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# Isolation, partial purification, characterization and inhibition of urease (E.C. 3.5.1.5) enzyme from the *Cajanus cajan* seeds

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Urease (urea amidohydrolase, E.C. 3.5.1.5), a nickel dependent metalloenzyme, catalyzes the hydrolysis of urea and one molecule of urea results in the release of two molecules of ammonia and one molecule of carbon dioxide. The objective of present study was to characterize urease enzyme from *Cajanus cajan*. The partial purification of urease enzyme was done by acetone fractionation method. The optimum temperature for urease enzyme in the present study was found to be 60°C and the optimum pH was 7.5. Partial purification of *Cajanus cajan* showed the fold purification to be 5.17 and the per cent recovery was found to be 56.6. Further, the effect of various inhibitors including $CuSO_4$, $AgNO_3$, $SnCl_2$ and $HgCl_2$ on the activity of urease enzyme was determined.

**Key words :** *Cajanus cajan*, Urease, Partial purification, Inhibition

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## INTRODUCTION

Urease is a nickel dependent metalloenzyme which catalyzes the hydrolysis of urea to yield ammonia and carbamate, the latter compound spontaneously hydrolyzes to form carbonic acid and another molecule of ammonia (Andrews *et al*., 1984). The best-studied urease is that from jack bean (Blakeley and Zerner, 1984), which was identified as the first nickel metalloenzyme (Dixon *et al*., 1975) and urease from jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized. Sumner (1926) showed that urease is a protein. Urease is found in bacteria, yeast, and several higher plants. Urease is a cytosolic enzyme. Its major activity with some exceptions is associated with the soluble fractions of the cells (Mobley *et al*., 1995). The best genetic data of plant ureases are available for soybean (*Glycine max*) (Polacco and Holland, 1993; 1994). Two urease isoenzymes, a tissue-ubiquitous and embryo-specific encoded by two separate genes, as well as regulatory proteins encoded by unlinked genes were identified in soybean (Meyer-Bothling and Polacco, 1987; Torisky *et al*., 1994). 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).

For activation, urease needs to bind two nickel ions per subunit (Benini *et al*., 1999). The enzyme inhibitors can interact with enzymes and block their activity towards natural substrates (Amtul *et al*., 2002). Because of instability of enzyme, its use is limited. This problem can be overcome by recent developments in the field of biotechnology for immobilizing enzymes. Many methods exist for the immobilization of enzymes but usually used methods include entrapment; physical adsorption; co-polymerization; and covalent attachment. The immobilization of urease in nylon tubes, carboxymethyl-cellulose, polyacrylamide and gelatin has been done. Calcium alginate is commonly used for enzyme entrapment (Sunger *et al*., 1992; Das *et al*., 1998).

A number of medical and ecological significances of ureases have been described. The significance of the enzyme includes: to serve as a virulence factor in human and animal infections of the urinary and gastrointestinal tracts, play role in recycling of nitrogenous wastes in the rumens of domestic livestock, and its application in environmental transformations of nitrogenous compounds, involve urea based fertilizers (Mobley and Hausinger, 1989). During storage of wines the wines treated with preparation of killed cells containing an acid urease, remove urea from wine which is the potential source of ethyl carbamate (carcinogen) and thus prevent the

formation of ethyl carbamate (Fidaleo *et al*., 2006; Andrich *et al*., 2010).

*Cajanus cajan* is commonly known as Arhar Dal. *Cajanus cajan* (L.) Millsp. is a leguminous shrub. A variety of cultivars and the many ways they can be used in farming systems have made pigeonpea (*Cajanus cajan*) popular to small scale farmers. It is the major pulse crop of the semiarid tropics and has been used for centuries in intercropping systems, and is an ideal source of fodder, food and firewood in agroforestry systems. Leaves are trifoliate and spirally arranged on the stem. Flowers occur in terminal or axillary racemes, are 2-3cm long (Purseglove, 1968). Pigeonpea is hardy, widely adaptable and more tolerant to drought and high temperature than that of most other crops. It grows on acid sands in the Sahel and in alkali clays in India. Frost or excessive soil salinity is not tolerant and water logging for 3-4 days severely reduces yields (Chauhan, 1987).

