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Steady state kinetics and inhibition studies of urease from pumpkin (*Cucurbita moschata*)

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Abstract

Urease was extracted from the dehusked seeds of pumpkin (*C. moschata*). The enzyme was stored at two different temperature 4 °C and 37 °C and it was found that the self-life ($t_{1/2}$) was 70 days at 4 °C where as it was 12 days at 37 °C. Under steady state kinetics, the optimum pH and optimum temperature were found to be 8.4 and 80 °C respectively. The value of K_m and V_{max} were 12.50 mM and 62.50 $\mu\text{mol/min}$, respectively. Furthermore, urease was assayed in presence of various compounds to explore the potential inhibitors. The I_{50} value for boric acid inhibition was found 7.5 mM. The value of inhibition constant (K_i) was found to be 2.50 mM and the competitive type of inhibition was revealed. Further, I_{50} value for Cu^{2+} was 4.0×10^{-3} mM. The I_{50} values for Hg^{2+} was observed at 4.8×10^{-4} mM revealing its highest inhibition potency. Based on I_{50} values, the heavy-metal ions were found to inhibit urease in the following order $\text{Hg}^{2+} > \text{Cu}^{2+}$. The inhibition studies are important as elevated levels of soil/microbial ureases have been known to decrease the efficiency of urea fertilizer. It is well known that low concentration of inhibitors could inhibit the urease activity in soil. Thus, such studies may have medical or agronomic importance, as well as provide insight into the urease catalytic mechanism.

Keywords: Urease, steady state kinetics, pumpkin, *Cucurbita moschata*, inhibition

1. Introduction

Urease (urea amidohydrolase; EC 3.5.1.5) is an important enzyme present in many organisms, including plants. This enzyme plays diverse roles and mainly involved in the nitrogen metabolism. This enzyme is responsible for the hydrolysis of urea and its conversion into carbon dioxide and ammonia^[1]. Both urea and urease were subjects of early scientific investigations. Urea was the first organic molecule to be synthesized and jack bean urease was the first enzyme ever to be crystallized. Fungal and plant ureases are homo-oligomers of 90-kDa subunits, bacterial ureases are multimers of two or three subunit complexes. They have similar structures and mechanisms of catalysis. In agriculture, high urease activity causes significant environmental and economic problems by releasing abnormally large amounts of ammonia into the atmosphere during urea fertilization^[2-4].

All fermented foods contain a naturally occurring compound, ethyl carbamate, which is carcinogenic in nature and it is removed by using urease^[5]. Urease also plays an essential role in the defense mechanism of plants as it has antifungal property and provides protection against phytopathogens and predators^[6-8]. Urease is also used to estimate the amount of urea present in blood or urine samples^[9]. The enzyme can be used to detect the presence of urea in the milk samples^[10]. Urease plays an essential role in the treatment of urinary tract infection caused by *Proteus mirabilis*^[11].

Most of the studies have utilized urease obtained from jack bean which being expensive. Therefore, there still exists a need to have urease from a non-conventional, unutilized and cheaper source for versatile applications. Therefore pumpkin (*C. moschata*) was selected as a source of urease due to its easy availability throughout the year besides being the cheaper source. All urease, irrespective of their source, have identical structure of their active site and therefore exhibit similar catalytic mechanism. In present study, the pumpkin urease has been characterized biochemically and investigations mainly involve enzyme stability, steady state kinetics and inhibition studies. Such studies are important for refinement in the overall understanding of the catalytic mechanism of urease and have application in industries.

Inhibition studies have important role in agriculture and environment and could be used to solve the problem like pollution and higher alkalinity of soil.

2. Materials and Methods

2.1 Chemicals and Enzyme

Urease was isolated from dehusked seeds of pumpkin procured from the local market. Urea (Enzyme grade), bovine serum albumin (BSA), tris buffer, NH_4Cl , boric acid, Nessler's reagent (NR) and trichloro acetic acid (TCA) were from Himedia, India. Glacial acetic acid, salts of heavy metals (HgCl_2 , CuSO_4), were purchased from Merck, India. All other chemicals were of analytical grade obtained from SRL or Merck, India. All solutions were prepared in double distilled water (DDW). Unless and otherwise mentioned, each experimental point represent the mean of three determinations.

