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Thermal Inactivation Kinetics and Inhibition Studies of Watermelon (*Citrullus vulgaris*) Seeds Urease

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Abstract:
Urease was isolated from the seeds of watermelon (*Citrullus vulgaris*). The optimum temperature and pH was found to be 50°C and 8.3. Urease shows no loss in their activity when treated overnight with assay buffer of different pH. Watermelon urease thermal inactivation studies revealed a variation in kinetic pattern at different temperatures. At temperature of 50ºC and 60ºC, urease shows almost no loss in activity i.e. their stable nature. Thermal inactivation (time dependent) shows biphasic kinetics at 70°C, 80°C and 85°C. Investigation studies strongly support the oligomeric nature of the enzyme. The Km and Vmax of *Citrullus vulgaris* urease was found to be 10 mM and 142.85 µmole of urea/min/mg of protein. The plot of percent residual activity versus the number of days gave a $t_{1/2}$ of 70 days for urease stored at 4°C and 16 days for urease that stored at 37°C. The I_{50} values for Hg^{2+} and NaCl was found to be 6.9 nM and 240 mM which shows strong inhibitory nature of Hg^{2+}.

Keywords: urease, watermelon, kinetics, inhibition, *Citrullus vulgaris*

Introduction

Urease (EC 3.5.1.5, urea amidohydrolase), a nickel-dependent metalloenzyme, catalyzes the hydrolysis of one molecule of urea results in the release of two molecules of ammonia and one molecule of carbon dioxide (Dixon *et al*., 1975). Urease was the first ever enzyme crystallized by Sumner *et al.,* (1926) while urea was the first organic molecule synthesized in the chemical laboratory. The structure of urease was first solved by P. A. Karplus *et al.,* (1995). It appears to be found in most, if not in all plants and is an abundant seed protein in many members of Cucurbitaceae, Fabaceae, Asteraceae and Pinaceae. Watermelon is an edible fruit belong to family Cucurbitaceae which was thought to be originated in Southern Africa. Now it's different variety widely cultivated in different parts of the world. Some investigations suggest that the seeds of water melon (*Citrullus vulgaris*) which belongs to Cucurbitaceae had high urease content (Damodaran and Shivaramakrishan, 1937) (Fahmy, Magda and Mamdouh, 1993). Urease was identified as the first nickel metalloenzyme (Dixon, Gazzola, Blakeley and Zerner, 1975). Its major activity with some exceptions is associated with the soluble fractions of the cells (Mobley, Island and Hausinger, 1995). Its sub cellular location was resolved by immuno-cytochemical and biochemical methods and proposed to be a cytosolic enzyme (Faye *et al.,* 1986). The best genetic data of plant ureases are available for soybean (*Glycine max*) (Polacco and Holland, 1993). Bacterial ureases play an important role in the pathogenesis of a number of bacterial species including *Proteus mirabilis, Staphylococcus saprophyticus, Yersinia enterocolitica, Ureaplasma urealyticum* and others (Mobley *et al.,* 1995). Due to urease activity, bacteria (e.g. *Klebsiella aerogenes*) are able to use urea as

