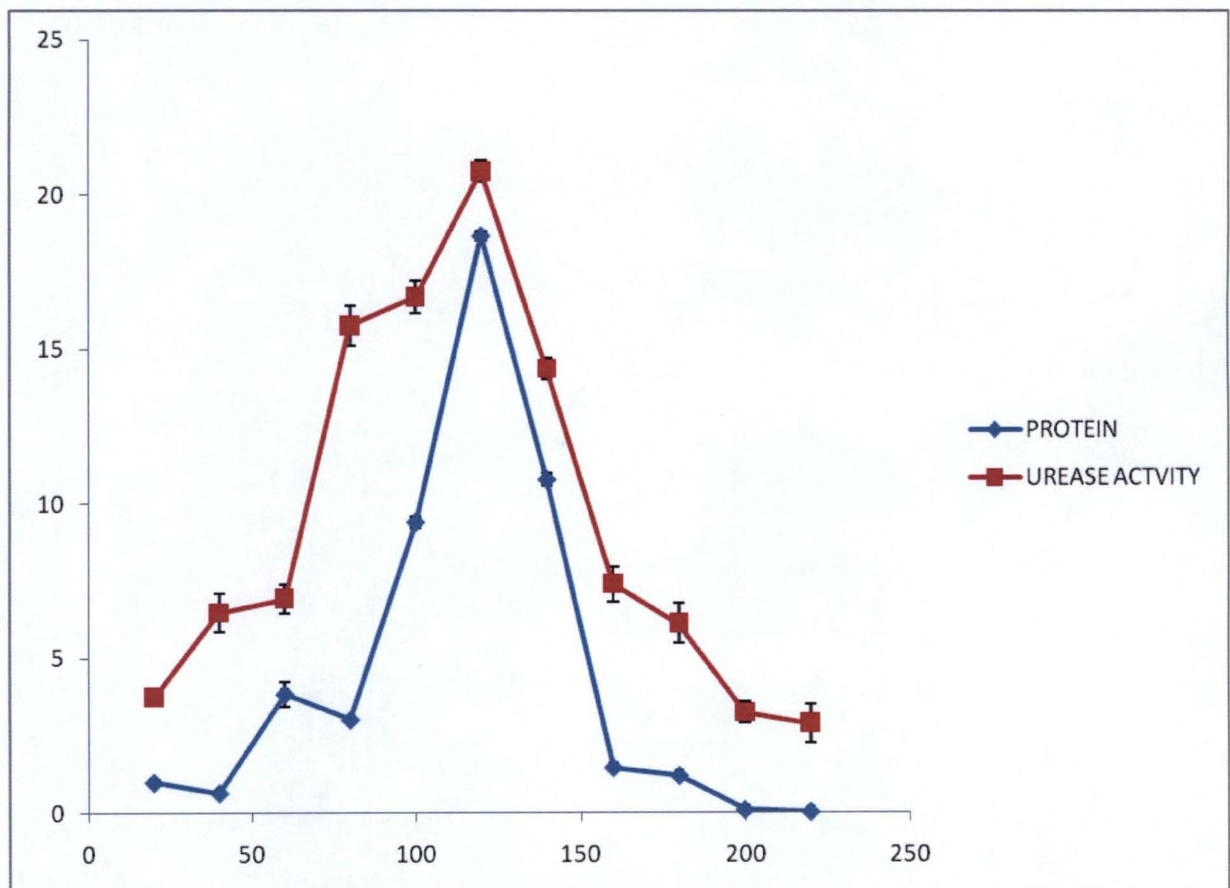


Figure 4.9: Specific activity and protein level of urease.

Table 4.4: Purification of urease from *Mucuna pruriens*

Fraction	Protein (mg/g)	Urease activity (U/mg)
Acetone	**95.57 ± 7.68^{a}**	**1.17 ± 0.03^{a}**
Sephadex G-100	**20.77 ± 0.35^{b}**	**18.69 ± 0.12^{b}**

Within columns, values with different letters (superscrpts) are statistically different at $p<0.05$. Each data is mean±SD of three replicates.

4.5 Characterization of Immobilized Urease

The results of optimum temperature, substrate concentration profile, storage ability and reusability of the immobilized and free urease enzyme are shown below.

4.5.1 Optimum Temperature

The results of optimum temperature for immobilized and free urease enzyme are shown in Figure 4.10. Results showed that, optimum temperature for both free and immobilized urease enzyme is $40^{0}C$.

