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Effects of various factors on urease activity in germinating seeds of *Macrotyloma uniflorum* (Lam.) Verdc

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Abstract
Enzyme activity is influenced by various environmental factors which may alter the three dimensional structure of an enzyme changing its rate of activity or its ability to bind substrate. The present investigation has been carried out to study the effect of germination time, temperature, substrate and enzyme concentration, promoters and inhibitor on the urease activity in germinating seeds of *Macrotyloma uniflorum*. The partial purification of urease enzyme has been done by acetone fractionation method. The optimum germination time for urease activity in the present study has been found to be 4 hours and the optimum temperature 60ºC. The optimum substrate (urea) concentration of urease has been found to be at 3ml of substrate and the optimum enzyme concentration at 1ml of urease enzyme. Further urease activity has been assayed in presence of various promoters like cobalt (Co), calcium (Ca) and manganese (Mn); and also an inhibitor Copper (Cu).

Keywords: urease, *Macrotyloma uniflorum*, temperature, enzyme activity, promoter, inhibitor, germination

1. Introduction
Urease (urea amidohydrolase, E.C. 3.5.1.5) is a nickel dependent metalloenzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide (Varner *et al.*, 1976) [27]. This enzyme has been isolated and characterized from bacteria, fungi and plants (Mobley *et al.*, 1995; Hausinger, 1993; Hausinger and Karplus, 2001; Follmer, 2008) [18, 13, 14, 11]. Urease was first reported from the leaves of legume, soybean (Takeuchi, 1909) [25]. Urease has been found to have a high specificity for its primary substrate, urea (Smith *et al.*, 1993) [24]. The primary function of urease is to allow the organism to use urea as a nitrogen source. In plants, urease has a function in systemic nitrogen transport pathways and is thought to behave as a toxic defense protein (Polacco and Holland, 1993; Pervin *et al.*, 2013) [21, 20].

The enzyme urease plays a major role in seed germination by degrading urea formed from arginase activity (Zonia *et al.*, 1995) [28]. The best studied urease is from jackbean (*Canavalia ensiformis*) (Blakeley and Zerner, 1984) [9] and it was the first enzyme to be crystallized (Banerjee *et al.*, 2012) [4]. The embryo-specific urease is a seed germination protein present abundantly in many plant species such as soybean (*Glycine max*) (Polacco and Holland, 1993; 1994) [21, 22], jackbean (Polacco and Holland, 1994) [22], *Arabidopsis* (Zonia *et al.*, 1995) [28] and *Cajanus cajan* (Banerjee *et al.*, 2012) [4]. The urease enzyme plays a major function during the germination process where the protein stored within the seed is mobilized to nourish the seedlings (Goldraij *et al.*, 1995) [12]. Urease is a cytosolic enzyme. Its major role is associated with the soluble fractions of the cells (Mobley *et al.*, 1995) [18].Ureases from plants are homo-oligomeric proteins consisting of identical subunits. Urease also plays an essential role in the defense mechanism of plants due to its anti-fungal properties and provides protection against phytopathogens and predators (Becker-Ritt *et al.*, 2007; Menegassi *et al.*, 2008) [5, 17].

