Synergistic effect of urease and nitrification inhibitors in the reduction of ammonia volatilization

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Abstract

Nitrogen (N) is deficient in more than 90% of soils of Pakistan mainly because of low organic matter contents. The use of nitrogenous fertilizers is a common practice for sustainable and profitable crop yields. A significant portion of added fertilizers is lost through volatilization, leaching, and denitrification. Low use efficiency of these fertilizers in our climate is a serious concern because of high costs and environmental issues. The present study evaluated the novel synergistic effect of urease and nitrification inhibitors such as ammonium thiosulfate (ATS) and 2-Chloro-6-(trichloromethyl)pyridine (Nitrapyrin) to reduce the urea hydrolysis in the soil of Faisalabad, Gujranwala, and Sheikhupura to manage the ammonia as well as N loss. Three different combinations such as A1, A2, and A3 of both inhibitors were prepared with different ratios of 1:1, 0.25:0.75, 0.75:0.25, respectively. Results showed that the minimum urea hydrolysis of about 2.41, 2.79, and 4.68 IU/g soil with A1 combination after 4^{th}-day observation with the rate of 0.50% concentration for Faisalabad, Gujranwala, and Sheikhupura, respectively. In addition, results showed the better urease activity at a pH value of 6.50, incubation time of 30 min, and temperature of 37 ℃ for all A1, A2, and A3 combinations with 0.50% concentration. Moreover, inhibitors treated urea showed the plant maximum height of 111, 101, and 101 cm, and root length of 15, 11, and 5 cm, number of tillers of 14, 16, and 19 per panicle, and number of spikes of 37, 21 and 38 per panicle with A1, A2, and A3 combination at 0.50% dose respectively in Faisalabad soil. Overall, it is concluded that 0.50% inhibitor concentration showed the much impressive urease inhibition results followed by 0.25 and 0.10%. However, the application of inhibitors was a good practice to reduce the N loss from soil.

Keywords: Urease inhibitor; ATS; Nitrapyrin; Ammonia volatilization; Nitrification inhibitor; Urea.

1. Introduction

With increasing the world population, the demand for quality life and food also increased. But during the past few years, the approaches for the efficient management of fertilizers for the production of required food quantity are facing persistent challenges in view of the world population especially in developing countries like Pakistan, India, and Bangladesh. In Pakistan, most farmers used nitrogen-based fertilizer due to the deficiency of nitrogen in soil [1, 2]. Nitrogen (N) is found abundantly in the atmosphere but in this form, it is inaccessible for plants. It became only available when primary producers such as plants are converted dinitrogen gas into ammonia (NH_3). Though, nitrogen-containing fertilizers gained special attention in this regard because nitrogen (N) is one of the vital and mandatory plant elements for crop development and growth. However, granular urea is one of the commonly used fertilizers in the agriculture section because it was economical and easy to produce, which is almost five times greater frequently used fertilizer than ammonium nitrate. During the past few decades, the utilization of urea has become very high due to their nitrogen-based fertilizer containing ability (which is 46% of world consumption), high foliage production, low corrosion capacity, and high-water solubility [3, 4]. Accumulated data revealed that urea is hydrolytically very stable and possesses a non-enzymatic half-life of about 3.6 years [5].

Naturally, urea is hydrolyzed by urease enzyme (urea aminohydrolase E.C.3.5.1.5) that is frequently found in several organisms including bacteria, plant, algae, fungi, and invertebrates, but a higher amount occurs in soil microorganisms [6]. Urease is a multi-subunit nickle containing metalloenzyme that is generally homo hexamers and each subunit contains an active site with two Ni^{2+} ions [7]. Urease has a vital role in nitrogen cycling in plants and improved the urea conversion rate about 10^{14} times in soil [8]. The emerging evidence revealed that the surface application of urea leads to its massive loss through several ways including leaching, immobilization, volatilization, and denitrification. However, after the surface application of

urea into soil, urea is rapidly hydrolyzed within 24-48 h by naturally occurring urease into ammonium (NH_4^+), carbonate (CO_3), and hydroxyl ions (OH-) [9]. NH_4^+ further hydrolyzed into nitrite (NO_2) by Nitrosomonas species and nitrite oxidized into nitrate (NO_3) by soil living organism such as nitrobacter bacteria (see Eqs. 1-3) [10]:

$$(NH_2)_2CO + 2H_2O \rightarrow (NH_4^+)_2CO_3 \quad 1)$$

$$(NH_4^+)_2CO_3 + 2H \rightarrow 2NH_4^+ + CO_2 + H_2O \quad 2)$$

$$NH_4^+ + OH \rightarrow NH_3 + H_2O \quad 3)$$

The excessive production of NH_3 caused toxicity for seed germination due to the escalation of soil pH [11]. However, the ammonium that produces nitrite becomes negatively charged and soluble in soil that is ultimately subjected to the accumulation adjacent to granular urea and soil leaching [12]. While NO_3 accumulation further increased the N loss through the production of powerful greenhouse gas such as N_2O by the nitri-or-denitrification process under wet conditions [13]. On the other hand, due to escalation of soil pH (alkaline condition) adjacent to granular urea through the removal of H^+ ions lead to the activation of volatilization process in the form of ammonia (NH_3) [14]. However, the continuous deposition of NH_3 into the atmosphere leads to environmental pollution by acidification as well as eutrophication [15]. A recent study reported that deposition of NH_3 largely depends on different soil parameters including organic matter, temperature, climate, pH, and soil texture [10, 16]. Globally, it is estimated that about 10% of applied N is lost through volatilization, but losses from individual fields could approach 60% under different conditions [17, 18].

However, rapid hydrolysis of urea by urease not only caused a quantitative loss of nitrogen (N) in the form of NH_3 but also increase, soil leaching, water pollution, and greenhouse gas emissions. Resultingly, the required amount quantity of N is decreased [17, 19, 20]. According to an estimation, only 33% of surface applied nitrogen-based fertilizer is used by plants worldwide, while the rest of all is destroyed by these processes [21]. However, the placement

of N is a critical factor to mitigate potential N losses. For example, a surface application followed by incorporation of the applied N reduces the risk of volatilization, whereas surface applications without incorporation increase the potential for N loss [22, 23]. Thus, the input of significant moisture, in the form of irrigation or rainfall after surface application could move the applied N down into the soil profile, thus reducing potential volatilization-induced N losses [24].

Therefore, these rapid hydrolyses and loss of N urged scientists to find out a solution to slow down the hydrolysis of urea not only to save the economic loss but also environmental as well as water pollution. Conventionally, different types of urease inhibitors like ammonium thiosulphate, sodium thiosulphate, thiourea, boric acid, hydroquinone, phenyl phosphorodiamidate, and n-butyl thiophosphoric triamide (NBPT) have been manufactured and applied into the soil. These inhibitors generally slowdown the urea hydrolysis and help in enhancing the absorption of urea in soil by irrigation and rain [25-27]. Urease inhibitors took the intention to gradually slow the hydrolysis of urea for a period of 7 to 14 days by suppressing the activity of urease. During this process, the surface applied urea could be moved into the soil profile effectively by lowering the attention of NH_4^+ on the soil surface [28]. Urease inhibitors (NBPT) have the potency to reduce ammonia volatilization and nitrite (NO_2) accumulation in soil and influenced the kinetic and thermodynamic behavior of urease in soil [29]. The efficiency of inhibitors mainly depends upon the temperature and pH of soil, and low concentration of inhibitor. Most of the inhibitors including NBPT are highly effective in neutral soil with a small range of organic matter [29, 30]. In addition to chemical inhibitors, some natural products such as phenolic compounds (methyl gallate, stilbenoids, and flavonoids) have the ability to suppress the urease efficiency [28].

In Pakistan, no significant work regarding urease inhibition potential under our local conditions has been reported. Therefore, it was planned to screen a combination of urease inhibitors

including ammonium thiosulfate (ATS) and 2-Chloro-6-(trichloromethyl) pyridine (Nitrapyrin) which would also be useful to reduce NO_3 leaching in soil and local climate condition. To the best of our knowledge, for the first time, the present study examined the inhibition activity of the collective effect of ammonium thiosulfate and 2-Chloro-6-(trichloromethyl)pyridine inhibitors in rice crops of three different districts of Pakistan including Faisalabad, Sheikhupura, and Gujranwala. Moreover, the present study discussed different factors' effects and kinetic assessment for a better understanding.

2. Materials and methods

2.1. Chemicals

Urea (≥99.5% pure; CH_4N_2O), 2-Chloro-6-(trichloromethyl) pyridine (≥98.0% pure; $C_6H_3Cl_4N$), sodium hydroxide (≥47.7-51% pure; NaOH), toluene (≥99.0% pure; C_7H_8), phenol (≥99.0% pure; C_6H_6O), ethanol (≥99.2% pure; C_2H_6O), sodium hypochlorite-(containing at least 0.9% active chlorine; NaOCl), ammonium sulfate (≥97.0% pure; $(NH_4)_2SO_4$) was obtained from Sigma-Aldrich (USA). Ammonium thiosulphate (≥95.0% pure; $(NH_4)_2S_2O_3$) was purchased from Uni-chem (USA). Citric acid (≥99.2% pure; $C_6H_8O_7$) was obtained from Riedel-deHaen. Methanol (≥99.8% pure; CH_3OH) was purchased from Chem-Lab, while acetone (≥99.5% pure; $CH3COCH_3$) was acquired from Merck (Germany).

