Standardization of assay method of urease activity in Inceptisol

Abstract

Urea is the most important nitrogen fertilizer widely used in crop production and its fate in soils is regulated largely by the soil enzyme urease. The use of preciseassay method for ureaseis important aspect in regulating activity of soil urease which isthe most useful to improve use efficiency of urea, and minimize the problems related to use of urea. In this context, the present laboratory investigation was undertaken with objective to standardize the assay media components of soil urease activity in Inceptisol of MPKV, Rahuri soils. The *TypicHaplustept* was selected as a representative of Inceptisol and used for present study of optimizing assay media components for*in vivo* assay method for measuring activity of urease from Inceptisol. All the assay components *viz.*, substrate concentration (0.2 M), buffer strength (0.05 M), weight of soil (5 g), amount of toluene (0.2 ml) and incubation time (2 h) were found optimum as per Tabatabai and Bremner (1972) except THAM buffer pH. The THAM buffer with a pH 8.5 was found to be optimum for measuring the activity of urease from Inceptisol. The values of urease activity from the 2g weight of soil and 1 h incubation time also found statistically at par with values of 5 g weight of soil and 2 h incubation time, respectively. Hence, the use of 2 g soil and 1 h incubation time in assay procedure are also sufficient for measuring urease activity, if the soil sample size and time are limiting factors.

Keywords: Assay media, Inceptisol, Soil urease, Standardization

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Introduction

Soil enzymes are central soil ecosystem processes and environmental research,~~whose usual inhabits~~ in soil and are very essential and continuously playinga key role in maintaining soil ecology, physical and chemical properties, overall soil fertility, and soil health.Among the many enzymes related to nitrogen (N), the enzyme urease (Urea amidohydolase, EC 3.5.1.5) is an important and responsible enzyme for breakdown of C-N bonds in various organic nitrogen compounds in soil, particularly it regulates the fate of applied urea to soil. The study of urea and various factors regulating its use efficiency deserves much attention since the urea is the important input in crop production and most preferred synthetic nitrogen fertilizers by the majority of the farmers.When the urea is applied to soil as a fertilizer, which is rapidly hydrolysed to ammonia and carbon dioxide in the most of the soils though the action of soil urease (Conrad, 1940; Bremner and Krogmeier, 1988; Yang *et al*., 2006).The efficiency of urea fertilizer is greatly regulated by urease in soil. The use of urea and its hydrolysis in soil causes the most of problems like volatilization of urea N as ammonia, nitrite toxicity, increase in soil pH and damage to germinating seedlings and young plants (Gasser,

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1964; Hutchinson and Viets, 1969; Bremner and Krogmeier, 1988; Bremner, 1995). The growing importance of the more use of urea as a nitrogen fertilizer in world agriculture necessitates research to reduce these problems. Thus, the study of soil urease and its activity is very important in research area of profitable use of urea, its efficiency and to reduce the related problems in soils, crops and environment with use of urea. For research on urea and urease, it is very essential to have satisfactory and precise site specific methods of assaying the existing and potential urease activity in soils and also to assess or develop the suitable soil amendments/inhibitors that restrict the excessive or fast hydrolysis of urea in soil.

~~The~~ several analytical methods to determine the soil urease activity have been published and majority methods are based on the detection of released ammonia during the urease reaction in soil.Based on survey of literatures appeared in India on the studies of soil urease research, it is evidenced that the majority (≈60%) of Indian researchers (for example;Chakrabarti *et al*., 2004; Chaudhuri *et al.*, 2009; Vahed*et al.*, 2011; Aruna *et al*., 2017) have adopted as such the Tabatabai and Bremner (1972) method for assaying urease activity from various soils without doing site specific standardization of assay media/components like buffer (molarity and pH), substrate concentration, amount of soil, amount of toluene and incubation time in their laboratories and for their soil types. The use of perfect enzyme assay method is a main challenge in study of enzymes. The use of imperfect assay method (without standardization) leads to misinterpretation of soil urease activity data. In the context of soil enzyme assay protocol, recently Nannipieri*et al.*, (2018) suggested to the researchers in soil enzymology to develop optimum assay methods for individual specific site, soil and their own laboratory conditions for measuring the activities of soil enzymes. In Maharashtra, the Inceptisol soils are the major soil group and most suitable for profitable crop production.