2.2 Preparation of Reagents

Assay buffer (50 mM Tris-acetate buffer) was prepared by dissolving 6.05 g of tris salt in 1 liter of double distilled water and pH was adjusted to 7.3 with glacial acetic acid. Extraction buffer (25 mM Tris-acetate buffer) was prepared by dissolving 3.03 g in tris salt in 1 liter of double distilled water and pH was adjusted to 6.5 with glacial acetic acid. Urea solution (0.2 M, pH 7.3) was prepared by dissolving 1.2 g urea in 100 ml of assay buffer. TCA solution (10%) was prepared by dissolving 10 g of TCA in 100 ml of DDW.

2.3 Extraction of enzyme

The pumpkin seeds (25 g) were soaked overnight in 50 ml of 50 mM tris-acetate buffer, pH 6.5 and stored at 4 °C. after about 24 hours, the soaked seeds were crushed in a kitchen blender to form slurry. The slurry was filtered through a muslin cloth (cheese cloth) and filtrate was then centrifuged at 12,000 rpm for 15 min at 4 °C. All the operations were carried out at 4 °C unless stated otherwise. The clear supernatant (crude preparation of urease) was obtained and stored at 4 °C till further use.

2.4 Calibration of Nessler's reagent

Nessler's reagent (NR) is a complex compound of mercury and is used extensively in estimation of ammonia. NR is commercially available and when it reacts with NH_3 to form a tetramine complex (Yellow Brown Colored Complex), which is colored and the intensity of color can be measured by a spectrophotometer. The stranded solution of NH_4Cl was prepared by dissolving 100 mg of NH_4Cl in 100ml of DDW. Different concentration of NH_4Cl were made and added to corresponding volumetric flasks (50ml capacity). The volumetric flask had already contained 15ml of DDW. One ml Nessler's reagent was added to each volumetric flask with constant shaking to avoid formation of yellow precipitate. The Nessler's reagent is used in activity measurement of urease and asparaginase and several other systems which releases ammonia. After mixing the volume was made up to 50ml. The absorbance was recorded at λ_{max} 405nm and standard curve was drawn.

2.5 Urease activity assay

Urease was assayed in 50mM Tris-acetate buffer (pH 7.3). An aliquot (0.9 ml) of assay buffer and 1.0 ml of 200mM urea in the same buffer were brought to 37 °C. The reaction was started by adding 0.1 ml of suitably diluted enzyme. After 10 min, 1 ml of 10% TCA was added to stop the

reaction. The 1ml of reaction mixture was transferred to a measuring flask (50 ml) and the volume was made to 50 ml with distilled water after adding 1 ml of Nessler's reagent as described earlier. The amount of ammonia liberated was measured at 405 nm in a Spectronic 21 UVD spectrophotometer. An enzyme unit has been defined as the amount of urease required to liberate 1 μmol of ammonia per min under our test conditions (0.1 M urea, 0.05 M Tris-acetate buffer, pH 7.3, 37 °C).

2.6 Storage stability studies

The temperature stability of pumpkin urease was investigated by storing the urease at two different temperatures and assayed at regular intervals. The enzyme was stored separately at 4 °C and room temperature (37 °C) for 80 days.

2.7 Steady state kinetics

2.7.1 Determination of optimum pH

The optimum pH was determined by preparing assay buffer of varying pH values (pH 5.0 to pH 9.0). Activity assay was performed at different pH and percent residual activity was determined.

2.7.2 Determination of optimum temperature

The effect of temperature was studied by varying the temperature from 20 °C to 100 °C in a multitemperature water bath (SL-M-WB-70) during the activity assay. The percent relative activity was plotted against temperature.