2.2. Soil sampling and properties

The soil was collected from three different districts of Pakistan including Faisalabad, Sheikhupura, and Gujranwala. Soil samples were taken during the crop growth period to assess the nutrient status of the soil in which plants are actively taking up nutrients. Soils were collected at depth of about 20 cm. However, in some cases, especially those areas in which irrigation continuously effect, the sample was collected to a depth of 60-100 cm for monitoring nitrate (NO_3-N) leaching. Soil samples were poured into pots (6 cm) for each district. The

146 average temperature of soil was 20.5 ℃ with an annual rainfall of 1470 mm. The mean pH,
147 bulk density, soil total nitrogen, and soil organic matter were measured as 7.10, 1.25 g/cm^3, 1.2
148 g/kg, and 28.9 g/kg, respectively. While the mean ammonium and nitrate content was recorded
149 as 3.90 and 1.37 mg N/kg, respectively [31].
150 Rice plants were sowed in pots (27 m^2 area; 4.5 × 6 m) with soil samples of all three districts
151 including Faisalabad (FSD), Gujranwala (GUJ), and Sheikhupura (SHK). Each district has 10
152 pots in number and each pot contains 6 kg soil. Plants were irrigated at different intervals of
153 time in a week and placed in sunlight to grow better.

154 2.3. Application of urease inhibitors

155 Urea and inhibitors are weighed and mixed with each other to make different
156 combinations and applied in a solution form. A combination of two inhibitors including
157 ammonium thiosulphate (ATS) and 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin) were
158 prepared with different ratio of 1:1, 0.25:0.75, and 0.75:0.25, which named as A1, A2,
159 and A3, respectively. 100 mL solution of each combination was prepared by dissolving
160 0.21, 0.51, and 0.90 g of inhibitors into 2.34 g of a fixed quantity of urea with the
161 addition of water to get the desired volume to make 0.10, 0.25, and 0.50% combinations,
162 respectively. After sowing rice crop into the pots, these prepared combinations of urease
163 inhibitors such as 0.10, 0.25, and 0.50% were applied in the soil of all three districts. Alone
164 urea was used as a control to compare the results. After the application of urease inhibitor with
165 urea, the readings were taken with the difference of one day in a consecutive manner up to 36
166 days to check the hydrolysis of urea [32].

167 2.4. Collection of cultivated soil samples for analysis

168 Soil samples of cultivated pots were collected at different intervals of days from 1st to 37th to
169 examine the urease enzyme activity. Cultivated soil samples were put in plastic bags and tagged
170 properly. Then depending upon the subsequent analysis samples and avoid any type of

contamination, samples were kept under cool conditions until further analysis. The fresh soil samples received in the laboratory were dried in wooden or card trays in the air. These trays were numbered, and care was taken to maintain the identity of each sample at all stages of preparation. After that sample containing trays were dried placed in racks in a hot air cabinet at 35 °C and humidity of 30-60%. In general, excessive oven-drying of the soil affects the availability of most of the nutrients present in the sample, therefore samples were dried in hot air instead of oven to maintain the total N content, $NH_4^{+,}$ and NO_3 content in the soil samples. After drying, the samples of all three districts were ground into fine powdered through a pestle and mortar. After grinding, the soil samples were screened through a 2 mm sieve and about 20 g of powdered samples of each district were stored in labeled plastic bags for future analysis.

2.5. Determination of urease potential assay

The concentration of ammonia (NH_3) in the cultivated soil after degradation of urea was determined through the calorimetric method with little modification Askin and Kilzikaya [33]. First, different solutions like citrate buffer, 10% w/v urea, 12.5% w/v solution of sodium phenolate, and sodium hypochlorite solution were prepared. Citrate buffer (pH 6.7) was prepared by dissolve 24.09 g (M=0.0819) sodium citrate and 3.47 g citric acid (M= 0.0181) in 800 mL distilled water. Solution was well shaken for 5 min and more distilled water was added to make the volume of 1000 mL and adjust the pH of solution 6.7. 10% w/v urea was prepared by dissolve 10 g of urea in 100 mL of d.H_2O. Similarly, 12.5% w/v solution of sodium phenolate was prepared by make solution (a) and (b). Solution (a) was prepared by dissolve 62.5 g of phenol in 1 mL ethanol, 2 mL methanol, and 18.5 mL acetone, and dilute to 100 mL with the addition of d.H_2O. Then, Dissolve 27 g. NaOH in distilled water and makeup to 100 mL. Solution (b) was made by dissolve 27 g NaOH in d.H_2O to adjust volume up to 100 mL. Just before use, the 20 mL mixture

of solutions (a) and (b) were mixed and made volume up to 100 mL with d.H_2O. The prepared solutions were stored in a refrigerator for further analysis.

The commercial sodium hypochlorite solution was a dilute solution with 50 mL d.H_2O so that it contains 0.9% active chlorine. The solution was stable, allow to stand for 20 min until the maximum color was obtained. The optical density was measured within 60 min. According to an estimation, 1 mg of enzyme contains 45 IU enzyme units (0.01 g/1 mL) or 1 mg/1000. So, different enzyme standard curves were prepared by dissolving the enzyme stock solution into citrate buffer by following the **Table 1**.

2.5.1. Urease inhibition potential of ATS and nitrapyrin

Urease activity was determined by using the method of Askin and Kizikaya [33] with few modifications. Different enzyme concentrations including 1, 2, 4, 6, 8, and 10 IU were added into a specific amount of citrate buffer to each tube to make the volume 1 mL. Then 150 µL toluene was to each tube and incubated the sample for 15 min at 37 ℃. Then 1mL urea and 2 mL citrate buffer were poured into each test tube and again incubated the samples for 3 h at 37 ℃. After the incubation time, the samples were filtered using Whatman No. 1 filter paper and took 1 mL filtrate from each tube and transfer to other tubes and added 100 µL H_2SO_4 to each tube and placed on ice for 10 min. After 10 min, the samples were removed from the ice, and 4 mL sodium phenolate and 3 mL sodium hypochlorite solutions were added to each tube and incubated for 15 min at 37 ℃. Absorbance was measured at 580 nm using a UV-Vis spectrophotometer (V-730). The urease hydrolysis activity was calculated from the standard curve of N corresponding to the difference in optical density between the sample and the reagent blank [32].

2.6. Kinetic potential assessment of ATS and nitrapyrin

After measuring urease inhibitor potential, different urease kinetic parameters including temperature, pH, and incubation time were calculated. The colorimetric procedure was used to

evaluate the effect of temperature by changing the temperature of the incubation period as 10, 20, 30, 40, and 50 ^{o}C for soil samples. The effect of pH on urease activity in soil samples was evaluated by changing pH as 3.50, 4.50, 5.50, 6.50, and 7.50 [34]. Similarly, the effect of incubation time was observed by changing incubation time from 5, 10, 15, 20, and 30 min by adopting the method of Khan et al. [35] with little modifications.

2.7. Agronomical parameters measurement

Different agronomical parameters including plant height, number of tillers, number of spikes, number of grains, and root length of rice were examined for both controls as well as inhibitors treated samples [36].

2.8. Statistical analysis

The results were analyzed by analysis of two-way ANOVA by using IMB SPSS software (Version 26.00; IBM Corp., USA). In addition, the mean SD was calculated for all the obtained results of kinetic parameters and urease activity [37].

3. Results and discussion

The whole study plan was alienated into two phases. In the 1st phase, the effect of combined inhibitors (2-Chloro-6-(trichloromethyl)pyridine + ammonium thiosulphate) was evaluated on urease activity in rice crops of selected three districts (Faisalabad, Gujranwala, and Sheikhupura). While, in the 2nd phase kinetics parameters, including the effect of pH, temperature, and incubation time on urease activity were investigated. Different concentrations of inhibitors such as 0.10, 0.25, and 0.50% were blended with urea granules and were applied to rice crops. In addition, three different combinations such as A1, A2, and A3 of both inhibitors including 2-Chloro-6-trichloromethyl pyridine (nitrapyrin) and ammonium thiosulphate (ATS) were prepared with different ratio of inhibitors like 1:1, 0.25:0.75, 0.75:0.25, respectively.

Results showed that 0.50% concentrations of inhibitors with a 1:1 ratio showed better inhibition, the detailed results are presented in the following sections.

3.1. Determination of urease inhibitors activity

3.1.1. Enzyme inhibition activity of A1 combination

Fig. 1 (a,b,c) showed the urease inhibition results of A1 combination (ammonium thiosulphate + 2-Chloro-6-(trichloromethyl)pyridine, 1:1) for all three districts (Faisalabad, Gujranwala, and Sheikhupura) of Pakistan. The inhibition activity of inhibitors was assessed continuously for up to 37 days until complete hydrolysis of urea for all three districts. Results showed the urease activity of about 29.54, 26.62, and 29.54 IU/g soil for control of Faisalabad, Gujranwala, and Sheikhupura soil, while the activity of the inhibitor was noted as 6.07, 8.53 and 7.07 IU/g soil for Faisalabad soil, 18.69, 22.22, 9.88 IU/g soil for Gujranwala, and 18.79, 18.71, 17.88 IU/g soil for Sheikhupura soil with 0.10, 0.25 and 0.50% concentration respectively after 1^{st}-day analysis. 1^{st}-day results exposed the control (alone urea) showed the greater enzyme activity as compared to A1 combination of inhibitor for all three districts, which indicated that A1 combination of inhibitors significantly reduces the hydrolysis of urea, especially with 0.50% concentration followed by 0.10 and 0.25% concentration.

