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EFFECT OF INOCULATION WITH PHOSPHATE-DISSOLVING BACTERIA AND FUNGI WITH HUMIC ACIDS IN THE KINETIC PARAMETERS OF THE ALKALINE PHOSPHATASE ENZYME IN CADMIUM-POLLUTED SOIL

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Abstract:

A laboratory experiment was carried out in the Department of Soil Sciences and Water Resources, College of Agriculture / University of Basra, to identify the role of the bacteria (*Bacillus subtilis*), the fungus (*Aspergillus niger*) isolated from agricultural areas, and humic acids extracted from cow dung in the kinetic parameters of the alkaline phosphatase enzyme in cadmium-polluted soil and compare it with soil unpolluted cadmium.

The soil was treated with a cadmium sulfate solution up to the critical limit (3 mg Cd L^{-1}), with the addition of humic and fulvic acid at a level of 50 L ha^{-1}, individually, then the soil was inoculated with bacterial *B. subtilis* and fungal *A. niger* vaccines, both individually and with a mixture of vaccines, and the activity of the enzyme alkaline phosphatase was measured under different levels of the substrate (0.010, 0.025, 0.050, 0.075 and 0.100 M) and kinetic parameters (Vmax and Km) were calculated using the Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee equation.

The results showed that the best concentration of the substrate was 0.075 M, which gave the highest activity for the alkaline phosphatase enzyme and both soils. The values of the kinetic parameters of the enzyme differed according to the pollution treatment, as the Km values ranged between 0.019-0.030 molar and the Vmax values ranged between 367.647-714.286 µg P. nitrophenol gm^{-1} soil 1 h^{-1} in unpolluted soil, but in polluted soil the Km values ranged between 0.021-0.033 molar and the Vmax values range between 294.985-663.150 micrograms P. nitrophenol gm^{-1} soil 1 h^{-1}.

Keywords: Inoculation, Humic acids, Kinetic parameters, Alkaline phosphatase, Cadmium.

INTRODUCTION:

Soil pollution is defined as any physical or chemical change in the soil that makes it unsuitable for agricultural exploitation. This may be the result of misuse of the land, its neglect, or chemical additions to it (Cachada *et al.,* 2018). Soil pollution with heavy metals can be defined as those elements that are found in the soil in abnormal quantities and above its critical limits due to various additions resulting from industrial and human activities, which affect the quality and characteristics of the soil and the biological activities in it (Patinha *et al.,* 2017). Heavy elements are elements that exhibit metallic properties such as ductility, expansion, conductivity, stability, and ionic bonding (Chibuike and Obiora, 2014). Heavy metals are characterized by an atomic mass greater than 20 and a relatively high density (more than 5 gm cm^{-3}), which is five times the density of water (Li *et al.,* 2019, Jiang *et al.,* 2020, and Nkwunonwo *et al.,* 2020), Cadmium is one of the main heavy metals polluting the environment because it has a large atomic number of 48, an atomic mass of 112.4, and a high density estimated at 8.64 µg m^{-3}. It is generally found in soil in the form of a divalent cation that forms complexes with other elements such as $CdCl_2$ and $CdSO_4$ (Tian *et al.,* 2017) Cadmium is considered a dangerous element that is widely distributed in the environment and can cause toxic effects even at low concentrations due to its mobility in the soil as well as its bioaccumulation in plants and thus in animal tissues (Selvi *et al.,* 2019; Kicińska *et al.,* 2022).

The activities of soil enzymes change rapidly and over time because they are sensitive to the surrounding environmental conditions and are used as indicators to evaluate soil quality, fertility, and biodiversity. They also work to remove toxins and pollutants through the decomposition of aromatic compounds and dangerous pesticides. They are also considered a measure of the level of soil pollution with heavy metals due to the speed of enzymes' response to these effects. It is

also possible to determine enzymatic activity easily, accurately, quickly, and at a lower cost (Nannipieri *et al.,* 2018; Novosyolova *et al.,* 2018; Kravkaz Kuşçu, 2019; Soni *et al.,* 2021). Enzyme activities in soil are affected by the physical and chemical properties of the soil, such as the quantity and type of substrate, mineral elements, organic materials (organic and mineral phosphorus for the phosphatase enzyme), clay content, soil moisture, soil depth, temperature, degree of soil interaction, and the C: N ratio), as well as biological factors such as aggregates biotic organisms and their activities (Jaworska and Lemanowicz, 2019; Sharma *et al.,* 2020; Łukowski and Dec 2021).