2.7.3 Effect of substrate concentration on urease activity

For effect of substrate concentration, the activity assay was performed at different substrate concentration. 1 M stock solution of urea was prepared in assay buffer and different concentration of same were made. Initial rates were measured and plotted against corresponding substrate concentration. The Lineweaver-Burk plot was drawn to determine K_m and V_{max} .

2.8 Inhibition Studies

2.8.1 Urease inhibition with boric acid

The stock solution of boric acid was prepared in 50 mM Tris-acetate buffer, pH 7.3 and was suitably diluted for the experiment. The activity assay was performed at standard conditions as described earlier. At first, the I_{50} value of inhibitor was determined. Then K_i value was determined from Dixon plot. Appropriately diluted urease was mixed with varying concentrations of inhibitor and in presence of either 0.1 M or 0.3 M urea, during the activity assay.

2.8.2 Effect of heavy metal ions on urease activity

The 1 mM stock solution of HgCl_2 was made in 50 mM Tris-acetate buffer (pH 7.3) and diluted with the same buffer as required. The activity assay was performed at standard condition and I_{50} value of the inhibitor was determined. The activity of suitably diluted enzyme was determined in the presence of varying concentrations of HgCl_2 at standard assay conditions (0.1 M urea, 50 mM Tris-acetate buffer, pH 7.3, at 37 °C). The solution of 1mM CuSO_4 was prepared in 50 mM Tris-acetate buffer (pH 7.3). Activity of suitably diluted enzyme was determined in the presence of varying concentrations of CuSO_4 at standard assay conditions (0.1 M urea, 50 mM Tris-acetate buffer, pH 7.3, at 37 °C). The I_{50} value of the inhibitor was determined.

3. Results and Discussion

In plants, urease is abundant in the seeds (embryo-specific urease) [12] and is also found at lower in the vegetative tissues of the most species urease (tissue-ubiquitous urease) [13]. The best biochemistry characterized plant urease is that from jack bean (*Canavalis ensiformis*). The Fig., 4.1 was drawn by plotting absorbance versus amount of NH_4Cl and calibration curve was found to be a straight line. From the Fig. 4.1, it is clear that 0.1 optical density corresponds to 150 μg NH_4Cl . Nessler's reagent is commercially available and when it reacts with NH_3 it forms a tetramine complex, which is colored and the intensity of color is measured by spectrometer.

3.1 Storage stability studies

The stability temperature of the pumpkin urease was determined by storing the urease separately at 4 °C and 37 °C. The plot of percent residual activity versus the number of days gave $t_{1/2}$ of 70 days for urease stored at 4 °C and 12 days for urease that was stored at 37 °C (Fig 1). *Ureaplasma urealyticum* urease was fully stable more than 20 days when stored at 4 °C in pH 7.2 buffer containing 1% bovin serum albumin [14].

3.2 Steady state kinetics

The rate of hydrolysis of urea by pumpkin urease at 37 °C showed optimum pH at 8.4 in the pH range 5 to 9 (Fig. 2). It is clear that pumpkin urease is only 50% efficient at pH 5.6 as compared to 100% activity at pH 8.5; whereas jack bean urease [15] has been reported to retain 68% activity at pH 5.2 compared to 100% activity at pH 7. Watermelon urease showed a sharp pH optimum at 8.0 [16]. While the optimum pH for mulberry leaf urease is 9.0, being the highest value among the urease purified so far [17]. Most ureases possess an optimum pH of near neutrality and are irreversibly denatured by exposure to pH values below 5.0.

The optimum temperature was determined by assaying the urease at different temperatures (20-100 °C). It is clear from the Fig 3., that the activity of urease at first increases with rise in temperature till 80 °C and then starts declining until its denaturation. It is evident that the optimum temperature of pumpkin urease is 80 °C. *Lactobacillus reuteri* urease was found to have a maximum activity and stability at 60-70 °C [18]. Palinska and coworkers (2000) [19] have shown that *Prochlorococcus marinus* urease was stable at for 15 min, at temperature 40-60 °C.