Results also exposed that the minimum urea hydrolysis of about 2.41, 2.79, and 4.68 IU/g soil was observed after 4^{th}-day incubation, with the rate of 0.50% for Faisalabad, Gujranwala, and Sheikhupura, respectively. While the control showed 25.43, 19.13, and 25.87 IU/g soil inhibition for the same districts, which indicated that A1 combination presented the minimum urea hydrolysis results as compared to control for all three districts. Hydrolysis of urea decreased as the incubation period increased. The average reduction in urea hydrolysis was observed between 1-15^{th} days, after that urease enzyme slow downed its activity. From 14^{th} to 37^{th} days, no significant ($P > 0.05$) results were obtained because the activity of urease in soil

267 treated samples was restored due to inhibitors' potential losses. However, control (untreated)
268 disclosed a high amount of urea hydrolysis as compared to 0.50% concentration of inhibitors
269 in all three districts treated soil from day 1^{st} to 15^{th}.

270 Our findings are also in correlation with the results of Liu et al. [38], who revealed that the
271 synergistic effect of urease and nitrification inhibitor (DMPP and NBPT) showed the optimum
272 urea hydrolysis of 4.57 mg N/kg d at acidic pH (5.18) and 9.39 mg N/kg d at alkaline pH (7.83).
273 Dawar et al. [39] stated that the application of agrotain treated urea showed a significant
274 reduction of urea hydrolysis within the first 7 days of application as compared to control, which
275 showed the almost complete hydrolysis of urea within the first 2 days of application. It is
276 estimated that the utilization of nitrification (nitrapyrin) and urease inhibitors could
277 significantly reduce the annual reduction of about 15-50% N_2O emission in rice and wheat
278 field, followed by more than 50% NH_3 volatilization reduction [31]. Ni et al. [40] reported that
279 the combination of urease and nitrification inhibitor (DCD and 2-NPT) significantly reduced
280 the production of 98.12 mg N/m^2 ammonia within 0-19 days observation.

281 Overall, it is concluded that 0.50% inhibitor concentration showed the much impressive urease
282 inhibition results followed by 0.25 and 0.10%. However, the application of inhibitors was a
283 good practice to reduce the N loss from soil.

284 **3.1.2. Effect of combination A2 on urease activity in rice cultivated soil**

285 **Fig. 2 (a,b,c)** presented the results of A2 combination of inhibitors (2-Chloro-6-
286 (trichloromethyl)pyridine + ammonium thiosulfate, 0.25:0.75) against rice cultivated soil of
287 Faisalabad, Gujranwala, and Sheikhupura. Based on results, it was observed that on the 1^{st} day
288 the urease activity with A2 inhibitor combination was found as 22.56, 18.87, and 19.66 IU/g
289 soil for Faisalabad cultivated soil, 21.69, 29.49, and 19.39 IU/g soil for Gujranwala, and 27.94,
290 18.47, and 22.64 IU/g soil for Sheikhupura soil samples with 0.10,0. 25, and 0.50% A2

inhibitors concentration, respectively. While the control showed 29.54, 26.62, and 29.54 IU/g soil enzyme activity for the same districts. Results showed the minimum enzyme activity of about 5.65, 5.54, and 5.53 IU/g soil for Gujranwala cultivated soil with 0.10, 0.25, and 0.50% A2 inhibitors concentration even after 3rd day of observation, while the control showed 27.63 IU/g soil enzyme activity. But in the case of Faisalabad cultivated soil, the minimum enzyme activity of about 5.89, 5.33, and 3.68 IU/g soil was noted on the 12th day of observation for 0.10, 0.25, and 0.50% concentration, respectively. While the control exhibited 25.21 IU/g soil enzyme activity. However, the difference in the days of inhibition was due to the difference of region from where soil samples were taken. The by-products of thiosulfate after oxidation are more inhibitory than thiosulfate itself, as little nitrification occurred from 12 to 26 days in treatments receiving soil samples. The addition of 1 mmol/S kg^{-1} (32 µg/g^{-1} thiosulfate-S) resulted in 60% of inhibition after 28 days [41].

Ammonium thiosulfate (ATS) significantly retarded the urea hydrolysis in the soil for 4-6 days when applied rates as high as 25,00 to 5,000 µg/g. After the 10th day of application ATS loss their inhibition potential due increase in substrate concentration and structure confirmation [42]. Yang and co-workers also reported that the synergistic effect of Azolla and urease inhibitor significantly reduced the NH_3 emission by about 61.1-63.6% in the rice field [43]. Similarly, Yusop et al. [44] demonstrated that the synergistic effect of nitrification and acid-based urease inhibitor (DMPP/Cu/Zn) significantly reduced the ammonia emission of 221.73 and 242.41 mg/kg for Selangor and Cempaka soil respectively.

Based on results, it is concluded that the minimum enzyme inhibition activity was observed from day 1st to 12th with A2 combination of inhibitors for all three districts with 0.50% inhibitor concentration followed by 0.25 and 0.10%. From the 17th-day urease activity in inhibitor-treated samples was gradually increase as compared to control till the 37th days. It was also observed that the combination of nitrification and urease inhibitor (A2) showed better results

316 regarding the reduction of N loss in the form of ammonia as well as the emission of NO_2. The
317 highly significant ($p<0.05$) results were recorded from 1-30th days when 0.50% inhibitor dose
318 was applied.

319 **3.1.3. Enzyme inhibition activity of A3 combination**

320 Nitrogen fertilizer plays an important role in increasing crop output. According to the Food and
321 Agricultural Organization of the United Nations (FAOUN), around 35–55% of production
322 increase was credited to N fertilizer [45]. Therefore, the use of various types of N fertilizer is
323 common agricultural practice for increasing crop yields all over the world. Extreme use of N
324 fertilizers results in ammonia volatilization which causes toxicity in the environment [46].
325 However, the combination of nitrification and urease inhibitor could significantly reduce the
326 N loss and improve the environment. **Fig. 3 (a, b, c)** represented the results of A3 inhibitor
327 combination (2-Chloro-6-(trichloromethyl)pyridine + ammonium thiosulfate, 0.75:0.25) for all
328 three districts with 0.10, 0.25, and 0.50% concentration. Based on results, it was noted that the
329 urease activity on the 1st day in control was 29.54, 26.62, and 29.54 IU/g soil for Faisalabad,
330 Gujranwala, and Sheikhupura respectively, while the inhibitors applied soil samples showed
331 32.25, 24.01, and 18.94 IU/g soil activity for Faisalabad cultivated soil, 13.91, 15.27, and 10.47
332 IU/g soil for Gujranwala, and 18.84, 16.48, and 14.09 IU/g soil for Sheikhupura with 0.10,
333 0.25, and 0.50% concentration.

334 The hydrolysis rate of urea increased in control as compared to inhibitors treated samples with
335 an increase in incubation time, which reported that A3 combination presented the better
336 reduction of urea hydrolysis as compared to control (urea alone). However, the minimum urea
337 hydrolysis of about 1.58 IU/g soil was observed for Faisalabad cultivated soil after 10th day of
338 observation, while control (urea alone) value was noted 18.24 IU/g soil, which showed that A3
339 combination presented much exceptional inhibition results than control. Similarly, the
340 minimum urea hydrolysis of about 2.54 and 2.13 IU/g soil was observed after 4th and 6th day

of observation, with the rate of 0.50% for Gujranwala and Sheikhupura, respectively. While the control showed 19.13, and 27.65 IU/g soil inhibition for the same districts, which indicated that A3 combination presented much more prime urea hydrolysis results as compared to control for all three districts. Generally, when soil was not treated with ammonium thiosulfate (ATS), nitrate rapidly increased throughout the two weeks of incubation period; however, using ATS delayed the initial nitrification rate and significantly reduced NO^{-3} formation. It was reported that ATS has the ability to delay nitrate formation [47].

ATS (ammonium thiosulphate) had retarded the hydrolysis of urea when added in a higher amount. It was observed that in soil, ATS converted into active tetrathionate to reduce the activity of urease. Tetrathionate is also required in a higher amount (2500-5000 µg/mL) to inhibit the activity of urease [48]. Soares et al. [18] reported that DCD (10%) treated urea showed that 80% N recovery at a pH value of 6.8, while NBPT treated urea presented an N recovery of 70%. Overall, it is concluded that 0.50% inhibitor concentration showed better results for all three districts. It means that based on results, it could be observed that the 10% urea could be saved in the future with the application of 2-Chloro-6-(trichloromethyl)pyridine and ammonium thiosulfate in combined form. This is not only a big innovation regarding the save of urea and N loss but also for environmental cleanliness.

3.2. Assessment of kinetic parameters

After the urease inhibition study, the effect of different kinetics parameters including pH, temperature, incubation time, and substrate concentration were analyzed for a better understanding of ammonia reduction. Effect of all parameters was evaluated on urease activity in the presence of urease and nitrification inhibitors like 2-Chloro-6-(trichloromethyl)pyridine ammonium thiosulphate in three different combinations as A1 (1:1), A2 (0.25:0.75), and A3 (0.75:0.25). Based on urease inhibition activity, only one concentration (0.50%) was selected for further analysis of kinetic assessment due to its better urea hydrolysis results.