Humic substances are considered a suitable environment for microorganism populations in the soil, as humic acids work to reduce the degree of alkaline soil reaction and reduce salinity, which provides a suitable environment for the growth and activity of microorganisms (Mosa *et al.,* 2020; Yang *et al.,* 2021; Ampong *et al.,* 2022) Humic acids also contribute to biological redox activities in soil because they contain (hydro)quinone, sulfhydryl, and the carboxyl and phenol functional groups which give humic acids the ability to form chelating aggregates with ions such as Mg^{+2}, Ca^{+2}, Fe^{+2}, Fe^{+3} and some other microelements that microbes use in respiratory activities and the formation of membranes and cell walls (Kögel-Knabner, 2002; Weber, 2020; Gautam *et al.,* 2021)

MATERIALS AND METHODS :

The soil sample was taken from the surface layer 0-30 cm from the Naher Saleh area of Al-Madina District/Basra Governorate, with coordinates 30°56˙39.9"N 47°11˙09.5"E. The samples were collected randomly and in the form of composite samples in November 2021. The samples were placed in bags, brought to the laboratory, air-dried, ground, and passed through a sieve with a diameter of 2 mm to study some of the primary physical, chemical, and biological properties of the soil (Table 1), according to Jackson, (1958), Black, (1965), Lindsay and Norvell, (1978) and Page *et al.* (1982):

Table 1: Some properties of the study soil

Field capacity	Sand ratio	Loam ratio	Clay ratio	Soil texture
%				
29.30	27.40	34.70	37.9	Clay loam

pH	EC	$CaCO_3$	Organic carbon	Organic matter	Cd	P
	ds m^{-1}	gm Kg^{-1} soil			mg Kg^{-1} soil	
7.89	4.73	364.53	2.48	4.27	0.00	13.68

Bacteria numbers	Fungi numbers	The activity of the alkaline phosphatase enzyme
cfu gm^{-1} soil		µg P-nitrophenol gm^{-1} soil 1 $hour^{-1}$
6.32×10^6	4.89×10^4	257.30

Extraction of humic acids

Humic acids were prepared after fermenting cow waste for two months. After the end of the fermentation period, humic acids were extracted by taking a certain weight of the fermented organic waste treating it with 0.1 molar of NaOH and leaving it for the next day. It was observed that a precipitate was formed, which is human, and it was disposed of. As for the filtrate, it was It is + Humic acid Fulvic acid. Concentrated HCl acid was added until the pH of the soil reached the limits of 2 and was left for the next day. After that, it was observed that a precipitate was formed, which is humic acid, while the filtrate was fulvic acid, and the pH value of humic and fulvic acid was adjusted to 6.5 according to the method described by Page *et al.* (1982).

Soil preparation and inoculation

A weight of 100 gm of soil was placed in plastic containers, and then the soil samples were polluted with cadmium salts at a concentration of 3 mg kg^{-1} soil and symbolized as Cd1. The treatment was left without adding cadmium and symbolized as Cd0. Cadmium was added to the soil by dissolving the salt in an amount of water equivalent to the field capacity for the soil. Then add 2% of peat moss sterilized in the incubator at 121°C and 15 pounds ng^2 with the bacterial and fungal inoculum at a rate of 10 ml to each container, with a population density of the fungi added to the soil of 40×10^3 cfu ml^{-1} soil and a population density of bacteria of 20.06×10^6 cfu ml^{-1}. Single and the bacterial inoculum sample is symbolized by I_B and the fungal inoculum is symbolized by I_F. As for the mixture of bacteria and fungi, it is symbolized by I_{Mix} with the addition of humic acid, which is symbolized by A_H, and fulvic acid, which is symbolized by A_F, at a level of 50 L ha^{-1}, leaving a treatment without addition for control, and incubated treatments were at a temperature of 2±28 °C for 30 days, while maintaining soil moisture within the limits of the field capacity by compensating the weight difference with deionized water, taking into account stirring the soil for aeration during the duration of the experiment.

Measurement of Enzyme alkaline phosphatase activity

Alkaline phosphatase activity was measured according to the method of Tabatabai and Bremner (1969), by incubating 1 gm of soil with 0.2 ml of talwin solution and 4 ml of alkaline buffer solution consisting of (12.1 gm of THAM + 11.6 gm of Maleic acid + 14 gm of Citric acid + 6.3 gm of Boric acid dissolved in 488 ml of 1 M ammonium hydroxide and raising the pH to 11 using 0.1 M NaOH and completing the volume to a litre with distilled water) and adding 1 ml of di-

Sodium-4-nitrophyenyl phosphate Hexahydrate solution (NO2C2H4OPO2Na2.6H2O) dissolved in the solution Substrate and incubated at a temperature of 37 °C for an hour. After incubation, 1 ml of a 0.5 M solution of $CaCl_2$ and 4 ml of a 0.5 M solution of NaOH were added. The solution was filtered through Whatman filter paper No. 42, and then 1 ml of the filtrate was taken to estimate the colour yellow using a spectrophotometer at a wavelength of 420 nanometers.

