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RESEARCH ARTICLE

INVESTIGATION OF AMMONIUM THIOSULFATE, SODIUM THIOSULFATE AND 1,2,4-TRIAZOLE (UREASE INHIBITORS) ON THE ACTIVITY OF UREASE IN WHEAT SOIL OF FAISALABAD, SHEIKHUPURA AND GUJRANWALA REGION

Mahnoor Qayyum Khan[1], Khalil-Ur-Rahman[1], Irha Basit[1], Safoora Shabbir[2], Ayesha Aziz[1], Rabia Azam[1], Hoda Zahoor[1], Marium Hayat[1], Saba Maruiam[1] and Usman Ghani[1]

1. Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.
2. Department of Biochemistry, Government College University, Faisalabad, Pakistan.

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Abstract

This paper describes the inhibition effect of different urease inhibitors i.e. Ammonium Thiosulfate, Sodium Thiosulfate and 1,2,4-Triazole on the activity of urease enzyme in wheat soil of 3 different districts of Punjab, Pakistan. Urease is a nickel dependent metalloenzyme that is involved in the hydrolysis of urea, forming Carbon dioxide and ammonia. Urea hydrolysis leads to the excessive nitrogen loss in the form of ammonia volatilization. Main purpose of the study was to check the potential of different blended inhibitors (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) on the activity of urease and to overcome the loss of nitrogen. For this purpose, soil samples were collected from three different districts of Punjab i.e. Faisalabad, Sheikhupura and Gujranwala. The whole work was divided into two phases. During phase Ⅰ, the effect of different inhibitors on the activity of urease was determined. Kinetic behavior of urease enzyme in the presence and absence of urease inhibitors was evaluated in phase Ⅱ. The data acquired was statistically analyzed by using two-way ANOVA. Kinetic study of urease enzyme was carried out to evaluate the effect of temperature, pH, incubation time and substrate concentration on the activity of enzyme.

Introduction:-

Pakistan is an agricultural country and 22 Mha (Million hectares) out of 80 Mha of the total land is currently used for agricultural production. Agricultural zone in Pakistan is also facing some of the most serious problems like drought conditions, water deficiency, high price of N fertilizers, lack of land reforms, non–utilization of cultivatable land and some other socio economic and environmental challenges that have intense agricultural productivity implications and playing negative role in agriculture. There is a need to highlight and resolve these problems at first priority (Ahmad et al., 2013). In crop production, nitrogen is a most important component that is well known due to the presence of a billion-euro fertilizer industry. Economic importance of nitrogen is well documented about hundred years ago (Lohnis, 1913). Nitrogen is an essential nutrient for all living organism, occupies a prominent role in plant metabolism system (Erisman et al., 2008).

Corresponding Author:-Irha Basit
Address:-Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.

Urease which is urea amidohydrolase (EC 3.3.1.5), is a nickel dependent enzyme (Dixon et al., 1975) that catalyzes the hydrolysis of urea to form carbon dioxide and ammonia at a rate 8×10^{17} times more rapidly than an uncatalyzed reaction (Callahan et al., 2005). Rate of soil urease activity varies between different soils (Freney et al., 1981). High soil urease activity leads to loss of nitrogen by volatilization. Low soil urease activity leads to the loss of urea by leaching and less availability of inorganic nitrogen to the crops (Baligar et al., 1988). Urease naturally occurring in soil is more stable than urease added to the soil. Organic soil constituents have ability to stabilize urease (Conrad, 1940). Urease inhibitors not only inhibits the breakdown of urea but can also affects the released NH_4^+ in soil (Zhao et al., 1992).

During urea fertilization in agriculture, high enzyme (urease) activity causes significant economic and environmental problems by releasing a huge amount of NH3 in to the atmosphere. This further leads to plant damage by depriving essential nutrients to plants, increase in soil pH by releasing CO_2 and NH_3 toxicity (Bremner, 1995). Young plants, germinating seeds and seedlings are damaged by accumulation of NH4+ ions, NH3 toxicity and loss of urea nitrogen as NH3 and nitrite (Radel et al., 1988). The above mentioned problems highlights the need of further research to find out effective methods to overcome the problems that belongs to urea as a fertilizer.

Materials and Methods:-

Main purpose of the study was to check the inhibition potential of different inhibitors on the activity of urease and to minimize the nitrogen loss. The whole work was divided into two phases. During phase Ⅰ, the effect of different inhibitors on urease activity was determined. Kinetic behavior of urease enzyme in the presence and absence of urease inhibitors was evaluated in phase Ⅱ.

Phase I: Effect of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitors on urease activity

Effect of Ammonium Thiosulfate, Sodium Thiosulfate and 1,2,4-Triazole inhibitors blended with urease enzyme was evaluated in this phase.

Collection of soil from different areas:

Soil samples were collected from 3 different districts of Punjab i.e. Faisalabad (FSD), Sheikhupura (SKP) and Gujranwala (GUJ) for evaluating the effect of different urease inhibitors on the activity of urease enzyme on December 10, 2018. Lysimeters were prepared and labelled on December 11, 2018. All the lysimeters were labelled as T1, T2, T3 for each district to show different ratios of the 3 selected inhibitor combinations as shown in Table 1. Soil was poured in to the lysimeters on December 13, 2018 and sowing of wheat seed was done on December 14, 2019 in these lysimeters. Plantlets attain a specific height up to 30 cm till February 26, 2019.

Preparation and application of urease inhibitors:

When plantlets attained a specific height of 30 cm, different inhibitors (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) blended with urease enzyme were prepared and applied to the soil on February 26, 2019. Urea applied to the lysimeter of each district was considered as control.

Table 1:- Composition of urease inhibitors.

	FSD	**SKP**	**GUJ**
T1	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (1:1:1)	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (1:1:1)	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (1:1:1)
T2	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (1.5:0.75:0.75)	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (1.5:0.75:0.75)	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (1.5:0.75:0.75)
T3	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (0.75:1.5:0.75)	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (0.75:1.5:0.75)	Ammonium Thiosulfate+ Sodium Thiosulfate+1,2,4-Triazole. (0.75:1.5:0.75)

Processing of collected soil:
Soil samples was processed by using pestle and mortal in five different steps of Drying, Grinding, Sieving, Packaging and Storage.

Collection and drying of wheat soil samples:
After application of inhibitors soil samples were collected from day 1 to day 37. Samples were collected up to 37th day from inhibitor application as shown in Table 2. The collected soil samples from three different districts of Punjab were air dried by following the procedure of Dick et al. (1996).

Table 2:- Day-wise sample with sample collection date.

Sample	Sample collection date
Day 1	Feb 27, 2019
Day 2	Feb 28, 2019.
Day 3	March 1, 2019
Day 4	March 2, 2019
Day 6	March 4, 2019
Day 8	March 6, 2019
Day 10	March 8, 2019
Day 12	March 10, 2019
Day 14	March 12, 2019
Day 17	March 15, 2019
Day 20	March 18, 2019
Day 23	March 21, 2019
Day 30	March 28, 2019
Day 37	April 4, 2019.

Processing of wheat soil samples:
The collected soil samples were grinded uniformly with the help of pestle and mortal by following the procedure of Dick et al. (1996). All the collected soil samples were sieved carefully by following the procedure of Dick et al. (1996). To avoid sample contamination soil samples was carefully packed in polythene bags for further analysis. All the polythene bags were labelled according to inhibitors applied e.g. T1, T2 and T3 for each district by following the procedure of Dick et al. (1996). 1g of soil sample is weighed from each district by using digital electronic balance by following the procedure of Dick et al. (1996).

Reagent preparation:
Different chemicals were used for the preparation of reagents to evaluate the urease activity by using the calorimetric method.

1. 10g of urea solution was weighed by using digital electronic balance, added in a beaker and volume was made up to 100mL with distilled water.
2. Citric acid (9.6g) and sodium citrate (14.7g) was dissolved in 500mL of distilled water separately. On the other hand, citric acid solution (186mL) and sodium citrate solution (15mL) were added in to the beaker and mixed well. pH is maintained up to 6.7 through pH meter by adding sodium hydroxide dropwise.
3. Sodium hydroxide (32g) and phenol (83mL) were mixed in a measuring flask and volume was made up to 500mL by distilled water.
4. Sodium hypochlorite (0.9mL) was measured and then dissolved in to the 100mL of water.

Standard urease curve:
To make standard curve, jack bean urease of Alfa Aeser was used. Some equations were also used to convert absorbance in to activity units. Standard graph represents the activity units of urease. 1U to 10U units of urease enzyme were used. Results show the activity of enzyme (IU/g soil) that was present in 1 gram of soil. The activity units in soil were find out by comparing jack bean standard urease. Standard curve of jack bean urease enzyme was drawn and standard equations were made to convert the absorbance of soil urease in to the enzyme activity units (IU/g soil). These activity units show the amount of urease present in to the soil as shown in Figure 1.