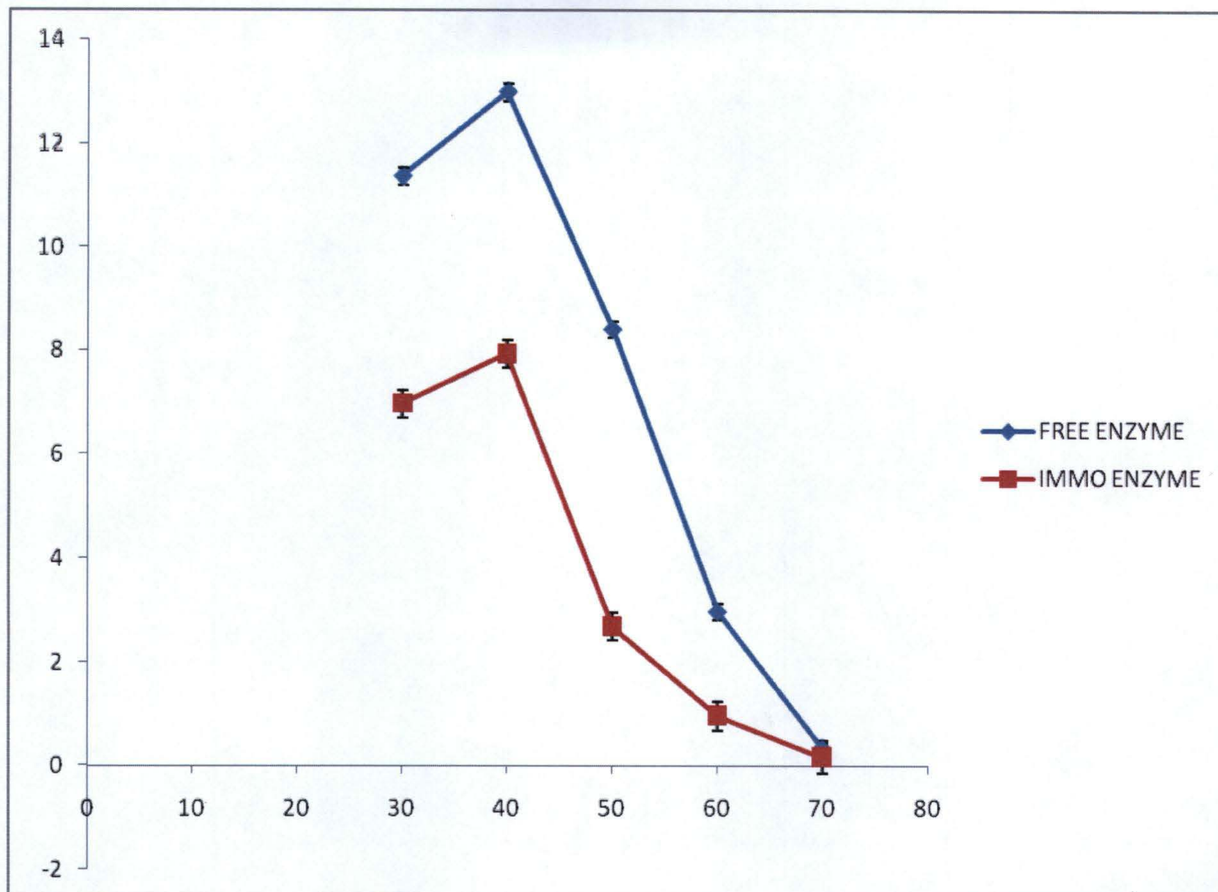


Figure 4.10: Effect of temperature on free and immobilized urease

4.5.2 Substrate Concentration and Enzyme Kinetics (V_{max} and K_m)

The result of the effect of substrate concentration on specific activity of free and immobilized urease enzyme are presented in Figure 4.11. From the results, urease activity was signicantly ($p<0.05$) higher in free urease enzyme compared to the immobilized. The results of reaction velocity (*Vmax*) and the Michelis-Menten constant (*Km*) are presented in Table 4.5. The partial purification step significantly ($p<0.05$) increase *Vmax* and decrease *Km* in free and immobilized urease compared to crude.