With this background, the present study was conducted with objective to standardize the assay media for developing the suitable *in vivo* assay method for measuring activity of urease from Inceptisol soil.

Material and Methods

TypicHaplustept soil type from the Post Graduate Instructional Farm, MPKV, Rahuri(MS) was selected as a representative of Inceptisol soil order for standardization of assay method of urease. The surface (0-15cm) soil sample at field capacity condition was collected, processed and used 2 mm sieved soil for various laboratory experiments conducted for optimizing assay media/components for standardization of method for*in vivo*assay of urease from Inceptisol.

The buffer method for *in vivo*assay of soil urease developed by Tabatabai and Bremner (1972) was used as base method for conducting various laboratory experiments on standardization of assay methods for optimizing the assay media/components for measuring activity of urease in Inceptisol soils. The Layout of laboratory experiments on standardization of various assay media are given in table 1.

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The optimum conditions of assay media/components *viz.*, substrate concentration, buffer strength, buffer pH, weight of soil, amount of toluene and incubation time were standardized by conducting various 6 laboratory experiments (Table 1).

Table 1 Layout of laboratory experiments on standardization of various assay media

Lab Expt. No.	Assay components/media for laboratory experiments	Concentrations/Levels of assay components as treatments						
		T1	T2	T3	T4	T5	T6	T7
1	Substrate conc. (M)	0.0	0.05	0.10	**0.2**	0.25	0.30	0.35
2	Buffer molarity (M)	0.0	0.025	**0.05**	0.10	0.15	0.20	0.25
3	Buffer pH	7.5	8.0	8.5	**9.0**	9.5	10.0	10.5
4	Toluene amount (ml)	0.0	0.05	0.1	**0.2**	0.25	0.30	0.35
5	Wt. of soil (g)	1	2	4	**5**	7	8	10
6	Incubation time (h)	0	0.5	1	**2**	4	6	8

Note: 1. Bold figures in treatment table indicate the assay components in the method given by Tabatabai and Bremner (1972)

2. Numbers of replications taken in each experiments-3

E.g. Experiment No 1- Influence of exogenously added various substrate concentration (0.0, 0.05, 0.10, 0.20, 0.25, 0.30 and 0.35 M) added into assay medium on *in vivo* urease activity in Inceptisol by using other assay components as per method of Tabatabai and Bremner (1972).

Similar types of other laboratory experiments as laid out in table 1, were conducted for optimizing the concentration of buffer, buffer strength, amount of toluene, weight of soil and reaction time for *in vivo* assay of urease activity in Inceptisol. All the laboratory experiments conducted for standardization of method for *in vivo*assay of soil urease were conducted in triplicate and the data generated from each laboratory experiment were statistically analyzed by using CRD and respective experimental SE and CD values were reported in tables 2 to 7.

Results and Discussion

1.1 Effect of Exogenously added Substrate Concentration on Activity of Urease in Inceptisol

The result of effect of varying substrate (urea) concentration on *in vivo*activity of urease from Inceptiol is presented in table 2 and depicted in figure 1. The study showed that urease activity increased linearly with an increasing exogenously added substrate concentration from 0.0 to 0.35M with a production of NH_4^+ in the range of 1.17 to 8.81µg of NH_4^+ g^{-1} of soil h^{-1}(Table 2). The line graph in figure 1 revealed that the rate of increase in the urease activity start decreasing after addition

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of 0.2 M substrate concentration, which indicated that 0.2 M substrate concentration, is found sufficient and optimum to saturate the active sites of enzyme for maximum products formation by the urease from Inceptisol. The urease activity with the use of substrate concentration beyond 0.2 M concentration *viz*., 0.25 M, 0.30 M and 0.35 M was found statistically at par with 0.2 M substrate concentration.

