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Research Paper

Effect of the substrate and inoculation of phosphate-dissolving bacterial and fungal with humic acids in the activity of the alkaline phosphatase enzyme in cadmium-contaminated soil

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ABSTRACT

A laboratory experiment was carried out in the laboratories of the Department of Soil Sciences and Water Resources, College of Agriculture, University of Basrah, to identify the role of the bacteria Bacillus subtilis and the fungus Aspergillus niger, with humic acids extracted from fermented cow dung, the effectiveness of the alkaline phosphatase enzyme in soil contaminated with cadmium and compare it with uncontaminated soil. The soil was treated with a cadmium sulfate solution up to the critical limit (3 mg Cd L-1) and humic and fulvic acid were added at a level of 50 L ha-1 separately, leaving the treatment without addition for control. Then the soil was inoculated with bacterial B. subtilis and fungal A. niger isolates individually and with a mixture of isolates, leaving a treatment without inoculation for control, the treatments were incubated at a temperature of 28±2 °C. Different levels of substrate concentration were used (0.010, 0.025, 0.050, 0.075, and 0.100 M). The results showed that cadmium contamination decreased alkaline phosphatase enzyme activity by 12.28%. There was also an increase in the activity of the alkaline phosphatase enzyme at all inoculation treatments compared to the control treatment, with the bio-mixture treatment being superior to the rest of the treatments, reaching 436.944 µg P. nitrophenol gm-1 soil 1 hour-1 in uncontaminated soil and 402.558 µg P. nitrophenol gm-1 soil 1 hour-1 in cadmium-contaminated soil. The humic acid treatment showed a similar increase in the activity of the alkaline phosphatase enzyme compared to the control treatment the highest increase was in the fulvic acid treatment. As for the effect of the substrate, the concentration of 0.075 M recorded the highest increase in alkaline phosphatase enzyme activity in unpolluted and cadmium-polluted soil for all treatments.

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1. INTRODUCTION

Soil pollution in recent years is one of the most prominent environmental problems, the most complex, and the most difficult to solve. The development of the global economy and the increase in human activities have led to an increase in soil pollution, especially pollution with heavy metals, as it is colourless odourless and difficult to notice. Studies estimate that there are more than 5 million hectares of soil around the world that have hazardous metals exceeding their critical limits in soil [1]. The resulting damage may not appear directly or over short periods, but when environmental conditions change, these metals become active in the soil and cause serious environmental damage over time [2]. 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. These include lead (Pb), cadmium (Cd), nickel (Ni), mercury (Hg), zinc (Zn), arsenic (As), chromium (Cr), cobalt (Co), copper (Cu), and manganese (Mn) [3]. Cadmium is considered one of the main heavy elements polluting the environment, as high cadmium concentrations can have toxic effects on biological activities in the soil and can be easily transferred to vegetation and ultimately enter the human food chain [4]. Heavy metals arise from natural and human sources and are eventually introduced into the environment. The main natural sources are volcanic eruptions, weathering of rocks containing these metals, and sea spray [5]. As for human sources of these metals, It occurs through various human activities such as mining, smelting, electroplating, coal combustion, vehicle exhaust, factories, chemical compounds, electric power, electronic devices, batteries, medical waste, and oil derivatives, which increases the occurrence of disturbance in the biological environment with the accumulation of large quantities of heavy metals in the environment, especially in the soil [6], [3], [7].