Pigeonpea is modulated with *Rhizobium* of the cowpea type and is effective green manure crop. Whiteman and Norton (1981) recommended incorporating high density planting at or about the time of flowering. When allowed to perennialize, pigeonpea can drop 1.6 dry tons/ ha/ yr of litter in the first year (Sheldrake and Narayanan, 1979). It is used in folk medicine in West Africa and has been proposed as a nurse crop in India (Purseglove, 1968).

**Protein structure of urease :**

In 1989, nucleotide sequence of urease gene of *Proteus mirabilis* was the first publication for nucleotide sequence of urease gene (Jones and Mobley, 1989). The plant and fungal ureases are homo-oligomeric proteins, while the bacterial ureases are multimers formed from a complex of two or three subunits (Mobley *et al*., 1995). From the sequence, it has been predicted that the structural subunits of the urease (Fig. A) are encoded by the *P. mirabilis ureA*, *ureB*, and *ureC* genes. Significant amino acid similarities were observed between all known ureases. In this the smallest subunit is homologous to the amino-terminal amino acids (aa) of jack bean (*Canavalia ensiformis*) urease, the medium-size subunit is homologous to internal sequences, and the large subunit is homologous to the carboxy-terminal sequences of the plant enzyme. When urease structural gene sequences appeared for *Helicobacter pylori* (Clayton *et al*., 1990), *Proteus vulgaris* (Morsdorf and Kaltwasser, 1990), *Klebsiella aerogenes* (Mulrooney and Hausinger, 1990), *Ureaplasma urealyticum* (Blanchard, 1990), it became clear that all ureases are related and high sequence similarity shows that all ureases are variants of the same enzyme and likely to possess similar tertiary structures and catalytic mechanism. Thus, urease from non-*Helicobacter* bacterial species are composed of three distinct subunits (Fig. A) encoded by three contiguous genes; ureases from *Helicobacter* species are composed of two distinct subunits encoded by two adjacent genes; and the jack bean enzyme is made up of only one distinct subunit encoded by one gene. 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 *Klebsiella aerogenes* (Jabri *et al*., 1995). Jack bean urease exits as a homotrimer able to aggregate to a hexamer. Bacterial ureases possess structures similar to the jack bean urease. They are either trimers or hexamers of subunit complexes. They can also exit in aggregated forms.

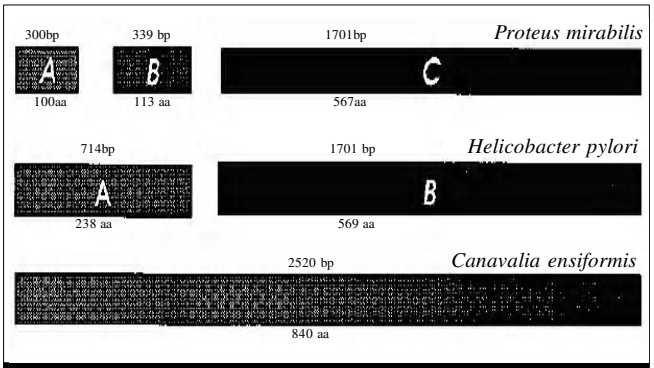


**Fig. A: Structural subunits of urease**

Although urease can have one, two or three distinct subunits, these enzymes are nevertheless closely related. This diagram showing a representative three subunit urease encoded by the *P. mirabilis ureA*, *ureB*, and *ureC* genes, the smallest subunit is homologous to the amino-terminal amino acids (aa) of jack bean urease, the medium-size subunit is homologous to internal sequences, and the large subunit is homologous to the carboxy-terminal sequences of the plant enzyme. The same relation holds true for the two subunit enzymes of *H. pylori* (Mobley *et al*., 1995).

**Inhibitors on urease activity :**

Urease inhibitors are used to the control urease-related pathogenesis of bacterial infections or excessive rates of ureolysis in soil. There are a number of urease inhibitors and some of them have been examined for pharmaceutical research in a variety of physiological conditions. Heavy metals were tested for their inhibitory effects on urease enzyme activity in many cases. Urease from seeds of pigeonpea showed a time-dependent and irreversible inactivation at very low concentrations of heavy metal ions. For 50 per cent inactivation (10 min of incubation) concentration of $Cu^{(2+)}$, $Hg^{(2+)}$ and $Ag^{(+)}$ were found to be $2.2 \times 10^{(-6)}$, $2.9 \times 10^{(-8)}$ and $6.3 \times 10^{(-12)}$ M, respectively. The kinetics of inactivation with each of these metal ions was found to be biphasic, with half of the activity being lost in a fast phase and remaining in a slow phase (Srivastava *et al*., 2002). Pigeonpea urease was competitively inhibited by boric acid and boronic acids (butylboronic acid,