Our results are similar with earlier results reported by various researchers (Douglas and Bremner, 1971; Tabatabai and Bremner, 1972 and Kandeler and Gerber, 1988) with respect to the trends in increase in urease activity with addition of increase in the concentration of substrate from 0.0 to 0.2 M urea solution. These results also indicate that urease activity is dependent on substrate concentration, as the rate of urea hydrolysis in soil increases with an increase in urea concentration until the amount of urea added is sufficient to saturate the active sites on enzyme molecules with substrate. The data from table 2 and figure 1 reveals that the 0.2M substrate concentration is optimum for the maximum activity of urease from Inceptisol soil order.

Table 2Effect of substrate concentrations on urease activity

Treatments	Substrate concentrations (M)	Urease activity (µg of NH_4^+/g of soil/h)
1	**0.0**	**1.17**
2	**0.05**	**3.21**
3	**0.10**	**4.67**
4	**0.20**	**8.52**
5	**0.25**	**8.63**
6	**0.30**	**8.69**
7	**0.35**	**8.81**
SE m(±)=0.080	**CD at 1% =0.339**	

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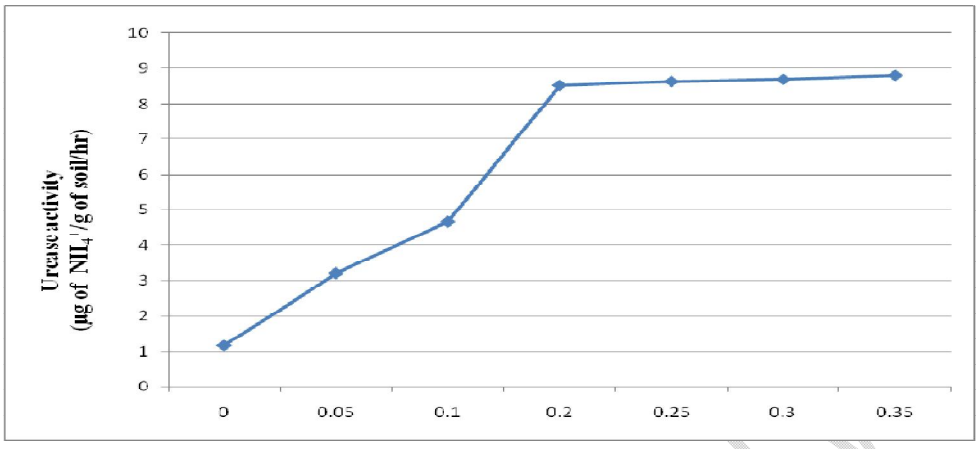

Figure 1: Effect of substrate concentrations on urease activity

1.2. Effect of Molarity of THAM Buffer (pH 9.0) on Urease Activity in Inceptisol

The data on study ofeffect of molarity of THAM buffer (pH 9.0) on urease activity is presented in table 3 and figure 2 showed that, with varying concentration of THAM buffer (0.0 to 0.25M) shows the urease activity in the range of 3.15 to 7.70 µg of NH_4^+/g of soil/h. Even though the activity of urease from buffer concentration 0.05 M and 0.10 M are statistically at par, the line graph depicted in figure 2 reveals that 0.05M concentration of THAM buffer used was found to be optimum for assay of urease activity from Inceptisol.

Table 3Effect of molarity of THAM buffer (pH 9.0) on urease activity

Treatments	Molarity of THAM buffer (M)	Urease activity (µg of NH_4^+/g of soil/h)
1	**0.0**	**3.15**
2	**0.025**	**4.20**
3	**0.05**	**7.70**
4	**0.10**	**7.67**
5	**0.15**	**7.29**
6	**0.20**	**7.17**
7	**0.25**	**6.94**
SE m(±)=0.087 CD at 1% =0.367		