a sole nitrogen source (Mulrooney *et al.,* 1989). Urease activity of an infectious microorganism can contribute to the development of urinary stones, pyelonephritis, gastric ulceration, and other diseases. In contrast to its pathogenic effects, microbial ureases are important enzymes in ruminant metabolism and in environmental transformations of certain nitrogenous compounds (Mobley and Hausinger, 1989). 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). In comparison to plant ureases, functions of bacterial ureases is known and have been shown to be important virulence determinants in the pathogenesis of many clinical conditions in human and animals (McLean *et al.,* 1986). Urease is known to be the major cause of pathologies e.g. induced by *Helicobacter pylori*, gastric and peptic ulcers which in some cases may progress to cancer (Kusters *et al.,* 2006). Urea represents an assimilable nitrogen source for bacteria that can colonize the human body. For example, a significant proportion of the urea produced in the liver ends up in the intestines, where it can be hydrolyzed and assimilated by several different species of anaerobic, ureolytic bacteria (Mobley, Island and Hausinger, 1995). Urease also plays an important role in germination and in seedlings nitrogen metabolism (Zonia, Stebbins and Polacco, 1995). Externally applied as fertilizer, urea becomes accessible to plants only through urease activity (Witte *et al*., 2002).More than 90% of world industrial production of urea is destined for use as a nitrogen-release fertilizer (Meessen *et al.,* 2005). Urea has the highest nitrogen content of all solid nitrogenous fertilizers in common use. Urea is highly soluble in water and is, therefore, also very suitable for use in fertilizer solutions. Since urea is one of the world's most widely used nitrogen fertilizers, its enzymatic hydrolysis is a process of great agricultural importance. Decreased activity of urease lead to accumulation of urea in plant leaves, which is responsible for leaf tip necrosis (Balch *et al.,* 1986; Balch, Olmstead and Guimerane, 1984). On the other hand excessive ammonia, released from urea adversely affect germination and seedling growth (Olmstead, Guimerans and Balch, 1983; Balch, Fossett, Guimerans, Olmstead, 1985). In agriculture, high urease activity causes significant environmental and economic problems by releasing abnormally large amounts of volatile ammonia inducing plant damage by ammonia toxicity and increase in the pH of soil. Because of mentioned reason it is need to appropriately control the urease activity (Mobley *et al.,* 1995). Vincent H. Varel *et al.* (1997) demonstrated the use of urease inhibitors to control nitrogen loss from livestock waste. According to them current waste management systems for cattle feedlots and swine facilities result in nitrogen losses of approx. 75%. Most of this loss occurs through the rapid hydrolysis of urinary nitrogen (urea) to ammonia, which volatilizes into the atmosphere. This contributes to odor, environmental problems, and loss of a valuable fertilizer resource. Hydrolysis of urea in untreated cattle or swine waste (controls) was complete within one day. Addition of the inhibitors once per week was the most effective method of preventing urea hydrolysis.

These problems can be successfully tackled by undertaking the inhibition studies of urease from any other source as model system. These inhibitors can then be successfully applied in conjunction with fertilizers. A number of studies have demonstrated that this approach can be very fruitful. In addition to their potential value in medicine and agriculture, the study of urease inhibitors can provide insight into selected aspects of the enzyme mechanism and active-site structure. Since, the amino acid sequence alignment reveals that all known ureases, isolated from different sources, are highly similar and shares a common phylogenetic relationship and proposed to have common structure and catalytic mechanism (Zerulla *et al.,* 2000). In present studies the urease from watermelon (*Citrullus vulgaris*) has been isolated and characterized biochemically along with thermal inactivation studies.

Material and Methods

Chemicals and enzyme

Urea (enzyme grade), Tris acetate buffer, Ammonium chloride (NH_4Cl), Sodium chloride (NaCl), Mercuric chloride ($HgCl_2$), Nesseler's reagent (N.R) and Trichloroacetic acid (TCA) were from HiMedia, India. Glacial acetic acid was purchased from Merck, India. All solutions were prepared in double distilled water.

Extraction of enzyme

Approximately, 25 gm. seeds of *Citrullus vulgaris* (Watermelon) were soaked overnight in 25 ml of 25 mM Tris acetate buffer, pH 6.5 and stored at 4°C temperature. Next day, the seeds of *Citrullus vulgaris* (Watermelon) were grinded in a kitchen blender to form slurry. Filtration of Watermelon seeds slurry through a four layered muslin cloth (Cheese cloth) to remove cell debris. Filtrate obtained was then centrifuged at 12,000 rpm for 15 minute at 4°C temperature. If required then re centrifuged at 10,000 rpm for 10 minute. The clear supernatant (crude preparation of Watermelon Urease) was obtained and stored at 4°C temperature in refrigerator for further studies. All the operations were carried out at 4°C temperature unless stated otherwise.

Urease activity assay

Urease was assayed by determining the amount of ammonia liberated in a fixed time interval on incubating the enzyme urease and urea. 0.9 ml assay Buffer (50 mM Tris acetate buffer) poured in 2ml test tube. Then properly diluted 0.1 ml urease enzyme solution with assay buffer was preincubated at 37°C. This is sufficiently high enough temperature to affect rapid enzyme hydrolysis, but not high enough for inactivation of enzyme. Substrate solution (0.2 M Urea solution) was added and incubated at 37°C for 10 minutes. 1.0 ml of 10% Trichloroacetic acid (TCA) solution was added to stop the assay reaction.1 ml of urease activity assay reaction mixture was transferred to 50 ml of volumetric flask, which already contained 10-15 ml of double distilled water.1.0 ml Nesseler's reagent (N.R) was then added to the 50 ml volumetric flask. The urease activity assay reaction mixture volume was made up to 50 ml with double distilled water in 50 ml of volumetric flask. The absorbance of urease activity assay reaction mixture was measured at 405 nm (Path length: 1 cm) on the spectrophotometer. A blank without enzyme was run side by side and correction was applied for the same.

