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Effect of humic acids extracted from different organic sources and inoculation with *Bacillus subtilis* or *Aspergillus Niger* on urease activity in calcareous soil

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Abstract: Humic substances have received a large share of research and development as amendments to poor fertility soils that are poor in fertility and have deteriorating physical properties, since the process of extracting is a common process and easy to implement, as well as diluting these acids with water enables them to be used at economically acceptable levels over large areas. It is necessary to noticed that the properties of these acids depending on the organic source and the concentration added to the soil. A laboratory experiment was conducted at the Department of Soil Sciences and Water Resources, College of Agriculture, University of Basrah ,IRAQ to demonstrate the effect of humic substances extracted from different sources and inoculation with *Bacillus subtilis* or *Aspergillus niger* on the activity of the urease in calcareous soil. Wheat straw, alfalfa leaves, goat waste and poultry waste were composted aerobically for 3 months by filling a hole of 2×2×1.5 m by layers of such waste ,turned weekly for aeration. Each pile was extracted for humic acids (humic acid + fulvic acid) . Humic acid mixed with to soil at levels of 0, 25, and 50 L h^{-1}. The soil was also inoculated with *Bacillus subtilis* or *Aspergillus niger* isolated from local soil at rates of 20.06×10^{6} and 40×10^{3} cfu ml^{-1}, respectively The samples were incubated at field capacity level 28±2°C for 30 days. Urease activity was estimated after 7 or 30 days by determining NH_4^+ released using steam distillation method . A decrease in urease activity was observed when treated with different types of humic acid compared to the control treatment. The lowest activity was obtained at humic acids extracted from poultry waste, reaching 312.0 and 188.72 μg N-$NH_4^+$$g^{-1}$soil.2$h^{-1}$ at incubation periods of 7 and 30 days, respectively .The results also indicated that increasing the level of humic acids significantly inhibited the activity of the enzyme. However, in soil treated with *Bacillus subtilis* or *Aspergillus niger*, an increase in urease activity was observed compared to the non-inoculation treatment. Treatment with the fungal inoculant had higher activity than the bacterial inoculum, reaching 324.5 and 297.2 µg N-NH4+ g^{-1} soil 2 $hours^{-1}$ for both inoculants at a period of 7 days, and 178.09 and 150.31 μg N-$NH_4^+$$g^{-1}$soil.2$h^{-1}$at a period of 30 days, respectively.It is proposed that the inhibition of urease by humic acid addition may benefit for increasing urea fertilizer efficiency.
Keywords: *Aspergillus niger, enzyme, Bacillus subtilis, Humic acid, Urease.*

1. Introduction

Soil quality and health depend on a large number of physical, chemical, biological, microbiological and biochemical factors. Soil microbial activity directly affects ecosystem and soil fertility, and it is widely recognized that a good level of microbial activity is necessary to maintain soil quality and health. Enzymatic activity in soil plays a role. It plays a major role in soil nutrient cycling and its activity is essential in both the mineralization and transformation of organic matter in soil ecosystem [1] Urease

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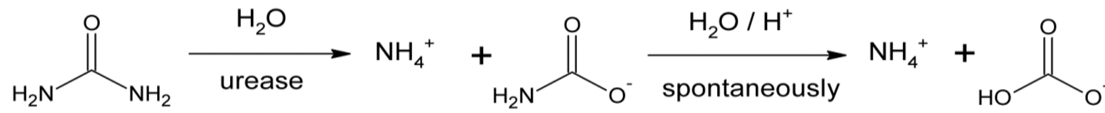
(Urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea into ammonia and carbon dioxide with high efficiency. The hydrolysis takes place in two stages, the first is the conversion of urea into carbamate and ammonia, the next stage decomposes carbamate into carbonate and another ammonia molecule as the final product [2], according to the following equation explained by Maroney and Ciurli [3].



This process is one of the sources of available nitrogen for plants, The rate of urea hydrolysis depends on the soil pH, since the soil pH is optimal for the enzyme's effectiveness. is between 6 and 7 [4]. Urease also strongly related humic substances and clay minerals [5]. The activity of urease in the soil is of great importance because it works to make nitrogen available for uptake by the plant after using urea as fertilizer. Urease enzyme is an ideal candidate that can be widely used to explore changes in soil fertility, as its activity increases with the use of organic fertilizers or amendments [6].