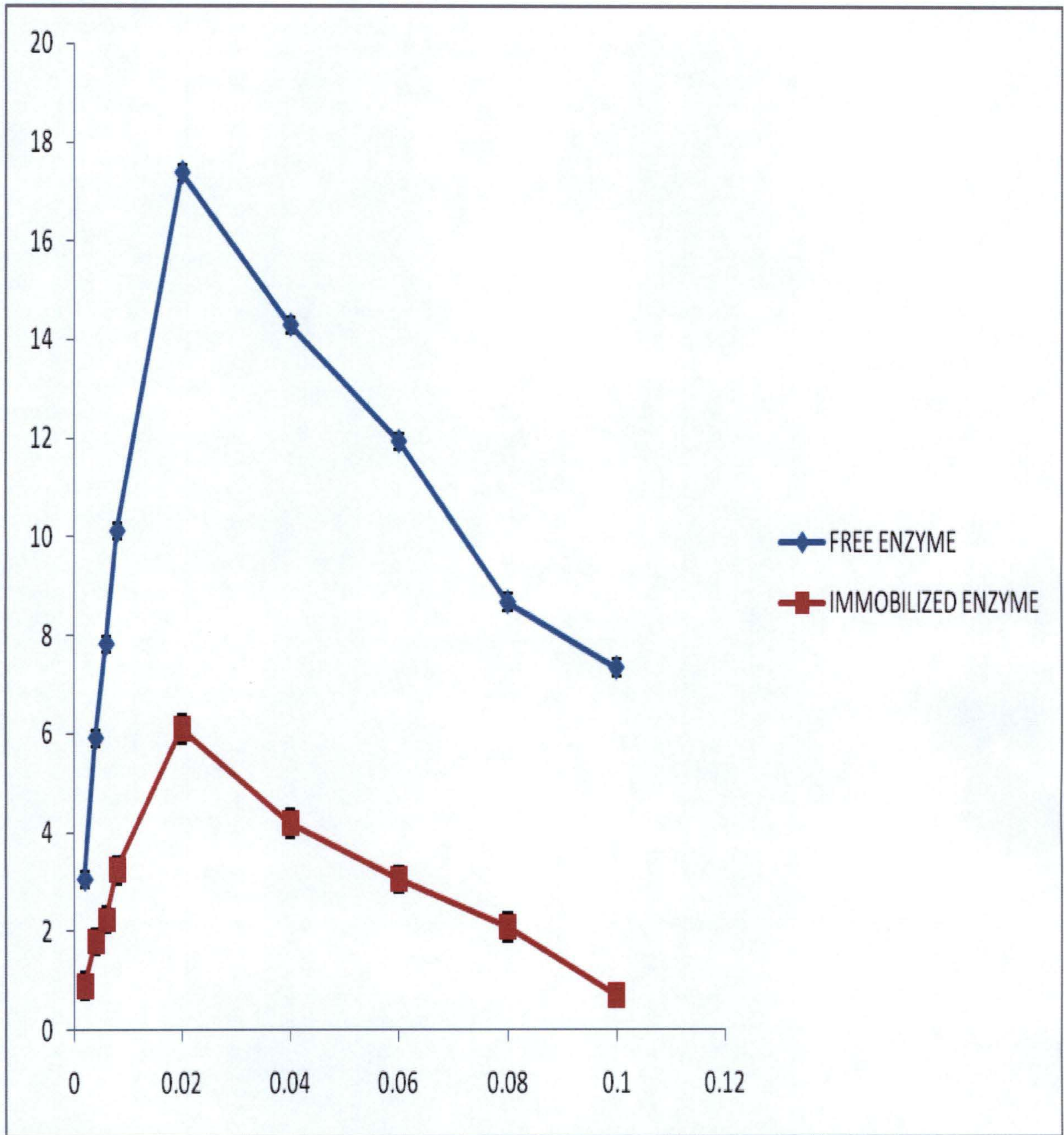


Figure 4.11: Effect of substrate concentration on free and immobilized urease

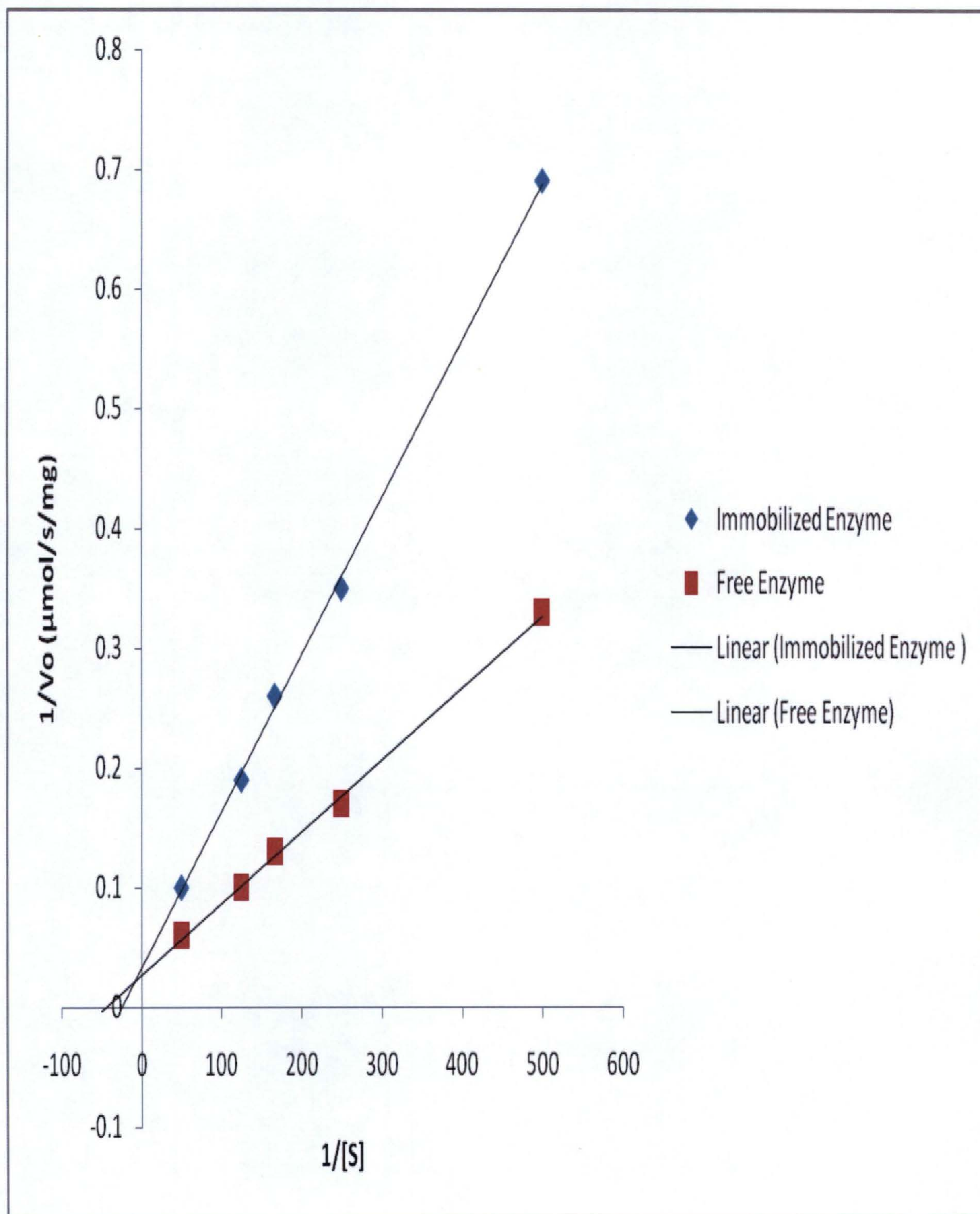


Figure 4.12: Double reciprocal plot of rate V^{-1} *vs* $1/[S]$ of immobilized and free urease

Table 4.5: Kinetic parameters of free and enzymes immobilized on chitosan beads

Urease Enzyme	K_m (mM)	V_{max} (U/mg)	Catalytic Efficiency
Immobilized	0.04	19.23	480.75
Free	0.02	38.46	1923

4.5.3 Storage ability and Reusability

The results of storage ability of free and immobilized urease are shown in Table 4.6. The results indicated that, free and immobilized urease enzyme retained 50% and 25% activity respectively, after one week. The result of reusability is presented in Figure 4.13. The result showed that, immobilized urease lost activity completely after third cycle of reuse.

Table 4.6: Storage ability of free and immobilized urease

Storage period	Free enzyme (Us^{-1})	Immobilized enzyme (Us^{-1})
Week 1	17.38±0.17	9.79±0.26
Week 2	8.69±0.17	1.99±0.00