The sensitivity of urease to heavy metal ions is due to the presence of multiple cysteine residue of which one conserved to all known ureases and is located in the mobile flap of the active site of enzyme (Hausinger, 1993; Magomya *et al.*, 2017) [13, 16]. Heavy metal ions inhibit the catalytic activity of urease by binding with a sulfhydryl group in the active centre of the enzyme in a reaction similar to the formation of metal sulphides (Bhattacharya *et al.*, 2007; Magomya *et al.*, 2017) [7, 16]. There will be a dramatic decrease in catalytic activity once the metal atom is bound and the sulfhydryl group cannot show catalysis. Very low ratios of inhibitor can have a dramatic effect on the enzyme activity thus, indirect determination of trace concentrations of the inhibitor is possible by monitoring the enzyme activity (Magomya *et al.*, 2017) [16]. Seeds of *Macrotyloma uniflorum* (Lam.) Verdc. has been selected as source of enzyme as it has high levels of urease. Enormous studies have been reported on urease activity of *Glycine max* and *Cajanus cajan* but the activity of urease in *Macrotyloma uniflorum* has not been studied much. *Macrotyloma uniflorum* (Lam.) Verdc. (English name- horse gram), is an annual or perennial herb, climbing, prostrate, rarely erect belonging to family Fabaceae. It is an underutilized (Aiyer, 1990) [1] and unexplored (Reddy *et al.*) [23]. It is found in Africa, Australia, India, Indonesia, Bhutan, Myanmar, Nepal and Sri Lanka (Nasir *et al.*, 1977) [19]. Horse gram termed as poor man's pulse and has more utility as cattle feed than human consumption. The seeds are rich in protein, calcium and polyphenols (Bharathi *et al.*, 2015) [6]. It is considered as a good source of carbohydrates, energy (Bravo *et al.*, 1998) [10]. Seeds of *M. uniflorum* are used in the traditional method for the treatment of heart disease, asthma, bronchitis, diabetes, obesity, kidney stones, etc. (Kirtikar et., 1976;Alok *et al.*, 2014; Bhuvaneshwari *et al.*, 2014) [15, 2, 8]. Horsegram has medicinal properties to remove kidney stones. It inhibits the formation of calcium oxalate stones in kidneys. The seed helps in breaking the stones into smaller pieces that would come out of the urinary tract easily. Horse gram seed possess anti-urolithiatic activity (Atodariya *et al.*, 2013) [3]. The seeds are used to control cholesterol, glucose level and has antioxidant properties (Tiwari *et al.*, 2013) [26].

The three dimensional shape of a protein is governed by its primary structure and its environment. The environmental factors which change the shape of the enzyme or which inhibits the substrate to bind to its active site, will affect

enzyme activity. Such environmental factors include temperature, germination time, pH, substrate concentration, activators, inhibitors, etc. The main objectives of the present study was to characterize the activity of partially purified urease enzyme extracted from germinating seeds of *Macrotyloma uniflorum* with respect to germination time, temperature, substrate and enzyme concentration and effect of promoters and inhibitor.

2. Materials and methods

Seeds of *Macrotyloma uniflorum* (Lam.) Verdc. (Horse gram) were soaked in glass distilled water, germinated for 1h, 2h, 4h, 8h, 16h and 32h. The seeds were used for further experimental purpose. All the chemicals were of analytical grade and purchased from Sigma Chemical Company U.S.A. The other chemicals were prepared using distilled water.

2.1 Enzyme Extraction

1g of germinated seeds of *M. uniflorum* were weighed and crushed in a mortar and pestle by adding 10ml of glass distilled water. The suspension was then filtered through muslin cloth and the filtrate was centrifuged in cold centrifuge at 8000 rpm for 15 minutes at 4ºC. The supernatant was again centrifuged at 8000 rpm for 15 minutes at 4ºC. Volume of supernatant was noted. This was the crude enzyme extract.

Partial purification of enzyme by acetone fractionation: The crude enzyme extract was placed in an ice-bath maintaining its temperature to about 4ºC. Pre-cooled acetone was slowly added under constant and gentle stirring (1/3rd of the volume of supernatant) and then centrifuged at 8000 rpm for 15 minutes at 4ºC. The resulting precipitate was collected and dissolved in 2ml of glass distilled water. This was again centrifuged, the clear supernatant was taken and the volume was noted. The supernatant so obtained was the partially purified enzyme solution.

Enzyme Assay

Following assays have been done with the help of partially purified enzyme extract of germinated *M. uniflorum* seeds.

2.2 Optimum germination time of the enzyme urease

To study the effect of germination time on urease activity of *M. uniflorum* seeds, reaction sets were prepared, each having three replicas.

Reaction mixture: 10 ml distilled water + 1 ml 1% urea solution + 1 ml enzyme extract.