Humic substance are a natural organic materials containing a negativ charged polyelectrolyte with a hydrophobic core [7]. Humic acids consist of a heterogeneous association of small molecules or subunits containing many functional groups, including quinone, aldehyde, carboxyl, phenolic hydroxyls, alcohols, and ethers, which vary greatly depending on the sources extracted from[8].So, the effect of humic acids on soil properties and plant growth varies depending on the source, which is mainly due to the concentration of the acid, its chemical characteris, molecular weight, and the type and quantity of the active groups prevailing [9]. This in turn affects the activity of soil microorganism and the enzymes released from it due to the interaction of humic acid with Clays and changing the nature of their surfaces affect the absorption and stabilization of enzymes. Humic acid is also a source of carbon and nutrients benefit for the growth and activity of soil microorganisms, in addition to the direct effect of this acid on enzyme activity in terms of its absorption or interacted with the active site of the enzyme. Lui *et al.* [10] pointed out that the inhibition of urease activity as a result of adding humic acid is due to the presence of aldehydes in the acid, which are converted into phenolic and carboxyl substances that interfere with the active site of the enzyme. While Al-Taweel and Abo-Tabikh [11] indicated that the reason for the inhibition is due to the change in soil pH after treating with humic acid. As for Marzadori *et al.* [12], they concluded that the inhibition of urease enzyme activity is due to the humic acid's content of heavy metals such as Hg and Cu. Matsubara *et al.* [13] reported that the presence of polyphenolic oxides in humic acids is capable of inhibiting the activity of urease.

Bacillus subtilis is of high environmental as pect as it has been used in agricultural ecosystems for decades [14],[15]. The use of soil beneficial bacteria in biofertilization significantly increased because providing a promising way to reduce the chemical fertilizers demands, even though their role as biofertilizer is beneficial in terms of physical properties. In the study of Charles *et al.* [16]the sterilized soil was inoculated with *Bacillus subtilis* and *Pseudomonas fluorescens* to improve soil properties. It was found that the content of available nitrogen and ammonium in the soil significantly increased. The filamentous fungi (*Aspergillus niger*) are widely used to produce hydrolysis enzymes. *Aspergillus niger* is one of the most important fungi used worldwide for biotechnology applications [17].

After inoculants application, soil produces hydrolytic enzymes which enhance decompose organic materials and improve the availability of nutrients to plants in the root zone [18] Biofertilization enhances the enzymatic activity in soil by improving the microbial activity and physical and chemical properties of the soil, which leads to increased availability of nutrients to plants [19] . Charles *et al.* [16] that inoculating the soil with *Bacillus subtilis* and showed a significant increase (34%) in nitrogen content of soil, compared to the non-inoculated soil. This is due to enhancing urease activity in soil with

Bacillus subtilis increased urease activity in the soil. Therefore, it was concluded that enhancing enzymatic activities in the soil led to increased regulation of soil fertility due to the growth of bacteria. Al- Hrkane [20] obtained an increase in urease activity in soil inoculated with *Aspergillus niger*, for 22.61% compared to the control treatment and the reason for this was the ability of the fungi to secrete most soil enzymes [21].

This study aimed to compare the effect of humic acids (humic acid and fulvic acid together) extracted from different organic wastes on the activity of the urease belong with. Bacillus *subtilis* and the *Aspergillus niger* as a biofertilizer that enhances bioactivities of soil.

2. Materials And Methods

Four organic wastes were collected: wheat, alfalfa Leaves, goat waste and poultry waste, cleaned, cut into small pieces then composted aerobically in a hole of 2 x 2 x 1.5 dimensions for 3 months. The piles in each hole moisted for 60% and treated with soil suspension and urea solution to accelerate microbial activity, then coverd with plastic. Piles were turning weekly to enhance aeration. After that, the organic waste was removed, air-dried and transported to the laboratory. The humic acids were extracted (humic acid and fulvic acid together) as described in Page *et al.* [22] by using 0.5 *M* NaOH twice then adjusted pH at 6. The initial samples were analyzed for different properties and listed in table (1).

Table 1.
Quantity, ionic analysis and E4/E6 values for humic acids extracted from various organic sources.