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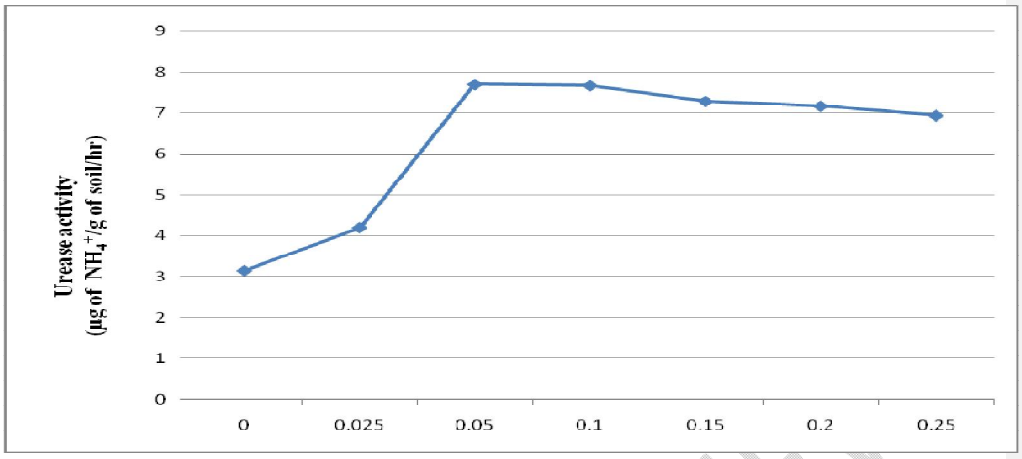

Figure 2: Effect of molarity of THAM buffer (pH 9.0) on urease activity

Overall, these findings are in accordance with the earlier findings reported by Tabatabai and Bremner (1972) and Naheem *et al*., (2003) who reported that the 0.05M concentration of THAM buffer is sufficient to maintain optimum soil-buffer mixture pH for a present assay condition. Hence, the 0.05M THAM buffer concentration was found to be optimum for activity of urease from Inceptisol of MPKV, Rahuri and laboratory conditions.

1.3 Effect of Weight of Soil on Urease Activity in Inceptisol

The present study on the effect of varying amount(weight) of soil in the assay of urease activity indicated the linear relationship between amount of soil from 1.0 to 10.0g in assay media and urease activity was observed in the range of 7.0 to 75.83 µg of NH_4^+/h (Figure 3). The study on the effect of amount of soil on urease activity revealed that increasing weight of soil beyond 5 gram results in decline in the urease activity *i.e.*, per gram release of NH_4^+ with increase in weight of soil and is in the range of 7.0 to 9.10 µg of NH_4^+/g of soil/h (Table 4).

The present results/trends with respect to linear increase in activity of urease with increase in amount of weight of soil in assay and decrease in per gram soil activity of urease beyond 5 gram of soil are very similar with the earlier results reported by Tabatabai and Bremner (1972) and Naheem *et al*. (2003). The decline in the per gram release of NH_4^+ with increase in weight of soil beyond 5g weight of soil is due to other assay of components seems to be limiting factor. Hence, 5g of soil in assay of urease from Inceptisol soil was found to be optimum. The observed values of the activity of urease by taking 2, 4 and 5 gram weight of soil in assay media are statistically at par, which suggest to use of 2 gram of soil is sufficient to measure the activity of urease under limited soil sample.

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Table 4 Effect of weight of soil on urease activity

Treatments	Weight of soil (g)	Urease activity (µg of NH_4^+/g of soil/h)	Urease activity (µg of NH_4^+/h)

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1	**1.0**	**7.00**	**7.00**
2	**2.0**	**8.60**	**17.21**
3	**4.0**	**8.67**	**34.71**
4	**5.0**	**9.10**	**45.50**
5	**7.0**	**8.12**	**56.87**
6	**8.0**	**8.09**	**64.75**
7	**10.0**	**7.58**	**75.83**
SE m(±)=0.20 CD at 1% =0.874			