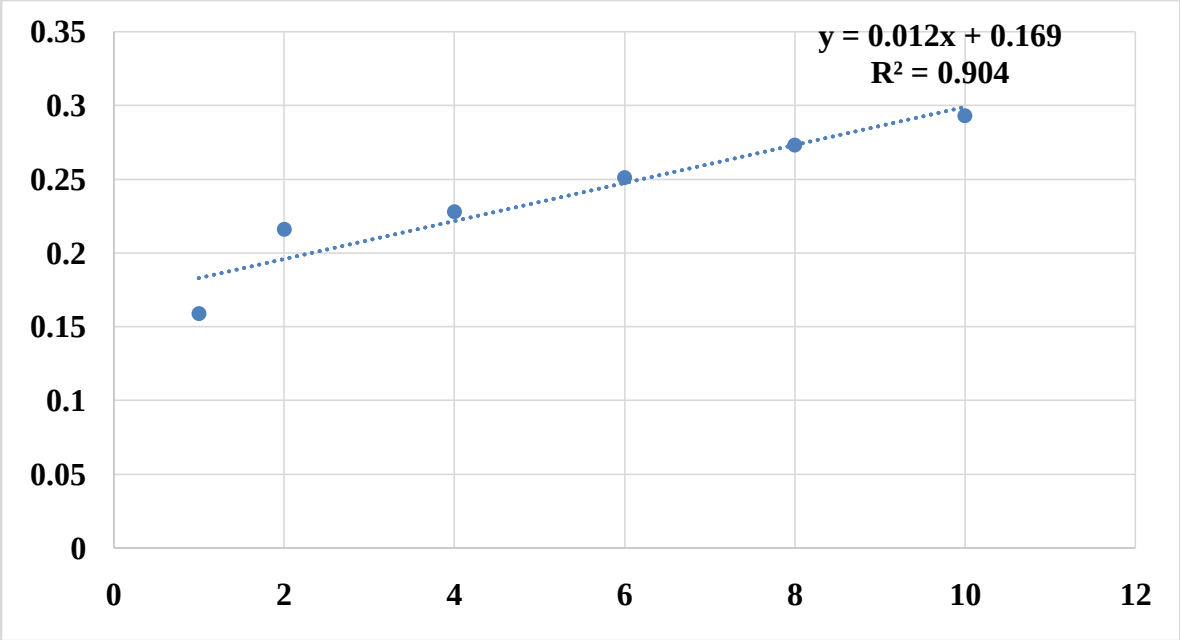

Figure 1:- Graph representing the absorbance of jack bean urease enzyme.

Procedure for standard curve:
1 mg of enzyme contains 45 IU enzyme units (0.01g/ml) or 1mg/1000 µl contains 45 IU.

For the preparation of enzyme standard, enzyme units are calculated as:
1. 01 IU = 22.22 µl enzyme sol. + 977.78 µl citrate buffer.
2. 02 IU = 44.44 µl enzyme sol. + 959.56 µl citrate buffer.
3. 04 IU = 88.88 µl enzyme sol. + 911.12 µl citrate buffer.
4. 06 IU = 133.32 µl enzyme sol. + 866.68 µl citrate buffer.
5. 08 IU = 177.77 µl enzyme sol. + 822.24 µl citrate buffer.
6. 10 IU = 222.2 µl enzyme sol. + 777.78 µl citrate buffer.

Test tubes were prepared for enzyme solution by stock enzyme solution as mentioned above.

1 IU, 2 IU, 4 IU, 6 IU, 8 IU and 10 IU were taken and citrate buffer was added to these test tubes to make volume 1ml. After this, 150µl toluene was added to each test tube and let the solution set for 20 minutes. Then 1000µl urea and 2000µl citrate buffer was added to these test tubes and incubated them for 3 hours at 37^0C. Then 100µl of incubated solution was diluted with 900µl of distilled water. 300µl sodium hypochlorite and 400µl phenolate solution was added to these test tubes. Let the solution stand for 20 minutes until the blue color appeared. Absorbance was taken at 580 nm by using spectrophotometer.

Procedure for soil analysis:
A blank containing distilled water was prepared. 1 gram sieved soil sample was taken in test tube. 150 microliter toluene was added to the soil sample. Mixed well and allowed to stand for 15 minutes. Then 1000 microliter urea and 2000 microliter citric acid buffer was added. Mix thoroughly and covered these test tubes with aluminum foil paper. This solution was incubated for 3 hours at 37^0C. After incubation, 100 microliter of filtrate was taken in another test tube from this incubated solution and mixed with 900 microliter of distilled water, 400 microliter phenolate solution and 300 microliter sodium hypochlorite solution. This solution was allowed to stand for 20 minutes until the maximum blue color obtained. Finally, the absorbance was taken at 580nm to evaluate the urease activity by using spectrophotometer by following the procedure of McGarity and Myers, (1967).

Urease inhibition potential of urease inhibitors:
Procedure followed for the activity of urease analysis was as such followed to evaluate the inhibition potential of urease. 0.1% blended inhibitors (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) were added to the

soil of Faisalabad, Sheikhupura and Gujranwala districts. For analysis, soil sampling was done on 1st day, 2nd day, 3rd day, 4th day, 6th day, 8th day, 10th day, 12th day, 14th day, 17th day, 20th day, 23rd day, 30th day and 37th day after the application of inhibitors. Mostly inhibitors were used to minimize the breakdown of urea by decreasing the activity of urease enzyme that results in nitrogen loss as NH_3. The main goal was to minimize the loss of nitrogen in the form of NH_3 by using different inhibitors (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) blended with urease enzyme. Three replicates of each soil sample were made to evaluate the effect of these inhibitors blended with urease enzyme by following the procedure of Hoffman and Teicher. The amount of released ammonia by adding blended inhibitors was measured at 580 nm by using spectrophotometer by following the procedure of Aşkin and Kizilkaya, (2005).

Phase II: Kinetic study of different inhibitors on urease activity

Inhibition effect of different urease inhibitors (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) was evaluated in this phase by applying blended inhibitors to all the lysimeters. Inhibitors were again applied to the wheat soil on April 9, 2019 for kinetic studies. Sample collection for this phase study was done on the 7th day after the application of inhibitors on April 16, 2019. Effect of pH, temperature, substrate concentration and incubation time was evaluated in this phase by following the procedure of Hoffman and Teicher (Aşkin and Kizilkaya, 2005).

Effect of pH on inhibitors treated soil samples:

The effect of pH on the activity of urease enzyme was examined by changing pH values (3.5, 4.5, 5.5, 6.5 and 7.5). All other parameters were kept constant by following the procedure of Frankenberger and Johanson, (1982).

Effect of temperature on inhibitors treated soil samples:

The effect of temperature on the activity of urease enzyme was examined by changing temperature values (10^0C, 20^0C, 30^0C, 40^0C and 50^0C). All other parameters were kept constant by using the method of Sahrawat, (1984).

Effect of incubation time on inhibitors treated soil samples:

The effect of incubation time on the activity of urease enzyme was examined by changing time (5 minutes, 10 minutes, 15 minutes, 20 minutes and 30 minutes). All other parameters were kept constant by following the method of Kandeler and Gerber, (1988).

Effect of substrate concentration on inhibitors treated soil samples:

The effect of substrate concentration on the activity of urease enzyme was examined by using five different concentrations (2%, 4%, 6%, 8% and 10%) while all other parameters were kept constant. Line weaver Burk was used to determine Vmax and Km kinetic parameters by using the method of Kandeler and Gerber, (1988).

Procedure for enzyme kinetics:

In a test tube, 1 gram sieved soil was mixed with 150 microliter toluene and stand this solution for 15 minutes. After this, 1000 microliter urea and 2000 microliter citrate buffer of pH (3.5, 4.5, 5.5, 6.5 and 7.5) was added to each sample individually and incubated for 3 hours at 37^0C. After incubation, 100 microliter filtrate was taken from incubated solution in a separate test tube and mixed with 900 microliter distilled water + 400 microliter phenolate solution + 300 microliter sodium hypochlorite solution. This solution was allowed to stand for 20 minutes and then absorbance was measured at 580 nm by using spectrophotometer. The same procedure was followed for different substrate concentrations (2%, 4%, 6%, 8%, 10%), incubation time (5 minutes, 10 minutes, 15 minutes, 20 minutes and 30 minutes) and temperatures (10^0C, 20^0C, 30^0C, 40^0C and 50^0C) respectively by keeping all other parameters constant by following the procedure of Kandeler and Gerber, (1988), Frankenberger and Johanson, (1982) and Sahrawat, (1984).

Statistical analysis:

All the values that were obtained from enzyme assay and enzyme kinetics were expressed in mean ± standard deviation of 3×3 data. Level of significance between different groups of inhibitors was measured by using two-way ANOVA multiple comparison test that was based on the analysis of variance. Analysis of variance was performed by using Graph pad prism 7.0 at p-value ≤ 0.05 (Montgomery, 2017).

Results and Discussions:-

This study explored the best inhibitor that control the activity of urease enzyme at maximum rate. Results showed that the urease activity was significantly low in the soil treated with inhibitors as compared to control group. Results

obtained from kinetic study indicates that at optimum temperature (37^0C) and pH (6.7) urease enzyme showed the best activity. This research work was planned and performed in Clinico-Medical research laboratory of Biochemistry department, University of Agriculture Faisalabad.

Phase Ⅰ: Effect of different inhibitors on urease activity

Effect of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitors on the activity of urease enzyme in wheat soil of Faisalabad region:

Increased urease activity is responsible for ammonia volatilization. Urease activity was measured till day 37. A significant reduction in urease activity was observed on day 1 and day 2 by T1 as compared to control, T2 and T3 (Figure 2). On day 3 urease activity again showed a significant high urease reduction in T1 as compared to Control, T2 and T3. From day 8 to day 12, high significant inhibition activity of urease enzymes was observed. Maximum urease inhibition activity was observed on day 14. After that inhibitors became ineffective. On day 17, effect of inhibitors begins to decrease as compared to day 14. A little increase in urease activity was also observed on day 17. On day 20 and day 23, a significant elevation in urease enzyme activity was observed as compared to day 17 (Figure 2).

Table 3:- Effect of (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) inhibitors on the activity of urease enzyme on Faisalabad wheat soil.