Kinetic Parameters of Alkaline Phosphatase Enzyme

100 gm of soil placed in plastic containers were treated with study agents. The treatments were incubated at a temperature of 30 °C in the incubator for two weeks. Then the enzyme activity was estimated using five concentrations (0.010, 0.025, 0.050, 0.075, and 0.100 M) of the substrate (di-Sodium-4-nitrophyenyl). phosphate Hexahydrate) with three replicates. Then the kinetic parameters of the alkaline phosphatase enzyme were calculated, which included the maximum activity of the enzyme (Vmax) and the Michaelis Constants (Km), according to the mathematical formulas shown below:

1- Lineweaver-Burk transformation derived from the Michaelis-Menten (1913) equation by plotting the linear relationship between 1/[S] /V as follows: $\frac{1}{V} = \frac{1}{Vmax} + \frac{Km}{Vmax} \cdot \frac{1}{[S]}$

2- Hanes-Wolf transformation derived from the Michaelis-Menten equation (1913) doing the linear relationship between [S] and [S]/V as follows: $\frac{[S]}{V} = \frac{Km}{Vmax} + \frac{1}{Vmax} . [S]$

3- Eadie-Hofstee transformation derived from the Michaelis-Menten equation (1913) by drawing, ear relationship between V/[S] and V as f, $= Vmax - Km . \frac{V}{[S]}$

Where:

V :represents the reaction speed (µg p-nitrophenol gm^{-1} soil 1 h^{-1})

Vmax :maximum enzyme activity (µg p-nitrophenol gm^{-1} soil 1 h^{-1})

Km: represents the Michaelis constant (mol L^{-1})

[S] :Concentration of the subject substance (mol L^{-1})

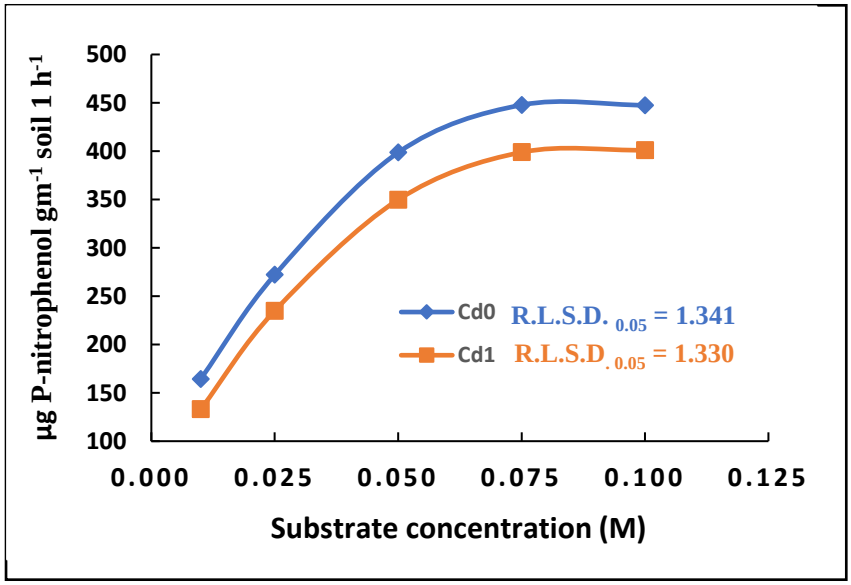
The kinematic measures Vmax and Km were calculated from the straight-line equation by extracting the slope for each of the equations used above, where the slope in the Lineweaver-Burk equation represents $\frac{Km}{Vmax}$and the intercept represents $\frac{1}{vmax}$. In the Hanes-Woolf equation, the slope represents $\frac{1}{Vmax}$ and the intercept represents $\frac{Km}{Vmax}$ In the Eadie-Hofstee equation, the slope represents -Km and the intercept represents Vmax.

RESULTS AND DISCUSSION

1- The effect of the substrate in the activity of the alkaline phosphatase enzyme:

In Figure 1, it can be observed that as the substrate concentration increases, the activity of the alkaline phosphatase enzyme also increases for both unpolluted and cadmium-polluted soils. This reaction follows a First-order reaction until the substrate concentration reaches the maximum activity of the enzyme. After that, it follows a Zero-order reaction. The highest activity of the alkaline phosphatase enzyme was observed at a substrate concentration of 0.075 M for both unpolluted and cadmium-polluted soils. The amount of enzyme activity was 447,733 and 398,839 µg P-nitrophenol gm^{-1} soil 1 h^{-1}, respectively. This is due to the enzyme's active sites being saturated with the reactant, as reported by Kumari and Padmasr (2021).