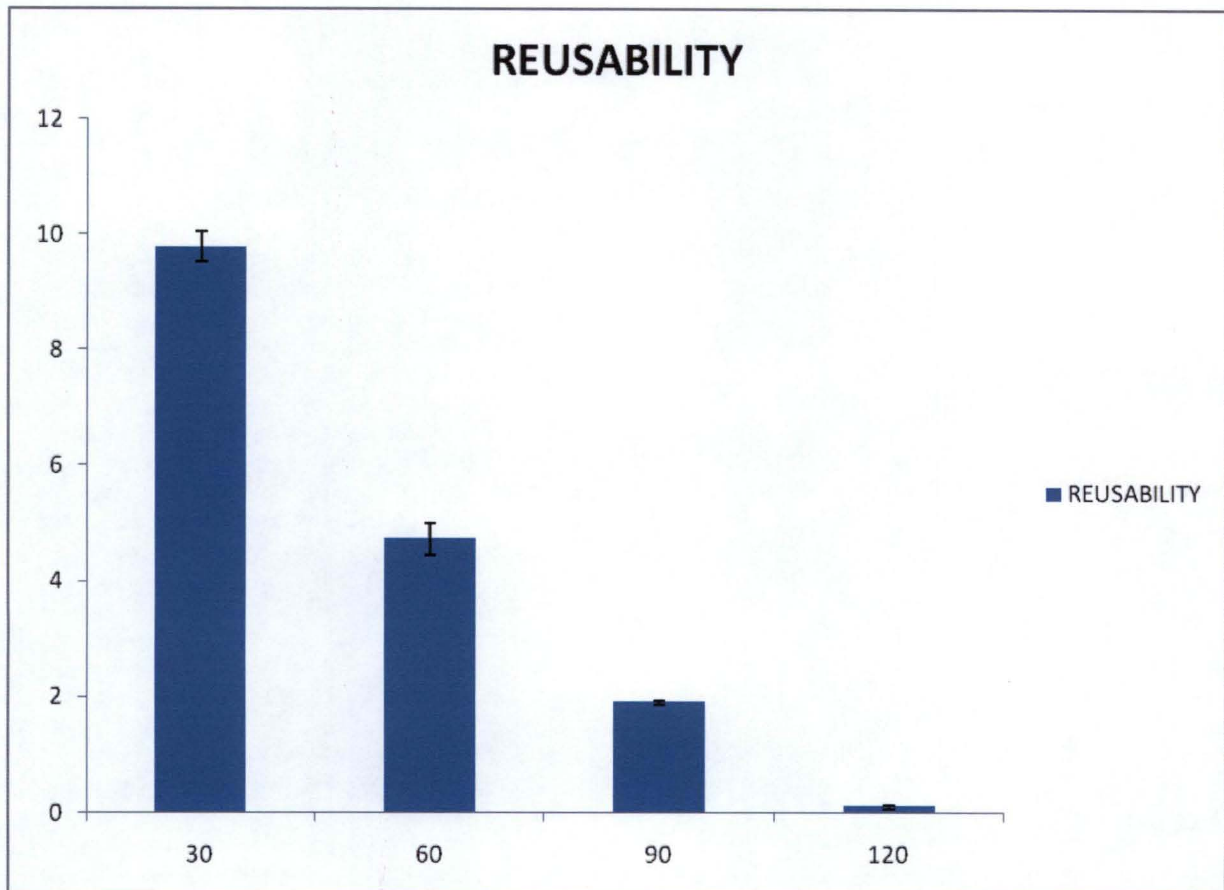


Figure 4.13: Reusability of urease immobilized on chitosan beads

4.6 Discussion

The results of protein level in germinated and ungerminated beans are presented in Table 4.1. From these results, it was observed that germination significantly ($p<0.05$) reduced the level of protein in beans samples studied. Polacco and Holland, (1993) had shown that, urease in conjunction with arginase (Figure 2.3) utilizes seed protein reserve during germination. Additionally, according to Mobley *et al.*, (1995), proteins, also act as urease-specific chaperones (Figure 2.5) in the process of nickel fixation (activation) into urease active site during germination (Polacco and Holland, 1994; Hausinger, 1997; Salomone *et al.*, 2007) thereby making free protein unavailable. The pathways shown in Figures 2.6 provide genetic information

of how seedling reserved proteins is utilized in the process of nickel incorporation at the active site of urease during germination. It could therefore be accepted that, naturally, as plants grow the protein levels of the seedlings reduce as a result of biological handling of nickel and nickel trafficking in the urease system. However, the information about the role of proteins in nickel incorporation during urease activation is not limited to plants only. Bacteria such as *K. aerogenes, Helicobacter spp.* e.t.c and any other urea metabolizing organism make use of their accessory protein for urease maturation (Jabri *et al.*, 1995; Tange and Niwa, 1997). Based on these facts, there was reduction in protein level in germinated beans when compared to the ungerminated as shown in this study.

In the same vein, the pattern of decrease (folds and %) in protein level differs in the beans samples studied. These variations might be depended on protein level and the rate of urease utilization of the protein for maturation in each of the beans samples studied. The protein level of both germinated and ungerminated *Mucuna pruriens* is somewhat higher compared specifically to *Glycine max* as well as *Sphenostylis stenocarpa, Vigna mungo, Cajanus cajan, Kerstings geocarpa, Phaseolus lunatus and Parchyrhisus tubberrosus* (Table 4.1). This suggested its high nutritional value. Arinathan *et al.*, (2009) had shown that, the protein level of *Mucuna prureins* is far higher than some of the well known pulse such as, black beans, green gram, pigeon pea, chick pea and cow pea. This nutritional profile of *Mucuna pruriens* makes it a good and cheap source of plant protein for animal feed and other industrial processes requiring protein.

The result of effect of germination on urease specific activity can be seen in Table 4.2. From the result, germination increased urease specific activity in the beans samples studied. It has been

reported that, urease specific activity is increased during palnt germination, and this was attributed to the role of seedling accessory protein involved in nickel incorporation at the urease active site (Polacco and Holland, 1994; Mobley *et al.*, 1995). Urease maturation is very necessary because of its involvement in seedlings' nitrogen matebolism (Claus-Peter *et al.*, 2002). Increase in urease specific activity is also associated with plants urea metabolism, most especially when urea is supplied as fertilizer (Mobley and Hausinger, 1989; Mobley *et al.*, 1995; Callahan *et al.*, 2005 and Ciurli, 2007). Ordinarily, the nitrogen present in urea is unavailable to the plant unless hydrolyzed by urease. Therefore, plants urease activity needed to be significantly increased to metabolize the bulk flow of urea which, if in excess could lead to leaf necrosis (Eskew *et al.*, 1983; Krogmeier *et al.*, 1989; Stebbins *et al.*, 1991). Therefore, there was significant ($p<0.05$) increase in urease activity in germinated beans compared to the ungerminated samples (Table 4.2).

Comparing the urease activity profile in the various beans samples as shown in Table 4.2, there was no significant ($p<0.05$) difference in *Mucuna prureins* and *Kerstings geocarpa* among others. Whereas urease specific activity in *Glycine max* (Polacco and Holland, 1993, 1994) and *Cajanus cajan* (Kayastha and Das, 1998) are very high compared to others, although they are well studied for their urease activity. However, *Mucuna pruriens* could be a better source of urease due to the fact that, it has limitations as a food material.

Additionally, from the result Table 4.2, *Spenostylis stenocarpa* showed a different pattern. The urease enzyme activity in germinated *Sphenostylis stenocarpa* was significantly ($p<0.05$) lower than the ungerminated (Table 4.2). It was observed that the germination did not have a significant effect on urease enzyme activity in the bean sample, though with lower protein level

(Table 4.1). This might be due to the genetic supression of urease gene of the beans whose inactivation might lead to reduction in urease activity. This observation could be supported by report on genetic analysis of urease from soya beans, where several genes became mutated and urease activity was lost despite reduction in protein level (Polacco and Holland, 1993; 1994). Torisky *et al.*, (1994) observed that, soybean expresses a urease essential for assimilation of urea, either endogenously generated or environmentally available. This urease is encoded by *Eu4* (Glyma11g37250), which is expressed in all tissues examined, including callus and the developing embryo (Torisky *et al.*, 1994). Loss of urease activity leads to accumulation of urea in seeds and in leaves to the extent that leaf tips become necrotic (Stebbins *et al.*, 1991). Similar trait was also observed in soybean plants deprived of nickel (Eskew *et al.*, 1983), a component of the urease active site. The loss of urease activity in soya beans is however similar to S*phenostylis stenocarpa* where loss of urease activity was expressed after germination, although the genetic analysis of urease expression in this legume in not studied yet.