In a study conducted by [8] in the Rumaila oil field in Basrah Governorate that the ready concentration of heavy metals (Cd, Cu, Fe, Zn and Pb) is 0.015, 0.787, 0.021, 0.515 and 4.304 µg gm^{-1}, respectively. Traditional agricultural practices are among the most important human sources of heavy metals in the soil, as the use of pesticides and organic fertilizers such as liquid fertilizer and solid materials of biological origin, as well as inorganic fertilizers, have contributed to the continuous accumulation of these elements [9]. Phosphate fertilizers usually contain Cd and Zn, and excessive use of these fertilizers leads to the deposition of higher concentrations of heavy metals in the soil [10], [11], [12]. Soil microbial activity is often considered an effective indicator of contaminated ecosystems because of its sensitivity to soil environmental conditions more than molluscs, microorganisms, or plants. The extent of soil pollution and its effects can be determined through microbial changes in the soil, mainly through the impact on biodiversity, soil microbial activity, and enzyme activity in Soil [13]. Soil enzymatic activities decrease or even inhibit with increasing concentrations of heavy metals because they cause negative effects on the main biological functions in the soil such as the decomposition of organic matter, and enzymatic and biotic activities and thus affect soil quality [14]. When these metals reach the cell, they can combine with proteins and the DNA base and hinder the functional groups of many enzymes, as the heavy elements bind to the active sites of the enzyme and the electron-donating groups, which leads to the displacement of some of the cations necessary for the cell's structure and functions, and thus Disrupting the function of these vital molecules and inhibiting the reproduction and growth of soil organisms [15], [16]. The common fungi that produce basic phosphatase are from the genera Aspergillus sp. especially A. niger, as well as Penicillium sp. Many bacteria produce phosphatase from the genera Bacillus sp. such as B. subtilis as well as Pseudomonas sp. [17], [18], [19], [20]. Found a significant increase in the activity of the alkaline phosphatase enzyme in soil inoculated with T. hamatum and A. niger, reaching % 47.05 and % 58.80 for both fungi, respectively,

compared to the control treatment noting that A. niger was superior to its counterpart in increasing enzyme activity .

Humic acids are of great importance in improving the vital properties of the soil as a result of the changes they cause, which are represented in encouraging the growth and reproduction of beneficial microorganisms in the soil due to their high carbon and nitrogen content. They have a major role in the biotic and abiotic interactions that occur in the plant roots which reflect positively on the soil various chemical and biological processes, including enzyme activities in soil. The ability of microbes to decompose humic substances is usually linked to enzymes because extracellular enzymatic hydrolysis is the first step required for microbes to adsorption of humic substances. The enzyme is stabilized by organic colloids and trapped by humus particles.

Table 1. Soil Microbial Population and Alkaline Phosphatase Activity

Bacteria Numbers	Fungi Numbers	The Activity of the Alkaline Phosphatase
cfu gm^{-1} soil		µg P. nitrophenol gm^{-1} soil 1 $hour^{-1}$
257.30	4.89×10^4	10^6

2. METHODOLOGY

A soil sample was taken from the surface layer 0-30 cm from a site planted with wheat crops in the Nahr Saleh area of the Medina district in 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 October 2021. Soil samples were placed in plastic bags and brought to the laboratory, Part of them was 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 2 According to what was Mentioned in [21], [22], [23], [24].

Table 2. Some Properties of the Study Soil

Ph	Organic Matter	Organic Carbon	$Caco_3$	EC	Ions Avail	
					P	Cd
	Ds M^{-1}	Gm Kg^{-1} Soil			Mg Kg^{-1} Soil	
13.68	0.00	4.27	2.48	364.53	4.73	7.89

2.1 Extraction of Humic Acids

Humic acids were extracted after fermenting cow dung for two months. After the end of the fermentation period (two months), humic acid was extracted by taking a certain weight of fermented organic materials, treating it with 0.1 M sodium hydroxide (NaOH), and leaving it for the next day. It was observed that a precipitate, which is human had formed and was disposed of, while the filtrate was humic and fulvic acids, then concentrated hydrochloric acid (HCl) was added until the pH of the soil reached about 2 and left until the next day, after which a precipitate formed, which was humic acid as for the filtrate it was fulvic acid, and the pH value of acid was adjusted humic and fulvic levels to 6.5 according to the method described by [24].