3.2.1. Effect of pH on urease activity

The high urea concentrations caused a considerable pH change in the soil. The effect of pH on urease activity in soils was studied in buffered urea solutions. Urea hydrolysis consumes two protons (H^+) for each mole of urea hydrolyzed. This reaction tends to increase the pH around urea-granules, and thus increases the rate of urea hydrolysis [49]. **Fig. 4 (a,b,c)** presented the results of the effect of pH on A1, A2, and A3 inhibitor combinations against all three districts (Faisalabad, Gujranwala, and Sheikhupura). The enzyme inhibition activity was processed at five different pH values including 3.50, 4.50, 5.50, 6.50, and 7.50 for all three districts. Results showed the maximum enzyme activity was observed as 17.78, 23.14, and 26.28 IU/g soil for A1, A2, A3 inhibitor combinations for Faisalabad at a pH value of 6.5 with 0.50% concentration, while the control (alone urea) showed 38.53 IU/g soil enzyme activity. Similarly, the maximum inhibition of about 17.75, 18.76, and 23.34 IU/g soil was observed for Gujranwala, and 16.98, 18.92, and 30.17 IU/g soil for Sheikhupura cultivated soil at pH value of 6.5 for A1, A2, and A3 combination respectively with 0.50% concentration, while the control was observed as 41.79 and 42.79 IU/g soil for same districts and same pH.

Similarly, the minimum enzyme activity of about 1.71, 6.82, and 5.66 IU/g soil was noted for Faisalabad cultivated soil, 7.21, 6.07, and 3.41 IU/g soil for Gujranwala, 1.31, 2.17, and 3.03 IU/g soil for Sheikhupura cultivated soil with A1, A2, and A3 combinations respectively, at pH value of 3.5 and 0.50% concentration. While the control presented 15.12, 12.25, and 9.93 IU/g soil inhibition value with the same reaction condition, which indicated. When soil pH increases to 6.5, the hydrolysis rate of urea increases, urease (optimum pH 6.67) showed maximum activity in control while applied inhibitors were found less active. At pH 7.5, the activity of urease decreased due to little conformational changes in their structure. Soares et al. [18] revealed that 5% NBPT and DCD treated urea showed 28% reduction of NH_3 and 74% of N recovery, while 10% NBPT and DCD treated urea disclosed 33 and 77% of NH_3 reduction

and N recovery respectively at pH value of 6.8 between 7-9 days of observation. Longo et al. [50] measured the rate of urea hydrolysis under laboratory conditions using a range of soil pH from 2.2 to 8.0. They found that as the soil pH increases the rate of urea hydrolysis increases almost exponentially. In addition, they found that the highest rate of urea hydrolysis was at pH 8.0.

Overall, it is concluded that A3 combination showed better enzymatic activity results in all three districts (Faisalabad, Gujranwala, and Sheikhupura) soil at a pH value of 6.5. So, based on results, it was observed that 6.50-6.67 is the optimum pH for enzymatic action of urease, while urease showed the minimum urea inhibition at a pH value of 3.5. However, the effect of pH on urease activity could be clarified in terms of the changes in the state of ionization of the enzyme.

3.2.2. Effect of temperature on urease activity

The effect of temperature on urea hydrolysis rates in soil was studied at different temperatures (10, 20, 30, 40, and 50 ℃) by keeping the other factors like urea concentration, pH, and incubation time constant for all three districts soil. To reduce the activity of urease inhibitors like 2-Chloro-6-(trichloromethyl)pyridine and ammonium thiosulphate were used in a combined form with different ratio of 1:1 (A1), 0.25:75 (A2), and 0.75:0.25 (A3). Combined inhibitors were used with 0.50% concentration. **Fig. 5 (a.b,c)** showed the results of temperature effect on the enzymatic activity of A1, A2, and A3 inhibitor combinations for all three districts. Results showed that the maximum enzymatic activity of urease was observed as 18.68, 19.23, and 23.03 IU/g soil for Faisalabad, 15.48, 18.92, and 15.12 IU/g soil for Gujranwala, and 18.61, 27.91, and 21.93 IU/g soil for Sheikhupura with A1, A2, and A3 inhibitor combination at 40 ℃. While the control (alone urea) showed the maximum activity of 44.50 IU/g soil for the same districts at the same condition with 0.50% concentration.

Similarly, the minimum enzymatic activity of urease was noted as 2.11, 6.51, and 1.55 IU/g soil for Faisalabad, 8.53, 4.81, and 8.53 IU/g soil for Gujranwala, and 6.75, 7.91, and 9.31 IU/g soil for Sheikhupura with A1, A2, and A3 inhibitor combination at 10 ℃. This indicated that with increasing the temperature the enzyme activity also increased towards the hydrolysis of urea. Overall, it is concluded that 37-40 ℃ is the optimum temperature for the maximum enzymatic inhibition of urease. It was also noted that at 50 ℃, the enzymatic activity suddenly decreased for all three districts. As temperature range increase enzyme was deactivated and did not stabilize their 3D conformation. High temperatures for long periods led to a decrease in crop yield [51]. Sha and coworkers demonstrated that NBPT urease inhibitor effectively reduced the ammonia loss of 31.6% at 30 ℃ that was better than lower temperature (20 ℃) [52]. Ding et al. [53] reported that temporal variations of surface soil moisture (WFPS) during maize growing season changed from 75.41 to 20.21% with increasing the temperature ranges from 15 to 30 ℃ for urease and nitrification inhibitor working (NBPT + DCD).

The results also revealed that the inhibitor combinations showed less activity as compared to control, which means inhibitors have the ability to slow down the hydrolysis of urea to minimize the N loss. It was known that the temperature needed to deactivate enzyme activity in soils is about 10 °C. This has been generally attributed to the immobilization of soil enzymes on soil colloids and cell debris [54].

3.2.3. Effect of incubation time on urease activity

Urease present in the soil breaks the urea into ammonia and carbon dioxide. The maximum activity of the enzyme depends on the incubation periods [55]. Enzyme activity was determined at different incubation periods as 5, 10-, 15-, 20-, and 30-min. Effect of different incubation periods on urease activity was determined by using combined inhibitors A1, A2, A3 in the ratio of 1:1, 0.25:0.75, and 0.75:0.25 with 0.50% concentration against Faisalabad, Gujranwala, and Sheikhupura cultivated soil (see **Fig. 6**). At 5 min of the incubation period, the activity of urease

in control was recorded as 23.03, 26.28, and 26.82 IU/g soil for Faisalabad, Gujranwala, and Sheikhupura cultivated soil respectively. While the soil treated samples with A1, A2, and A3 inhibitors showed the urease activity of 6.75, 3.83, and 7.06 IU/g soil for Faisalabad, 10.78, 16.51, and 7.06 IU/g soil for Gujranwala, and 11.07, 9.01, and 9.26 IU/g soil for Sheikhupura soil respectively at 5 min incubation time and 0.50% concentration (see **Fig. 6**).

The highest urease activity of about 21.09, 23.94, and 18.21 IU/g soil was noted for Gujranwala soil as compared to Faisalabad and Sheikhupura cultivated soil samples with A1, A2, and A3 combinations at 30 min incubation time and 0.50% concentration. Results reported that with increasing the incubation time the urease activity also increased. However, the minimum hydrolysis of urea was observed at 5 min incubation time. The activity in control (untreated) samples was recorded high as compared to inhibitor-treated samples, because of the production of more urease from plants and micro-organism due to substrate stimulation. Sha et al. [52] stated that 336 h incubation of urease inhibitor (NBPT) significantly reduced the cumulative HN_3 loss from soil as compared to control (alone urea). They also performed an experiment with mixing the urea with DAP fertilizer and amended with inhibitor, they found that the coating of urea with DAP reduced the overall efficiency of inhibitor after 84 h incubation time.

3.3. Mechanism of action of urease

Urease is a Ni-containing enzyme, so to understand the proper mechanism of urease with an amazing 10^{14} rate, it is necessary to examine the characteristics of urease-Ni ion [56]. In 2019, Mazzei and co-workers [57] reported that urease has a binuclear active site, and two pseudo-octahedral paramagnetic nickel ions (Ni^{2+}), which are separated by the carboxylate group of the carboxylated $Lys^{\alpha 217}$ residue by 3.5 Å. Once the urea arrives into the active site cavity of urease, it opens the conformation in such direction to have the suitable fit of a substrate, and caused the replacing of a substrate with three water hydroxide molecules at active site that change its dimension and molecular shape, and resulting in the formation of hydrogen bonding

which provides a tight anchor to stabilize the interactions between the active site of urease and orient the urea in the catalytic activity (**Fig. 7**) [58]. Urea forms a bridge of two metal ions in such a way that one of its amino group binds to Penta-coordinated Ni (I) ion, while hexa-coordinated Ni (II) binds to its other amino group with carboxyl oxygen which is stabilized by hydrogen. This bidentate ligation causes the conformation to change back from an open to a closed position, and this arrangement stimulates the inert urea atom by the nucleophilic attack via polarizing the C=O and C-NH_2 bonds of the urea molecule. To eliminate the NH_2 group from the C-N bond in urea, a proton is required which could support the carboxylate group of $Asp^{\alpha 323}$ and resulting in the reduction of pKa value by the formation of C=O bond (**Fig. 7**). Finally, the C-N interaction is broken, and urea collapses into NH_3 and a nickel-containing carbamate, and ammonia is released from the active site and mobile flexible flap again open and ready for another cycle [59, 60]. The three-dimensional structure of the urease active site and interaction of (AST + Nitropyrin) inhibitors with urease are presented in **Fig. 8**.

3.4. Assessment of agronomical parameters

The different agronomical parameters such as plant height, root length, number of tillers, and number of spikes were analyzed for all three districts cultivated soil with A1, A2, and A3 inhibitor combinations. Three different inhibitor concentrations including 0.10, 0.25, and 0.50% were used for agronomical parameters.