Figure 1: Effect of substrate concentration on the activity of alkaline phosphatase enzyme in unpolluted and cadmium-polluted soil



2- Kinetic parameters of alkaline phosphatase enzyme:

Figure 2 shows a plot of the linear relationship according to the Lineweaver-Burk equation between substrate concentrations and alkaline phosphatase enzyme activity1/[S] in molar and 1/V, as it represents the enzyme activity measured in units of µg P-nitrophenol gm^{-1} soil 1 h^{-1} for unpolluted and cadmium-polluted soil. It is clear from the values of the correlation coefficient (r) in Table 2 that there is a positive correlation between 1 / [S] and 1 / V for all coefficients, and from the straight-line equations (Table 2) the values of Vmax and km were calculated from the slope and intercept shown in Table 3.

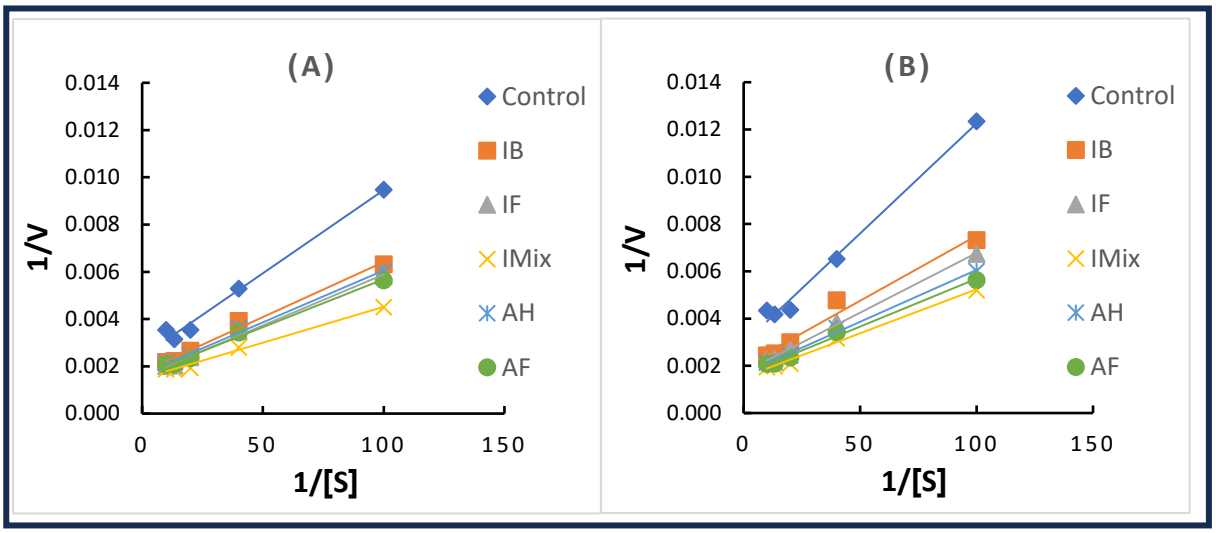


Figure 2: The linear formula of the Lineweaver-Burk equation for the activity of the alkaline phosphatase enzyme under the influence of bio inoculation and humic acids for soil A) unpolluted and B) cadmium-polluted (V): activity of the alkaline phosphatase enzyme measured in micrograms P. nitrophenol gm^{-1} soil 1 h^{-1}, S: concentration of the substrate in molars

Table 2: Straight line equation and correlation coefficient (r) for the relationship between substrate concentration (Molar) and alkaline phosphatase enzyme activity for unpolluted and cadmium-polluted soil under the influence of bioinoculation and humic acids according to the Lineweaver-Burk equation.