Steady state kinetics

Determination of optimum pH: The optimum pH was determined by preparing assay buffer of varying pH values (pH 5.3-10.3). The activity assay was performed at the different pH and percent relative activity was determined.

Determination of optimum temperature: The effect of temperature was studied by varying the temperature from 20° - 90°C in a multi temperature (TC-120, Grant instruments, England) water bath during activity assay. The percent relative activity was plotted against temperature.

Effect of substrate concentration on urease activity: For effect of substrate concentrations, the activity assay was performed in different substrate concentrations as discussed above. The stock solution of substrate (1.0 M urea) was prepared in assay buffer and different concentrations of the same were made. Initial rates were measured and plotted against corresponding substrate concentrations. The Line weaver-Burk plot was drawn to determine the *Km.*

Storage stability studies: The temperature stability of the watermelon urease was determined by storing urease, separately at two different temperatures and assayed at regular intervals. The enzyme was stored at 4°C and 37°C, separately for 80 days.

Time dependent thermal inactivation studies: For time thermal inactivation studies, 0.2 ml urease was incubated in water bath at the indicated temperatures for a fixed period of time. The aliquots drawn at the different time intervals were rapidly cooled in ice water and checked for residual activity. The activity measurement of the sample was executed after 5 minutes storage in ice water. The experiments were carried out at four different temperatures, like 50°C, 60°C, 70°C and 80°C.

Inhibition studies

Inhibition with NaCl and $HgCl_2$: The 5M stock solution of NaCl was prepared. Different concentrations of inhibitor were made by adding different volumes of NaCl to assay buffer and the final volume was made to 0.9 ml. Urease (0.1ml) was added to 0.9 ml of assay buffer containing inhibitor and the volume was made up to 1 ml. Next, urea (1.0ml) was added and incubated with 10 min at 37°C. After stopping reaction with TCA the absorbance was recorded at 405 nm for each sample. The I_{50} value of inhibitor was determined. Next, the solution of 1.0 mM $HgCl_2$ was prepared. The activity assay was carried out at standard conditions as described earlier in the presence of varying concentration of inhibitor and I_{50} value of the inhibitor was determined.

Results and discussion

Steady state kinetics

The rate of hydrolysis by watermelon urease at 37°C showed optimum pH at 6.3 in pH range of 5.3 – 10.3. (Fig.1). Urease from plant sources has been purified from the seeds of jack bean (*Canavalia ensiformis*) showed optimum pH at 7.0 (Sumner, 1926) while leaves of soybean (*Glycine max*) reported to show optimum pH at 7.0 (Polacco and Havir, 1979), pigeon pea (*Cajanus cajan*) showed optimum pH at 7.3 (Das *et al.,* 2002) and leaves of mulberry showed optimum pH at 9.0 (*Morus alba*) (Hirayama *et al.,* 2000).

A bell shaped curve (Fig. 2) was obtained when Watermelon (*Citrullus vulgaris*) urease was assayed at different temperatures from 20°C – 90°C. The optimum temperature was found to be 50°C. The results showed that the temperature optima of watermelon (*Citrullus vulgaris*) urease shifted directly towards high temperature. Ureases from different sources have been reported to be most efficient in the temperature ranges between 30°C to 70°C. Ureases of jack bean (*Canavalia ensiformis*) showed optimum temperature at 60°C (Sumner and Hand, 1929) while urease from leaves of soybean (*Glycine max*) reported to show optimum temperature at 65°C (Singh, Dwevedi and Kayastha, 2009), pigeon pea (*Cajanus cajan*) urease at 47°C (Reddy , Srivastava, Dey, Kayastha, 2004), *Cheopodium album* leaf urease exhibits optimum temperature between 30°C-40°C (Shora *et al*; 2001), urease purified from the pea seeds (*Pisum sativum*) display optimum temperature at 40°C. Chickpea (*Cicer arietinum*) urease at 48°C (Pervin, Jahan, Rana, Sana, Rahman, Shaha, 2013), Syrian mesquite (*Prosopis farcta*) urease at 45°C (Hamzah *et al.,*2014). Other microorganisms ureases also shows varying optimum temperature ranges viz. *Yersinia enterocolitica* urease possess optimum temperature at 65°C (Bhagat and Virdi, 2009) while *Helicobacter pylori* urease at 43°C (Mobley, 2001). *Camphylobacter pylori* urease was found to have a maximum activity and stability at 45°C (Mobley *et al.,* 1988), *Lactobacillus reuteri* urease display maximum activity at optimum temperature 60°C-70°C (Kakimoto *et al., 1989*) which is

greater than that of *Prochlorococcus marinus* urease exhibits optimum temperature between 40℃-60℃ (Palinska *et al.,* 2000).