2.2 Preparation of Bacterial and Fungal Inoculum

The bacterial inoculum was prepared by growing it in tubes containing Slant nutrient Agar medium (N.A.), then the growing bacterial cells were added to the liquid Nutrient broth medium and incubated at a temperature of 30 °C for five days. As for the fungal inoculum, it was grown in Potato Dextrose Broth medium (PDB). by adding 0.5 cm diameter discs of pure colonies to the sterile liquid nutrient medium (PDB) and incubating at a temperature of 28±2 °C for five days.

2.3 Soil Preparation and Inoculation

A certain weight of the study soil was placed in clean plastic containers, then the soil samples were contaminated with cadmium salts at a concentration of 3 mg kg^{-1} soil, which represents %100 of the critical limit concentration of cadmium according to [25]. It was symbolized as Cd1 and the treatment was left without adding cadmium for control It has Cd0. Cadmium was added to the soil by dissolving the salt in an amount of water equivalent to the field capacity of the soil, then mixing % 2 of peat moss sterilized in an autoclave at 121 °C and 15 pounds ng^2 with the 5-day-old bacterial and fungal, inoculum at a rate of 20 ml per container, with a numerical density of the fungi added to the soil of 40 × 10^3 cfu ml^{-1} of soil with a bacterial population density of 20.06 × 10^7 cfu ml^{-1} individually. for the control sample is denoted by I_0, the bacterial inoculum is denoted by I_B, and the fungal inoculum is denoted by I_F. As for the mixture of bacteria and fungi, it is denoted by I_{Mix} with the addition of humic acid, which is denoted by A_H. Fulvic acid, which is symbolized by A_F at a level of 50 L ha^{-1}, leaves a treatment without humic acids and is symbolized by A_0 for control. The soil moisture was brought to the limits of field capacity and incubated at a temperature of 28±2 °C for 30 days. While maintaining soil moisture at Field capacity limits by compensating the weight difference with distilled water, taking into account stirring the soil for aeration during the experiment period.

Table 3. Physical Properties and Texture Classification of Soil

Clay Ratio	Loam Ratio	Sand Ratio	Field Capacity	Soil Texture
%				
Clay loam	37.90	34.70	27.40	29.30

2.4 Measurement of Alkaline Phosphatase Activity

Enzyme activity was measured according to the method of [26]. By incubating 1 gm of soil with 0.2 ml of toluene solution and 4 ml of basic buffer solution consisting of (12.1 gm of THAM, 11.6 g of Maleic acid, 14 gm of Citric acid, 6.3 gm of Boric acid dissolved in 488 ml of 1 M ammonium hydroxide and adjusting the pH to 11 using 0.1 M sodium hydroxide (NaOH) and completing the volume to a litter with distilled water) and adding 1 ml of di-Sodium-4-nitrophyenyl phosphate Hexahydrate solution ($NO_2C_2H_4OPO_2Na_2.6H_2O$) dissolved in the basic buffer solution. As a substrate, it was incubated at a temperature of 37 °C for an hour. After incubation, 1 ml of a solution of 0.5 M calcium chloride, $CaCl_2$, and 4 ml of a solution of 0.5 M sodium hydroxide, NaOH, was added as an inhibitor. Then the solution was filtered through Whatman filter paper No. 42 and 1 ml of the filtrate was taken. To estimate the yellow colour using a Philips PU8670 spectrophotometer at a wavelength of 420 nm. Different concentrations of substrate were used in the study (0.010, 0.025, 0.050, 0.075, and 0.100 M).

3. RESULTS AND DISCUSSION

3.1 The Effect of Biological and Organic Reclamation on the Activity of Alkaline Phosphatase Enzyme in Cadmium-Contaminated Soil

The results of Figure 1 show that treating the soil with cadmium led to a decrease in the activity of the alkaline phosphatase enzyme compared to natural, uncontaminated soil. The lowest value when contaminated with cadmium was 303.488 µg P. nitrophenol gm^{-1} soil 1 h^{-1}, with a decrease rate of % 12.284 compared to uncontaminated soil. The reason may be because cadmium, when it enters the microbial cell, combines with proteins and the DNA base and obstructs the work of the functional groups of many enzymes, as cadmium binds primarily to the active sites of the enzyme and the electron-donating groups which leads to the displacement of some of the cations necessary for the cell's structure and functions thus disrupting the work of the enzyme These biomolecules inhibit the reproduction and growth of soil microorganisms [16].