Traits	Unit	Wheat straw	Alfalfa leaves	Goat waste	Poultry waste
Quantity of humic acids	%	8.6	8.9	3.0	9.6
EC	dS m^{-1}	15	19.2	13.9	20.2
C	%	60.41	43.26	42.06	45.58
H	%	4.95	4.83	0.96	4.56
Total N	%	1.39	3.88	2.79	4.07
Total S	%	0.85	0.77	1.9	0.71
Total P	%	0.042	0.09	0.10	0.12
C/N	----------	43.46	11.14	15.07	12.67
C/P	----------	1438.33	480.66	420.62	379.83
E4/E6	----------	5.0	4.5	1.8	1.4
Total acidity	meq g^{-1}	5.7	5.3	7.3	7.1
Carboxyl groups	meq g^{-1}	3.0	2.9	4.2	3.9
Phenolic groups	meq g^{-1}	2.7	2.4	3.1	3.2
Alcoholic groups	meq g^{-1}	3.0	2.9	4.2	3.9

Silty clay soil samples 0 - 30 cm were collected from agricultural research station, College of Agriculture , University of Basra, air-dried , grinded then passed through a 2 mm sieve. A part of sample was analyzed for initial properties using standard procedures described in Black [23] and Page *et al.* [22] (table 2). The reminded part of soil sample was used for incubation experiments.

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Table 2.
Initial characteristics of the slutied soil.

Traits	Unit	Reading
pH	____________	7.82
EC	dSm^{-1}	4.0
Total solid carbonate	$g\ kg^{-1}$	359
CEC	Cmol $+kg^{-1}$	26.73
Organic matter	$g\ kg^{-1}$	2.62
Available phosphorus	$mg\ kg^{-1}$	4.9
Available nitrogen	$mg\ kg^{-1}$	19.2
Available sulfur	$mg\ kg^{-1}$	0.62
Total bacteria	CFU	$10^6 \times 2.11$
Total Fungi		$10^5 \times 4$
Urease activity	$\mu g\ N\text{-}NH_4^+ g^{-1} soil.2h^{-1}$	50.03
Sand		11.2
Clay	%	54.6
Silt		34.2
Texture		Silty clay

The bacterial isolation (*Bacillus subtilis)* growing in test tubes containing nutrient agar media (NA) in the form of a slant and pre-insulated in the soil microbiology laboratory of soil and water resources department was taken. One ml of sterile distilled water mixed with bacterial colonies and using for inoculation of nutrition broth media and incubated at 30°C for five days. As for the fungal inoculum (*Aspergillus niger)* was grown in Potato Dextrose Broth (PDB) media by adding 0.5 cm diameter discs of pure colonies growing in PDA medium to the liquid nutrient medium (PDB) and incubating at 28°C for five days.

500 g of soil samples treated with 2%(w\w) of cow waste sterilized at 121C° and 15 pounds $inch^{-2}$ and left in the incubator for 14 days for balance. The samples were thoroughly mixed with humic acids at levels of 0, 25 and 50 L ha^{-1} and inoculated with 20 ml of *Aspergillus niger* at a density of 40 x 10^3 cfu ml^{-1} or with *Bacillus subtilis* at a density of 20.06 x 10^6 cfu ml^{-1} .Soil moisture was brought to the field capacity and incubated at 28 ± 2 °C .Control treatment without inoculation was conducted .After 7 and 30 days of incubation, a set of samples was taken, dried, ground, and sieved, then urease activity was measured.

Urease activity was measured according to the method of Tabatabi and Bremner [24] by incubating 5 grams of soil with 2.0 ml of toluene, 9 ml of Tris (amino hydroxymethyl methane (THAM) buffer solution) , and urea solution as a substrate at a concentration of 0.2 *M.* samples were the incubated at 37°C for two hours after that, 35 ml of 5.2 *M* potassium chloride solution containing 100 ppm silver sulphate was added as an inhibitor of enzyme activity, and the volume was completed with the same solution to 50 ml. Ammonium ion resulting from the enzyme reaction was estimated by steam distillation according to Bremner and Edwards [25] Using heavy (MgO) and receiving ammonia with boric acid, then titrated with standard hydrochloric acid.

Variance analysis of the urease activity was performed using GenStat program and the difference between means were obtained by RLSD test [26].