Urease was assayed in different concentrations of urea to determine the effect of substrate (Fig.3). The Line weaver-Burk plot was used to calculate the Km and Vmax for watermelon urease was found to be 10 mM and 142.85 µmole of urea/min/mg of protein respectively (Fig. 4).Km values of Jack bean urease is 2.9 mM, similar to values of 2.8 mM for *Klebsiella aerogenes* urease (Todd and Hausinger, 1987), 2.2 mM for *Selenomonas ruminatium* urease (Hausinger *et al.,* 1986) while *Ureaplasma urealyticum* Km of urea was reported to be 2.5 mM (Eng, Robertson, Stemke, 1986). Generally acid ureases show Km between 2.0-3.8. Some shows further high values of Km viz. 12.5 mM for *Arthrobacter oxydans* urease (Schneider, Kaltwasser, 1984); 17.3 mM for *Bacillus pasteurii* (Christians and Kaltwasser, 1986); 32 mM Km for the *Brevibacterium ammoniagenes* (Nakano, Takenishi and Watanabe, 1984).

The stability temperature of the watermelon urease was determined by storing the urease at 4℃ and 37℃. The plot of percent residual activity versus the number of days gave a $t_{1/2}$ of 70 days for urease stored at 4℃ and 16 days for urease that stored at 37℃ (Fig.5).The *Pisum sativum* urease enzyme activity decreased with time even when stored at −4°C. It represented 100% on the first day but decreased to 80% on the tenth day. However, it retained about 14.1% even after 2 months. The exponential decay fits showed a half life time of 22.4 days. (Hefnawy, Sakran, Ismail, Aboelfetoh, 2014).