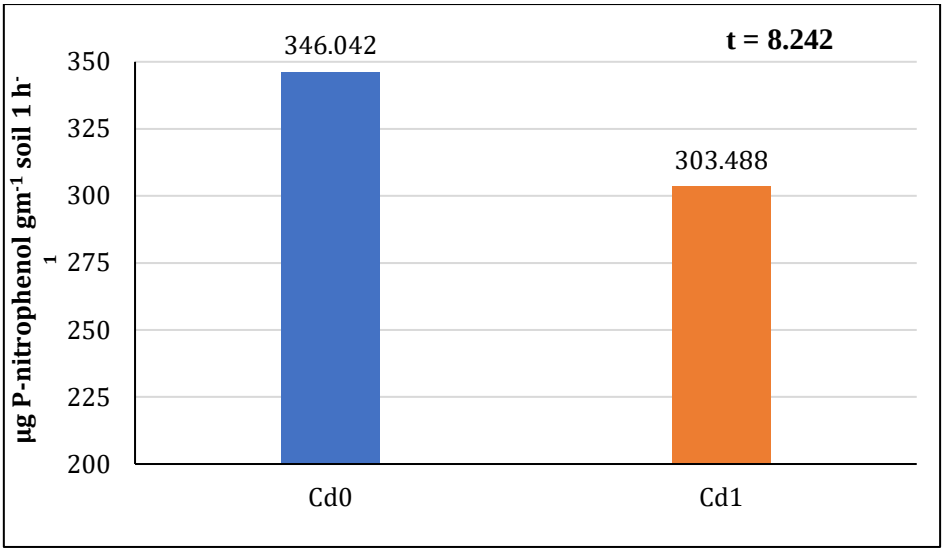


Figure 1. The Effect of Cadmium-Contaminated on the Activity of the Alkaline Phosphatase Enzyme

Figure 2 shows a significant increase in the activity of the alkaline phosphatase enzyme in uncontaminated soil treated with I_B, I_F, and I_{Mix}, as the percentage of increase reached % 44.065, % 58.354, and % 85.632, respectively, compared to the control treatment I_0. It is noted from the figure that there are significant differences between all biological species and the treatment excelled I_{Mix} is superior to its counterparts in increasing enzyme activity. This may be because bioinoculation increased the rate of enzyme activity, as the bacteria *B.* subtilis and the fungus *A. niger* are microorganisms that produce the enzyme alkaline phosphatase, and there is also a symbiotic relationship between bacteria and fungi, as bacteria grow on mycelium [27]. Figure 2 also shows a significant increase in the activity of the alkaline phosphatase enzyme in the cadmium-contaminated soil treated with I_B, I_F, and I_{Mix}, reaching 61.713%, 75.325%, and 116.828%, respectively, compared to the control treatment I_0. It is noted from the same figure that there are significant differences between the biological species, and the I_{Mix} treatment was superior on the rest of the parameters the reason may be because microorganisms have advanced methods that enable them to survive in the presence of heavy elements polluting the environment, as the vital cell wall consists of polysaccharides, peptides, proteins, and compounds with effective groups that bind the ions of the heavy elements, and these groups are carboxylates, hydroxyls, and amines. And phosphate groups [14]. Bacteria and fungi also depend on the participation of various internal and external enzymes in binding heavy metal ions and reducing their biological risks [28].