The total volume was 12 ml. All the prepared sets were incubated for 20 minutes, then 1 drop of 1% phenolphthalein was added. The mixture was titrated against N/50 HCl and the end point was determined by the point of disappearance of pink colour.

The optimum germination time was determined over the time ranging from 1h, 2h, 4h, 8h, 16h and 32h.

2.3 Optimum temperature of urease enzyme activity

The effect of temperature on urease activity was studied showing the optimum germination time at 4h.

The optimum temperature for urease activity was determined by incubating the enzyme with substrate (1% urea solution) at different temperatures (30ºC, 40ºC, 50ºC, 60ºC, 70ºC , 80ºC, 90ºC and 100ºC) for 20 minutes in incubator. The urease enzyme activity was assayed at each temperature.

2.4 Effect of substrate concentration on urease activity

The effect of substrate (urea) concentration on urease enzyme activity, different concentrations of urea (1ml, 1.5ml, 2ml, 2.5ml, 3ml, 3.5ml, 4ml, 4.5ml and 5ml) were used. The optimum substrate concentration for urease activity was determined by incubating the enzyme for 20 minutes at room temperature.

2.5 Effect of enzyme concentration on urease activity

For studying the effect of enzyme concentration on urease enzyme activity, different concentrations of enzyme (1ml, 1.5ml, 2ml, 2.5ml and 3ml) were used. The optimum enzyme concentration for urease activity was determined by incubating the enzyme for 20 minutes at room temperature.

2.6 Effect of promoters on urease enzyme activity

Standard solutions of different metal promoters (Cobalt, Calcium and Manganese) of different concentrations (1mM, 0.1mM, 0.01mM and 0.001mM) were prepared by serial dilution from their respective 1(M) stock solution and their promoting effects were tested on the germinated seeds of *M. uniflorum*.

The enzyme extract was assayed by adding 9ml distilled water, 1ml 1% urea (substrate), 1ml enzyme and 1ml of individual metal solution. The effect of each promoter at different concentrations ranging from 1mM to 0.001mM was examined for the seeds by incubating each enzyme solution at room temperature for 20 minutes and then titrating each reaction set against N/50 HCl. 1 drop of 1% phenolphthalein was used as an indicator, therefore the end point was determined by the point of disappearance of pink colour. A control set without promoter was run side by side.

2.7 Effect of inhibitor on urease enzyme activity

Standard solution of a metal inhibitor, Copper of different concentrations (1mM, 0.1mM, 0.01mM and 0.001mM) were prepared by serial dilution from its respective 1(M) stock solution and the inhibitory effect at different concentration was observed on the germinated seeds of *M. uniflorum.*

The enzyme extract was assayed by adding 9ml distilled water, 1ml 1% urea (substrate), 1ml enzyme and 1ml metal solution. The effect of the inhibitor at different concentrations ranging from 1mM to 0.001mM was examined by incubating each reaction sets at room temperature for 20 minutes and then titrating each reaction set against N/50 HCl. 1 drop of 1% phenolphthalein was used as an indicator, therefore the end point was determined by the point of disappearance of pink colour. A control set without inhibitor was run side by side.

3. Results & Discussion

3.1 Effect of germination time on urease activity

Different germination time (1h, 2h, 4h, 8h, 16h and 32h) were carried out to optimize the germination time for urease enzyme activity of *Macrotyloma uniflorum* seeds.

Urease activity increases with an increase in the germination time. The study revealed that urease activity increased gradually and showed maximum activity at 4h after germination and then declined rapidly (Fig-1). In the present study, germination time of 4h was optimized for the germinated seeds of *M. uniflorum* for further experimental purpose.

3.2 Effect of Temperature on Urease Activity

Different temperature (30ºC, 40ºC, 50ºC, 60ºC, 70ºC, 80ºC, 90ºC and 100ºC) were carried out to optimize the temperature for urease enzyme activity of *Macrotyloma uniflorum* seeds. The urease enzyme activity increases with an increase in temperature. The study of effect of different temperature on urease activity has revealed that urease activity increased up to 60ºC and gradually declined above 60ºC. At 100ºC no urease activity has been observed (Fig-2). As urease enzyme is proteinaceous in nature, it loses its three-dimensional conformation at such a high temperature and enzyme is disintegrated.