Pollution level	Cd_0		Cd_1	
Treatments	**Equation**	**r**	**Equation**	**r**
Control	Y=0.00007x+0.0024	0.9946	Y=0.00009x+0.0029	0.9959
I_B	Y=0.00005x+0.0018	0.9950	Y=0.00006x+0.0020	0.9878
I_F	Y=0.00004x+0.0016	0.9974	Y=0.00005x+0.0017	0.9992
I_{Mix}	Y=0.00003x+0.0014	0.9961	Y=0.00004x+0.0015	0.9960
A_H	Y=0.00004x+0.0017	0.9977	Y=0.00005x+0.0018	0.9984
A_F	Y=0.00004x+0.0016	0.9973	Y=0.00005x+0.0017	0.9980

Table 3 shows the variation in the values of kinetic parameters depending on the type of inoculation and the type of humic acids in the soil. The results showed that the highest value of Vmax for bioinoculation in unpolluted and cadmium-polluted soil in the IMix treatment reached 714.286 and 662.252 µg P. nitrophenol gm^{-1} 1 h^{-1} over Consecutively, the coefficients can be arranged in terms of the superiority of the Vmax values as follows: $I_{Mix} > I_F > I_B$. The reason may be that vaccination increased the activity of the alkaline phosphatase enzyme, because the fungus *A. niger* and the bacteria *B. subtilis* are organisms that produce the enzyme (Bhattacharjee *et al.,* 2018; Nosalj *et al.,* 2021), while in the humic acid treatments, the highest value of Vmax appeared in the A_F treatment for unpolluted and cadmium-polluted soil, reaching 617.284 and 588.236 µg P. nitrophenol gm^{-1} h^{-1}, respectively, which is a similar value to the A_H treatment. Which amounted to 598,802 and 555,556 µg P. nitrophenol gm^{-1} 1 h^{-1}, respectively. The reason is that treating the soil with humic acids led to an increase in the production of extracellular enzymes and an increase in its ability to extract nutrients and dissolve them, making them available to microorganisms. It also stimulates their growth by providing Energy and carbon to increase biological activity and thus increase enzymatic activity (Wu *et al.,* 2017), as Gianfreda and Rao (2014) explained. There is a direct proportionality between the increase in Vmax values with the increase in

the amount of enzyme in the soil, and high or low enzyme values for the subject matter are affected by the molecular structure of the enzyme, which varies according to the biological source of that enzyme.

Table 3 shows the variation of the values of the affinity constant (Km) depending on the type of inoculation and the type of humic acids. The lowest value of Km appeared in the I_{Mix} inoculation treatment compared to the rest of the treatments and amounted to 0.022 and 0.027 M in the unpolluted and cadmium-polluted soil, respectively, while the highest values of Km were In the control treatment, it amounted to 0.029 and 0.031 M, respectively, while in the humic acids treatment, the highest values of Km appeared in the A_F treatment, and amounted to 0.025 and 0.029 M, respectively, for unpolluted and cadmium-polluted soil, as these treatments with low values of Km show increased affinity between the enzyme and the substrate, and this indicates that the treated soil was affected by cadmium, which led to a decrease in the affinity between the enzyme and the substrate, as indicated Juna *et al.* (2010) that high values of Km in cadmium treatments indicate the formation of a complex (cadmium-enzyme), and this leads to a decrease in the affinity between the enzyme and the substrate, or it may indicate a change that occurs in the enzyme protein, which makes the active sites less polarizing to the substrate. Marx (2005) showed that microorganisms are considered the primary source of most soil enzymes and that any change in their numbers and types leads to a change in the concentration and source of the enzyme, which changes the Km and Vmax values. Fitriatin *et al.* (2008) stated that the nature, numbers, and types of microorganisms in the soil are among the most important factors affecting enzyme kinetic parameters. The Km values for the alkaline phosphatase enzyme vary depending on the distribution of substrate molecules in the active sites of the enzyme, and this is related to the nature of the organic components and their partial weights, but in general, a decrease in the Km value in the soil indicates an increase in the affinity between the enzyme and the substrate in that soil. Tabatabai *et al.* (1994) indicated that the Km value does not depend on the concentration of the enzyme, but rather it is a property of it, and the value of Km is used as a relative measure of the affinity with the reactant, as the lower the value of Km, the greater the affinity or homogeneity between the enzyme and the reactant.

Table 3: Km (molar) and Vmax values (P. nitrophenol gm^{-1} soil 1 h^{-1}) for unpolluted and cadmium-polluted soil under the influence of bio inoculation and humic acids according to the Lineweaver-Burk equation

Pollution level	Kinetic parameters	Treatments					
		Control	I_B	I_F	I_{Mix}	A_H	A_F
Cd0	Vmax	413.223	568.182	636.943	714.286	598.802	617.284
	Km	0.029	0.028	0.026	0.022	0.024	0.025
Cd1	Vmax	340.136	500.000	581.395	662.252	555.556	588.236
	Km	0.031	0.030	0.029	0.027	0.028	0.029

Figure 3 shows a plot of the linear relationship according to the Hanes-Woolf equation between the concentrations of P. nitrophenol as a substrate for the alkaline phosphatase enzyme [S] in molar and [S] / V, where V represents the speed of substrate decomposition measured in units of µg P. nitrophenol gm^{-1} soil 1 h^{-1} for both Study soil (unpolluted and polluted with cadmium). From the straight-line equations in Table 4, the values of Vmax and Km were calculated from the slope and intercept shown in Table 5 through the relationship 1/Vmax and Km/Vmax.