3. Results And Discussion

It was noted from Figure (1) the effect of the source of humic acids on the urease activity. Control treatment was superior to all humic acid treatments, with an increasing percentes of 36.58, 50.81, 41.91 and 54.96 % at 7 days of incubation and 25.42 ,38.13, 40.13 and 46.7 % at 30 days of incubation, for wheat straw, alfalfa leaves, goat waste and poultry waste, respectively. The lowest values were obtained at humic acid extracted from poultry. The negative effect of humic acids on enzymes may be due to the interaction between humic acids and the urease enzyme, since humic acids may interacte with the active site of the enzyme or by forming complexes with urea, which reduces its availability as a substrate [27]. The result was similar to that of Lui *et al.* [10], who obtained an inhibition of urease activity due to the addition of humic acid extracted from organic waste, indicating that the reason is the presence of aldehyde groups in the humic acid that can be reduced to from phenolic and phenolic carboxylic substances interfere with the thiol group present in the cysteine of the enzyme, changing the structure of the enzyme and resulting in inhibition. Al-Taweel and Abo-Tabikh [11] also found inhibition of the activity of the urease in calcareous soil as a result of adding humic acids at high concentrations. They attributed this to the ability of humic acid to change the pH of the soil, which leads to a decrease in enzyme activity.

It is noted that the activity of the urease enzyme in the treatments containing humic acid is in the following order: poultry < alfalfa < goat < straw. Humic acids extracted from poultry, and to some extent extracted from goats contain a higher percentage of active compounds that can interact with enzymes and inhibit them. These compounds include phenolic compounds or complex organic substances (Table 1). The number of carboxylic groups and substances phenolic acids in humic acid extracted from poultry and goats is higher than other sources, as phenolic substances are among the effective substances in inhibiting the activity of urease and constitute the largest percent of the structure and stability of humic substances [28]. Abdulkareem [29] obtained an inhibition of 51.75. % in urease activity as a result of adding phenolic substances extracted from the myrtle plant. On the other hand, the difference in molecular weights of humic acids has a significant effect on the activity of the enzyme. Sun *et al.* [30] concluded that humic acid with a high molecular weight is able to inhibit enzyme activity. while that of ith low molecular weight do not have such.

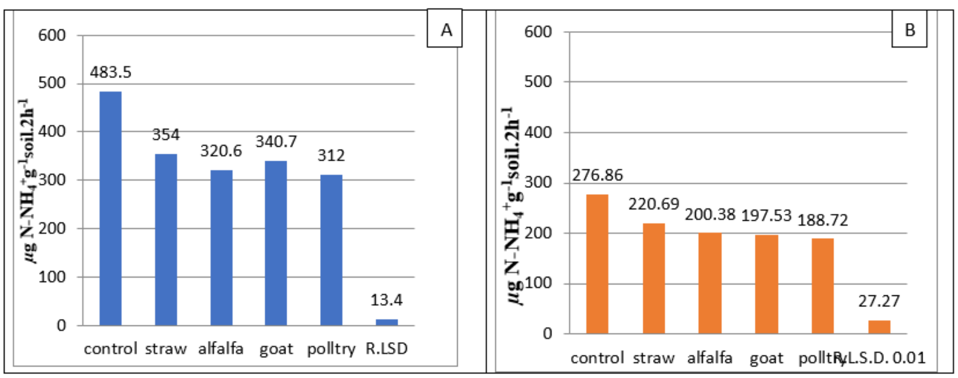

Figure 1.
Effect of humic acid source on urease activity in soil at 7 days (A) and 30 days (B) of incubation.

Increasing humic acids level significantly decreased urease activity at the two incubation periods (f.g2). The values were 483.63, 324.51 and 297.20 μg $N\text{-}NH_4^+ g^{-1} soil.2h^{-1}$ at the incubation period of 7 days and reached 276.86, 178.09 and 150.31 μg $N\text{-}NH_4^+ g^{-1} soil.2h^{-1}$ at the incubation period of 30 days for levels of 0, 25 and 50 L ha^{-1}, respectively. The reason for this is the increasing of the number of active compounds that reduced the activity of the enzyme, either by influencing the enzyme-producing organisms or by directly affecting the structure of the enzyme. This result is consistent with Gomes *et al.* [31], who observed a decrease of 10% in urease activity. With using humic acid compared to the control treatment indicating that the effect of humic acids on the enzymatic activity is due to lowering pH of reaction solution.