In the present study, the maximum urease enzyme activity was observed at 60ºC in *M. uniflorum* seeds.

3.3 Effect of substrate concentration on urease activity

Different substrate concentrations ranging from 1ml to 5ml of urea were carried out to determine the optimum substrate concentration for urease enzyme activity of *Macrotyloma uniflorum* seeds.

Urease enzyme activity initially increased with increasing substrate concentration but decreased with rising substrate concentration (Fig-3). At high concentration, urease activity became independent of substrate concentration (Banerjee *et al.*, 2012) [4]. The rate of urease activity increases on increasing substrate concentration until there are unsaturated binding sites of urease which can carry the enzymatic activity with the additional substrate.

In the present study, the optimum substrate concentration has been found to be 3ml.

3.4 Effect of enzyme concentration on urease activity

Various enzyme concentrations ranging from 1ml to 3ml of urease were carried out to determine the optimum enzyme concentration for urease enzyme activity of *Macrotyloma uniflorum* seeds.

The urease enzyme activity decreased with increasing enzyme concentration (Fig-4). After saturation of binding sites of urease to the substrate, addition of further amount of enzyme did not show increase in enzymatic activity as substrate concentration remained constant.

In the present study, the optimum enzyme concentration has been found to be 1ml.

3.5 Effect of promoters on urease activity

Effect of various metal promoters-Cobalt (Co), Calcium (Ca) and Manganese (Mn) on the activity of urease enzyme of *Macrotyloma uniflorum* seeds were studied. The results showed that Co, Ca and Mn had significant effects on urease activity of *M. uniflorum* seeds.

Metal promoters Co, Ca and Mn at different concentrations (1mM to 0.001mM) exhibited distinct role in urease action. The present study has revealed that urease activity was optimum in presence of these metal promoters at 1mM concentration and at higher concentration, the urease activity decreased significantly. The urease enzyme activity of the seeds was promoted maximally at 1mM concentration of Co. The promoting effect of Ca and Mn was comparatively less as compared to Co (Fig-5, 6 and 7).

3.6 Effect of Inhibitor on Urease Activity

Effect of a metal inhibitor, Copper (Cu) at different concentrations (1mM to 0.001mM) on the activity of urease enzyme of *Macrotyloma uniflorum* seeds were studied. The results showed that Cu had significant inhibitory effects on urease activity.

The present study has revealed that Cu at 1mM concentration showed the maximum inhibitory effect on urease enzyme activity and inhibitory effect gradually decreased with increasing concentration (Fig-8).

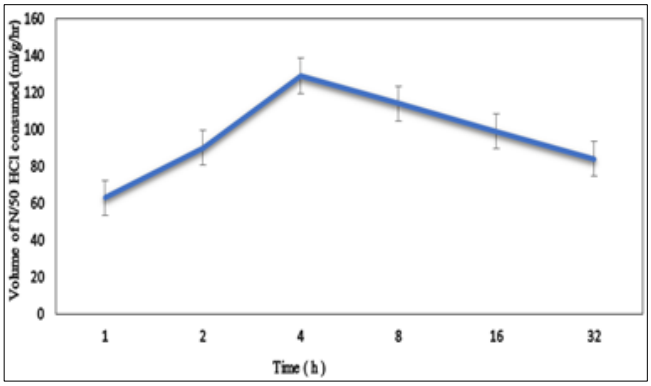

Fig 1: Effect of germination time on urease activity in *Macrotyloma uniflorum* seeds