Enzyme activity (IU/g soil) mean±S.D				
Treatments Days	**Control**	**T1**	**T2**	**T3**
Day 1	11.273±0.118	7.940±0.249 ****	9.620±0.232 ****	10.007±0.155 ****
Day 2	22.720±0.279	13.108±0.155 ****	18.664±0.118 ****	22.100±0.205 **
Day 3	21.868±0.232	8.948±0.118 ****	14.167±0.118 ****	14.968±0.155 ****
Day 4	16.105±0.249	5.692±0.118 ****	7.449±0.232 ****	7.785±0.161 ****
Day 6	15.821±0.232	5.356±0.077 ****	5.149±0.195 ****	6.467±0.195 ****
Day 8	4.322±0.195	1.609±0.195 ****	2.979±0.161 ****	3.961±0.155 ns
Day 10	7.449±0.232	0.524±0.118 ****	5.821±0.155 ****	3.031±0.155 ****
Day 12	3.341±0.155	2.359±0.118 ****	0.834±0.195 ****	1.351±0.236 ****
Day 14	8.121±0.161	0.653±0.313 ****	3.263±0.077 ****	5.356±0.155 ****
Day 17	3.160±0.118	10.059±0.089 ****	14.813±0.155 ****	9.749±0.236 ****
Day 20	2.100±0.155	8.276±0.249 ****	8.638±0.195 ****	3.987±0.118 ****
Day 23	1.480±0.155	6.329±0.121 ****	16.622±0.272 ****	7.708±0.118 ****
Day 30	4.090±0.118	12.074±0.195 ****	13.651±0.205 ****	14.710±0.195 ****
Day 37	1.403±0.077	16.416±0.236 ****	21.403±0.232 ****	17.759±0.155 ****

**** indicates high significance difference among control and treatment. ***indicates significance difference among control and treatments.

** indicates less significance difference among control and treatments. */ns indicates non-significant results among control and treatments. (GraphPad Prism 7.0)

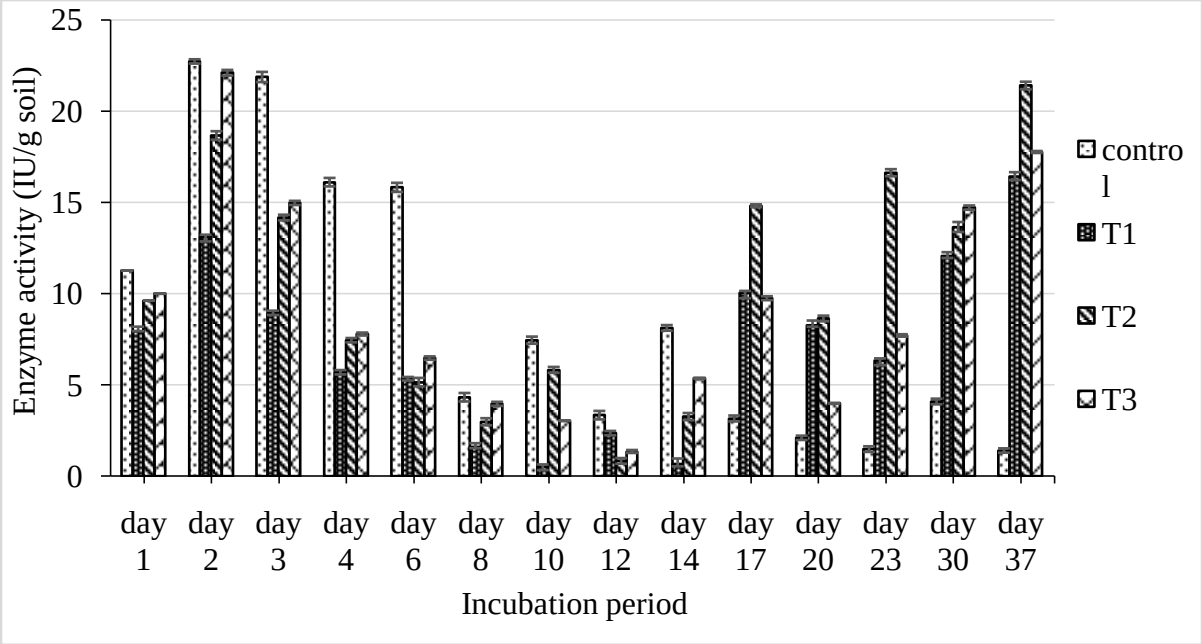

Figure 2: Effect of (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) inhibitors on the activity of urease enzyme in Faisalabad wheat soil.

The relative effects of Ammonium thiosulfate, Sodium thiosulfate and 1,2,4-Triazole at different ratios was observed on urease activity of Faisalabad wheat soil as demonstrated in Figure 2. Inhibition potential of Ammonium thiosulfate, Sodium thiosulfate and 1,2,4-Triazole was potentially high on day 8 and day 12. After that, urease inhibition activity decreases day by day till day 37. Ammonium thiosulfate (ATS) and Sodium Thiosulfate (STS) both are rich source of nitrogen and phosphorous. ATS has been shown to inhibit volatilization in combination with urea ammonium nitrate (Goos and Johnston, 1999). STS is also potent inhibitor it helps to control soil nitrification and improves the nitrogen loss in soil and crop production (Abbasi et al., 2011). Similarly, 1,2,4-Triazole is a non-volatile and water soluble compound (Bundy and Bremner, 1973). At moderate and high soil temperatures, 1,2,4-Triazole is equally effective to crops (Mahmood et al., 2008). Soil samples treated with Ammonium thiosulfate (ATS) + Sodium Thiosulfate (STS) + 1,2,4-Triazole at 1:1:1 (T1) shows high urease inhibition activity at day 12. But later on this inhibition pattern was decreased on day 17. High urease inhibition activity was observed by T1 combination as compared to T2 (1.5:0.75:0.75) and T3 (0.75:1.5:0.75).

Effect of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitors on the activity of urease enzyme in wheat soil of Sheikhupura:

The results on urease activity of the wheat soil of Sheikhupura region in Control, T1, T2 and T3 was shown in Table 4. A significant reduction in urease activity was observed up to day 3 by T1 as compared to control, T2 and T3 (Figure 3). On day 4, It again showed a significantly high urease reduction in T1 against Control, T2 and T3 as compared to day 3. Significantly Increased urease inhibition was observed on day 8 and day 10 as compared to day 6. Here T1 again shows higher efficiency as compared to T2 and T3. On day 12, T1 shows least urease inhibition as compared to T2 and T3 due to rain fall event occurs. On day 14, an increase in urease inhibition activity was observed. After day 14 inhibitors became ineffective. On day 17, effect of inhibitors begins to decrease as compared to day 14. A little increase in urease activity was also observed on day 17. On day 20 and day 23, a significant elevation in urease enzyme activity was observed as compared to day 17 (Figure 3). On day 37, maximum reduction in urease inhibition was observed. In this study, maximum urease inhibition was observed till day 10. On day 12 and day 14, urease inhibition was disturbed due to rain fall. Urease activity increases day by day till day 37. On day 37 no urease inhibition was observed.

Table 4:- Effect of (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) inhibitors on the activity of urease enzyme in Sheikhupura wheat soil.

Enzyme activity (IU/g soil) mean±S.D				
Treatments	**Control**	**T1**	**T2**	**T3**

Days				
Day 1	8.147±0.155	3.987±0.195****	4.374±0.161 ns	5.563±0.118****
Day 2	17.372±0.232	12.023±0.155****	13.366±0.195****	12.229±0.195****
Day 3	15.072±0.161	7.888±0.195****	10.162±0.155****	9.387±0.155****
Day 4	13.599±0.293	3.186±0.155****	4.297±0.236****	4.865±0.118****
Day 6	17.940±0.161	7.372±0.232****	14.038±0.155****	12.049±0.161****
Day 8	10.266±0.118	2.100±0.155****	1.454±0.195****	2.126±0.272****
Day 10	11.764±0.195	1.196±0.236****	3.651±0.232****	3.031±0.155****
Day 12	16.674±0.310	6.751±0.155****	5.046±0.155****	7.268±0.161****
Day 14	11.273±0.236	9.568±0.118****	7.656±0.118****	10.188±0.195****
Day 17	2.953±0.077	6.829±0.118****	13.444±0.118****	14.607±0.195****
Day 20	3.186±0.155	8.638±0.195****	7.268±0.118****	12.049±0.161****
Day 23	2.049±0.195	13.651±0.155****	19.361±0.195****	17.682±0.077****
Day 30	5.124±0.279	11.945±0.155****	13.754±0.272****	16.105±0.272****
Day 37	3.573±0.155	9.878±0.118****	12.333±0.155****	12.850±0.272****

**** indicates high significance difference among control and treatment. ***indicates significance difference among control and treatments.

** indicates less significance difference among control and treatments. */ns indicates non-significant results among control and treatments. (GraphPad Prism 7.0)

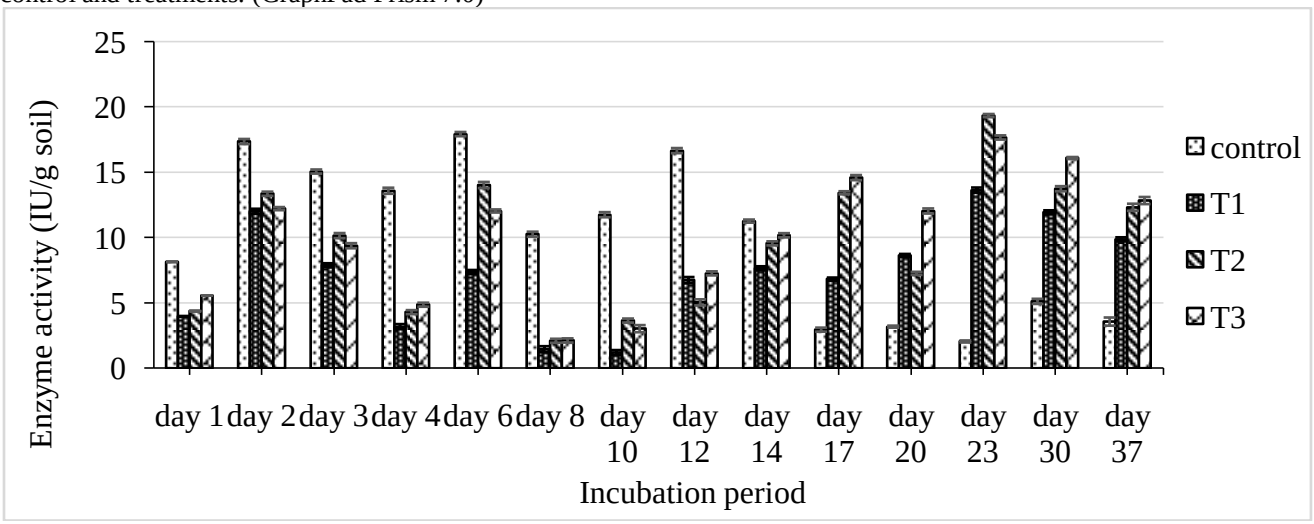

Figure 3: Effect of (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) inhibitors on the activity of urease enzyme in Sheikhupura wheat soil.