Spontaneous degradation of urea occurs with a half-life of approximately 3.6 years, but in the presence of urease, the hydrolysis of urea is 10^{14} times faster [20]. Fig. 4, shows the effect of substrate concentration on urease activity. Lineweaver-Burk plot was used to calculate the K_m and the value was 12.5 mM (Fig 5). The value of V_{max} for pumpkin urease was found to be 62.50 μmol NH_3/min . Kinetic studies of jack bean urease showed a K_m of 2.9 mM to 3.3 mM [15]. An apparent K_m of 0.85 mM for soybean leaf urease; 19 mM to 476 mM for the soybean seed urease, depending on the buffer systems chosen, has been reported [21]. From mulberry leaf urease K_m of urea was reported to be 0.16 mM; this is the lowest value among the plant urease purified so far [17]. In urea hydrolysis, plant and microbial ureases exhibit K_m values ranging from 0.1 to >100 mM urea; jack bean urease has a K_m value of 2.9 mM^2 . Although

urea is the major substrate of urease, this enzyme is capable of hydrolyzing (*albeit* poorly) other substrates such as acetamide, formamide, *N*-methylurea, semicarbazide, and hydroxyurea [22].

3.3 Inhibition studies of pumpkin urease

The study of urease inhibition is important due to many factors, urease is known to be a major cause of pathologies induced by *H. pylori*, which allows the bacteria to survive at the low pH of stomach during colonization and therefore plays an important role in the pathogenesis of gastric and peptic ulcer cancer also³. In infections with *P. mirabilis* and *Y. enterocolitica*, urease has been implication in urolithiasis (stone formation) and contributes to the development of acute phelonephritis and infection induced reactive arthritis, respectively [23-24]. Urease inhibitors possess a wide application in agriculture. These inhibitors play a significant role in reducing the loss of nitrogen from urea fertilizer in the form of volatile NH_3 and thus, improve the utility of urea fertilizers [25].

3.3.1 Inhibition with boric acid

Boric acid was investigated for its inhibitory effect on the pumpkin urease. The I_{50} value was determined by performing the activity assay in the presence of boric acid with varying concentration. The I_{50} value of boric acid inhibition for pumpkin urease was 7.5 mM (Fig. 6). Furthermore, the inhibition constant (K_i) was determined from the Dixon plot and the value was found to be 2.50 mM and the competitive type of inhibition was observed as evident from the Fig. 7. Boric acid has been reported as a potent competitive inhibition of Soybean urease with K_i value of 0.20 ± 0.05 mM at pH 7.0 [26]. It has also been reported to be a strong competitive inhibition in the case of jack bean, pigeonpea (*CajanusCajan*), *P. mirabilis* and *K. aerogenes* urease. The detailed mechanism of urease inhibition by boric acid cannot be established by kinetic studies alone. Benini *et al.* (1984) [27], reported the structure detail of the *Bacillus Pasteurii* urease boric acid complex and clarified the molecular details of the inhibition and the unique binding mode for this inhibitor and provided the insights into role of nickel ions in enzyme urea hydrolysis.

3.3.2 Inhibition with Heavy metal ions

The effect of Hg^{2+} ion (HgCl_2) on the activity of pumpkin urease was studied in the concentration range that had measurable inhibition (Fig 8). The desired concentration of inhibitor (10^{-3} to 10^{-4} mM) was added to the standard assay mixture and assayed. The results revealed a concentration-dependent inhibition of the activity. About 50% inhibition (I_{50}) was observed at 4.8×10^{-4} mM, which increased to about 70% at 3×10^{-4} mM of Hg^{2+} . Evidently the complete loss in enzyme activity was recorded at 7×10^{-4} mM of Hg^{2+} . Inhibition studies with Cu^{2+} ion (CuSO_4) at different concentrations (10^{-3} to 10^{-8} mM) revealed that the enzyme was inhibited maximally at 10^{-5} mM (Fig 9). The I_{50} was observed at 5×10^{-5} mM. Singh and Nye (2006) [28] 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. The results revealed a concentration-dependent inhibition of the activity.