3.4.1. Plants height and root length

Table 2 showed the results of plant height and root length for Faisalabad, Gujranwala, and Sheikhupura with different inhibitor combinations and concentrations. In Faisalabad soil, plants height and root length in control were recorded as 67 and 71 cm as compared to inhibitors treated samples which showed the plant height of 111, 101, and 101 cm, and root length of 15, 11, and 5 cm when treated with A1, A2, and A3 at 0.50% dose respectively. In Gujranwala soil, plants height in tested samples were 81, 96, and 80 cm at 0.50% concentration of A1, A2

and A3 treated soil as compared to control which presented was only 71 cm. While the maximum root length was observed as 11, 13, and 12 cm for Gujranwala soil with A1, A2, and A3 inhibitor combination respectively against a control of 9 cm at 0.50% concentration.

However, in Sheikhupura treated soil, plant height in tested samples was found as 98, 99, and 101 cm at 0.50% concentration against control of 79 cm. While root length in tested samples was observed as 11, 17, and 18 cm at 50% concentration against a control of 9 cm (see **Table 2)**. Overall, based on results, it is concluded that 0.50% inhibitor concentration showed impressive findings regarding plant and root length as compared to control (alone urea). So, it is stated that the practice of urease inhibitors not only helps in the reduction of N loss and clean climate but helped in the improvement of plant health and growth.

3.4.2. Number of tillers and spikes

Table 2 showed the results of a number of tillers and spikes for Faisalabad, Gujranwala, and Sheikhupura with different inhibitor combinations and concentrations Number of tillers was count high in Faisalabad treated samples such as 14, 16, and 19 per panicle as compared to control that only showed 10, when A1 and A2, and A3 inhibitors combinations used at 0.50% concentration. However, the maximum number of tilers were recorded as 9, 10, and 8 per panicle for Gujranwala, and 11, 10, and 10 per panicle for Sheikhupura soil with A1 and A2, and A3 inhibitors combinations against control of 6 and 9 per panicle respectively at 0.50% concentration. Results showed that the overall, number of tillers was higher in inhibitor-treated soil samples as compared to control.

A number of spikes from each district (Faisalabad, Gujranwala, and Sheikhupura) were found lower in control such as 7, 13, and 6 per panicle respectively. While in inhibitors treated samples number of spikes was counted as high when A1, A2, and A3 inhibitors applied. The maximum number of spikes in Faisalabad was 38 at 0.50% concentration (A3). While the

highest number of spikes in Gujranwala and Sheikhupura treated soil were recorded as 47 and 8 at 25 and 0.50% urease inhibitory dose respectively when A3 inhibitor was used (see **Table 2)**.

Yang et al. [43] reported that the utilization of *Azolla* in combination with urease inhibitor significantly increased the spikelet number per panicle by about 15.91%, panicle number 4.11%, and total biomass as 22.91%. Li and co-workers showed that the combination of CRU and SWD presented the better grain yield of about 9 Ib/ha with early rice season and 10 Ib/ha in late rice season as compared to control that only showed 7 and 8 t/ha yield respectively [61]. Li et al. [61] reported that the combination of a CRU and SWD significantly showed the tiller number of about 147 per/m^2 in early rice season and 154 per/m^2 in late rice season. Similarly, Ding et al. [53] revealed that the application of NBPT, DCD, NBPT + DCD effectively improved the maize grain yield of about 15.12, 14.21, and 8.42%. Galindo and coworkers stated that the 100 and 150 kg N/ha application of *Azospirillum brasilense* in combination with NBPT treated urea significantly increased the grain yield of 19.6 and 18.8% respectively [62].

Overall, it was found that a number of plant tillers and spikes, plants height, and root length were higher as compared to control. Data produced from the research showed a smaller number of spikes in Sheikhupura treated soil due to environmental changes and late rice plantation.

Conclusion

A major portion of urea applied for enhancing crop yield and quality is lost every year in the form of NH_3, N_2O, and NO_3 and contaminating air and water. These losses increase the economic burdens on farmers. The reduction in N losses in the form of ammonia volatilization into the air and NO_3 leaching in water are necessary for the safety of the environment throughout the world. The present study objective was to examine the combined effect of urease and nitrification inhibitors on rice crop of Faisalabad, Gujranwala, and Sheikhupura

(Pakistan) to minimize the urea hydrolysis to reduce ammonia emission. It was also observed that the combination of nitrification and urease inhibitor (A1 and A2) showed better results regarding the reduction of N loss in the form of ammonia as well as emission of NO_2. The highly significant ($p<0.05$) results were recorded from 1-14th days when 0.50% inhibitor dose was applied. Based on results, it was detected that 6.50-6.67, 35-37 ℃, and 30 min were the optimum pH, temperature, and incubation time for enzymatic action of urease in all three districts (Faisalabad, Gujranwala, and Sheikhupura) soil with A1 inhibitor combination. In addition, Faisalabad soil showed better plant height, root length, number of tillers, and spikes as compared to other districts due to rainfall and irrigation. However, it was well documented that 25-40% of the urea is lost in the environment and causing air and water pollution. In addition to the financial impact of (AST + Nitrapyrin) by saving >10% urea, this strategy could also be adopted to clean almost 60-100% of environmental and water pollution caused by urea losses. However, the present findings provide a scientific basic recommendation on how to apply urease and nitrification inhibitors for rice crop production.

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CRediT authorship contribution statement

A. Hazafa, **K. Rahman**: Conceived the presented data, Writing - original draft, Software, & Supervision. **A. Hussain, Z. Jabeen, N. Afshan, M. Naeem**: Developed the theory, Formal analysis, & Investigation. **H. Rafiq, Z. Huma**: Software.

Compliance with ethical standards

Conflict of interest

The authors declared no conflict of interest.

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Informed consent

For this type of study informed consent is not required.

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List of Tables

Table 1. The parametrs for the preparation of different urease enzyme solutions from a stock solution to analyse the enzyme standard curve.

Enzyme concentration (IU)	**Mixture**	
	Enzyme solution (µL)	**Citrate buffer (µL)**
1	22.22	977.78
2	44.44	959.56
3	66.66	933.33
4	88.88	911.12
5	111.11	889.91
6	133.32	866.68
8	177.77	822.24
10	222.20	777.78

776

777 **Table 2.** The assesment of different agronomical parameters of rice crop of all thee districts
778 with A1, A2, and A3 inhibitor combination.

Inhibitor comninat ion	**Treatm ent**	**Plant length (cm)**			**Root length (cm)**			**No. of tilers (per panicle)**			**No. of spikes (per panicle)**		
		FS D	**GU J**	**SK P**	**FS D**	**GU J**	**SK P**	**FS D**	**GU J**	**SK P**	**FS D**	**GU J**	**SK P**
	Control	67	71	79	10	9	9	11	6	9	7	13	8
A1	0.10%	77	75	80	13	8	8	14	5	11	12	25	2
	0.25%	88	78	86	12	10	7	16	8	10	17	20	3
	0.50%	111	81	98	15	11	11	19	9	11	37	29	7
A2	0.10%	97	53	80	13	8	11	11	6	9	27	44	4
	0.25%	88	104	94	14	13	6	16	9	11	9	41	5
	0.50%	101	96	99	11	13	10	17	10	10	21	38	8
A3	0.10%	77	84	99	7	6	9	15	6	10	15	30	3
	0.25%	85	76	108	16	16	16	8	5	7	14	47	4
	0.50%	101	80	101	5	12	13	18	8	10	38	15	8

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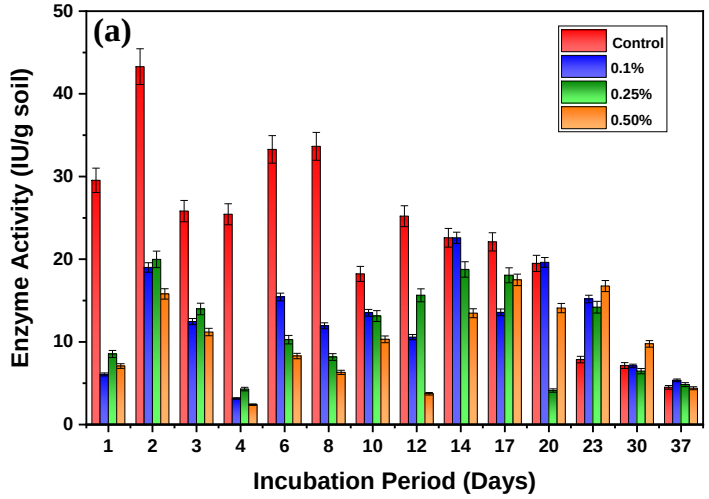
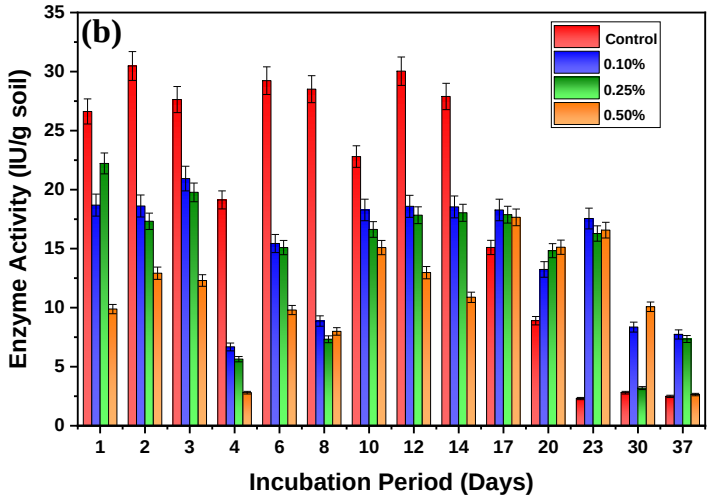
List of Figures