The optimum pH of crude urease enzyme from the beans samples studied are shown in Figure 4.3a-c and 4.4a-c. The result showed that, the pH optimum for crude urease in both germinated and ungerminated beans samples shifted towards neutral and was stable within the pH range of 6.5-7.0. The optimum pH of urease from both germinated and ungerminated beans samples is summarized in Appendix A. However, the specific activity of urease enzyme from germinated beans was significantly ($p<0.05$) higher than the ungerminated at the optimum pH of each urease, except urease extracted from both germinated and ungerminated *Sphenostylis stenocarpa*. The result in this work is supported by other researchers. For instance, optimum pH for urease activity in Jack beans (Anita, *et al.*, 2010 and Zu Pei LIANG *et al.*, 2005), *Rhyzopus oryzea*

(Neveen, 2006) and surface black soil (Lu, 2000) were found to be 7.0, 7.0 and 6.45 respectively. The stability of urease activity at these pH values may be explained by the structural changes occurring in enzyme proteins caused by pH variation. The tertiary structure of a protein depends on interactions such as hydrogen bonding between enzyme amino acids functional groups. A change in pH can alter the ionization of these amino acid side chains and disrupt the native conformation and in some cases denature the enzyme. Hence, each enzyme has an optimal pH range that helps the maintenance of its native conformation in an environment, where it operates. The degree of ionization of the surface functional groups of amino acid residues is a function of the medium's pH. The main factor involved here is the titration of the ionizable group which maintains surface charge, in the active site, or stabilizes the enzyme resulting in an optimum pH for the enzyme (Banu, 2001). Hence, from observation, germination did affect optimum pH for urease activity in germinated beans samples. Because, it has been shown that, its only the incorporation of nickel in the active site of urease during the process of germination that alters the conformational state of the inactive (apo-urease) urease enzyme to form the active urease (Holo-urease) (Figure 2.3 and 2.5) (Hausinger, 1997). Since the optimum pH of enzyme only depends on tertiary structure of protein, therefore, germination did not confer any special trait on urease that could influence the optimum pH, because there was no difference in optimum pH of urease activity in germinated and ungerminated beans samples (Appendix A).

This implies that, urease can be very useful in clinical chemistry, for determination of urea in biological fluid such as blood and urine as well as water treatment. Besides, microencapsulation of urease in the development of artificial kidney for those with renal failure is possible, because both blood and urease are stable at pH around neutral.

The optimum temperature of crude urease from the studied beans samples are shown in Figure 4.3a-c and 4.4a-c and summarized in Appendix B. The result showed that, optimum temperature ranged between 60-70°C for both germinated and ungerminated beans samples. From the result, germination did not have effect on the optimum temperature of urease when compared to the ungerminated (Table 4.4). Krajewska, (2009), observed that, urease is a thermostable enzyme. Published works on optimum temperature for urease activities in Jack beans (Anita *et al.,* 2010), *Rhizopus oryzae* (Neveen, 2006), *Yersinia enterocolitica* (Jagsharan and Neeru, 2009), purified *Chenopodium album* leaves (El-Shora, 2001) are 60, 55, 65, 60 and 40°C respectively. The temperature dependence of an enzyme-catalyzed reactions exhibit an optimum because the thermodynamic increase of reaction rate is followed by a steep drop caused by thermal denaturation of the enzyme (Wolfgang, 2007).

The high optimum temperature of urease makes it very relevant in any industrial process involving urease at high temperature. For example, urease is used as a reducing agent in alcoholic beverages, in the manufacture of glues, hair conditioners and plastics.

The substrate concentration on enzyme activity profile follows the normal Michelis-Menten rate of reaction which means that the enzyme do not deviate from the expected enzyme characteristics. The results of the effect of substrtate concentration on the enzyme activity and kinetics are shown in Figure 4.50a-c, 4.60a-c, 4.70a-b and 4.80a-b respectively. From the results germination significantly ($p<0.05$) increased V_{max} and there was no significant ($p<0.05$) difference in K_m of germinated compared to ungerimnated beans samples studied, except in *Sphenostylis stenocarpa* where reverse was the case. It has been shown ealier that, germination

influences proper assemblage of Ni^{2+} at the active site of urease enzyme. As a result of this activation, there was no significant ($p<0.05$) difference in K_m values of urease in germinated beans samples compared to the ungerminated, thereby increasing the affinity of urease to its specific substrate urea, without any conformational changes in enzyme protein making its active sites more accessible to its substrate. The K_m values varied between the urease in different beans samples studied. The values of the V_{max} were also different in each of the beans samples, germinated and ungerminated respectively. The effect of germination on K_m had a corresponding relationship with the V_{max}. That is, the activity of the enzyme depends on its affinity for urea. Therefore, the increase in maximum activity (V_{max}) observed in urease is due to effect of germination on the urease active site compared to the ungerminated samples.

This observation is further strengthened by the catalytic efficiency (V_{max}/K_m) of urease in germinated compared to ungerminated beans samples respectively. Results presented in Table 4.3 showed clearly that, germination increased the catalytic of efficiency of urease. It was also deduced from result that, with increased V_{max} and reduced K_m, the catalytic efficiency of the enzyme is heightened. Therefore, the catalytic efficiency of an enzyme is an index of the catalytic capacity of enzyme through enzymatic reactions (Juan *et al.*, 2010). That is, the higher the affinity of the enzyme for the substrate (K_m), the higher the maximum activity (V_{max}) and this will also increase catalytic efficiencyof the enzyme. Hence, the relationship of K_m and V_{max}, and the mechanism of nickel fixation into active site of urease by germination are in accordance with the principle of enzyme kinetics. Anita *et al.*, (2010) showed that, the K_m and V_{max} of urease from jack beans are 0.25mmol/L and 0.154mmol/mg respectively, whereas, Jugsharan and Neeru (2009) showed that, *Yersinia erocolitica* had K_m and V_{max} of 1.7 ± 0.4 mM and 7.29 ± 0.42 µmol

respectively. Comparing these results, urease from germinated beans samples is of better catalytic efficiency than urease from these sources.