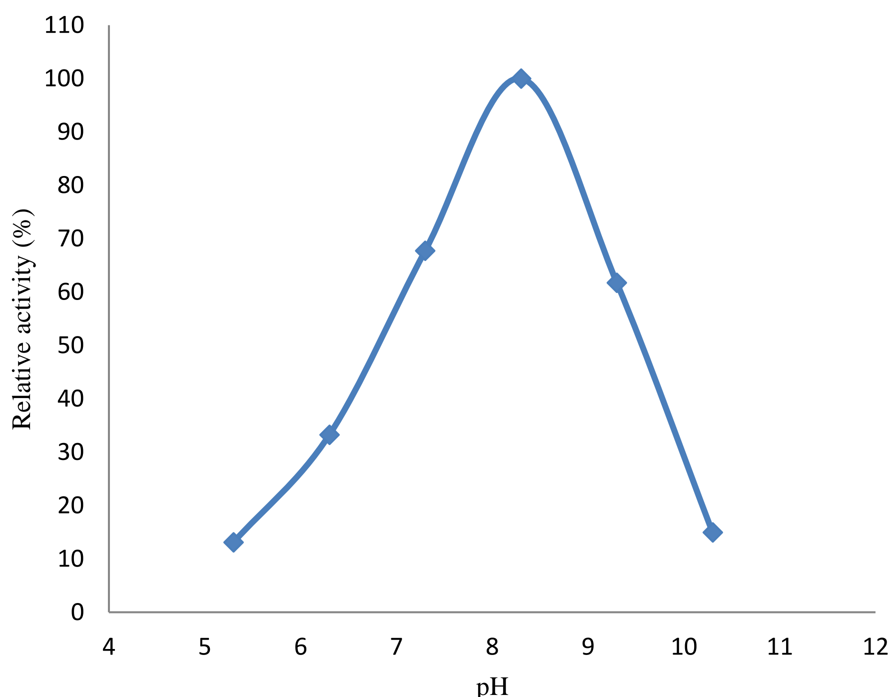

Fig 1. Effect of different pH on the activity of watermelon urease

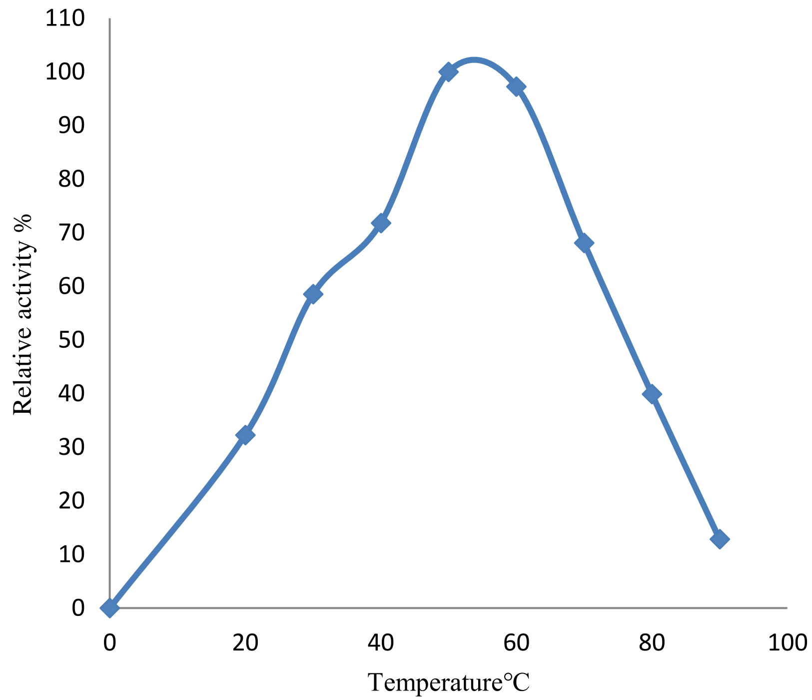

Fig 2. Effect of varying temperature on the Watermelon urease

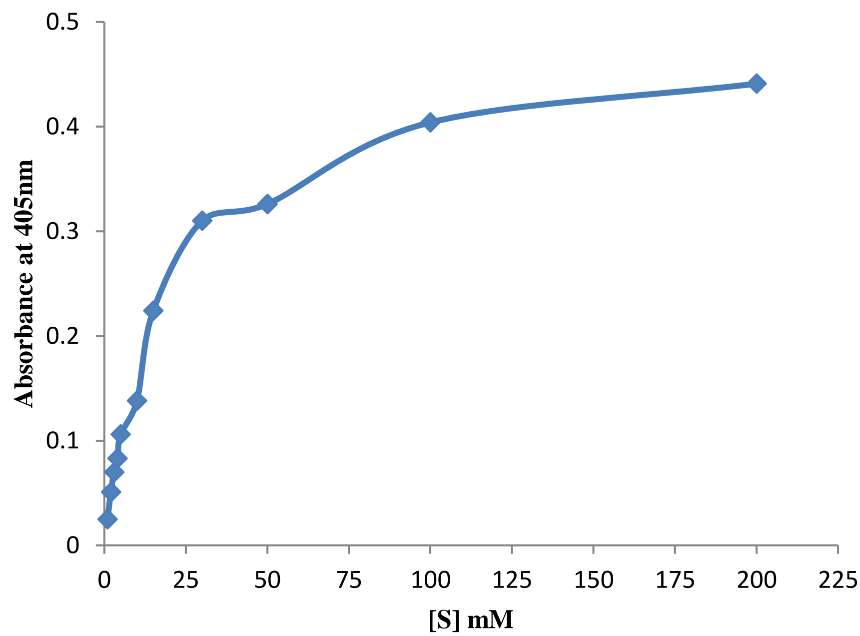

Fig 3. Effect of substrate concentration on the activity of Watermelon urease (*Citrullus vulgaris*)