The efficiency of ATS differs with soil type to reduce volatilization as it increases when temperature is high and soil moisture content is low. As reduced tillage, ATS urease inhibition tends to decreases by lowering the soil temperature and increasing the soil moisture content (Sullivan and Havlin, 1992). Ammonium thiosulfate (ATS), Sodium Thiosulfate (STS) and 1,2,4-Triazole regulates the breakdown of the urea from day 8 to day 14. After this hydrolysis of urea again increases urease inhibitors help to reduce nitrogen loss from ammonia. Results demonstrated that soil samples treated with Ammonium thiosulfate (ATS) + Sodium Thiosulfate (STS) + 1,2,4-Triazole at 1:1:1 (T1) shows high urease inhibition activity at day 10. But later on this inhibition pattern was decreased on day 17. High urease inhibition activity was observed by T1 combination as compared to T2 (1.5:0.75:0.75) and T3 (0.75:1.5:0.75).

Effect of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitors on the activity of urease enzyme in wheat soil of Gujranwala:

Efficiency of Ammonium Thiosulfate, Sodium Thiosulfate and 1,2,4-Triazole was determined by evaluating the urease activity level. To decrease the ammonia volatilization, these inhibitors shows more inhibition potential from day 8 to day 12. The results indicated that urease inhibition potential was significantly decreased after day 12 as compared to control (Table 5). The effect of Ammonium Thiosulfate, Sodium Thiosulfate and 1,2,4-Triazole on the

activity of urease enzyme was also observed. A significant reduction in urease activity was observed up to day 3 by T1 as compared to control, T2 and T3 (Figure 4). On day 4, It again showed a significant high urease reduction in T1 as compared to Control, T2 and T3. Significantly high urease inhibition was observed on day 8 to day 10. On day 12, T1 shows least urease inhibition due to rain fall event occurs. Maximum urease activity was observed on day 12 after that inhibitors became ineffective. On day 17, effect of inhibitors begins to decrease as compared to day 14. A little increase in urease activity was also observed on day 17. On day 20 and day 23, a significant elevation in urease enzyme activity was observed as compared to day 17 (Figure 4). On day 37, maximum reduction in urease inhibition was observed (Table 5).

Table 5:- Effect of (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) inhibitors on the activity of urease enzyme in Gujranwala wheat soil.

Enzyme activity (IU/g soil) mean±S.D				
Treatments **Days**	**Control**	**T1**	**T2**	**T3**
Day 1	9.284±0.195	6.545±0.195 ****	7.992±0.155 ****	7.191±0.118 ****
Day 2	15.744±0.155	4.633±0.236 ****	9.620±0.232 ****	9.749±0.236 ****
Day 3	10.395±0.232	6.286±0.155 ****	9.155±0.232 ****	6.958±0.195 ****
Day 4	5.925±0.161	2.798±0.155 ****	3.651±0.155 ****	4.581±0.155 ****
Day 6	7.578±0.195	3.961±0.232 ****	5.356±0.155 ****	5.976±0.155 ****
Day 8	4.167±0.195	1.196±0.195 ****	1.764±0.118 ****	2.669±0.118 ****
Day 10	5.950±0.195	2.100±0.155 ****	3.341±0.155 ****	3.186±0.155 ****
Day 12	5.175±0.118	4.426±0.155 ****	2.643±0.232 ****	3.625±0.118 ****
Day 14	6.441±0.155	4.891±0.155 ****	6.286±0.155 ns	5.950±0.195 **
Day 17	2.333±0.775	4.555±0.195 ****	7.423±0.195 ****	6.726±0.195 ****
Day 20	2.178±0.775	7.888±0.195 ****	3.031±0.155 ****	4.064±0.118 ****
Day 23	7.501±0.195	6.596±0.205 ****	13.237±0.195 ****	15.046±0.232 ****
Day 30	4.297±0.313	10.989±0.236 ****	14.167±0.161 ****	14.193±0.155 ****
Day 37	5.201±0.155	7.966±0.118 ****	17.372±0.251 ****	15.563±0.118 ****

**** indicates high significance difference among control and treatment. ***indicates significance difference among control and treatments.

** indicates less significance difference among control and treatments. */ns indicates non-significant results among control and treatments. (GraphPad Prism 7.0)

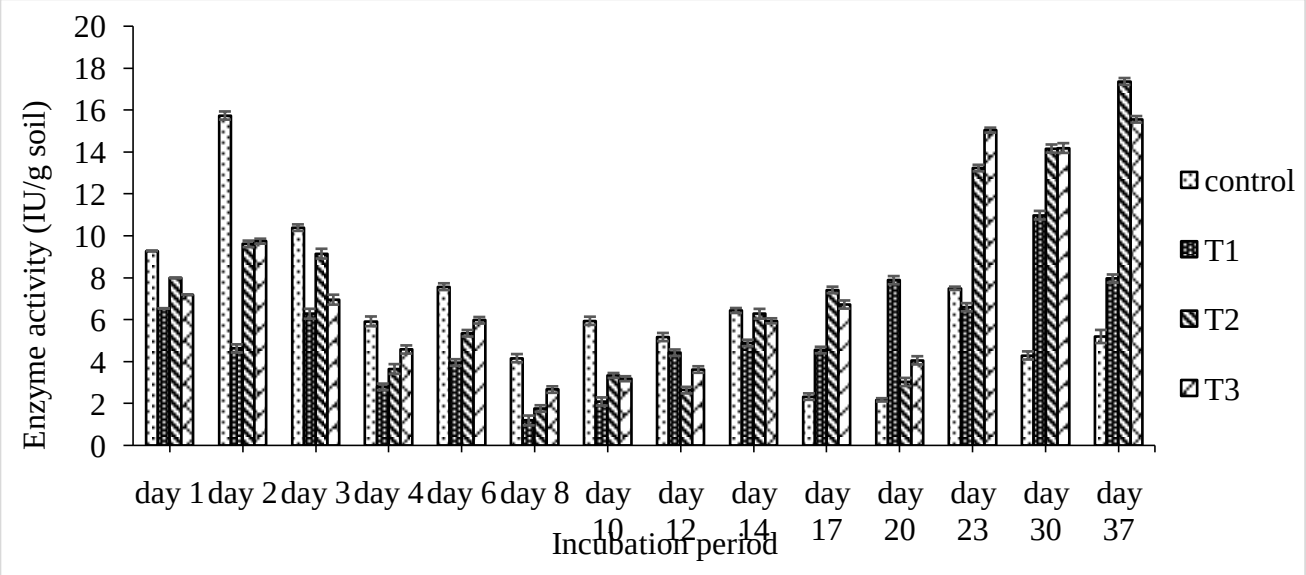

Figure 4:- Effect of (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) inhibitors on the activity of urease enzyme in Gujranwala wheat soil.

Results indicated that from day 4 to day 10 urease activity decreases in soil samples treated with Ammonium thiosulfate (ATS) + Sodium Thiosulfate (STS) + 1,2,4-Triazole at 1:1:1 (T1). But later on this inhibition pattern was decreased on day 17. High urease inhibition activity was again observed by T1 combination as compared to T2 (1.5:0.75:0.75) and T3 (0.75:1.5:0.75). Ammonium Thiosulfate and Sodium Thiosulfate contains thiosulfate ions as nitrification inhibitors (Goos, 1985).

Phase Ⅱ: Kinetic study of different inhibitors on urease activity

In soil, activity of any enzyme is a composite of activities linked with many abiotic and biotic factors (Burns, 1982). Enzymes found in soluble and insoluble organic matrices function completely different as compared to enzymes in free solution. Ureases immediately absorbed in to the soil matrix that is structured by many humic substances as well as clays in a heterogeneous system (Lai and Tabatabai, 1992). Soil ureases i.e. jack bean ureases show Michalies Menton kinetics (Boyd and Mortland, 1985).

In phase Ⅱ, kinetic behavior of urease enzyme in the presence and absence of urease inhibitors (Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole) was evaluated in the soil of Faisalabad, Sheikhupura and Gujranwala regions. Chemical reactions proceed on different rates. A little change in one or more factors can easily change the entire rate of a reaction. The effects on urease activity was determined by varying the conditions of the reaction. The results of this experiment confirms that the urease inhibition in soil varies widely in pH, temperature, incubation time and substrate concentration. Kinetic parameters i.e. Temperature, Substrate concentration, Incubation time and pH has a great impact on urease activity. To evaluate the effect of kinetic behavior on inhibitors treated soil, graphs were plotted by using SlideWrite program 7.01. Standard errors, Km and Vmax values were calculated.