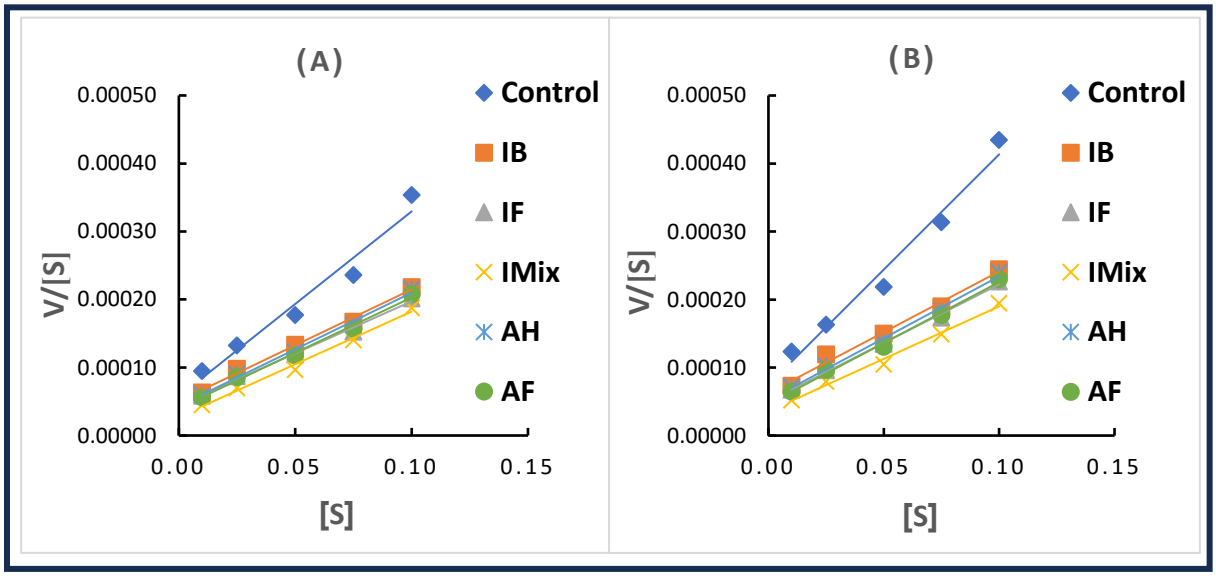


Figure 3: The linear formula of the Hanes-Woolf equation for the activity of the alkaline phosphatase enzyme under the influence of bio inoculation and humic acids for soil A) unpolluted and B) polluted

with cadmium (V): activity of the alkaline phosphatase enzyme measured in micrograms P. nitrophenol gm^{-1} soil 1 h^{-1}, S: substrate concentration in molar

Table 4: The equation of the straight line and the correlation coefficient (r) for the relationship between the substrate concentration (Molar) and the activity of the alkaline phosphatase enzyme (µg P. nitrophenol gm^{-1} soil 1 h^{-1}) for unpolluted and polluted soil with cadmium under the influence of bio inoculation and humic acids according to the equation Hanes-Woolf

Pollution level	Cd_0		Cd_1	
Treatments	Equation	r	Equation	r
Control	Y=0.00272x+0.00006	0.9797	Y=0.00339x+0.00007	0.9875
I_B	Y=0.00164x+0.00005	0.9962	Y=0.00179x+0.00006	0.9926
I_F	Y=0.00153x+0.00004	0.9967	Y=0.00172x+0.00005	0.9979
I_{Mix}	Y=0.00150x+0.00003	0.9951	Y=0.00154x+0.00004	0.9952
A_H	Y=0.00166x+0.00004	0.9967	Y=0,00182x+0.00005	0.9966
A_F	Y=0.00162x+0.00004	0.9968	Y=0.00179x+0.00005	0.9976