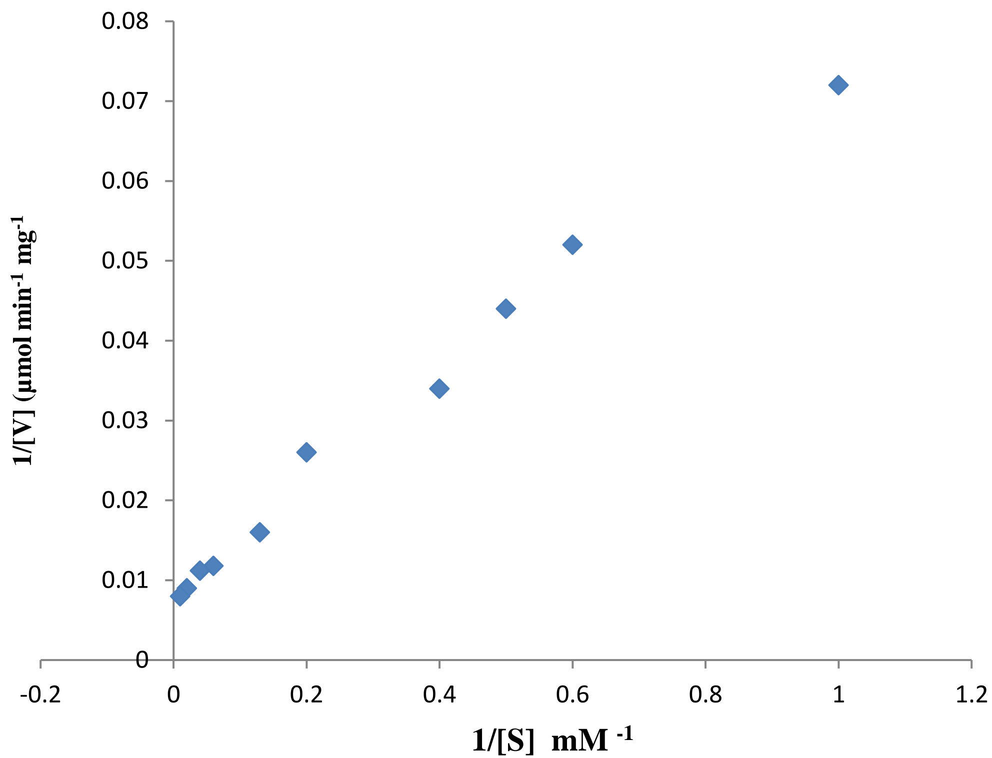

Fig.4. Lineweaver-Burk plot to determine the value of watermelon urease Km and Vmax.

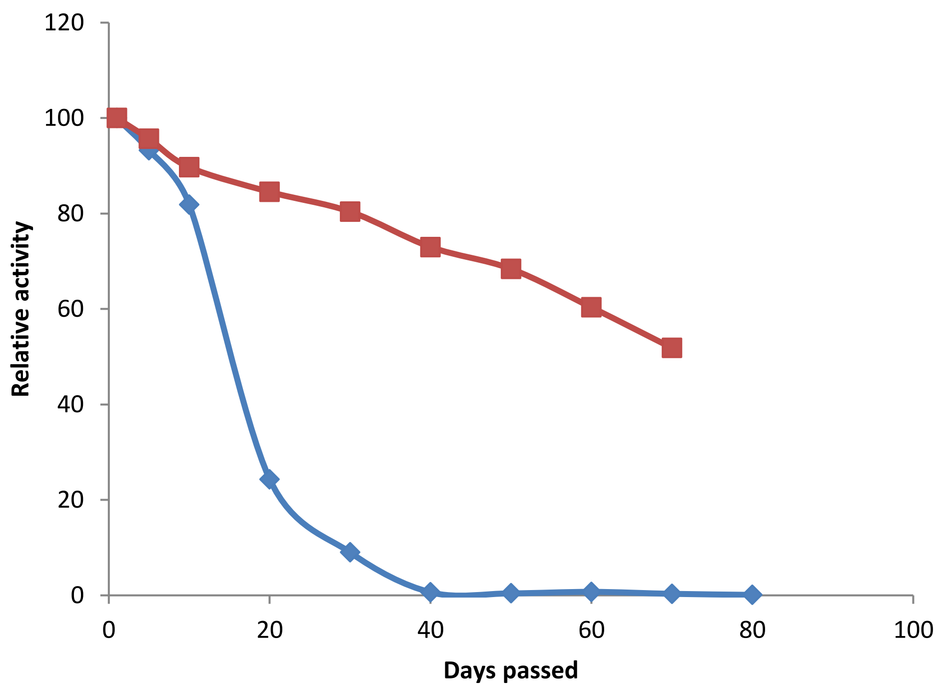

Fig.5. Graph shows comparison of storage stability of watermelon urease at two temperatures 4°C and 37°C.