Juan *et al*., (2010) showed the inhibitory effect of N-(n-Butyl) thiophosphoric triamide (NBPT), phenyl phosphorodiamidate (PPD) and hydroquinone (HQ) on K_m and V_{max} of urease from black soil. The K_m of soil urease in the presence of these inhibitors were significantly higher, compared with the control, which may be attributed to the formation of inhibitor-urease complex, thereby reduing the affinity of urease for its specific substrate urea (Vieth and Venkatasubramanian, 1973 and Juan *et al*., 2009). In general, the $Vmax$ values were lower in the presence of PPD and NBPT than HQ, indicating that PPD and NBPT had higher inhibitory effectiveness on soil urease. The inhibitory effect of these inhibitors on urease active site further confirmed that, the process of germination actually activated urease thereby increasing V_{max} and redued K_m.

Although, there were obvious differences in the values of V_{max} and K_m of urease from the various beans samples. The comparison of V_{max} values among the beans samples studied showed that, (Table 4.3), there was no significant ($p<0.05$) difference in V_{max} of germinated *Glycine max* and *Cajanus cajan*. However, the V_{max} of urease in germinated *Mucuna pruriens* was significantly ($p<0.05$) higher compared *Sphenostylis stenocarpa, Vigna mungo, Kerstings geocarpa, Phaseolus lunatus and Parchyrhisus tubberrosus*. In general, the V_{max} values were higher in germinated than the ungerminated beans samples because germination had activatory effect on plant urease. However, there was consistent trend of higher catalytic efficiency (V_{max}/K_m) as influenced by germination in all the germinated beans samples.

The effect of germination on the kinetic characteristics of ureae as discussed imply that, urease as a result of it catalytic efficiency would have desirable clinical and industrial applications most especially in the area of blood urea analysis and development of artificial kidney (Nose, 1990), as biosensor for the determination of heavy metal in surface and underground water and soil during environmental impact assessment due to industrial waste and also for biocementation.

Hence, since germination increased the catalytic capacity of urease, it indicated that, plants during germination can efficiently hydrolyze both internally generated urea and the one in the soil. It therefore means that, the bulk flow of urea from arginine (Figure 2.2) and soil into the plant increase the activity of the enzyme. That is, with an increased urea concentration, there is higher hydrolysis of urea (higher V_{max}) until the active site becomes saturated (optimum substrate concentration), thereby lowering the sensitivity of the enzyme to the substrate. As a result of this phenomenon, with increased substrate concentration, there is lower enzyme sensitivity to substrate at the optimum. Thus, the biochemical characteristics of urease during plant germination showed that the V_{max} is increased so as to metabolize urea generated in the plant and the urea supplied as fertilizer (external). This elucidates the importance of urea in agriculture most especially in the area of soil fertility. Consequently, enzyme kinetic parameter is a function of substrate concentration and enzyme activity, and Michelis-Menten constants for enzymes usually range between 10^{-2} and 10^{-5} mM for most enzymes (Wolfgang, 2007).

The result of partial purification of urease from *Mucuna pruriens* is presented in Figure 4.90a and summarized in Table 4.4. From the elution profile of sephadex G-100 column chromatography (Figure 4.90b), it can be seen that, urease from *Mucuna pruriens* was eluted

from the column in one peak of protein accompanied with one peak of urease activity. The protein level of partially purified urease was significantly ($p<0.05$) reduced when compared to the acetone-precipitated protein. This showed that, gel filtration removed about 78.23% of the contaminating proteins and reduced the protein by 4.60fold. The peak of activity was found in fractions 120ml with urease activity 18.67U/mg which is 94% and 15.97fold higher than the crude enzyme. El-Shora, (2001) reported on the activity of purified urease from leaves of *Chenopodium album*. He showed that, the activity increased form 1.6 to 1.9U/mg with1.2fold increase. This showed that, Mucuna pruriens contains higher urease activity than leaves of *Chenopodium album*. These results showed that, purification significantly ($p<0.05$) increased the urease activity. Therefore, purification of urease enzyme further increased the enzyme activity. Purified enzyme enhances higher product yield most especially during industrial application. As mentioned earlier, urease enzyme is very important in clinical chemistry, environmental pollution assessment to mention a few, which means that, application of purified enzyme will in no mean way improve the yield of products in the course of industrial processing.

The results of optimum temperature of both free and immobilized urease immobilized are shown in Figure 4.10.0. The result showed that, both free and immobilized enzyme possessed the same temperature optima (40°C). Usually, the optimum temperature of immobilized enzymes is higher when compared to the free enzyme (Yoshida *et al.*, 1989; Sadhukhan *et al.*, 1993), but the reverse effect was observed in immobilization under consideration (Sungur *et al.*, 1992). However, report of crude urease extracted from Jack beans and immobilized on calcium alginate showed that, both free and immobilized urease possessed the same temperature optima (55°C) (Anita *et al.*, 2010). Also, report of lipase immobilized on palm-base polyurethane foam showed

that, optimum temperature both free and immobilized lipase was (40°C) (Roila *et al.*, 2007). According to these reports, for both free and immobilized enzyme to have the same optimum temperature, it means, immobilization did not alter the general charater of these enzymes. Therefore, for immobilized urease enzyme to have the same optimum temperature as the free enzyme shows that, urease immobilized on chitosan beads in the present study retained its structural integrity and activity.

Immobilization technology is very important in the industrial/large scale application of urease. With this technology, under a very suitable condition, there is low cost of production with enzyme and reusability.

The result of substrate concentration profile and kinetic (V_{max} and K_m) behavior of partially purified urease is presented in Figure 4.11 and 4.12 respectively while the kinetic parameter is summarized in Table 4.5. From the result, the V_{max} increased from 3.39U/mg to 38.46U/mg with 11.35fold increase and K_m 0.29 to 0.02 with 14.5fold decrease. Additionally, the catalytic efficiency (V_{max}/K_m) of the partially purified urease increased from 11.69 to 1923 with 164.50fold higher than crude. This implies that, with purification enzyme activity is greatly enhance for desirable performance during application. The V_{max} and K_m (19.23U/mg and 0.04) of immobilized urease are less than the free urease. This might be due to mass transfer resistance and steric hindrance created by enzyme immobilization, which restricted the transport of substrate from the bulk solution to the catalytic sites and diffusion of products back to the solution (Anita *et al.*, 2010). Hence, only a little amount of substrate concentration was required by the immobilized enzyme to achieve the same rate of reaction obtained with free enzyme.