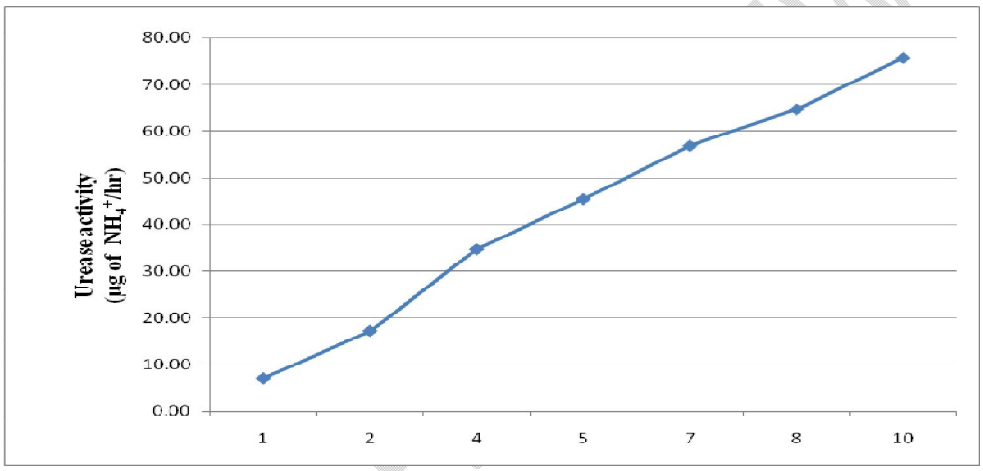

Figure 3: Effect of weight of soil on urease activity (µg of NH_4^+/h)

1.4. Effect of Amount of Toluene on Urease Activity in Inceptisol

The data of laboratory experiment of study on effect varying the amount of toluene added into assay media on urease activity presented in table 5 and depicted in figure 4 describes that the urease activity was ranged from 5.07 to 9.04 µg of NH_4^+/g of soil/h with use of varying the amount of toluene (0.0 to 0.30 ml) in assay media. The urease activity due to use of 0.2 ml toluene showed the highest value of activity of urease, which is at par with the use of 0.1 ml toluene in assay media. These results showed that 0.2 ml toluene used in assay media was found as the optimum amount of toluene for assay of urease activity in Inceptisol soil type and our laboratory conditions.

Tabatabai and Bremner (1972) and Naheem *et al.* (2003) also reported as the 0.2 ml toluene quantity as the optimum for assay of urease in their studies which confirm the present results in effect of amount of toluene on activity of urease in Inceptisol. Usually toluene is added to soil to inhibit microbial activity in the assay mixture soil. Galstyan (1965) found that addition of toluene led to a decrease in the values obtained in assay of soil urease activity in a buffer method, but he concluded

that toluene has an inhibitory effect on urease. On the contrary, Tabatabai and Bremner (1972) found that the results obtained by their buffer

Table 5 Effect of amount of toluene on urease activity

Treatments	Amount of toluene(ml)	Urease activity (µg of NH_4^+/g of soil/h)
1	0.0	5.07
2	0.05	5.66
3	0.10	8.87
4	0.20	9.04
5	0.25	8.52
6	0.30	8.05
7	0.35	7.52
SE m(±)=0.0798 CD at 1% =0.336		

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method of assaying urease activity in soils were increased by the addition of toluene. In present study the toluene showed stimulatory effect on the activity of urease with their addition from 0.0 to 0.2 ml amount of toluene and later showed inhibitory effect with addition from 0.2 to 0.35 ml, these observations on effect of varying amount of toluene on activity of urease showed that 0.2 ml of toluene is optimum in assay media for present soil type as a stimulatory effect.

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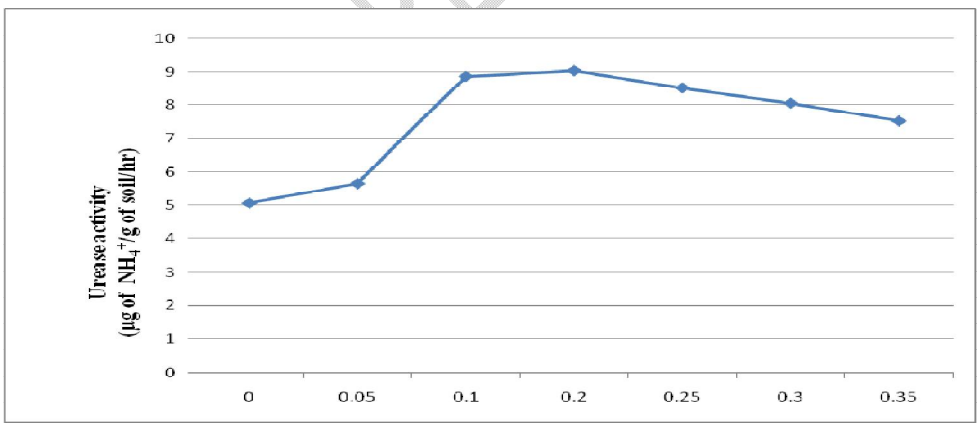

