International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 2s, 2025
https://www.theaspd.com/ijes.php

Environmental Effect of Native and Mutated *B. subtilis* And *P. fluorescens* On the Removal of Lead and Cadmium over Time

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ABSTRACT: This experiment was conducted in the laboratories of the Department of Soil Science and Water Resources, College of Agriculture, University of Basrah. Soil samples were collected from various areas of Basrah governorate . This study showed the effect of inoculation with *B. subtilis* and *P. fluorescens* (both native and physically mutated) on the removal rate of heavy metals (lead and cadmium) from the liquid nutrient medium. Native *B. subtilis* achieved a removal rate of 90.80% compared to 75.51% for *P. fluorescens*. For *P. fluorescens* mutated for 60 minutes, a significant improvement in removal rate was observed, reaching 80.47%. The study indicated that mutated bacteria enhanced their ability to remove heavy metals in the contamination at critical concentrations (100 and 3 ppm) did not show a significant impact on the removal rate . Furthermore, native *B. subtilis* outperformed *P.fluorescens* in both 2 and 4 week incubation periods in removing heavy metals. The mutated strains showed better results after two weeks compared to longer incubation period .The results support previous studies indicating that genetically mutated organisms enhance bioremediation by improving microbial activity and tolerance to heavy metals.

Key words: *B. subtilis*, *P. fluorescens*, heavy metals, lead, cadmium, mutation, Pollution, Bioremediation.

INTRODUCTION:

The environment is exposed to pollution due to improper disposal methods of industrial waste, such as those from the production of paper products, paints, dyes, rubber, plastics, detergents, pesticides, organic solvents, chemicals, fuel additives, and pharmaceuticals, These industries are major contributors to the use of heavy metals and as a result metallic waste is released as a byproduct globally each year (Al-Saadi *et al.,* 2016; Mehvish *et al.,* 2020; Hu *et al.,* 2023). Heavy metals are among the most toxic pollutants compared to organic pollutants in the soil , These metals exist in the environment at very low concentrations, which can harm living organisms due to their accumulation in the food chain , They can be natively present or added to the soil through human activities, High levels of these elements can lead to the destruction of soil microorganisms and some of them pose a risk to health and the environment (Annu and Urmila, 2016; Dutta *et al.,* 2020). Heavy metals can reduce microbial populations and plant diversity as long as they persist in the soil, The only effective method for removing these elements is through the extraction of the contaminated heavy metals, which can be achieved by various physical and chemical treatments of the soil, However, both methods are costly and negatively impact soil quality and biodiversity (Borymski and Piotrowska , 2014). There is an increasing need to develop a new effective and cost friendly environmental approach to treatment of inorganic elements such as lead released into the environment and to protect the ecosystem. Therefore, it is necessary to develop an effective strategy to treat water and soil contaminated with these various pollutants, which has led to recent developments based on pushing microbes to bioremediation as a potential alternative to traditional techniques (Igiri, 2018). Microorganisms, especially resistant bacteria, are considered a promising environmental solution to reduce the toxicity of these elements, as well as the need to explore bioremediation technology further to achieve better results by highlighting the sources of The importance of heavy element pollution and the mechanisms of bacterial

resistance to this pollution (Mehvish *et al.,* 2020). Microorganisms are characterized by their high efficiency and ability to trap heavy elements and reduce their concentration in complex solutions. This is attributed to their behavior as a metal ion absorber and to the chemical functional groups contained in microbial cells such as carboxyl, hydroxyl, phosphite, amine and amide, which are responsible for biosorption Ion exchange pattern, complex formation, rearrangement, adsorption, chelation, and microbial precipitation (El-Said *et al.,* 2016). Therefore, successful bioremediation involves the integration of environmental microbiology and genetic engineering techniques (Rajak, 2017). The use of native and genetically mutated isolates of microorganisms to remove heavy elements such as lead has been found that genetically mutated microorganisms have outperformed in removing lead when compared to native bacteria (Al-Masry and Amin, 2017). Mutagenesis is of great importance in genetic engineering, as it consists of changing the genetic information of a living microorganism in a fixed and stable manner in nature or in experimental laboratories using methods Physical such as ultraviolet rays (Al-Zubaidi and Al-Taie, 2008). Microorganisms are considered an important scientific attraction due to the ease of genetic modification and handling them ,and related to the modern biotechnology techniques, and the widespread exploitation of microorganisms to serve humanity, genetic engineering is a powerful tool for exploring biological diversity. Widely used in the environment, and focused on the adaptation of microbial organisms to harsh environmental conditions, the benefit of applying genetic engineering science used in microbial biotechnology is improving the microbial environment, biodegradation of foreign materials, and its impact on agricultural crops (Rangasamy *et al.,* 2020).

The aim of the experiment: The biological removal of heavy elements such as lead and cadmium and the reduction or elimination of pollution resulting from these elements from the environment, such as water and soil, which can pose a major threat due to their toxic and persistent effects, so living organisms are used Live bacteria such as *B. subtilis* and *P. flourescens*, both natural and genetically modified, are used to remove or convert these elements into non-toxic or less toxic forms to protect the environment and public health from environmental hazards resulting from heavy metal pollution.