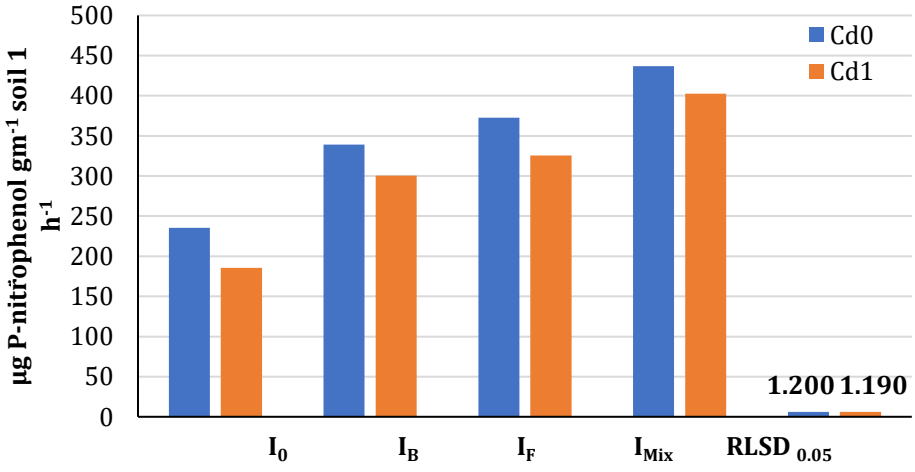


Figure 2. The Effect of Bioinoculation on the Activity of the Alkaline Phosphatase Enzyme in Uncontaminated and Cadmium-Contaminated Soil.

I_0: Control I_B: *B. subitils* I_F: *A. niger* I_{Mix}: *B. subitils* + *A. niger*

Figure 3 shows a significant increase in the activity of the alkaline phosphatase enzyme in soils not contaminated with cadmium and treated with A_H and A_F compared to the control treatment A_0, as the enzyme activity reached 356.635 and 371.627 µg P. nitrophenol gm^{-1} soil 1 h^{-1}, respectively, because humic acids It works to increase the stimulation of extracellular enzymes in the soil. It is also noted that there are significant differences between the two types of organic acid, as the treatment with fulvic acid was superior to humic acid. This may be because fulvic acid contains a low percentage of C: O compared to humic acid, which increases its solubility in water so that it is more readily available to microorganisms [29]. Figure 3 shows a significant increase in the activity of the alkaline phosphatase enzyme in soil contaminated with cadmium and treated with A_H and A_F, as the activity of the enzyme reached 316.992 and 331.505 µg P. nitrophenol gm^{-1} soil 1 h^{-1}, respectively, compared to the control treatment A_0. It is noted that there are significant differences between the two types of acid, as Fulvic acid treatment was superior to humic acid, and the reason may be that fulvic acid contains higher active sites than humic acid, such as carboxyl, phenol, and hydroxyl, which bind with cadmium and remove it from the soil solution, thus reducing its toxicity and effect [30].

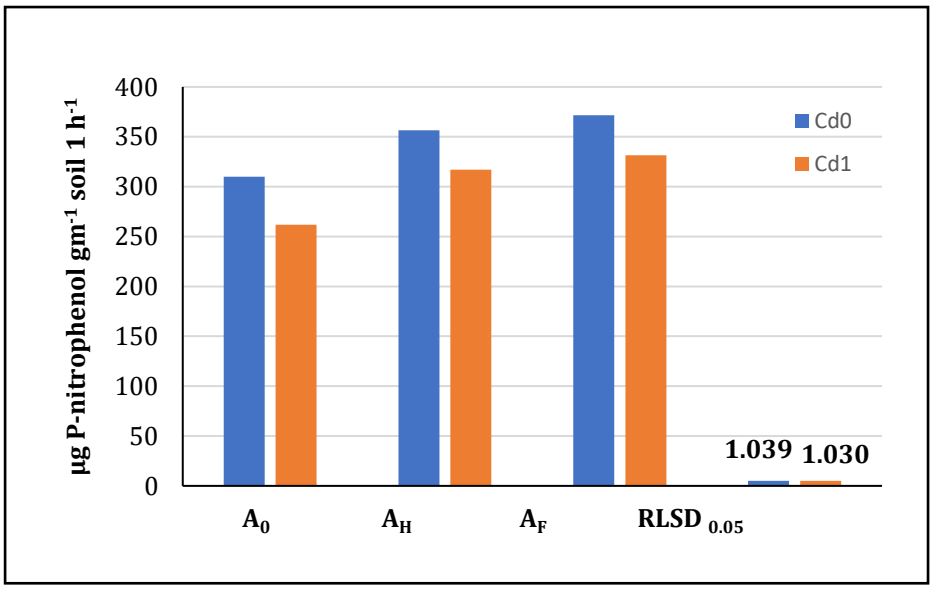


Figure 3. The Effect of Humic Acids on the Activity of the Alkaline Phosphatase Enzyme in Uncontaminated and Cadmium-Contaminated Soil.