phenylboronic acid, and 4-bromophenylboronic acid). 4-bromophenylboronic acid is the strongest inhibitor. Urease inhibition by boric acid is maximal at acidic pH (5.0) and minimal at alkaline pH (10.0), *i.e.*, the trigonal planar B(OH)3 form is a more effective inhibitor than the tetrahedral B(OH)4 -anionic form (Reddy and Kayastha, 2006). Acetohydroxamate (AHA) is the best hydroxamic acid that shows competitive inhibitory activity against urease from pigeon pea urease with K(i) of 0.041 mM at pH 7.3. This inhibition was found to be pH dependent. A reversible and time-dependent inhibition was observed with AHA. Kinetics of AHA inhibition was found to be biphasic as observed with the heavy metal ions (Srivastava *et al*., 2002). Fluoride ions were observed as competitive inhibitor of pigeonpea urease with the determined K(i) value for fluoride as 1.23 mM. These inhibition studies suggest the possible interaction of these inhibitors with active site thiol groups and Ni (II) ion (Srivastava *et al*., 2002). Acetohydroxymate (AHM) inhibited the allantoinase (E.C. 3.5.2.5) activity resulting in accumulation of allantoin and decreased allantoate levels in all parts of the pigeon pea plants. Inhibitory effects are consequence of blockage in ureide catabolism due to AHM (Reddy *et al*., 1995). The biosensor with urease entrapped in polyvinyl chloride (PVC) layer at the surface of pH-sensitive iridium oxide electrode was applied for testing of mercury and other metal ions inhibition on enzymatic reaction of urease. The calculation of inhibition effect was based on the measurement of initial rate of decrease of biosensor potential (proportional to the initial rate of enzymatic reaction) after addition of substrate after inhibition step (Krawczynski vel Krawozyk *et al*., 2000).

## RESEARCH METHODOLOGY

**Source of enzyme :**

Urease was extracted from *Cajanus cajan* (pigeonpea) seeds.

**Extraction of enzyme :**

20 g of seeds were weighed and soaked in 20 ml of extraction buffer and kept in refrigerator at $4^0$C. For extraction buffer, Tris- acetic acid buffer of 0.025 M concentration and pH 6.5 was used. Soaked seeds were crushed in ordinary mixer by adding 20 ml of extraction buffer. Suspension was filtered through muslin cloth and filtrate was then centrifuged at 8,000 rpm for 30 min at $4^0$C. Volume of supernatant was noted. This was the crude cell free extract. The supernatant of crude extract was analyzed for enzymatic activity and total protein content.

**Enzyme unit :**

One unit of urease activity is defined as the amount of urease that produces 1 µmole of ammonia in one minute at $60^0$C and pH 7.5.

**Nessler's reagent :**

Standard graph was prepared by taking different concentrations of ammonium chloride. To 1 ml ammonium chloride solution in each tube, 0.2 ml of 10 per cent trichloroacetic acid (TCA), 8.8 ml of distilled water and 1ml Nessler's reagent was added. O.D. was measured at 405 nm against blank, which contained all the reagents except ammonium chloride.

**Enzyme assay :**

The enzyme was assayed by adding 1ml of (0.2 M) urea to 1 ml enzyme solution (0.1 ml enzyme + 0.9 ml assay buffer). For assay buffer, tris- acetic acid buffer of 0.05 M concentration and pH 7.5 was used. After 10 min reaction was stopped by inactivating the enzyme with 1ml of 10 per cent TCA. The mixture was then centrifuged at 8000 rpm for 10 min and the supernatant was taken to estimate released ammonia by adding 1ml Nessler's reagent. An aliquot (1 ml) of the reaction mixture transferred to a 50 ml volumetric flask and 1 ml Nessler's reagent was added with swirling and the volume made up to 50 ml with distilled water. The yellow colour produced was measured at 405 nm to assay the urease enzyme activity.

**Total protein estimation :**

0.1 ml of crude extract was taken and diluted to 1ml with distilled water, 0.2 ml of 10 per cent TCA was added and centrifuged at 8000 rpm for 10 min. Supernatant was discarded and the precipitate was dissolved in 1 ml of 0.02 N NaOH. Total protein content was estimated as per method of Lowry *et al*. (1951), using Bovine Serum Albumin as standard.