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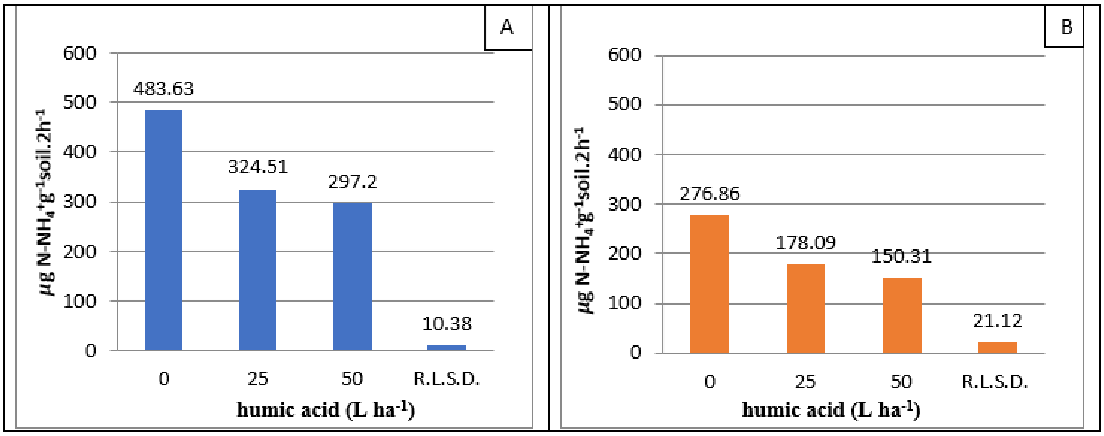

Figure 2.
The effect of humic acid level on urease activity at 7 days (A) and 30 days (B) of incubation.

As for the influence of incubation, it is noted from Figure (3) urease activity significantly increased when. Bacillus *subtilis* or *Aspergillius niger* were used. The mean values were activity 346.23, 391.59 and 367.14 μg N-$NH_4^+g^{-1}$soil.2h^{-1} at the incubation period of 7 days and 194.61, 247.16 and 208.74 μg N-$NH_4^+g^{-1}$soil.2h^{-1} at the incubation period of 30 days, for the control, *Aspergillius niger* and *Bacillus subtilis*, respectively, with a significant superiority of *Aspergillius niger* over *Bacillus subtilis*. Oluwaseun *et al.* [32] found that the improvement of urease enzyme activity in soil treated with *Bacillus subtilis* isolate is due to its role on increasing the availability of nutrients, which in turn improves the metabolic processes of microorganisms.

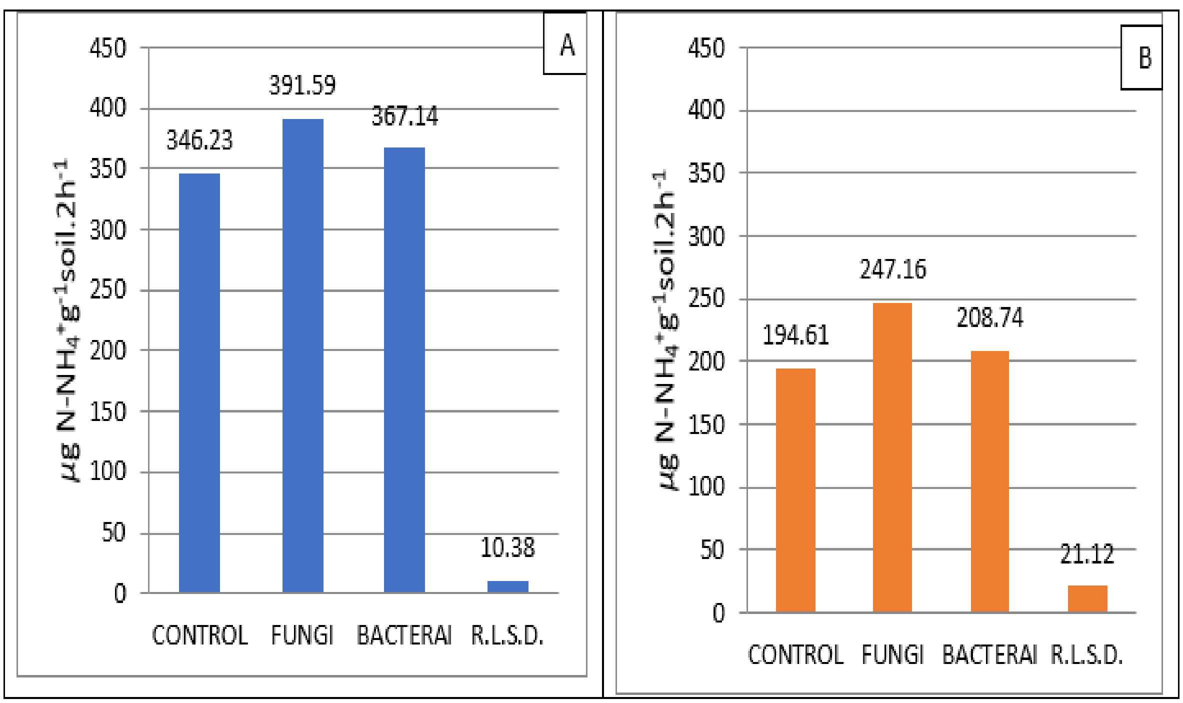

Figure 3.
Effect of inoculating soil with *Bacillus subtilis* or *Aspergillus niger* in urease activity at 7 days (A) and 30 days (B) of incubation.