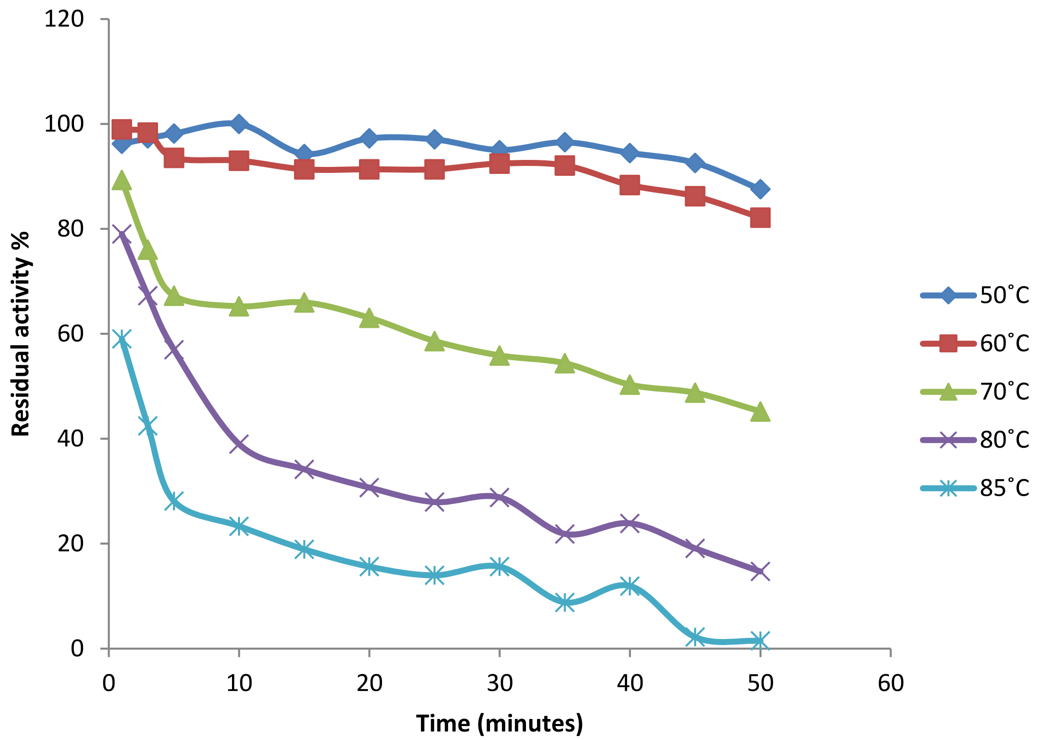

Fig.6. Time dependent thermal inactivation studies of watermelon urease

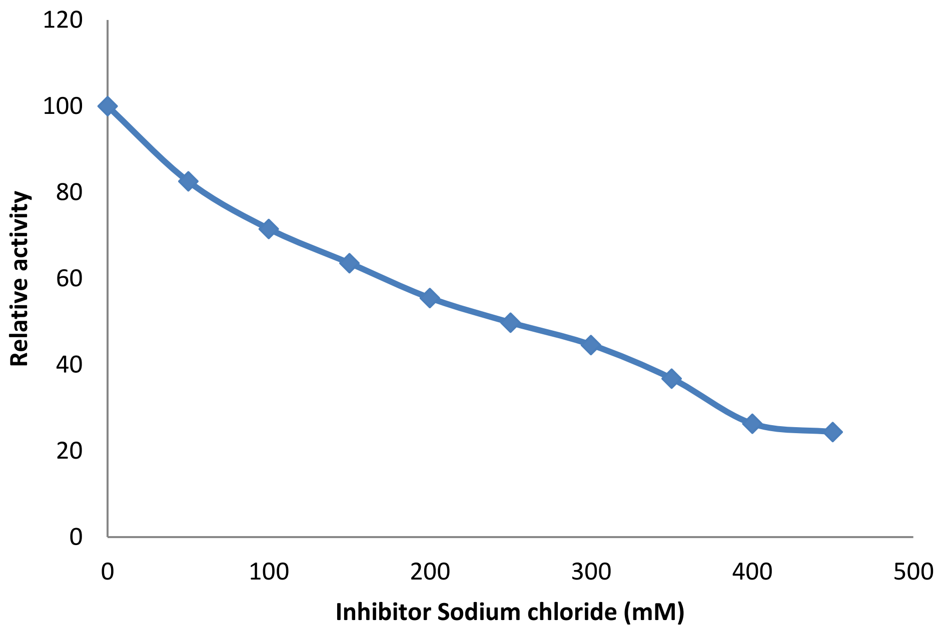

Fig.7. Graph shows the inhibitory effects of NaCl on the activity of the watermelon urease.