Partial purification of enzyme by acetone fractionation: The crude extract was prepared as described earlier. It was placed in an ice-bath maintaining its temperature to about $4^0$C. Pre-cooled acetone ($20^0$C temperature) was slowly added with regular swirling (1/3rd of volume of the supernatant) and then centrifuged at 8000 rpm for 20 min. Precipitate was collected and dissolved in 2 ml of extraction buffer. It was again centrifuged and the clear supernatant was taken and the volume was noted. This was the partially purified enzyme solution. Thus acetone fractionation of enzyme from *Cajanus cajan* was done.

**Characterization of partially purified urease :**

*Effect of temperature* :

To study the effect of temperature on urease activity, enzyme was incubated with substrate at different temperatures ($10^0$C, $20^0$C, $30^0$C, $40^0$C, $50^0$C, $60^0$C, $70^0$C, $80^0$C). At each temperature enzyme and other reagents were incubated separately for 10 min before starting the reaction. The enzyme activity was measured at each temperature.

**Effect of pH :**

To study the effect of pH on urease enzyme activity,

assay buffer of different pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) were used. At each pH, substrate and enzyme were incubated for 10 minutes separately prior to start of reaction and then enzyme activity was measured.

**Effect of substrate concentration :**

For studying the effect of substrate concentration on urease activity, different concentrations of urea (50 mM, 100mM, 150 mM, 200 mM, 300 mM, 350 mM, 400 mM, 450 mM) were used. Enzyme activity was measured as described earlier.

**Inhibition of urease :**

Different inhibitors ($CuSO_4$, $AgNO_3$, $SnCl_2$ and $HgCl_2$) of different concentrations ($10^{-3}$ M to $10^{-8}$ M) were prepared. The enzyme activity was assayed by adding 1ml of urea (0.2 M) to 1ml of enzyme solution (0.1 ml enzyme + 0.1 ml inhibitors + 0.8 ml assay buffer) having individual inhibitor in different concentrations. Then the inhibitory effect of each inhibitor at different concentrations was observed.

## RESEARCH FINDINGS AND ANALYSIS

The experimental findings of the present study have been presented in the following sub heads:

**Partial purification of urease :**

Urease from *Cajanus cajan* was partially purified by acetone fractionation and it was found that there was approximately 3 times increase in the specific activity of urease (Table 1). Fold purification and per cent recovery of the enzyme after acetone fractionation were approximately 5.17 and 56.6, respectively.

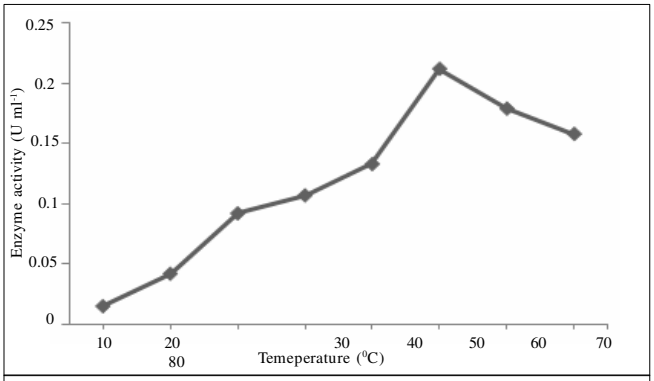


**Fig. 1: Activity of urease at different temperatures**

**Characterization of partially purified urease :**

*Effect of temperature on Cajanus cajan urease activity* :

Different temperatures ranging from 10-80$^0$C were carried out to optimize the temperature for urease enzyme. Study of temperature on urease activity revealed that urease activity increased up to the temperature 60$^0$C and started declining above 60$^0$C (Fig. 1).

Urease activity (partially purified from *Cajanus cajan*) was measured at various temperatures ranging from 10 to 80$^0$C. The optimum temperature was found to be 60$^0$C.

*Effect of* pH *on Cajanus cajan urease activity* :

Different buffer solutions of pH ranging from 4-9 were carried out to optimize the pH for urease enzyme. Study of pH on enzyme activity revealed that the enzyme hydrolyzed maximum substrate at pH 7.5 .The enzyme showed minimum activity at pH 4. The enzyme activity significantly increased up to pH 7.5 but declined as the alkalinity increased (Fig. 2). The pH of the reaction mixture strongly affects the enzyme activity.