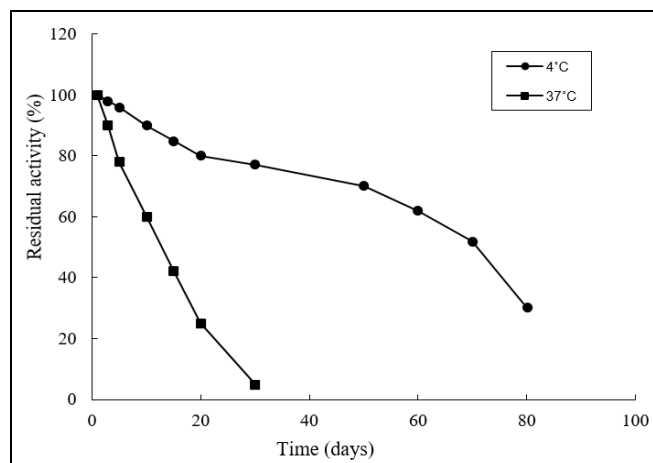


Fig 1: Comparison of stability of pumpkin urease when stored at 4 °C and 37 °C. Suitably diluted urease was assayed at standard conditions after incubating at the indicated temperatures.

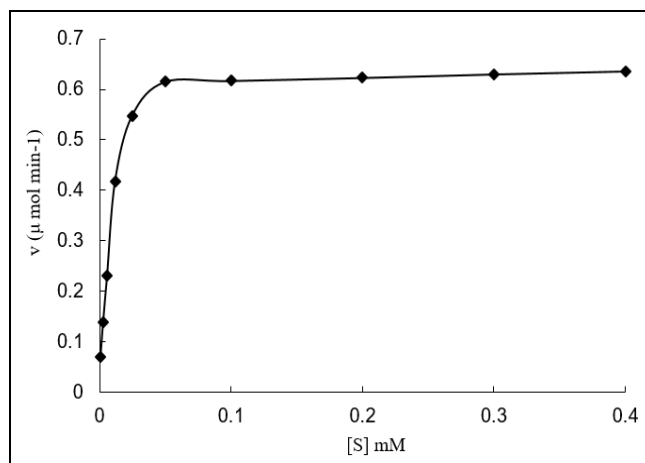


Fig 4: Effect of substrate concentration on the activity of pumpkin urease. Suitably diluted urease was assayed in the presence of varying concentrations of urea (1-50 mM) and at under standard conditions as described under materials and methods.

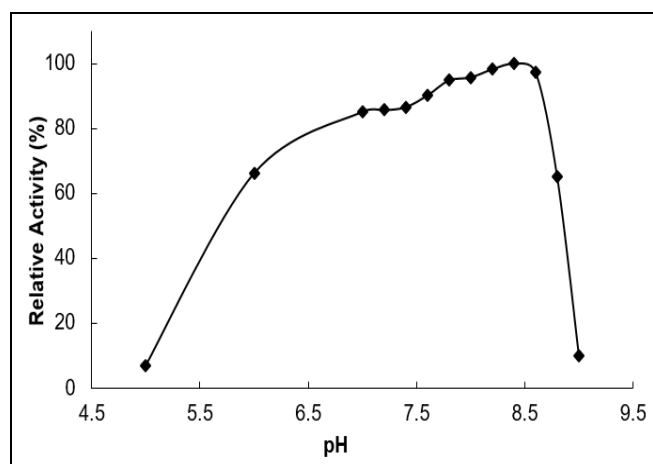


Fig 2: Effect of pH on the activity of pumpkin urease. Suitably diluted urease was assayed at various pH values under standard conditions as described under materials and methods.