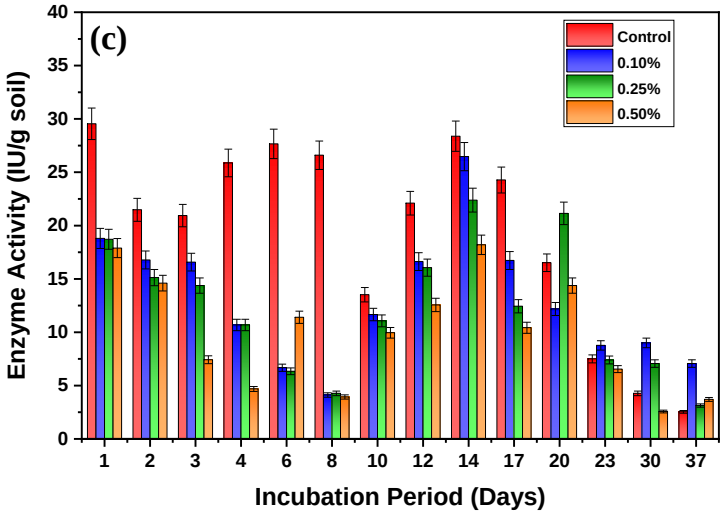
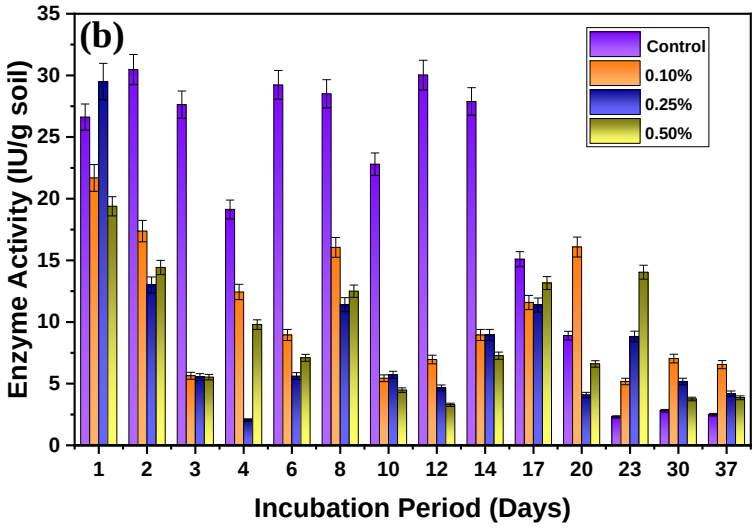


Fig. 1. The urease activity of **(a)** Faisalabad, **(b)** Gujranwala, and **(c)** Sheikhupura cultivated soil with A1 inhibitor combinavtion.



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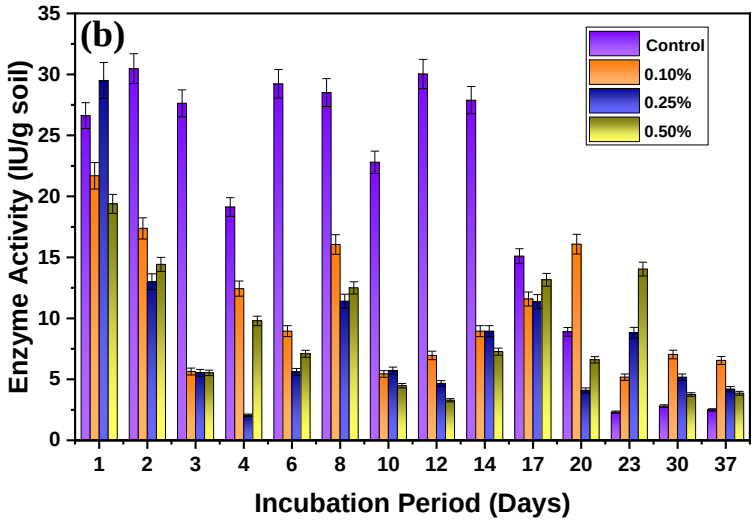

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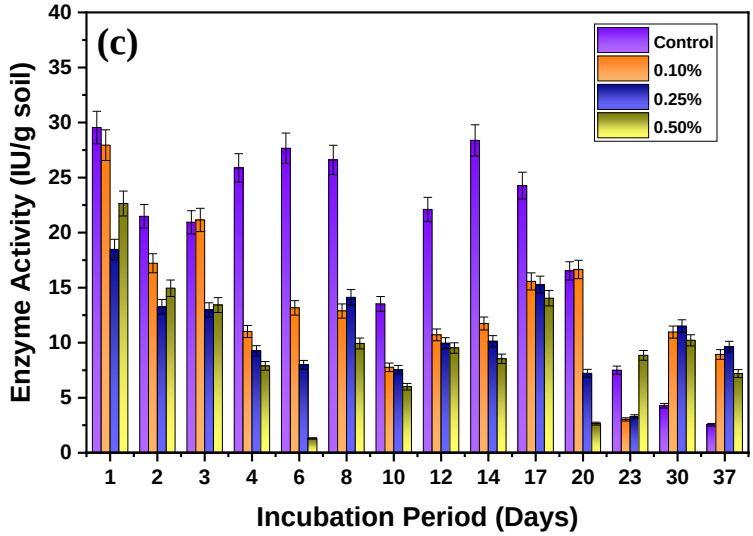

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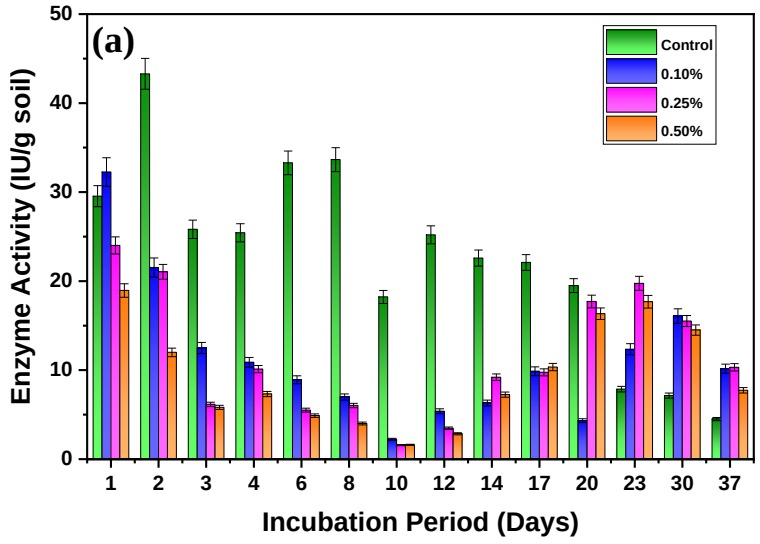
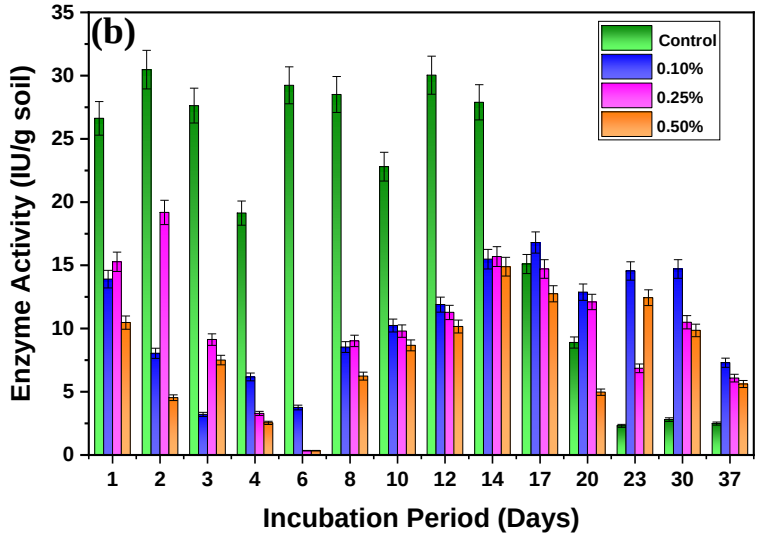
791 **Fig. 2.** The urease activity of **(a)** Faisalabad, **(b)** Gujranwala, and **(c)** Sheikhupura cultivated
792 soil with A2 inhibitor combination at different concentrations.