Regarding the effect of incubation period (7 and 30 days) on the urease activity, it has been observed that the activity decreased with increasing incubation period from 7 to 30 days. The values were 368.32 and 216.83 μg N-NH_4^+g^{-1}soil.2h^{-1} for the two periods, respectively. This may be due to the decrease in the concentration of substrate over time or the decrease in materials necessary for the growth and activity of organisms [33]. This result was similar to Al-Taweel and Abo-Tabikh [11].

Tables (3) and (4) showed the effect of humic acids and inoculation on urease activity after 7 and 30 days of incubation. It is noted that all humic acids led to decrease in urease activity compared to the control treatment, with a lowest value obtained in the soils treated with acids extracted from poultry, at all inoculation treatments. Increasing the level of adding acids significantly increased the inhibition of enzyme activity at all inoculation treatments and sources of acids. These results confirm the ability of humic acids extracted from the various organic sources under study to inhibit the enzyme at a wide range of levels.

This result is encouraging in terms of soil fertility, controlling rapid urea hydrolysis in calcareous soils, reducing ammonia volatilization rates, increasing the fertilizer use efficiency as well as reducing the possibility of soil and water pollution. The highest values of interaction were recorded at soil inoculated with at all acid treatments compared to inoculated with bacteria. The highest values were 506.0 and 329. μg N-NH_4^+g^{-1}soil.2h^{-1} at *Aspergilli's niger* and not adding humic acid for the incubation periods of 7 and 30 days, respectively, while the lowest values (185.5 and 126.0 μg N-NH_4^+kg^{-1}soil.2h^{-1}) were associated with using humic acids derived from 50 L ha^{-1} poultry waste without inoculation for incubation periods of 7 and 30 days, respectively.

Table 3.
Effect of source and concentration of humic acids and inoculation with *Bacillus subtilis* and *Aspergillius niger* on urease activity (μg N-NH_4^+g^{-1}soil.2h^{-1}) at 7 days of incubation.

Humic acid source	Humic acid level	Inoculation treatment		
		Non	Fungi	Bacteria
Control	0	471.3	506	473.6
	25	471.3	506	473.6
	50	471.3	506	473.6
Straw	0	471.3	506	473.6
	25	287.0	347.0	320.0
	50	206.0	298.0	277.6
Alfalfa	0	471.3	506	473.6
	25	240.3	324.3	310.0
	50	262.5	291.6	284.6
Goat	0	471.3	506	473.6
	25	263.2	317.3	291.6
	50	233.3	256.8	256.6
Poultry	0	471.3	506	473.6
	25	216.5	263.6	235.6
	50	185.5	233.3	224.0
RLSD	20.40			

Table 4.
Effect of source and concentration of humic acids and inoculation with *Bacillus subtilis* or *Aspergillius niger* on urease activity (μg N-$NH_4^+$$g^{-1}$soil.$2h^{-1}$) at 30 days of incubation.

Humic acid source	Humic acid level	Inoculation treatment		
		Non	Fungi	Bacteria
Control	0	242.6	329.0	259
	25	242.6	329.0	259
	50	242.6	329.0	259
Straw	0	242.6	329.0	259
	25	196.0	224.0	224.0
	50	143.0	198.3	170.3
Alfalfa	0	242.6	329.0	259
	25	161.0	191.3	168
	50	140.0	161.0	151.6
Goat	0	242.6	329.0	259
	25	168.0	177.3	170.3
	50	140	149.3	142.3
Poultry	0	242.6	329.0	259
	25	147.0	156.3	154.0
	50	126.0	147.0	137.6
RLSD	81.80			

4. Conclusions

It can be concluded that the properties of humic acids extracted from the organic wastes used in the study were able to inhibit the activity of the urease even at a low level, while the effect of inoculation with *Bacillus subtilis* or *Aspergillus niger* is the opposite and encouraged urease activity during the incubation period. In continuation of this study, we recommend implementing the treatments in a field to demonstrate the effect of the treatments on plant growth and, in return, the effect of the presence of the plant in controlling the effect of action of both humic acids and biological fertilization.

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