Effect of Substrate concentration on inhibitors treated soil samples:

Kinetic parameters (Km and Vmax) were calculated by using Lineweaver-Burk equation. For urease activity, kinetic constants (Km and Vmax) were calculated by changing the urea (substrate) concentration. Linear Michaelis Menten equation is:

$$V_0 = V_{max}\left(\frac{[Substrate]}{[Substrate] + K_m}\right)$$

Here V_0 is the initial rate at substrate concentration [S], Km is the concentration of substrate at which reaction rate is attained half of the Vmax (Michaelis constant) and Vmax is the maximum, rate at saturating substrate concentration. This Michaelis Menten equation helps to mathematically represent the mechanism that is involved in reversible inhibition in which a chemical reacts with the enzyme by either non-covalent association or non-covalent dissociation reactions (Torres and Santos, 2017).

In this study, effect of different substrate concentrations on inhibitors treated soil samples were determined. In the wheat soil of Faisalabad, Sheikhupura and Gujranwala region, Km value of soil ureases treated with inhibitors was high as compared to control while the value of Vmax is high in control as compared to T1, T2 and T3 as demonstrated in Table 6. If in mixed type of inhibition, the value of Km increases, the value of Vmax decreases (Khan et al., 2013). Sodium thiosulfate is a cheap inhibitor that controls the nitrification and gradually inhibits the urease activity and improves the crop production. Similar results were observed for mixed type inhibition of urease activity at different substrate concentrations (Singh and Nye, 2006). At optimum substrate concentration urease enzyme shows maximum activity. In the wheat soil of Faisalabad, Sheikhupura and Gujranwala region, the activity of urease enzyme increases as the substrate concentration raises i.e. 2%, 4%, 6%, 8% and 10% (Figure 5).

Table 6:- Effect of substrate concentration on inhibitors treated soil samples.

Vmax of enzyme					**Km of enzyme**				
	Control	**T1**	**T2**	**T3**		**Control**	**T1**	**T2**	**T3**
FSD	16.838	12.295	8.481	9.945	**FSD**	1.644	2.33	3.858	3.094
SKP	18.392	9.127	11.097	12.509	**SKP**	2.069	4.808	3.765	3.324
GUJ	14.330	7.618	10.298	8.411	**GUJ**	2.069	4.223	3.858	3.289

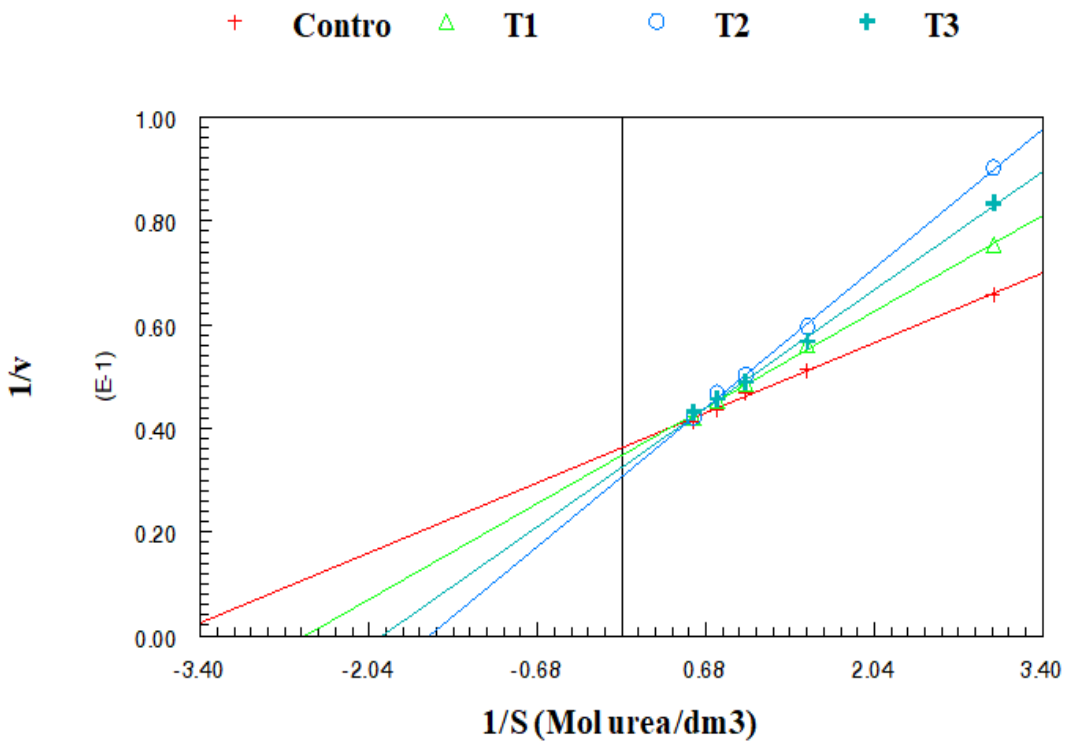

Figure 5(a):- Effect of substrate concentration on inhibitors treated soil samples of Faisalabad region.

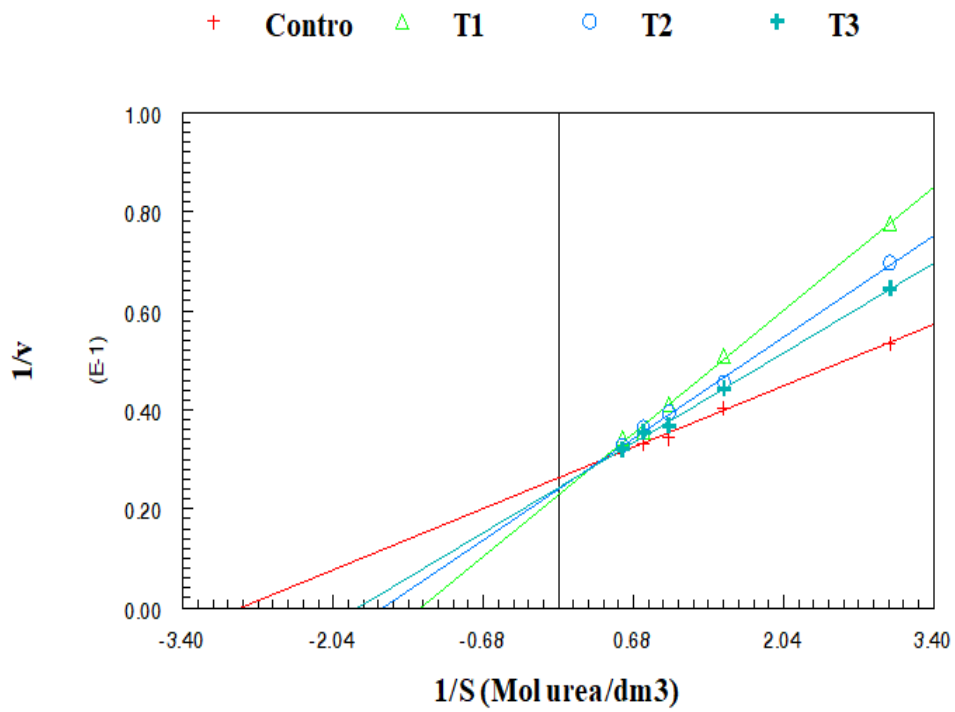

Figure 5(b):- Effect of substrate concentration on inhibitors treated soil samples of Sheikhupura region.

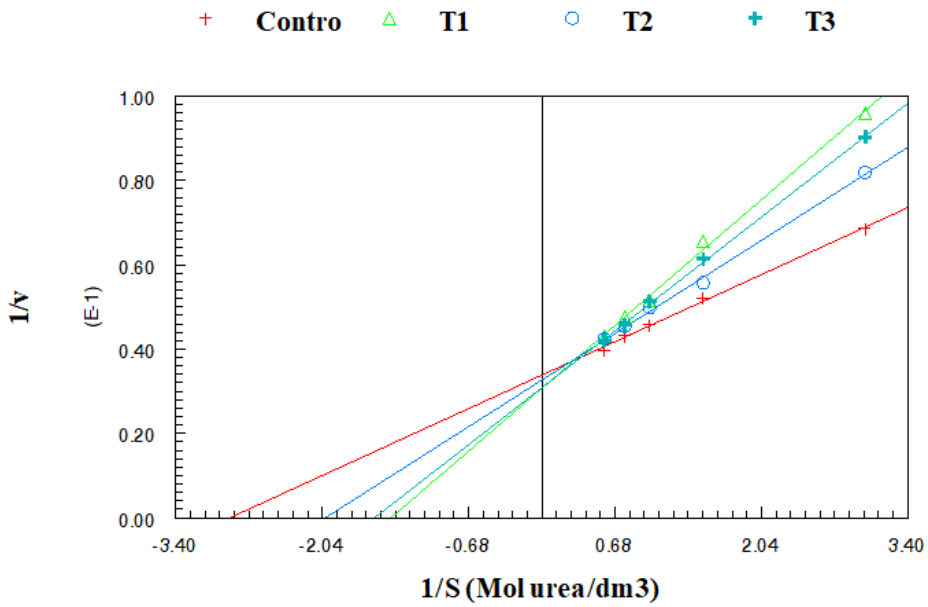

Figure 5(c):- Effect of substrate concentration on inhibitors treated soil samples of Gujranwala region.

Maximum activity of urease enzyme was observed at optimum substrate concentration. Urease activity increases as the substrate concentration increases until it attained an optimum substrate concentration. Further increase in substrate concentration results in decreased urease activity. At high substrate concentration, the activity of urease enzyme decreases due to the substrate inhibition. Urease enzyme exhibited high affinity for the substrate at low substrate concentration (Singh and Nye, 2006).Hence, results indicate that as the substrate concentration increases the activity of urease enzyme also increases in Faisalabad, Sheikhupura and Gujranwala soil (Table 6).