Table 5 shows the variation of the values of the kinetic parameters Vmax and Km depending on the inoculation and the type of humic acids. The results showed the highest value of Vmax for bioinoculation in unpolluted soil and soil polluted with cadmium in the I_{Mix} treatment, which amounted to 666.667 and 649.351 µg P. nitrophenol gm^{-1} 1 h^{-1} over Consecutively, the coefficients can be arranged in order of superiority of the Vmax values as follows: $I_{Mix} > I_F > I_B$. The reason may be that microorganisms are considered the primary source of most soil enzymes and that any change in their numbers and types leads to a change in the concentration and source of the enzyme, which works to change the values of Km and Vmax (Hui *et al.,* 2013), while in the humic acid treatments, the highest value of Vmax appeared in the A_F treatment of unpolluted and cadmium-polluted soil, reaching 617.284 and 558.660 µg P-nitrophenol gm^{-1} 1 h^{-1}, respectively, which is a similar value to the A_H treatment, which It reached 602,410 and 549,451 µg P. nitrophenol gm^{-1} 1 h^{-1}, respectively. The reason may be that fulvic acid has a low molecular weight and a relatively small particle size of (80-100) nanometers compared to humic acid (150-300) nanometers, which makes it more available for absorption by organisms, thus increasing the number of enzyme-producing organisms (Zhang *et al.,* 2009).

Table 5 shows the variation of the values of the affinity constant (Km) depending on the type of inoculation and the type of humic acids. The lowest value of Km appeared in the I_{Mix} inoculation treatment relative to the rest of the treatments and amounted to 0.019 and 0.026 M in the unpolluted and cadmium-polluted soil respectively while in the cadmium treatment. Humic acids the highest values of Km appeared when treated with fulvic acid, reaching 0.025 and 0.028 M, respectively, for unpolluted and cadmium-polluted soils, as these treatments with low values of Km indicate an increase in affinity between the enzyme and the substrate, and that the difference in the Km value between soils may be because these Values change depending on the amount of organic matter, its molecular weight, and the nature and types of microorganisms in the soil. It also does not depend on the concentration of the enzyme, but rather is a characteristic of that enzyme, and its value can be used to identify the behaviour of an enzyme in the soil (Tan *et al.,* 2020).

Table 5: Km (molar) and Vmax values (P. nitrophenol gm^{-1} soil 1 h^{-1}) for unpolluted and cadmium-polluted soil under the influence of bio inoculation and humic acids according to the Hanes-Woolf equation

Pollution level	Kinetic parameters	Treatments					
		Control	I_B	I_F	I_{Mix}	A_H	A_F
Cd0	Vmax	367.647	598.802	653.595	666.667	602.410	617.284
	Km	0.022	0.030	0.026	0.019	0.024	0.025
Cd1	Vmax	294.985	558.659	581.395	649.351	549.451	558.660
	Km	0.021	0.033	0.029	0.026	0.027	0.028

Figure 4 shows a drawing of the linear relationship according to the Eadie-Hofstee equation between the activity of the alkaline phosphatase enzyme V, where V represents the speed of decomposition of the substrate measured in µg P. nitrophenol gm^{-1} soil 1 h^{-1} and from the straight-line equations Table 6 The values of Vmax and km were calculated from the slope and intercept. Shown in Table 7 through the relationship V / [S], where [S] represents the molar concentration of both cadmium-polluted and unpolluted soils.