Figure 4: Effect of amount of toluene on urease activity

1.5 Effect of Incubation Time on Urease Activity in Inceptisol

The study on effect of varying the incubation (reaction) time in the assay of urease activity showed the linear relationship between incubation time and urease activity and which was ranged from 0 to 32.5 µg of NH_4^+/g of soil for 0 to 8 hour incubation and it is alsodepicted in figure 5. The

study on effect of incubation time on urease activity reveals that with an increase in incubation time beyond 2 h leads to decrease in the per hour activity of urease with the increase in incubation time is showed in the table 6.

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A similar pattern of results of urease activity as influenced by time of incubation or reaction time were also reported by Tabatabai and Bremner (1972), Naheem *et al.* (2003) and Cordero *et al.* (2019). The decrease in per hour release of ammonium N after 2 h incubation time may be due to limitation of substrate or other assay components for the reaction of urease. Thus, 2 hour of incubation time is found as optimum for assay of urease activity for our soil type and laboratory conditions. Although 2 hour incubation time is found to be optimum for the activity of urease, 1 hour incubation time is also statistically at par with 2 hour incubation time, which suggests short incubation time of 1 hour is useful for determination of the activity of urease from the large number of samples and where the time is the limiting factor. A short incubation time also reduces cost as it allows more samples analysis to be conducted in a set amount of time. The minimum incubation time is desired which reduces potential problems related to long incubation and also ensures enough product formed to detect the activity of urease at low concentration of enzyme in soil.

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Table 6 Effect of incubation time on urease activity

Treatments	Incubation time(h)	Urease activity (µg of NH_4^+/g of soil/h)	Urease activity (µg of NH_4^+/g of soil)
1	0	0	0
2	0.5	7.23	3.40
3	1	8.16	9.20
4	2	8.28	14.0
5	4	5.07	19.3
6	6	4.25	26.0
7	8	4.15	32.5
SE m(±)=0.106 CD at 1% =0.447			

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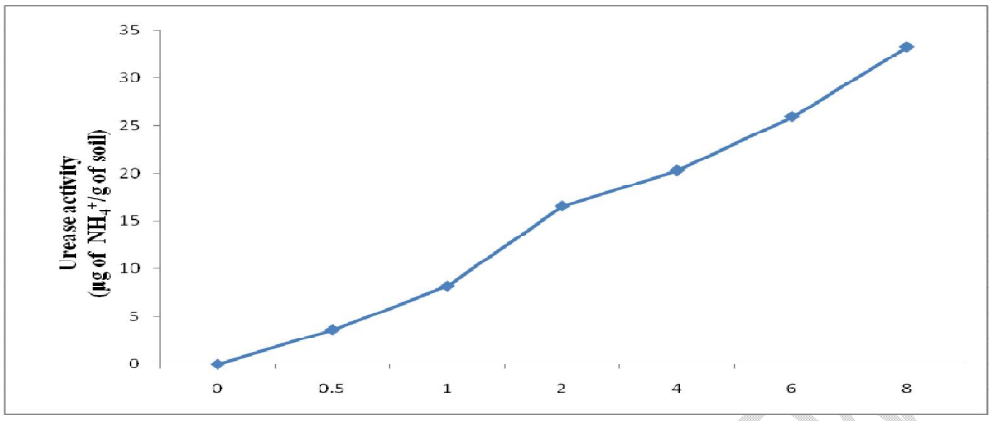

Figure 5: Effect of incubation time on urease activity (µg of NH_4^+/g of soil)

1.6. Effect of Buffer pH on Urease Activity in Inceptisol

Study of varying assay buffer pH from 7.5 to 10.5 in the assay media of urease activity showed the significant variations in the urease activity in the range of 5.60 to 8.92 µg of NH_4^+/g of soil/h (Table 7 and Figure 6). The data of study on effect of pH of THAM buffer displayed that urease activity with use of buffer pH 8.5 in assay media is found to be the highest and which is statistically at par with the urease activity obtained with buffer pH 9.0. These results indicate that the THAM buffer with 8.5 pH is optimum for assay of urease activity than the buffer pH 9.0 which is given by Tabatabai and Bremner (1972).