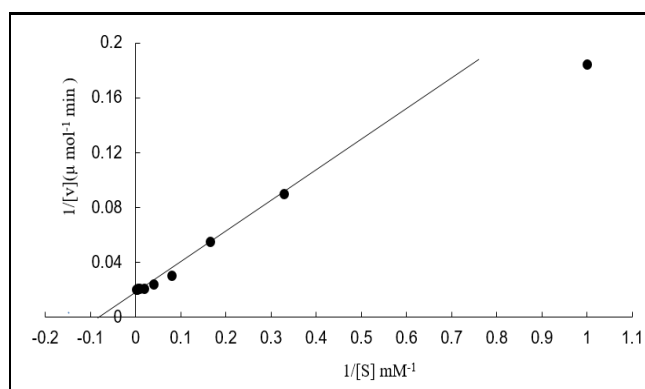


Fig 5: Lineweaver-Burk plot to determine the value of K_m and V_{max} . Suitably diluted urease was assayed in the presence of varying concentrations of urea (1-110 mM) and at under standard conditions as described under materials and methods.

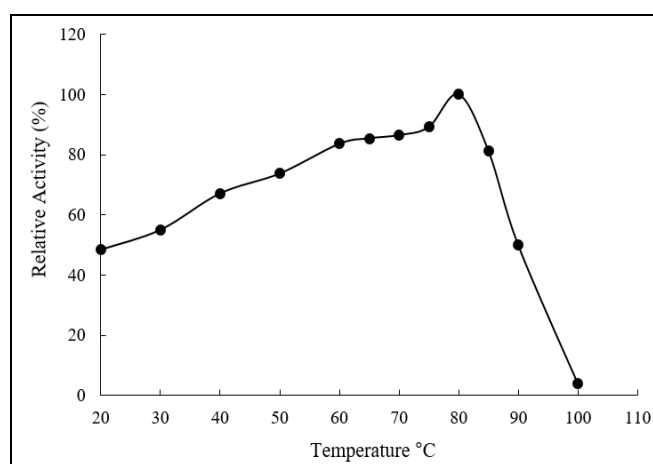


Fig 3: Effect of temperature on the activity of pumpkin urease. Suitably diluted urease was assayed at various temperatures under standard conditions as described under materials and methods.

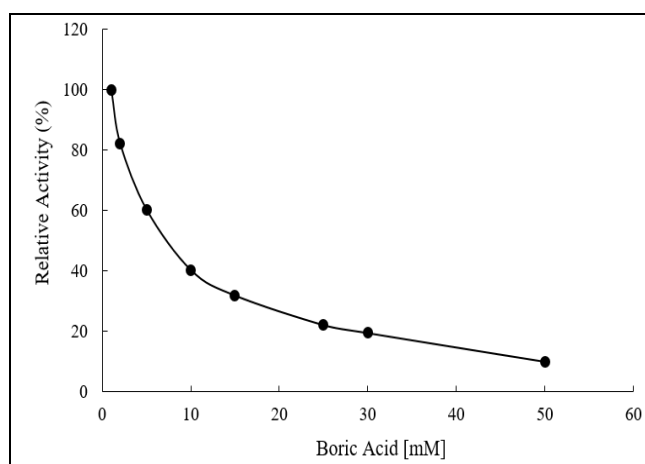


Fig 6: Effect of boric acid on the activity of pumpkin urease. Suitably diluted urease was assayed in the presence of varying concentrations of boric acid under standard conditions as described under materials and methods.

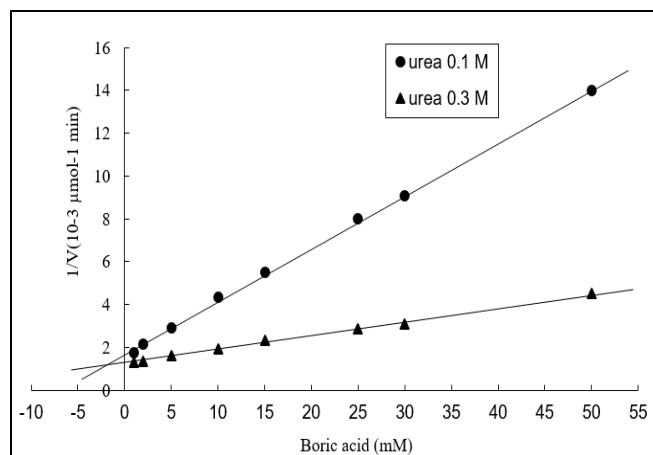


Fig 7: Dixon plot for the determination of K_i of boric acid inhibition of pumpkin urease. Suitably diluted urease was assayed in the presence of varying concentrations of boric acid and in presence of 0.1 M & 0.3 M urea under standard conditions as described under materials and methods.