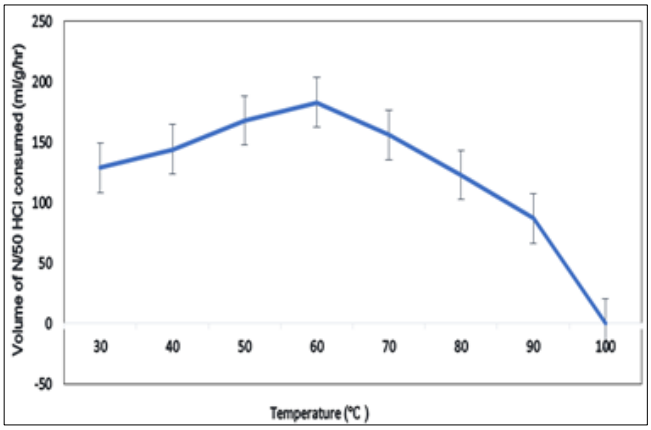

Fig 2: Effect of temperature on urease activity in *Macrotyloma uniflorum* seeds

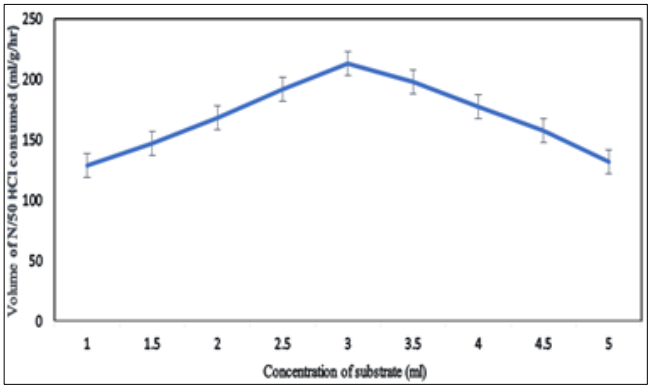

Fig 3: Effect of substrate concentration on urease activity in *Macrotyloma uniflorum* seeds

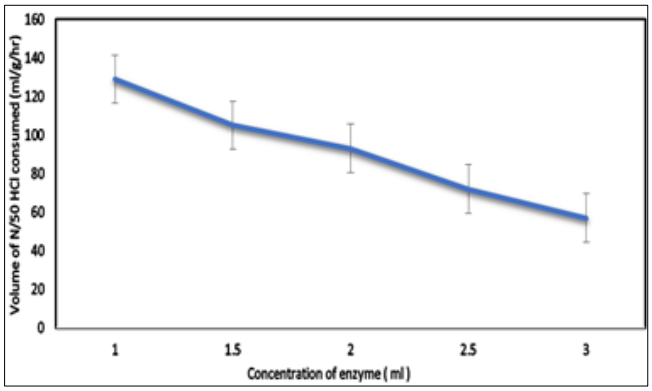

Fig 4: Effect of enzyme concentration on urease activity in *Macrotyloma uniflorum* seeds