However, the catalytic capacity of the immobilized urease enzyme is much higher than the crude enzyme and the kinetic behavior of free and immobilized are in accordance with the Michaelis–Menten rate equation.

The result of storage ability of free and immobilized urease is summarized in Table 4.6 and the reusability of the immobilized urease in Figure 4.13 respectively. From the result, both free and immobilized urease retained activity above one week. However, free and immobilized enzyme retained 50% and 25% of their activity after seven days respectively. Ordinarily, immobilized enzymes usually show higher storage stability, but in this case it was not.

From the result of reusability of immobilized enzyme, activity was lost after every use, and completely lost after three times of reuse. On a general note (Table 4.6), storageability and reusability of enzyme immobilized with physical adsorption on polysaccharide such as chitosan is low (Akşamoğlu, 1997). This is speculated to be due to the weak intermolecular interaction, such as hydrogen bonding, Van der Waal's forces e.t.c which anchors the enzyme amino acid residue to the polysaccharide. These characteristic features of physical adsorption method usually cause leakage of enzyme at ambient temperature (Wolgang, 2007 and Villeneuve *et al.*, 2000). Therefore there was reduction in the reusability and storageability of the enzyme immobilize on chitosan.

Zu pei LIANG *et al.*, 2005 reported that, urease immobilized on chitosan maintained high reusability and storage ability when cross-linked with glutaraldehyde. Adsorption followed by cross-linking with glutaraldehyde some times stabilizes the activity of immobilized enzyme by

anchoring the enzyme molecule properly, and thereby preventing leakage and thermoinactivation (Wolfgang, 2007). The immobilized urease retained 40% of its initial enzyme activity even after 10 repeated uses. The immobilized urease stored at 4°C retained 46% of its initial activity even after 35 days. This indicated that, as more aldehyde groups are available on the activated chitosan beads, multiple-point of attachments of urease molecules to the beads will likely occur and this leads to activation of urease (Wolfgang, 2007).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this study, it was observed that germination reduced the protein level and increased the specific activity of urease when compared to ungerminated beans samples. In view of what other researchers have done, seed reserved protein was used to assemble the active sites of urease by incorporation of nickel. It was discovered that, the optimum pH and temperature were not affected by germination, since both germinated and ungerminated beans samples have similar optimum pH and temperature. The results of this experiment also showed that, the K_m and V_{max} were greatly influenced by germination in that, germination increased V_{max} and reduced K_m. Therefore the catalytic efficiency of the enzyme was increased. These express the significance and application of urease in agriculture, clinical chemistry (blood urea analysis can efficiently be carried out with urease enzyme) and industry. The mechanism that increased urease activity during germination makes urease an important enzyme in agriculture

The urease kinetics observed in *Mucuna pruriens* compared to *Glycine max* and *Cajanus cajan* showed that, the legume could be a very good source of urease without any competition with food status of the society because, as for now *Mucuna pruriens* is not a staple food.

Finally, partial purification of urease from *Mucuna pruriens* showed that, with purification enzyme activity is greatly increased. However, immobilization is a very good means of making enzyme utilization cost effective because of it reusability and storage ability under suitable conditions and methods.

5.2 Recommendation

Activation of urease could be means of improving urease before extraction. *Mucuna pruriens* could be a very good and cheap source of animal protein, industrial processes requiring protein and urease without any effect on the nutritional status of the society. In the same vein, urea can be a very good fertilizer owing to the biochemical mechanism of its metabolism. In the same vein, immobilization of enzyme can reduce cost of production, but should be done with cross-linking if physical adsorption method will be used.

The only problem with *Mucuna pruriens* is the itching effect which poses a further area of research as regards its suitability for better plant technology. I therefore suggest the removal of this itching effect for further research.

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APPENDICES

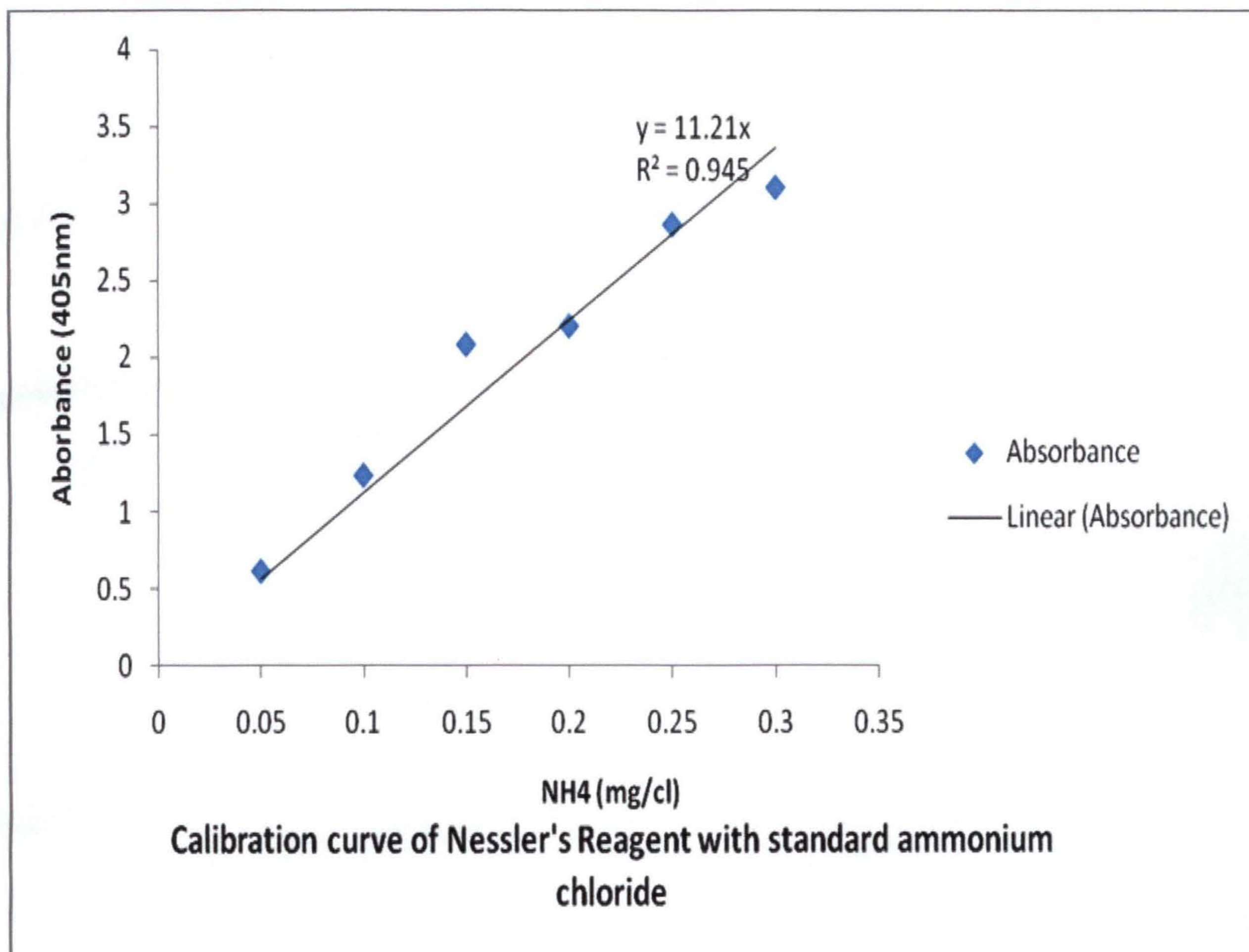
Appendix A: Optimum pH for urease activity in germinated and ungerminated beans samples