Effect of pH on inhibitors treated soil samples:

Optimum pH for soil ureases was 6.5-7.0 (Petit et al., 1976). Optimum pH of an enzyme activity depends on the buffer used. pH stability of any enzyme depends on many factors i.e. ionic strength, temperature, concentration of substrate, contaminating metal ions, enzyme cofactors and enzyme concentration (Segel, 1975). Change in H+ concentration due to either denaturation of enzyme or the reversible reaction of deionizing and ionizing proto-trophic groups present at the active site of the enzyme leads to the alteration in soil enzyme activity. Catalytic activity of soil enzyme decreases when these enzymes were exposed to extreme acidic or alkaline conditions. High H+ ions concentration (pH 1-2) and high OH- ion concentration (pH 12-14) disturbs the hydrogen and ionic bonds that are required to maintain the active conformation of an enzyme (Frankenberger and Johanson, 1982).

Effect of pH on the activity of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitors treated soil urease was determined. Ratio between Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitor was 1:1:1 in T1, 1.5:0.75:0.75 in T2 and 0.75:1.5:0.75 in T3 respectively. Urease enzyme activity was observed at different pH i.e. 3.5, 4.5, 5.5, 6.5 and 7.5. The activity of urease enzyme was found maximum near to the 6.7 pH. Increase or decrease in pH is responsible for decrease in enzyme activity. So, Optimum pH for urease enzyme was considered as 6.7. The results indicated that in Faisalabad, Sheikhupura and Gujranwala wheat soil, urease activity was increases as pH level increases as at 3.5 pH ureases activity was less as compared to pH 4.5. On the other hand, activity level significantly increases at a pH of 5.5. At pH 6.5 (near to optimum pH) activity of urease enzyme again increases. pH of the buffer is further increased to 7.5, that leads to significant decrease in enzyme activity as compared to optimum pH (Table 7).

Table 7:- Effect of pH on inhibitors treated soil samples.

	FSD				**SKP**				**GUJ**			
pH	**Control**	**T1**	**T2**	**T3**	**Control**	**T1**	**T2**	**T3**	**Control**	**T1**	**T2**	**T3**
3.	8.689	2.255	3.573	4.038	13.883	11.01	6.441	5.124	14.426	8.379	7.294	11.32

5						5						5
4.5	16.131	10.395	6.131	7.837	17.139	15.046	11.480	6.364	17.682	10.240	12.565	14.348
5.5	24.658	13.806	17.527	10.705	23.573	17.527	15.666	9.232	21.403	17.682	16.054	17.372
6.5	29.620	24.581	23.496	14.658	33.031	24.968	26.519	16.751	32.333	27.604	23.418	24.503
7.5	26.441	17.992	21.558	12.178	27.837	22.953	18.379	14.348	28.069	21.945	19.697	22.333

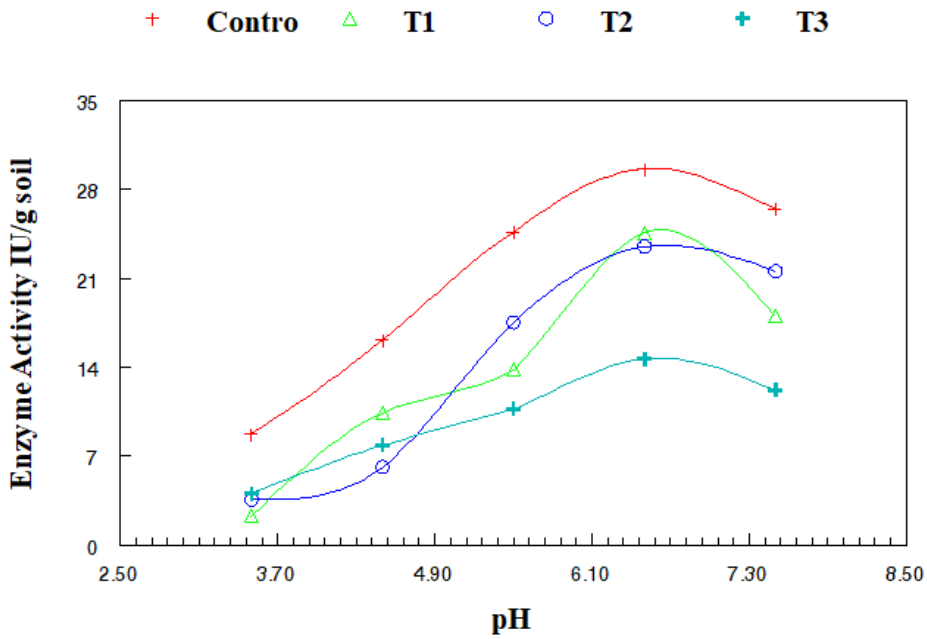

Figure 6(a):- Effect of pH on inhibitors treated soil samples of Faisalabad region.

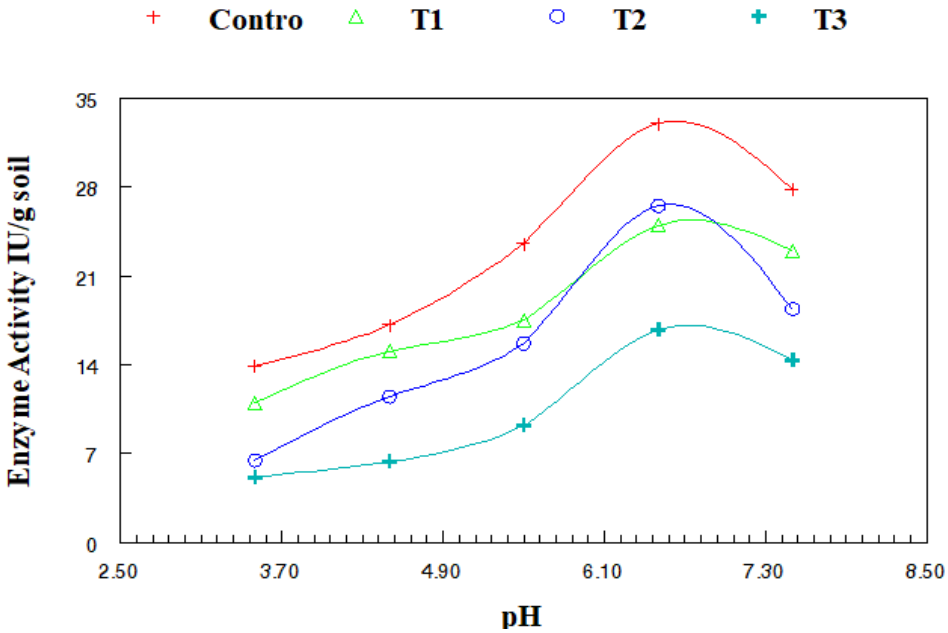

Figure 6(b):- Effect of pH on inhibitors treated soil samples of Sheikhupura region.

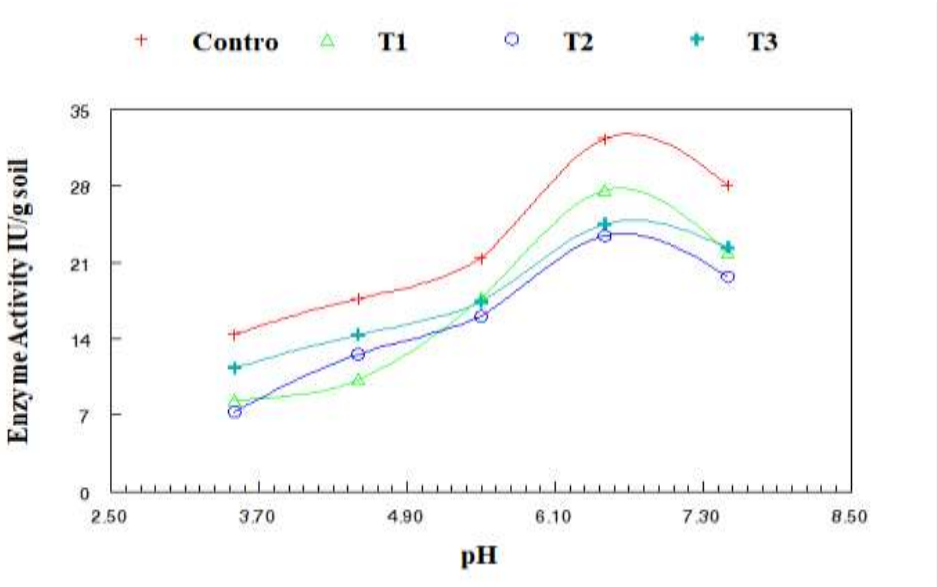

Figure 6(c):- Effect of pH on inhibitors treated soil samples of Gujranwala region.

Ammonia hydrolyses to bicarbonate ion at neutral pH. At a pH less than 6.3, ammonia hydrolyzes to carbonic acid. Ammonia release decreases at pH 5.2-6.0 due to the acidic pH that leads to the decrease in urea hydrolysis (Fan and Mackenzie, 1993). Hence, results indicate that at optimum pH, urease enzyme shows highest activity in Faisalabad, Sheikhupura and Gujranwala soil. As the pH decreases, activity of urease enzyme also decreases as shown in Figure 6. At pH 3.5, 4.5, 5.5 and 7.5, the readings obtained were low as compared to pH 6.5.

Effect of temperature on inhibitors treated soil samples:

Effect of temperature on the activity of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitors treated soil urease was determined. Ratios of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitor was 1:1:1 in T1, 1.5:0.75:0.75 in T2 and 0.75:1.5:0.75 in T3 respectively. Urease enzyme activity was observed at different temperatures i.e. 10^0C, 20^0C, 30^0C, 40^0C and 50^0C. The activity of urease enzyme was found maximum near to the 37^0C. Increase or decrease in temperature is responsible for decrease in enzyme activity. Results obtained were: In Faisalabad, Sheikhupura and Gujranwala wheat soil, urease activity increases with temperature. At 10^0C urease activity begin to increase significantly. As temperature raises to 20^0C the activity again increases respectively. Similarly, by increasing temperature to 30^0C, the activity of urease enzyme was also increased. Temperature is further increased to 40^0C (near to optimum temperature) activity of urease enzyme increases. Temperature is again increased to 50^0C, that leads to significant decrease in enzyme activity as compared to optimum temperature for control, T1, T2 and T3 (Table 8).