A_0: Control A_H: H. Acid A_F: F. Acid

Figure 4 (a, b) shows that the binary interaction between inoculation and humic acids had a significant effect on increasing the activity of the alkaline phosphatase enzyme, as the maximum activity of the alkaline phosphatase enzyme when treated with A_F I_{Mix} in uncontaminated and cadmium-contaminated soil reached 458.873 and 427.353 µg P. nitrophenol gm^{-1} soil 1 h^{-1}, respectively, while the I_{Mix} A_H treatment for uncontaminated and contaminated soil reached 442,980 and 413,500 µg P. nitrophenol gm^{-1} soil 1 h^{-1}, respectively. The reason for the superiority of the A_F I_{Mix} treatment over the I_{Mix} A_H treatment may be due to the small size of the particles. Fulvic acid, which is (80-100 nanometres) compared to the size of humic acid molecules, which is (150-300 nanometres), makes it more suitable for living organisms due to its ease of entry into the membranes of living cells [31]. Biovaccination also increased the effectiveness of the organisms that produce the enzyme alkaline phosphatase are a result of the existence of a symbiotic

relationship between the fungus *A. niger* and *B. subtilis*, where the bacteria grow on the mycelium and multiply, which increases the production of the enzyme [27]. The addition of heavy elements in the soil negatively affects the biological environment, including Decreased enzyme activity in the soil, as it was found that the activity of the alkaline phosphatase enzyme was negatively associated with heavy metals in the soil [32].

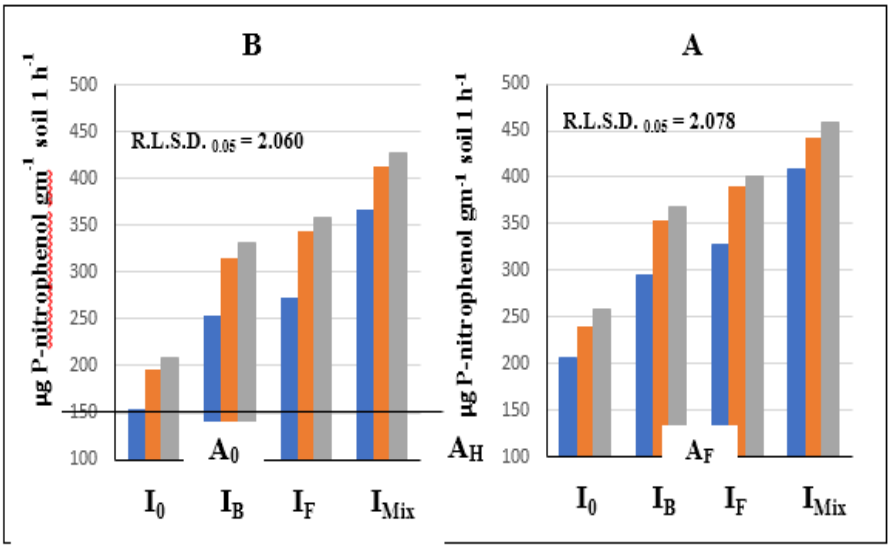


Figure 4. The Effect of the Binary Interaction Between Bio Inoculum and Humic Acids on the Activity of the Alkaline Phosphatase Enzyme in Soil: A) Uncontaminated B) Cadmium-Contaminated