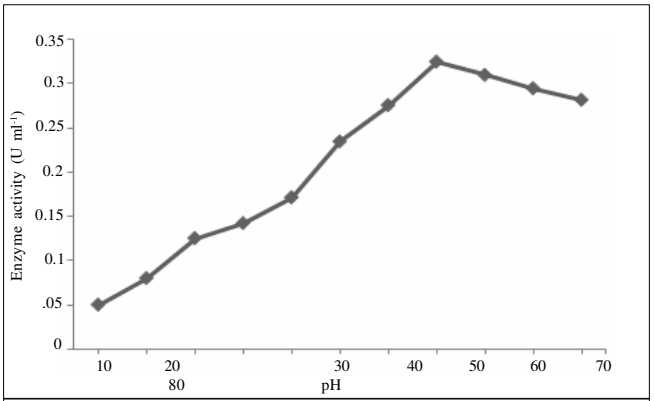


**Fig. 2: Effect of pH on urease enzyme activity**

Optimum pH of enzyme was determined by assaying urease activity (partially purified from *Cajanus cajan*) over the range from 4.0 to 9.0. The pH 7.5 was found to be optimum.

*Effect of substrate concentration on urease activity* :

Various substrate concentrations ranging from 50-400 mM of urea were done to find out the optimum substrate

**Table 1: Partical purification of urease from *Cajanus cajan* seeds**

| Purification step | Total volume (ml) | Protein concentration (mg/ml) | Total protein (mg) | Activity (mole/min/ml) | Total enzyme unit | Specific activity (units/mg of protein) | Fold purification | Per cent recovery (%) |
|---|---|---|---|---|---|---|---|---|
| Crude | 22.0 | 2.7 | 59.4 | 194.4 | 4726.8 | 72.0 | - | - |
| Acetone Fractionation | 5.0 | 1.3 | 6.5 | 484.3 | 2421.5 | 372.5 | 5.17 | 56.6 |

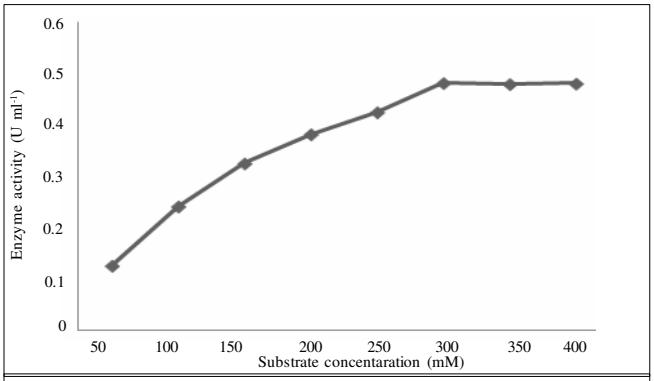


**Fig. 3: Effect of different substrate concentrations ranging from 50-400 mM on the urease activity (partially purified from *Cajanus cajan*)**

concentration for urease enzyme. Initially, the enzymatic activity increased with increasing substrate concentration but at high concentration the activity became independent of substrate concentration (Fig. 3).

300 mM was found to be the optimum substrate concentration.

*Inhibition of Cajanus cajan urease by different inhibitors* :

Inhibition study of urease activity using different inhibitors ($CuSO_4$, $AgNO_3$, $HgCl_2$ and $SnCl_2$) showed the following results.

Inhibition study using $CuSO_4$ at different concentrations ($10^{-3}$ to $10^{-8}$ M) revealed that the enzyme was inhibited maximally at $10^{-3}$ M concentration of $CuSO_4$ and showed 50 per cent inhibition at $10^{-5}$ M concentration (Table 2). Studies on inhibition of urease from *Cajanus cajan* by $Cu^{2+}$ ion showed that in the presence of $Cu^{2+}$ urease was functional for initial 2 h and thereafter decreased with continued decomposition. Singh (2006) has reported a drastic reduction in urease activity in the presence of $Cu^{2+}$ which may be the consequence of the blockage of energy to urea transporter. Inhibition study was similarly performed using $AgNO_3$ and at concentration of $10^{-3}$ M showed maximum inhibition and almost 50 per cent inhibition at $10^{-5}$ M concentration (Table 3). Then,