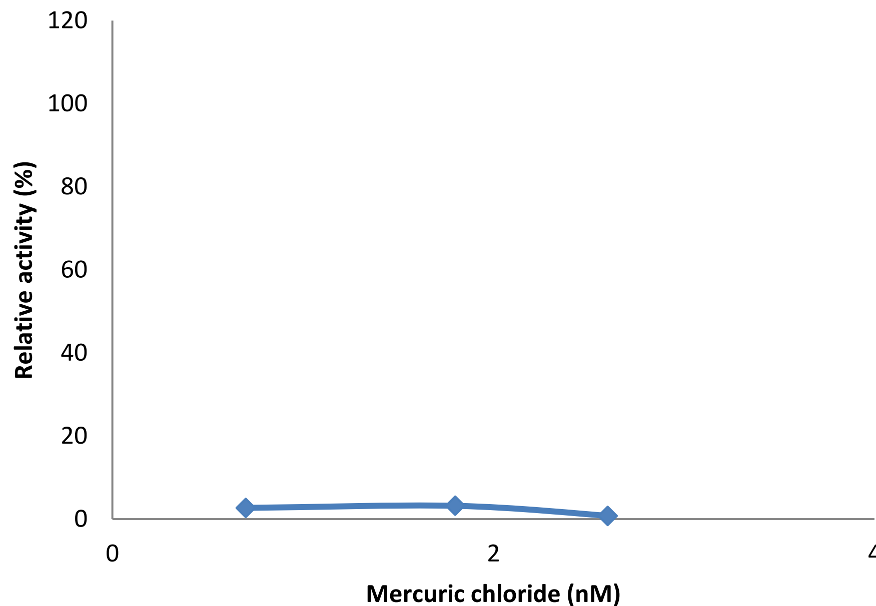

Fig.8. Graph shows the inhibitory effects of Mercuric chloride on the activity of the watermelon urease.

Time dependent thermal inactivation:
The thermal inactivation of watermelon urease was investigated at five different temperatures (50°C, 60°C, 70°C, 80°C and 85°C) (Fig. 6). At temperature 50°C and 60°C the watermelon (*Citrullus vulgaris*) urease shows stability until 35 min. which may be due to watermelon urease works best at these temperatures. At 60°C, 70°C and 80°C, thermal inactivation of soluble watermelon urease shows biphasic kinetics, in which the enzyme activity decreases in two phases, i.e. half of the initial activity was destroyed more rapidly than the remaining half. Thermal inactivation studies strongly support the oligomeric nature of urease, which is also suggested by earlier workers (Polacco and Havir, 1979).

Urease inhibition
The inhibitors of urease were extensively studied for therapy against bacterial ureases like *Helicobacter pylori* that induce human pathogenic diseases like urinary kidney stone formation, pyelonephritis, peptic ulcer, hepatic coma, gastric ulceration, hepatic encephalopathy, urinary and ammonia catheter encrustation etc. (Mobley H., Island M.D., Hausinger R.P., 1995; Ermler U., Grabarse W., Shima S., Goubeaud M., Thauer R.K., 1998). Increased levels of soil microbial urease have been known to decrease the efficiency of urea fertilizers. Due to increased use of urea as fertilizer this has been caused severe environmental and economic problems by continuously releasing high amount of urea in the environment (Upadhyay B.S.L., 2012). Urease from different sources has been found to be inhibited by heavy metal ions. Kumar and Kayastha (2004) investigated the heavy metal ions including Ag^{+}, Hg^{2+}, and Cu^{2+} showed strong inhibition on soybean urease viz. $I_{50} = 2.3 \times 10^{-8}$ mM, 7.1×10^{-5} mM, and 3.3×10^{-3} mM, respectively, with the silver ion being a potent inhibitor. Heavy metal ion $Hg^{2+}$was investigated for their inhibitory effect on the watermelon urease. Many literatures also proved the inhibitory effect of Hg^{2+} on urease (Zabroska *et al.*, 2004; Du N *et al.,* 2012). The I_{50} value for the Hg^{2+} was found to be 6.9nM. Due to low value of I_{50} as compare to other inhibitor mercuric chloride was

found to be strong inhibitor of watermelon urease. NaCl was investigated for its inhibitory effect on watermelon urease. The objective was to investigate the effect of constituent anion. The activity assay was carried out in the presence of varying concentrations of NaCl. The I_{50} value for the Sodium chloride was found to be 240 mM. Due to high value of I_{50}, NaCl was found to be poor inhibitor of watermelon urease.

Conclusion

The physiological role of watermelon urease or other plant urease in the cellular economy is not well known. But it has proved from the present studies that under proper physiological conditions, urease activity will be strongly inhibited by several inhibitors. Since, the amino acid sequence alignment reveals that all known ureases, isolated from different sources, are highly similar and shares a common phylogenetic relationship and proposed to have common structure and catalytic mechanism. Therefore, urease from any source, may be bacterial or plant, can be used as model system for inhibition studies and results would be equally applicable for any system or field of application.

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