Beans samples	**Optimum Temperature (^oC)**	
	Germinated beans	**Ungerminated beans**
Phaseolus lunatus	7.0	7.0
Parchyrhizus tuberrosus	7.0	6.5
Sphenostylis stenocarpa	6.5	6.5
Glycine max	6.5	6.5
Cajanus cajan	7.0	7.0
Mucuna pruriens	7.0	6.5
Kerstings geocarpa	6.5	7.0
Vigna mungo	7.0	6.5

Appendix B: Optimum temperature for urease activity in germinated and ungerminated beans samples

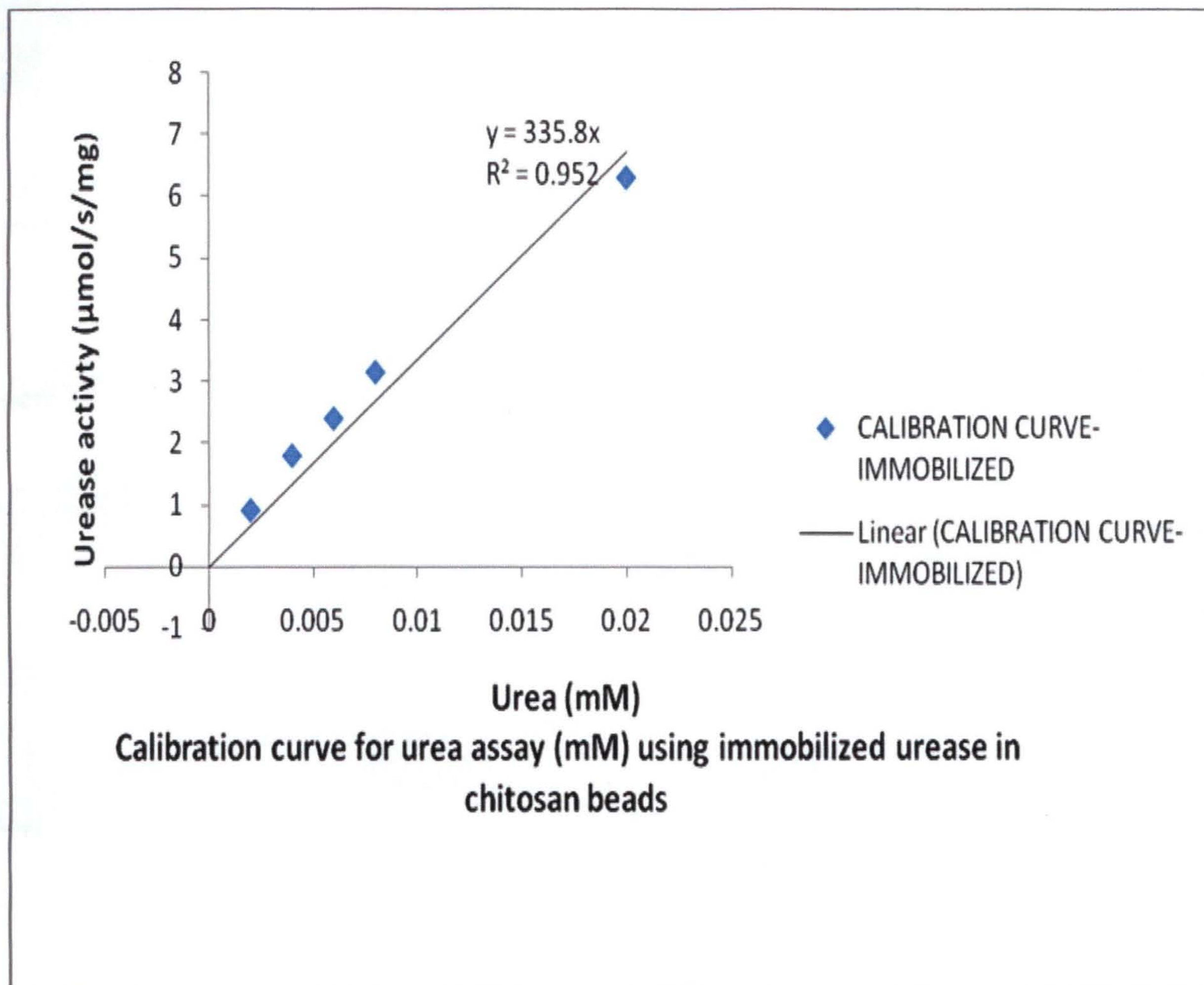
Beans samples	Optimum Temperature (°C)	
	Germinated beans	**Ungerminated beans**
Phaseolus lunatus	60	60
Parchyrhizus tuberrosus	60	60
Sphenostylis stenocarpa	70	70
Glycine max	60	60
Cajanus cajan	70	60
Mucuna pruriens	60	60
Kerstings geocarpa	70	60
Vigna mungo	70	60

Appendix C: Calibration curve for crude and partially purified urease assay



Calibration curve of Nessler's Reagent with standard ammonium chloride

Appendix D: Calibration curve for immobilized urease assay



Calibration curve for urea assay (mM) using immobilized urease in chitosan beads