**Table 2: Inhibition by $CuSO_4$ on urease enzyme from *Cajanus cajan***

| Sr. No. | Concentration of $CuSO_4$ (M) | Residual activity | Per cent inhibition |
|---|---|---|---|
| 1. | Blank | - | - |
| 2. | Control (0.0) | 100 | 00.00 |
| 3. | $10^{-3}$ | 23.45 | 76.55 |
| 4. | $10^{-4}$ | 42.39 | 57.61 |
| 5. | $10^{-5}$ | 52.32 | 47.68 |
| 6. | $10^{-6}$ | 74.12 | 25.88 |
| 7. | $10^{-7}$ | 87.09 | 12.91 |
| 8. | $10^{-8}$ | 91.28 | 8.72 |

**Table 3: Inhibition by $AgNO_3$ on urease enzyme from *Cajanus cajan***

| Sr. No. | Concentration of $AgNO_3$ (M) | Residual activity | Per cent inhibition |
|---|---|---|---|
| 1. | Blank | - | - |
| 2. | Control (0.0) | 100 | 00.00 |
| 3. | $10^{-3}$ | 16.00 | 84.00 |
| 4. | $10^{-4}$ | 23.97 | 76.03 |
| 5. | $10^{-5}$ | 46.94 | 53.06 |
| 6. | $10^{-6}$ | 78.95 | 21.05 |
| 7. | $10^{-7}$ | 80.79 | 19.21 |
| 8. | $10^{-8}$ | 86.38 | 13.72 |

**Table 4: Inhibition by $SnCl_2$ on urease enzyme from *Cajanus cajan***

| Sr. No. | Concentration of $SnCl_2$ (M) | Residual activity | Per cent inhibition |
|---|---|---|---|
| 1. | Blank | - | - |
| 2. | Control (0.0) | 100 | 00.00 |
| 3. | $10^{-3}$ | 42.62 | 57.38 |
| 4. | $10^{-4}$ | 51.45 | 48.55 |
| 5. | $10^{-5}$ | 62.72 | 37.28 |
| 6. | $10^{-6}$ | 71.35 | 28.65 |
| 7. | $10^{-7}$ | 78.86 | 21.14 |
| 8. | $10^{-8}$ | 90.76 | 9.24 |

inhibition study of urease was also performed using $SnCl_2$ as inhibitor. Maximum inhibition of urease by $SnCl_2$ was reported at $10^{-3}$ M concentration. It showed 50 per cent inhibition at $10^{-4}$ M concentrations (Table 4). Inhibition study of urease was also performed with $HgCl_2$ as inhibitor. Maximum inhibition of urease by $HgCl_2$ was at concentration of $10^{-3}$M and showed about 40 per cent inhibition at $10^{-5}$M concentration (Table 5).

**Table 5: Inhibition by $HgCl_2$ on urease enzyme from *Cajanus cajan***

| Sr. No. | Concentration of $HgCl_2$ (M) | Residual activity | Per cent inhibition |
|---|---|---|---|
| 1. | Blank | - | - |
| 2. | Control (0.0) | 100 | 00.00 |
| 3. | $10^{-3}$ | 18.87 | 81.13 |
| 4. | $10^{-4}$ | 26.62 | 73.18 |
| 5. | $10^{-5}$ | 60.56 | 39.44 |
| 6. | $10^{-6}$ | 68.26 | 31.74 |
| 7. | $10^{-7}$ | 74.42 | 27.58 |
| 8. | $10^{-8}$ | 91.08 | 8.92 |

## Conclusion :

Several assays, mainly based on the measurement of the amounts of products released during the reaction, are available for quantifying urease activity. In the present study *Cajanus cajan* seeds were used as source of urease enzyme. The urease

enzyme was partially purified from *Cajanus cajan* seeds by acetone fractionation. The maximum hydrolysis of urea by urease enzyme was determined at temperature $60^0$C and pH 7.5. The optimum substrate concentration for partially purified urease of *Cajanus cajan* was found to be 300mM. The partially purified urease enzyme from *Cajanus cajan* showed fold purification of 5.17 and per cent recovery of 56.6. The effect of using different inhibitors ($CuSO_4$, $AgNO_3$, $HgCl_2$ and $SnCl_2$) showed that $10^{-3}$ M concentration of various inhibitors causes maximum inhibition of urease activity. The order of inhibition of urease enzyme activity by the inhibitors used in the present study was found to be $AgNO_3 > HgCl_2 > CuSO_4 > SnCl_2$. Such studies provide important insight into the urease enzyme activity which is useful because urease finds wide applications in agriculture, pathology and industry.

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