Table 7 Effect of Buffer pH on urease activity

Treatments	pH of THAM buffer	Urease activity (µg of NH_4^+/g of soil/h)
1	7.5	6.01
2	8.0	7.76
3	8.5	8.92
4	9.0	8.86
5	9.5	7.58
6	10.0	6.30
7	10.5	5.60
SE m(±) =0.096		CD at 1% =0.404

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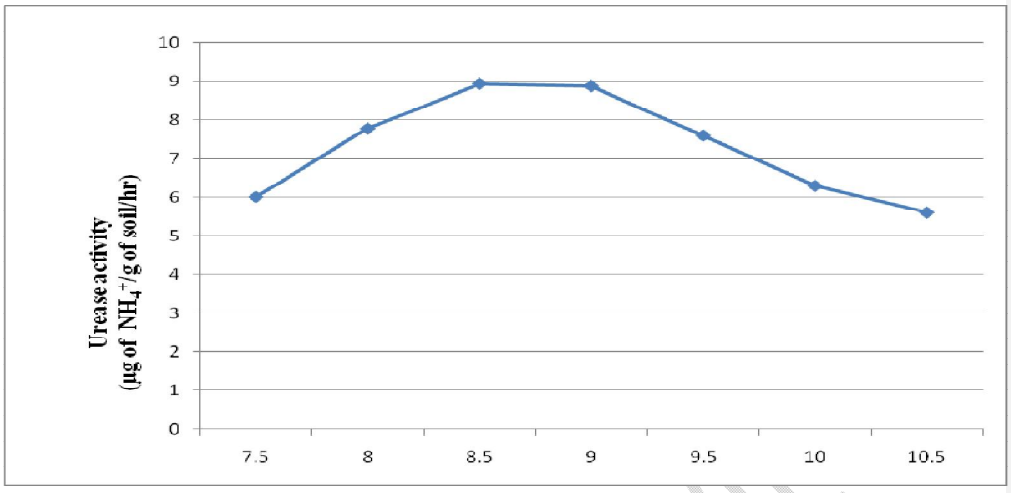

Figure 6: Effect of Buffer pH on urease activity (µg of NH_4^+/g of soil/h)

The result of present study with respect to effect of pH of THAM buffer on urease activity is little but interesting because the earlier researchers *viz*., Tabatabai and Bremner (1972),and Naheem *et al*., (2003) have reported optimum pH of THAM buffer as 9.0, however, the result of present study showed optimum pH of THAM buffer to be 8.5. The deviation of optimum buffer pH may be due to wide variation in soil type under the present study and earlier researchers' study. The earlier researcher Tabatabai and Bremner (1972) used soils having the pH range of 5.7 to 7.5 but in the present study Inceptisol soil having the pH 8.42. Therefore, the pH of buffer*i.e.*, 8.5 is found to be sufficient to maintain optimum soil-buffer mixture pH for a given assay condition. This study confirmed that the pH of assay THAM buffer 8.5 found optimum for assay of urease activity in soils of Inceptisol at our laboratory conditions.

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Conclusion

In conclusion, the assay media/ components as per Tabatabai and Bremner (1972),*viz.*, substrate concentration (0.2 M), buffer concentration (0.05 M), weight of soil (5 g), amount of toluene (0.2 ml) and incubation time (2 h), and modified component i.e., pH of THAM buffer 8.5 are optimum for measuring *in vivo* activity of urease from Inceptisol (TypicHaplustept) soils. This study also suggests that the 2 g soil and 1 h incubation time in assay procedure are also sufficient for measuring urease activity if the soil sample size and time are limiting factors, by using 2 g soil and 1 h incubation time urease activity from large number of samples can be done in a short time.

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