3.2 The Effect of the Substrate on the Activity of the Alkaline Phosphatase Enzyme

Figure 5 shows an increase in the activity of the alkaline phosphatase enzyme with increasing substrate concentration for both uncontaminated and cadmium-contaminated soils. This reaction is subject to a First-order reaction until the substrate concentration corresponding to the maximum activity of the enzyme is reached then it is subjected to a Zero-order reaction, and the maximum activity of the alkaline phosphatase enzyme was at a substrate concentration of 0.075 M and for all treatments for uncontaminated and cadmium-contaminated soil and amounted to 447.733 and 398.839 µg P. nitrophenol gm^{-1} soil 1 h^{-1}, respectively, and this is due to the saturation of the active sites of the enzyme with the reactant [33].

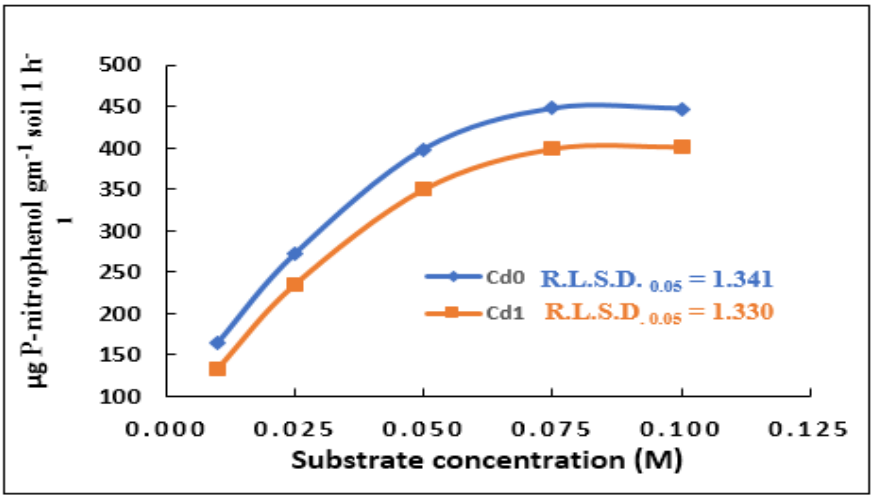


Figure 5. Effect of Substrate Concentration on the Activity of Alkaline Phosphatase Enzyme in Uncontaminated and Cadmium-Contaminated Soil

Figure 6 (a, b) shows that the binary interaction between inoculation and substrate concentration had a significant effect in increasing the activity of the alkaline phosphatase enzyme and that the maximum activity of the enzyme was in the I_{Mix} treatment with a substrate concentration of 0.075 M for both uncontaminated and cadmium-contaminated soils, as it reached 553.689 and 514.033 µg P. nitrophenol gm^{-1} soil 1 h^{-1}, respectively. This may be because the reacting materials combined with the active sites in the enzyme molecules and formed intermediate compounds. By continuing to add the reacting materials, it is possible to reach the state in which the active sites in the enzyme molecules are saturated, as a result of which the enzyme molecules do not increase. The speed of the reaction. On the contrary, increasing the concentration of the reacting materials may have an inhibitory effect on the enzymatic activity sometimes [34]. The reason may also be because the bacteria *B.* subtilis and the fungus *A. niger* are organisms that contribute to the production of the alkaline phosphatase enzyme in the soil and as a result of the relationship Symbiosis between the two species of organisms [35]. The I_{Mix} treatment outperformed the rest of the treatments.