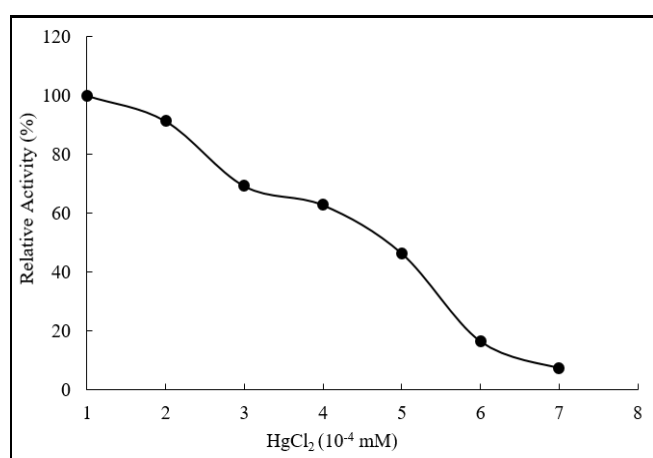


Fig 8: Effect of HgCl_2 on the activity of pumpkin urease. Suitably diluted urease was assayed in the presence of varying concentrations of HgCl_2 under standard conditions as described under materials and methods.

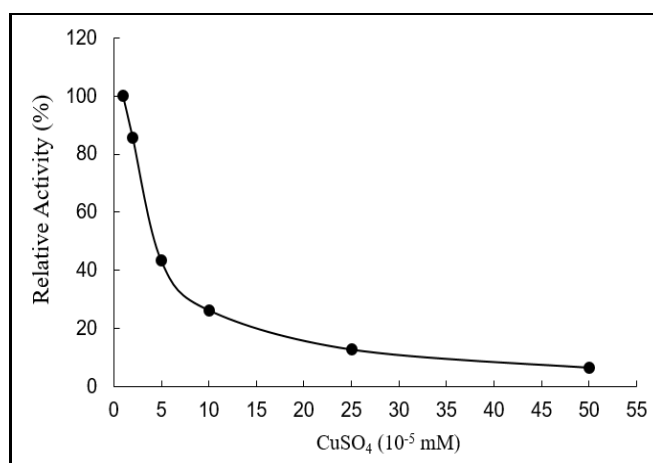


Fig 9: Effect of CuSO_4 on the activity of pumpkin urease. Suitably diluted urease was assayed in the presence of varying concentrations of CuSO_4 under standard conditions as described under materials and methods.

4. Conclusion

The physiological role of pumpkin or other plant urease in the cellular economy is not well known. However, it is apparent from the present studies that under physiological

condition, its activity will be strongly inhibited by several inhibitors. It is speculated that the low concentration used for the inhibitors can also inhibit any of the soil urease, which is important in plant agronomy. In agriculture, high urease activity during urea fertilization causes significant environment and economical problems by releasing abnormally large amount of NH_3 into atmosphere. This induces plant damage primarily by depriving plants of essential nutrient and secondly through NH_3 toxicity in agriculture, which accounts for about 46% of the total world nitrogen fertilizer consumption. Due to action of enzyme urease, urea nitrogen is lost as volatile NH_3 . For sufficient use of fertilizer nitrogen, urease inhibitor along with the urea fertilizer is one of the available options. Urease inhibitors also provide an insight in understanding the mechanism of enzyme catalyzed reaction, the role of various amino acids in catalytic activity present at the active site of enzyme and the importance of nickel to this metalloenzyme. Due to the similar catalytic mechanism exhibited by all ureases, the inhibition studied can be successfully used in conjunction with ureases of any origin for controlling/inhibiting urease activity in soil and pathogenic microbes to solve the various problems as stated earlier.

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Conflicts of Interest

All authors declare no conflicts of interest.

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