Table 8:- Effect of temperature on inhibitors treated soil samples.

	FSD				**SKP**				**GUJ**			
Temp.	**Control**	**T1**	**T2**	**T3**	**Control**	**T1**	**T2**	**T3**	**Control**	**T1**	**T2**	**T3**
10°C	8.612	2.023	4.348	3.651	6.891	4.271	3.108	4.581	3.263	1.868	1.480	2.565
20°C	11.713	8.922	5.976	5.124	9.147	7.674	7.217	5.589	9.930	4.116	6.519	4.736
30°C	17.682	13.170	9.232	8.379	15.341	12.100	12.705	9.930	15.046	8.767	8.612	11.015
40°C	24.581	21.015	14.969	17.294	23.108	16.441	19.852	18.689	21.480	19.310	13.883	15.279
50°C	21.635	17.217	11.403	15.046	17.604	13.806	15.046	16.209	18.844	13.186	11.325	13.806

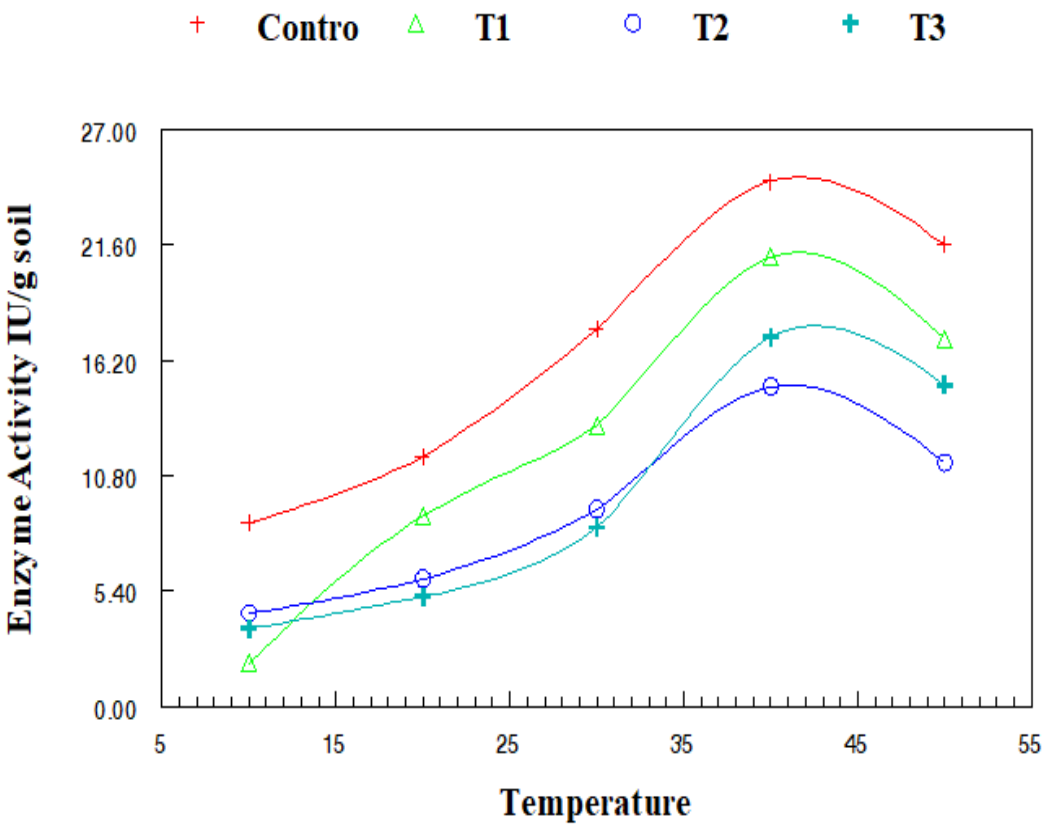

Figure 7(a):- Effect of temperature on inhibitors treated soil samples of Faisalabad region.

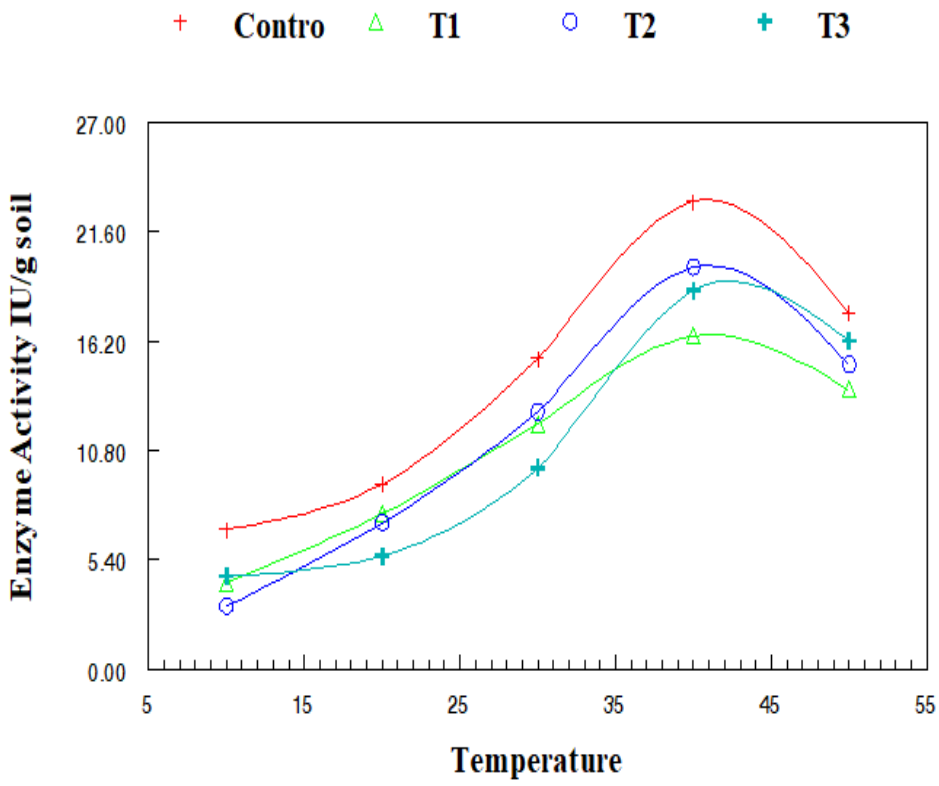

Figure 7(b):- Effect of temperature on inhibitors treated soil samples of Sheikhupura region.

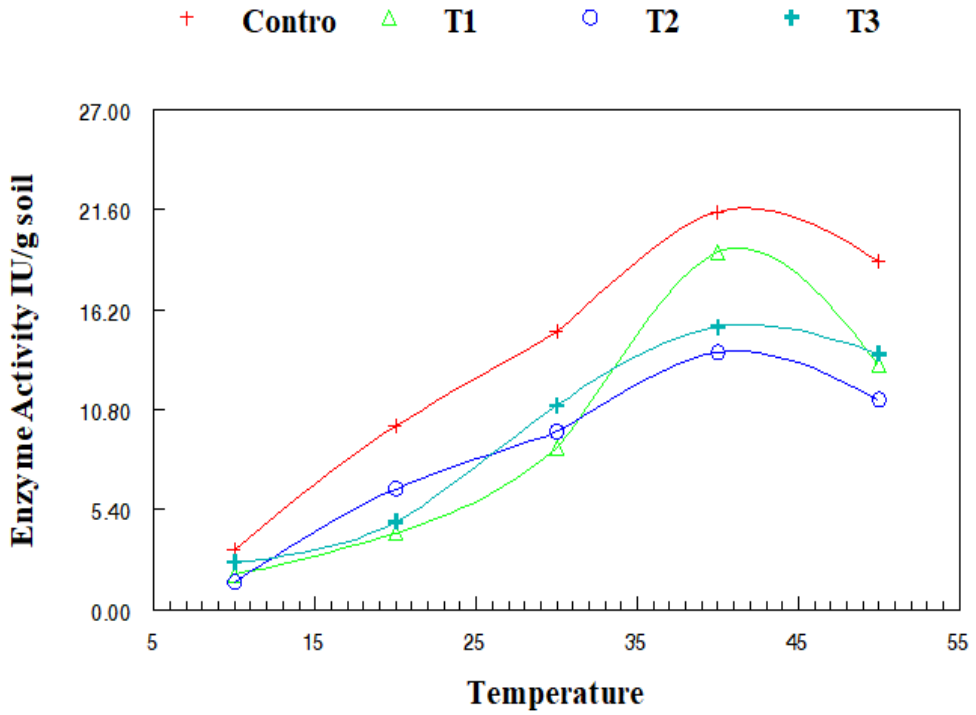

Figure 7(c): Effect of temperature on inhibitors treated soil samples of Gujranwala region.

Ammonium thiosulfate and Sodium thiosulfate has very little potential for retarding the hydrolysis of urea in soil. It retards soil ureases only when it is applied to the soil at a very high rate that leads to crop degradation or damage (McCarty et al., 1990). Inhibition by ATS was observed at two temperatures (20^0C and 30^0C). Maximum ATS inhibition was observed at high soil temperature (30^0C). The oxidation of thiosulfate in to $S_4O_6^{2-}$ was more rapid at drier environment. The efficiency of ATS differs with soil type to reduce volatilization as it increases at high temperatures and low soil moisture contents (Sullivan and Havlin, 1992). Results indicated that as temperature increases urease activity also increases till the optimum temperature. At 10^0C, 20^0C, 30^0C and 50^0C, the readings obtained were low as compared to 37^0C-40^0C (Figure 7).