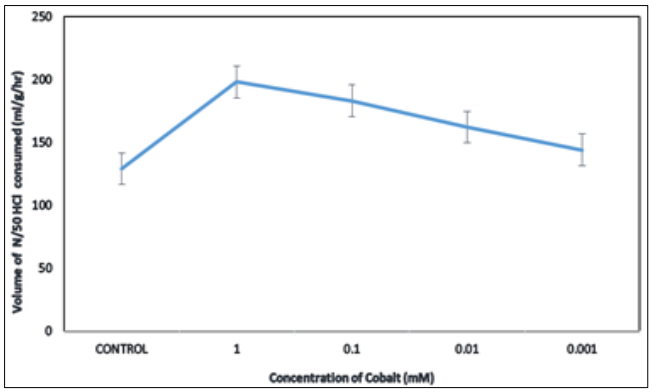

Fig 5: Effect of Cobalt on urease activity in *Macrotyloma uniflorum* seeds

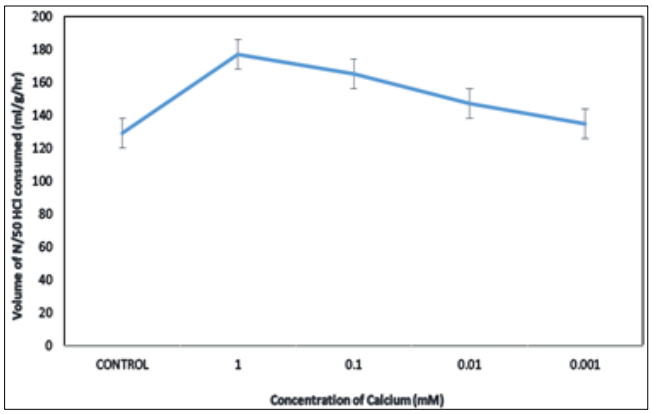

Fig 6: Effect of Calcium on urease activity in *Macrotyloma uniflorum* seeds

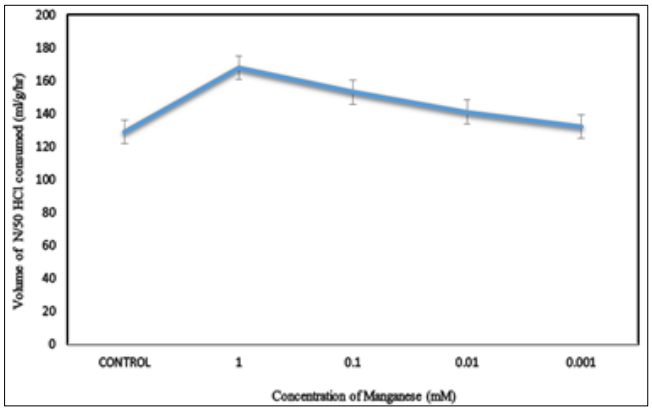

Fig 7: Effect of Manganese on urease activity in *Macrotyloma uniflorum* seeds

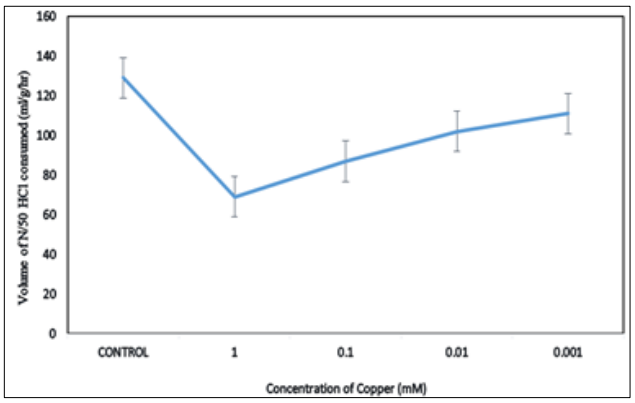

Fig 8: Effect of Copper on urease activity in *Macrotyloma uniflorum* seeds

4. Conclusion

The effect of several factors such as germination time, temperature, substrate concentration, enzyme concentration, promoters and inhibitor on urease enzyme activity of three different seeds has been evaluated in the present study. Germinating seeds of *Macrotyloma uniflorum* were used as source of enzyme and the urease enzyme was partially purified by acetone fractionation method. This study revealed that maximum urease activity has been obtained at 4h. The optimum temperature for maximum hydrolysis of urea by urease enzyme has been determined at 60ºC. The optimum substrate concentration and enzyme concentration was found to be 3ml and 1ml respectively.

The effect of various metal promoters like cobalt (Co), manganese (Mn) and calcium (Ca) revealed that at 10^{-3}M concentration of different promoters, maximum promotion of urease activity has been observed. The order of promoting activity of urease enzyme in *M. uniflorum* seeds used in the present study has been found to be Co > Ca > Mn.

Inhibition studies develops an enzyme-inhibition based system for detection of heavy metals. The effect of using a metal inhibitor, copper (Cu) showed that at 1mM concentration of inhibitor, maximum inhibition of urease activity occurred in germinated seeds of *M. uniflorum.*

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enzyme from the *Cajanus cajan* seeds. Asian J Bio. Sci. 2012; 7(2):203-209.
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