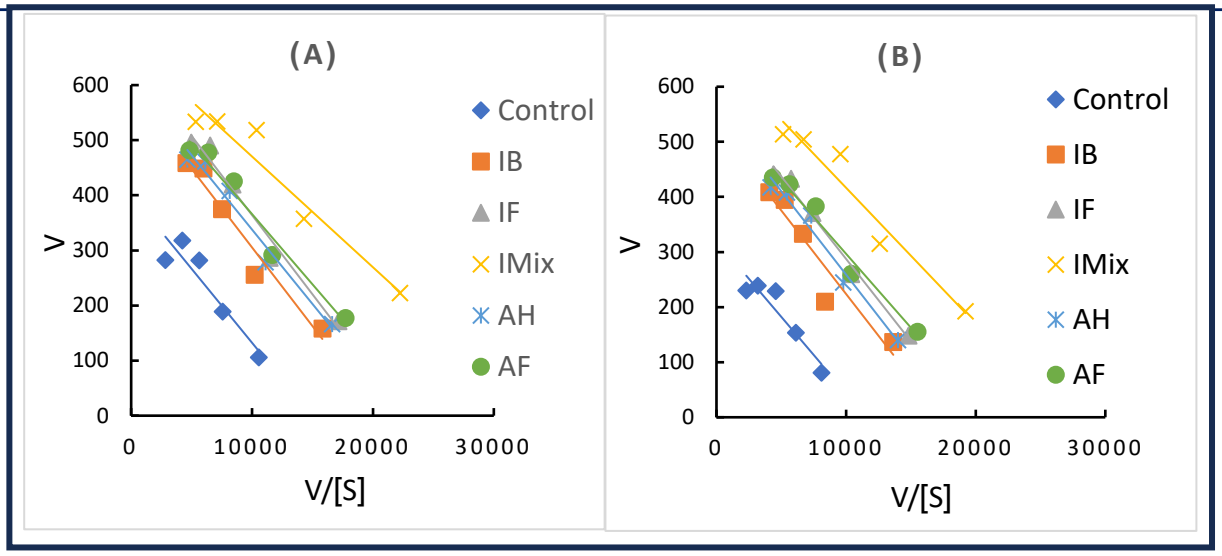


Figure 4: Linear formula of the Eadie-Hofstee equation for the activity of alkaline phosphatase for soil A) unpolluted and B) polluted with cadmium, under the influence of bio inoculation and humic acids (V: activity of alkaline phosphatase measured in micrograms P. nitrophenol gm^{-1} soil 1 h^{-1}, S: substrate concentration in molar)

Table 6: The equation of the straight line and the correlation coefficient (r) for the relationship between the substrate concentration (Molar) and the activity of the alkaline phosphatase enzyme (µg P. nitrophenol gm^{-1} soil 1 h^{-1}) for unpolluted and polluted soil with cadmium under the influence of bio

Pollution level	Cd_0		Cd_1	
Treatments	**Equation**	**r**	**Equation**	**r**
Control	Y=400.87-0.0269x	0.9311	Y=320.60-0.0277x	0.9389
I_B	Y=590.94-0.0286x	0.9765	Y=526.90-0.0303x	0.9512
I_F	Y=651.52-0.0287x	0.9847	Y=586.69-0.0299x	0.9932
I_{Mix}	Y=671.37-0.0201x	0.9688	Y=663.15-0.0247x	0.9718
A_H	Y=607.94-0.0270x	0.9850	Y=561.67-0.0303x	0.9859
A_F	Y=621.00-0.0255x	0.9842	Y=564.63-0.0268x	0.9871

Inoculation and humic acids according to the equation Eadie-Hofstee

Table 7 shows the variation of the values of the kinetic parameters Vmax and Km depending on the inoculation and type of humic acids in both unpolluted and cadmium-polluted soils. The maximum value of Vmax in the I_{Mix} treatment reached 671.370 and 663.150 µg P. nitrophenol gm^{-1} 1 h^{-1}, respectively. The treatments can be arranged in order. In terms of the superiority of the Vmax values, they are as follows: $I_{Mix} > I_F > I_B$. The reason for the superiority of the biological mixture treatment may be due to the existence of a symbiotic relationship between the fungus *A. niger* and the bacteria *B. subtilis*, which increased the secretion of the enzyme by these organisms (Kjeldgaard *et al.,* 2019), while In the humic acid treatments, the highest value of Vmax appeared in the AF treatment of unpolluted and cadmium-polluted soil, which amounted to 621,000 and 564,630 µg P. nitrophenol gm^{-1} 1 h^{-1}, respectively, and it is a similar value to the A_H treatment, which amounted to 607,940 and 561,670 µg P. nitrophenol gm^{-1} 1 h^{-1}, respectively. The reason may be that fulvic acid contains more functional groups such as carboxyls, phenols, and hydroxyls than humic acid, and it can chelate binary ions without precipitating them. They are also considered humic acids considered a nutritional source rich in energy for microorganisms (Sun *et al.,* 2020) .

Table 7 shows the variation of Km values according to the type of inoculation and the type of humic acids. The highest values of Km appeared in the comparison treatment and amounted to 0.029 and 0.031 M, respectively, while the lowest value of Km was in the I_{Mix} inoculation treatment and amounted to 0.020 and 0.025 Molar in for unpolluted and cadmium-polluted soil respectively, while the humic acid treatment recorded the highest Km values in the A_H treatment, which amounted to 0.027 and 0.030 M, respectively, for unpolluted and cadmium-polluted soil, as these treatments with low Km values show increased affinity between the enzyme and the substrate.

Table 7: Km (molar) and Vmax values (P. nitrophenol gm^{-1} soil 1 h^{-1}) for polluted and cadmium-polluted soil under the influence of bio inoculation and humic acids according to the Eadie-Hofstee equation

		Treatments

Pollution level	Kinetic parameters	Control	I_B	I_F	I_{Mix}	A_H	A_F
Cd0	Vmax	400.870	590.940	651.520	671.370	607.940	621.000
	Km	0.027	0.029	0.029	0.020	0.027	0.026
Cd1	Vmax	320.600	526.900	586.690	663.150	561.670	564.630
	Km	0.028	0.030	0.030	0.025	0.030	0.027

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