Effect of incubation time on inhibitors treated soil samples:

With time, all proteins suffer from catalytic activity loss or denaturation. If an enzyme uses much of the substrate during incubation, the activity of enzyme becomes inadequate and leads to the decreased product formation. Enzyme catalyzed reactions are reversible. Initially there is no product formation that is responsible for forward reaction but with the passage of time, significant product formation occurs that significantly proceed the reaction backward. As the incubation proceeds, the rate of the product formation became slow. If incubation time is too long, the activity of enzyme decreases (Robinson, 2015).

The effect of incubation time on the activity of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole treated soil ureasewas determined. Ratios of Ammonium Thiosulfate + Sodium Thiosulfate + 1,2,4-Triazole inhibitor was 1:1:1 in T1, 1.5:0.75:0.75 in T2 and 0.75:1.5:0.75 in T3 respectively. The effect of incubation time on the activity of urease enzyme was examined by changing time (5 minutes, 10 minutes, 15 minutes, 20 minutes and 30 minutes) as shown in Table 9. Results were discussed as: in Faisalabad Sheikhupura and Gujranwala wheat soil, urease activity increases by increasing the incubation period. As at 5-minute incubation, the activity of urease enzyme significantly increases. By raising temperature, the activity of urease enzyme again increases up to 30 minutes for control, T1, T2 and T3 (Table 9).

Table 9:- Effect of Incubation time on inhibitors treated soil samples.

	FSD				SKP				GUJ			
Tim	**Contr**	**T1**	**T2**	**T3**	**Contr**	**T1**	**T2**	**T3**	**Contr**	**T1**	**T2**	**T3**

e	ol				ol				ol			
5 min.	12.635	5.589	7.062	10.705	7.604	6.674	2.798	5.476	11.248	3.573	7.217	8.922
10 min	18.449	13.651	10.162	16.596	14.679	10.705	6.441	9.330	16.984	10.162	9.155	14.116
15 min	24.193	20.705	16.984	19.534	18.759	14.813	13.806	13.023	19.930	15.589	13.108	17.131
20 min	30.937	23.883	20.627	22.565	23.255	17.449	21.178	19.930	24.503	21.015	15.356	21.860
30 min	34.333	30.472	23.728	25.356	28.844	19.620	24.503	22.875	33.263	25.693	21.790	24.928

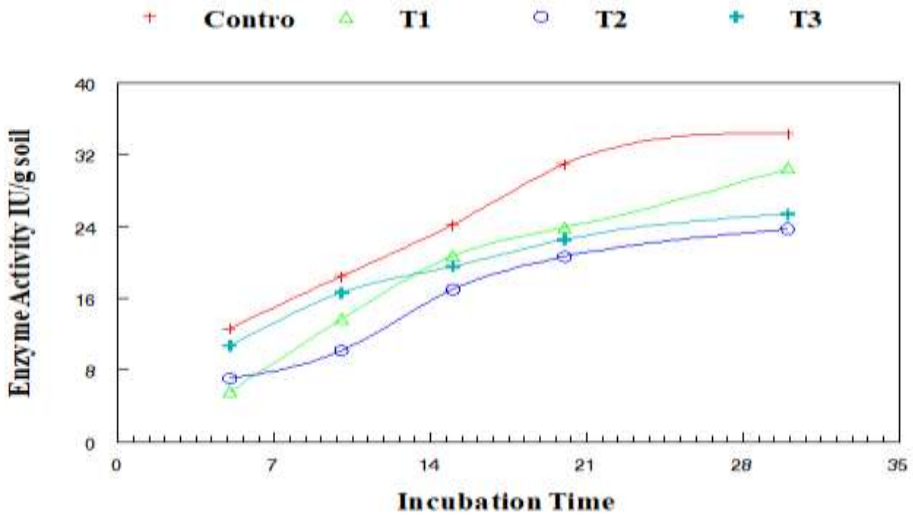

Figure 8(a): Effect of Incubation time on inhibitors treated soil samples of Faisalabad region.

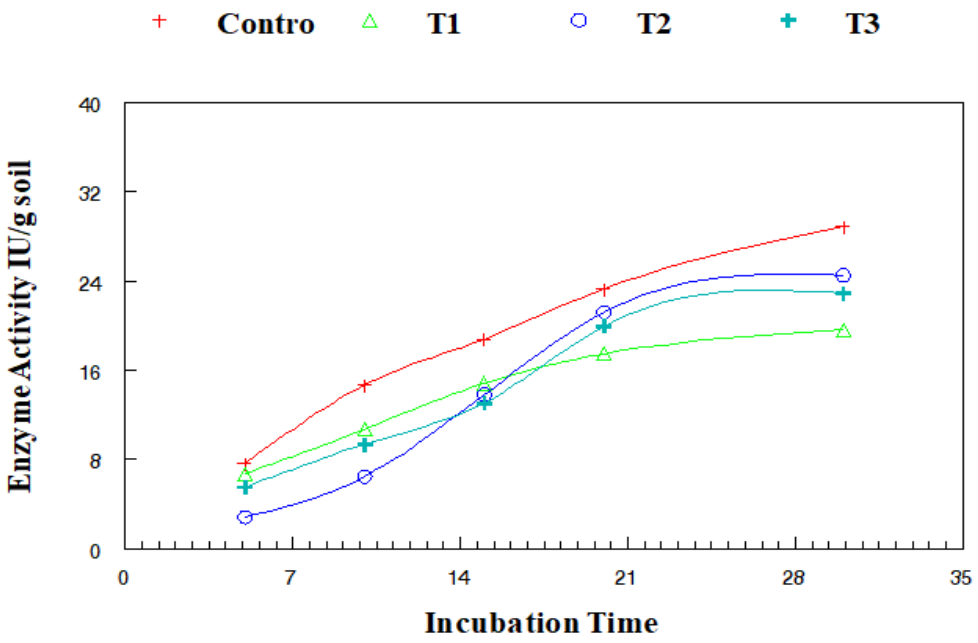

Figure 8(b): Effect of Incubation time on inhibitors treated soil samples of Sheikhupura region.

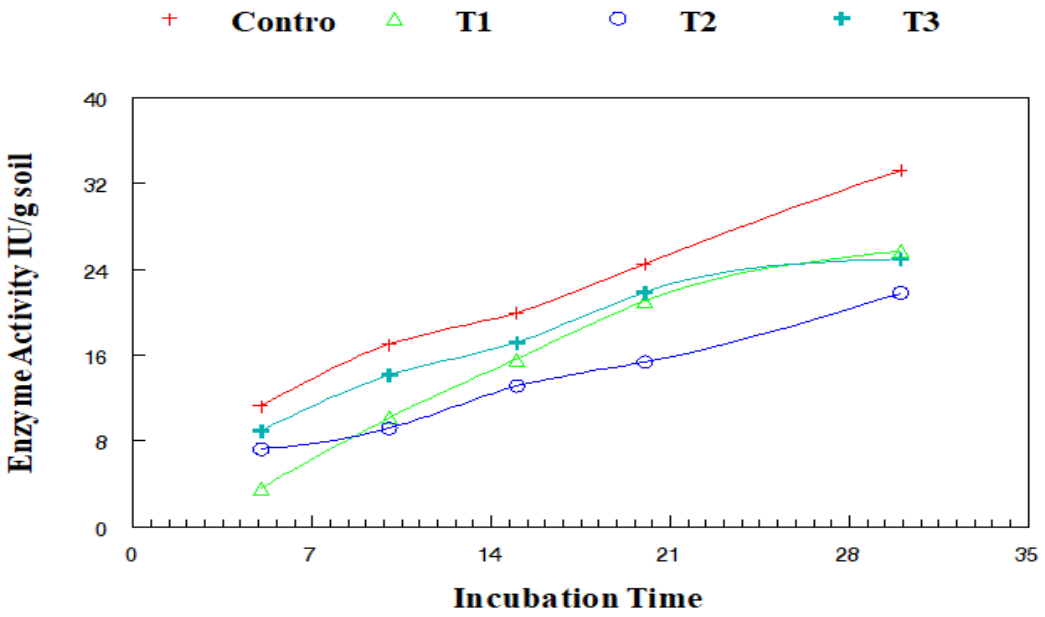

Figure 8(c):- Effect of Incubation time on inhibitors treated soil samples of Gujranwala region.

Inhibitory effect of many compounds decreases by increasing the incubation time of inhibitors treated soil. By increasing incubation time, Inhibitory effect of those inhibitors increases that eventually decompose in to the soil and has no effect on the hydrolysis of urea (Bremner and Douglas, 1971). Results indicated that with the passage of time, urease activity also increases till 3-hour incubation. After that enzyme activity begins to decrease. After 30-minutes incubation, maximum enzyme activity was observed as compared to 5-minutes incubation (Figure 8).

The overall results indicate that there are 4 major factors that influence the activity of urease enzyme e.g. substrate concentration, incubation time, temperature and pH of the soil. Level of urease activity was lower in inhibitors treated soil as compared to the untreated soil. These inhibitors potential was quite efficient in Faisalabad region samples as compared to the Sheikhupura and Gujranwala region. The inhibition potential of all applied inhibitors was higher in day 6^{th} to day $14^{th.}$ From day 17^{th} to day 37^{th} the inhibition potential decreases day by day. Effect of kinetic parameters showed that with an increase in temperature and incubation time, the activity of urease enzyme also increases up to 37°C while the results of pH demonstrated that as pH increases, the enzyme activity also increases up to the optimum pH (6.7), above this pH, the enzyme activity significantly falls. At optimum pH and Temperature, enzyme remains protected from denaturation. Above this pH and temperature, the enzyme becomes inactivated.

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