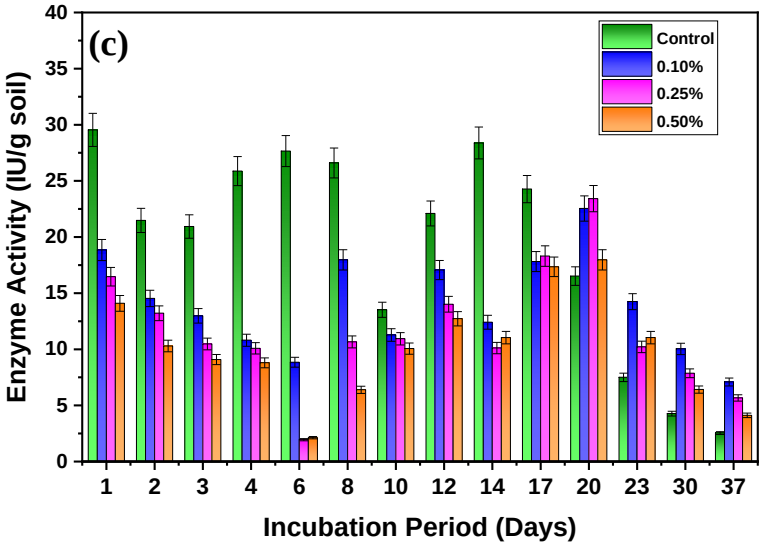
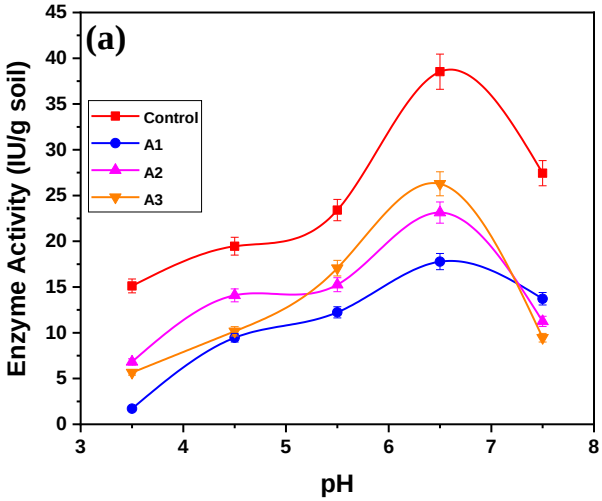


Fig. 3. The urease activity of **(a)** Faisalabad, **(b)** Gujranwala, and **(c)** Sheikhupura cultivated soil with A3 inhibitor combination at different concentrations and incubation period.



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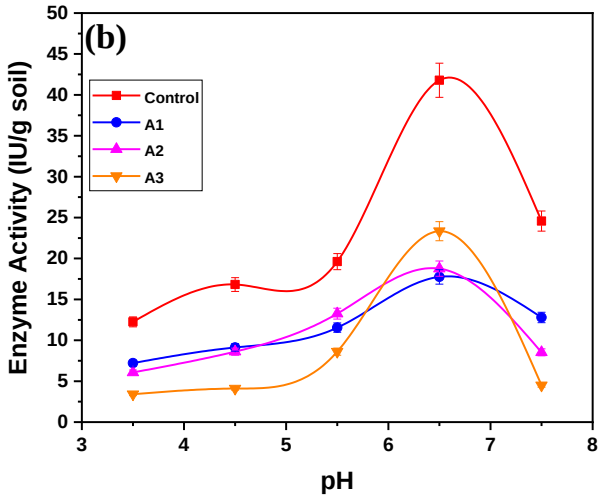

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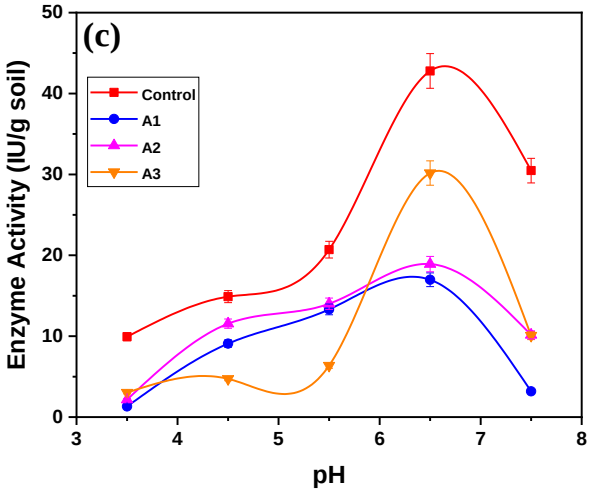

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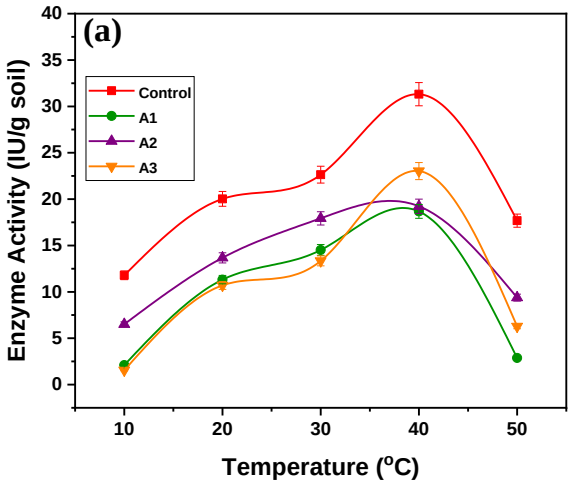
801 **Fig. 4.** The enzymatic activity of urease for **(a)** Faisalabad, **(b)** Gujranwala, and **(c)**
802 Sheikhupura cultivated soil at different pH level with 0.50% inhibitor concentration.



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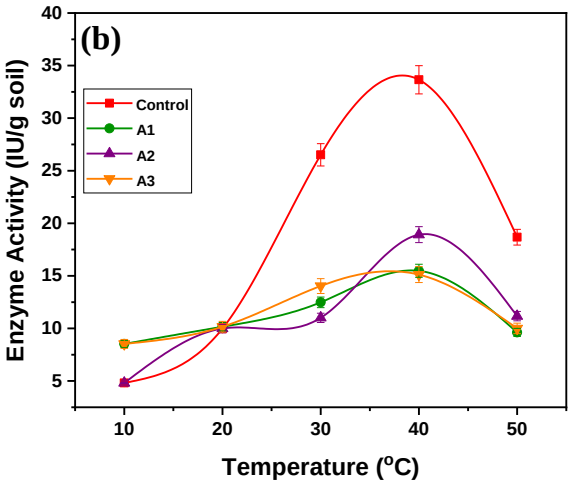

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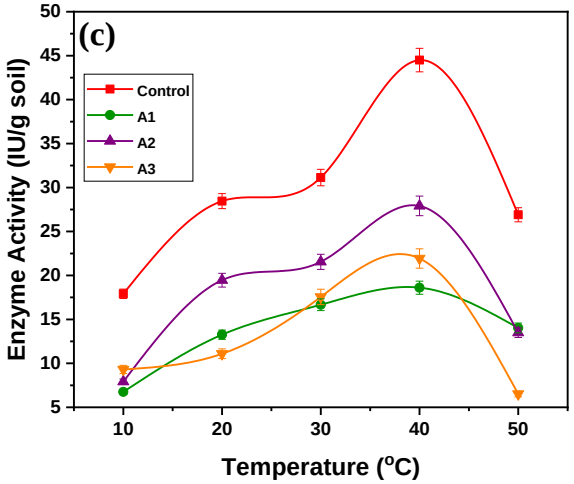

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806 **Fig. 5.** The effect of different temperatures ranges for **(a)** Faisalabad, **(b)** Gujranwala, and **(c)**
807 Sheikhupura cultivated soil at 0.50% inhibitor concentration.

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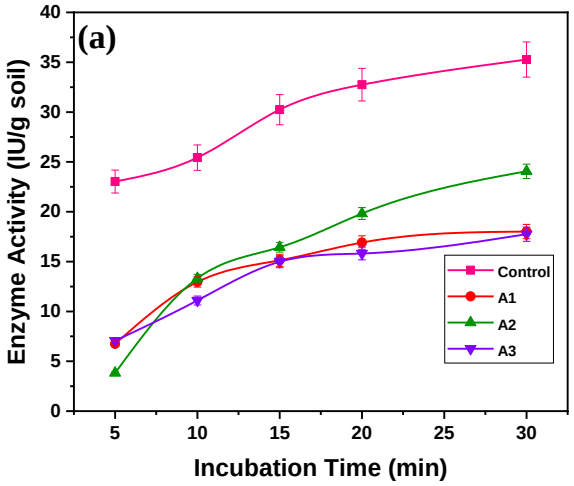

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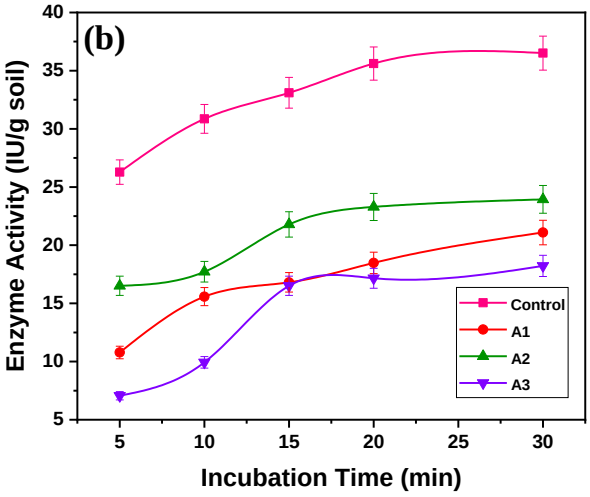

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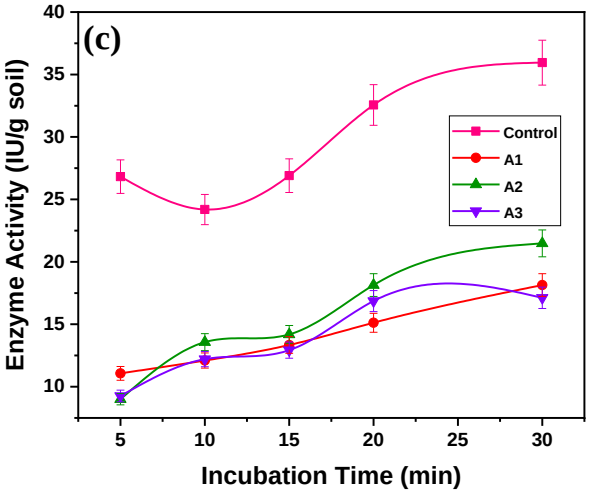

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812 **Fig. 6.** The effect of incubation time on urease activity for **(a)** Faisalabad, **(b)** Gujranwala, and
813 **(c)** Sheikhupura cultivated soil at 0.50% inhibitor concentration.