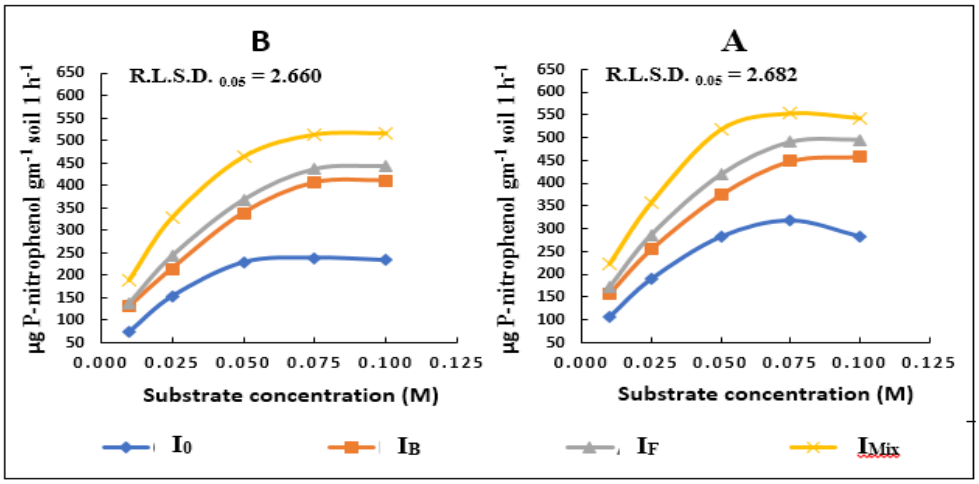


Figure 6. The Effect of the Binary Interaction between Bio Inoculation and Substrate Concentration on the Activity of the Alkaline Phosphatase Enzyme in Soil A) Uncontaminated B) Cadmium-Contaminated

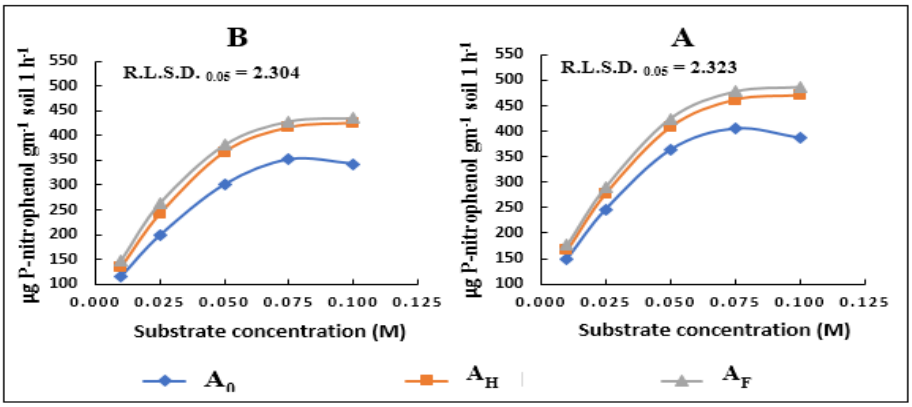


Figure 7. The Effect of the Binary Interaction between Humic Acids and Substrate Concentration on the Activity of the Alkaline Phosphatase Enzyme in Soil A) Uncontaminated B) Cadmium-Contaminated

Figure 7 (a, b) shows that the binary interaction between the concentration of the substrate and humic acids has a significant effect in increasing the activity of the alkaline phosphatase enzyme and that the maximum activity of the enzyme was in the A_F treatment at the substrate concentration of 0.075 M for uncontaminated and cadmium-contaminated soil, as it reached 477.683 and 428.942 µg P. nitrophenol gm^{-1} soil 1 h^{-1} respectively. The reason for the superiority of fulvic acid treatment may be due to it containing a

high percentage of nutrients that are considered an energy source for the growth and activity of microorganisms, which increases the production of the alkaline phosphatase enzyme. Fulvic acid also contains sites more effective than humic acid, thus increasing the binding of cadmium ions and reducing its effect on enzyme-producing organisms [36]. The reason for the stability of the activity of the phosphatase enzyme with increasing substrate concentration is due to the saturation of the active sites of the enzyme with the reactant [37], [38], [39], [40], [41].

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Author Contributions Statement

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Meiad Mahdi Al-Jaberi	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

Conflict of Interest Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Informed Consent

All participants were informed about the purpose of the study, and their voluntary consent was obtained prior to data collection.

Ethical Approval

The study was conducted in compliance with the ethical principles outlined in the Declaration of Helsinki and approved by the relevant institutional authorities.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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