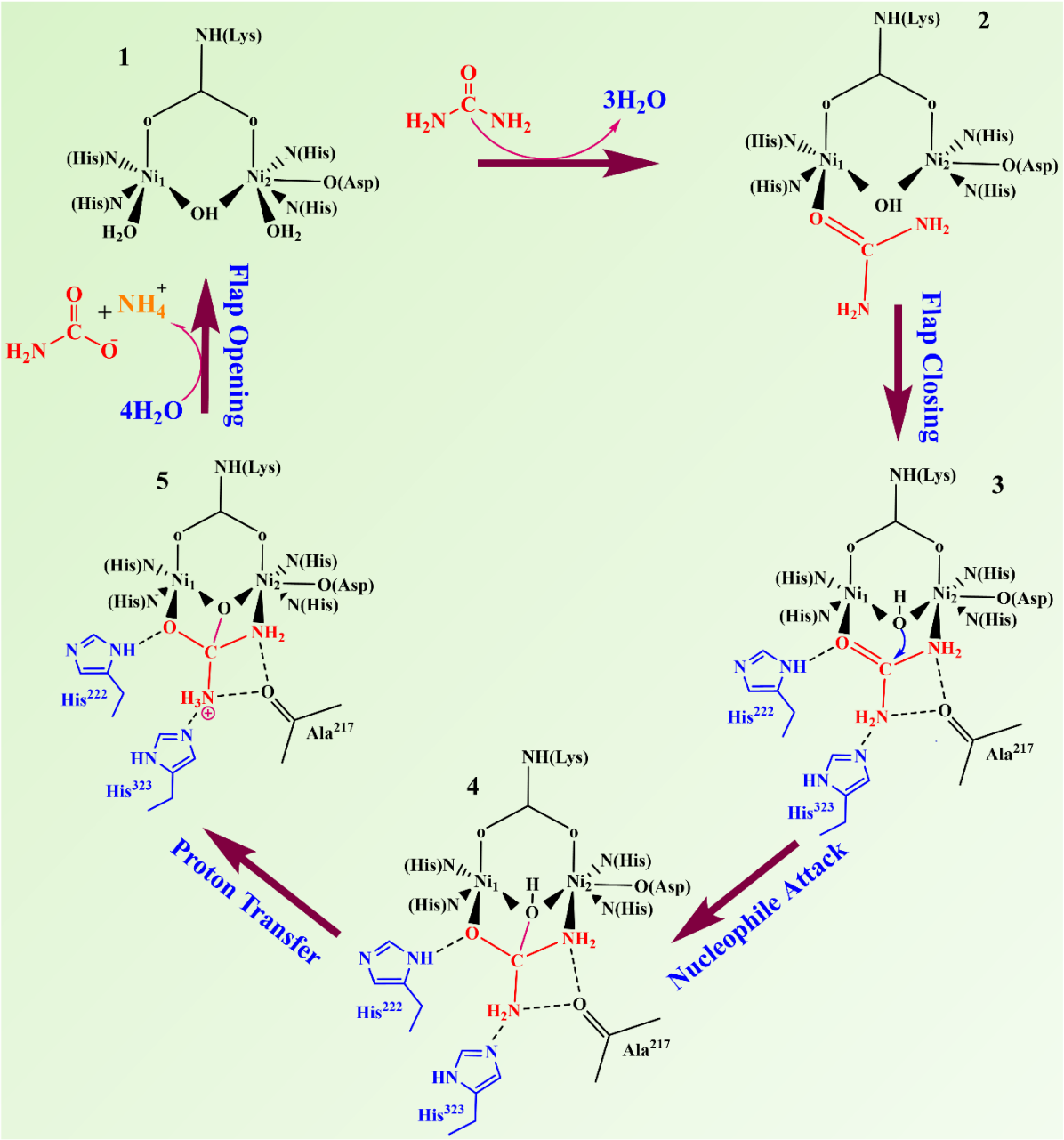


Fig. 7. Mechanism of action of urease enzyme by binding with urea.

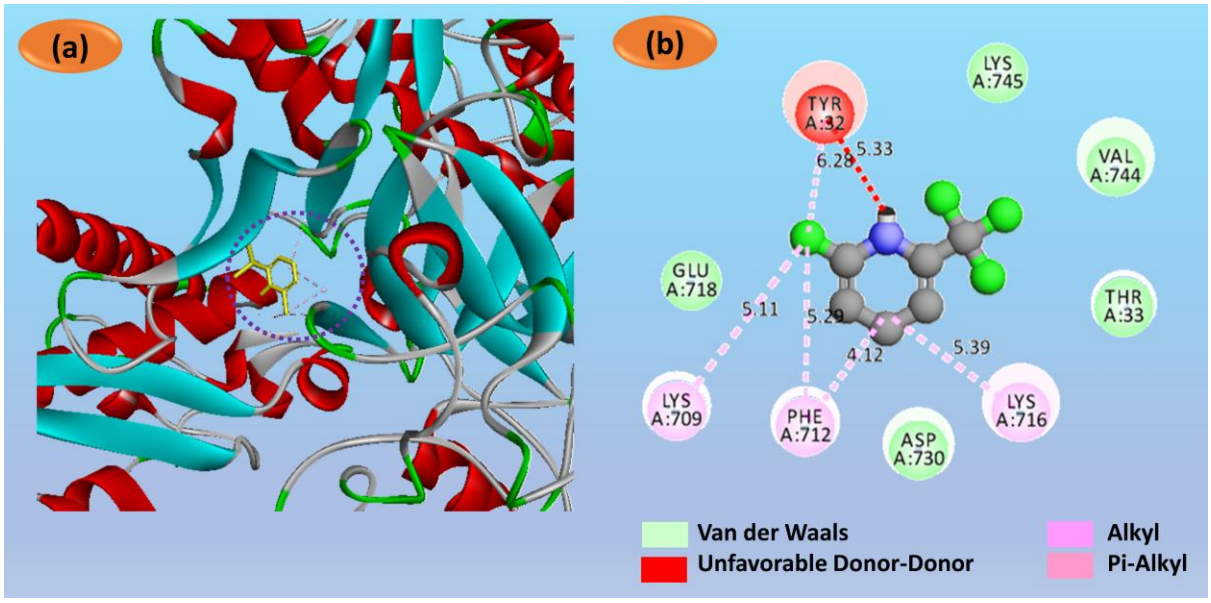
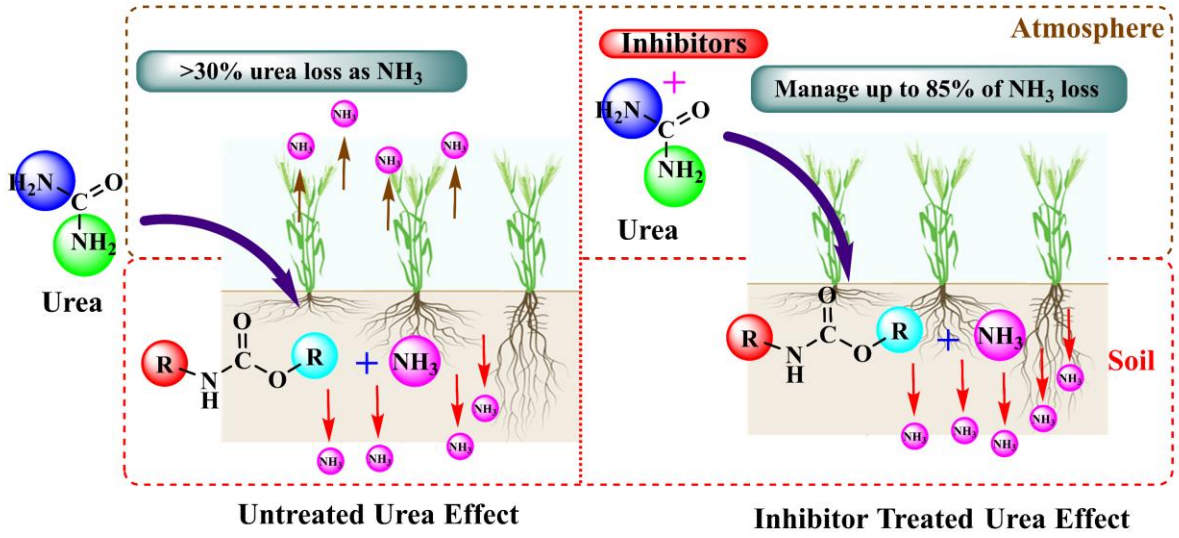


Fig. 8. The three-dimentional structures of **(a)** binding of inhibitor with urease and **(b)** active site of urease.

Highlights

- For the first time, the inhibition activity of ammonium thiosulfate and 2-Chloro-6-(trichloromethyl)pyridine inhibitors were examined.
- Results showed that the minimum urea hydrolysis of about 2.41 IU/g soil with A1 combination at 0.50% concentration.
- The better urease activity was observed at a pH value of 6.50, incubation time of 30 min, and temperature of 37 ℃.
- Results also showed the maximum plant height of 111 cm, root length of 15 cm, number of tillers 15 per panicle, and number of spikes of 38 per panicle.
- It is concluded that 0.50% inhibitor concentration showed the much impressive urease inhibition results followed by 0.25 and 0.10%.

852 **Graphical Abstarct**



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