MATERIALS AND METHODS:

Soil samples were prepared by adding 1 g of the soil sample to 9 mL of sterilized water. *Bacillus subtilis was* isolated by inoculated on (LB) agar according to (Dusane *et al.,* 2013). *Pseudomonas fluorescens* was isolate by inoculated on King's agar according to (Stephane and Jacques, 2000). The Petri dishes were incubated at 30°C ± 2°C for 3 days. The growing colonies were examined for bacterial identification, which served as an initial indicator of the growth of *B.subtilis* and *P.fluorescens*. The bacterial isolates and identified based on cultural, morphological, biochemical, and molecular characteristics ,salt solutions were prepared in the form of lead acetate and cadmium chloride at the critical concentration levels of the elements (100 and 3 ppm respectively) according to (Kloke, 1980).

Subsequently, the studied bacteria were mutated using ultraviolet (UV) radiation for tow durations. UV lamp was used, and 1 mL of a 48-hour-old bacterial culture was placed in a sterile Petri dish and irradiated in the dark at a distance of 30 cm high from the radiation source for 30 and 60 minutes, with a wavelength range between 200-280 nanometers. After the irradiation periods, the plates were covered in sterilized aluminum foil to prevent photo-reactivation and left for 5 minutes. Then, 2 mL of the irradiated suspension was taken and plated on sterilized Petri dishes containing nutrient agar. The dishes were incubated in an incubator at a temperature of 28°C±2 for 7 days (Al-Shammari and Taha 2017). After that, the natural bacteria were diagnosed using the Polymerase Chain Reaction (PCR) technique.

Table (1) Sequence of nitrogenous bases of *P. flourescens* and *B. subtilis*

Bacterial Type	Starter Name	Nucleotide sequence	
P. fluorescens	16SrRNA	GGGCGGTGCGGCGTGCTATAC	F
		TCAAATCTGTCACCTTAGGCG	R
B. Subtilis	16SrRNA	CGCAGTGGCGCAGCTATACATGCAAGT	F
		ACAAAATTCTGGTCACCTTCGGCGGCT	R

The efficiency of native and genetically mutated bacterial isolates to grow in liquid nutrient broth media contaminated with heavy elements (lead and cadmium) at a concentration of 100 and 3 ppm was tested by placing 150 ml of the contaminated liquid nutrient medium in a 250 ml flask. The flasks were sterilized in an autoclave at a temperature of 121 °C and a pressure of 15 pounds of inch-2 for 20 minutes and were inoculated with isolates of the natitve and mutated bacterial genera *B. subtilis* and *P. fluorescens*. 1 ml of a 48-hour-old fresh culture at a density of 1.5×108 cells/ml-1 of each bacterial isolate was added, and the flasks were incubated at 30°C±2 for *B. subtilis* and *P. fluorescens* for two periods of time (2 and 4 weeks) in a shaking incubator.

After incubation, 10 mL of the liquid medium withdrawn using a sterile pipette and centrifuged at 6000 rpm. (The centrifugation process was repeated three times to obtain a clear supernatant). The concentrations of lead and cadmium in the supernatant were measured using an Atomic Absorption Spectrometer (AAS) model Phoenix-986. The removal percentage of the studied elements were calculated as follows:

$$Removal\ percentage\% = \frac{initial\ concentration - remaining\ concentration}{initial\ concentration} * 100$$

Statistical Analysis:

Analysis of variance (ANOVA) was used to evaluate the effect of the two bacteria genes (native and mutated) removal percentage of Heavy metals from broth medium using SPSS ver. 19.0 program. Means were using Revised Least Significant Differences (RLSD) test at a significance level of 0.01 (Al-Rawi, and Khalaf ,1980).

RESULTS AND DISCUSSION:

Figure 1 shows that inoculation with *B. subtilis* and *P. fluorescens* (native and mutated) significantly p≤ 0.01 affected the removal percentage of heavy metals (lead and cadmium) from the liquid nutrient medium, The removal percentage from the medium inoculated with the native *B.subtilis* bacteria reached 90.80%, compared to the native *P. fluorescens*, which achieved a removal percentage of 75.51%. As for the treatment inoculated with mutated B. *subtilis* for 30 and 60 minutes, also showed significant difference, with removal percentage of 78.37% and 76.27%, respectively. The treatment inoculated with mutated P. *fluorescens* for 60 minutes showed a significant p≤ 0.01 improvement, with a removal percentage of 80.47%, compared to the 30-minute mutated and native bacteria, which had removal percentage of 75.67% and 75.51%, respectively. This suggests that the mutation of *P. fluorescens* for 60 minutes improved its ability to remove heavy metals.

This improvement could be attributed to the bacterial assistance in removing accumulated heavy metals through bioremediation. These microorganisms are used as biosensors to control heavy metal contamination in ecosystems. This is facilitated by genes encoded to resistant to heavy metals, which are located on plasmids, and the removal occurs through biosorption, which aids in removing heavy metals from the environment (Igiri *et al.,* 2018).

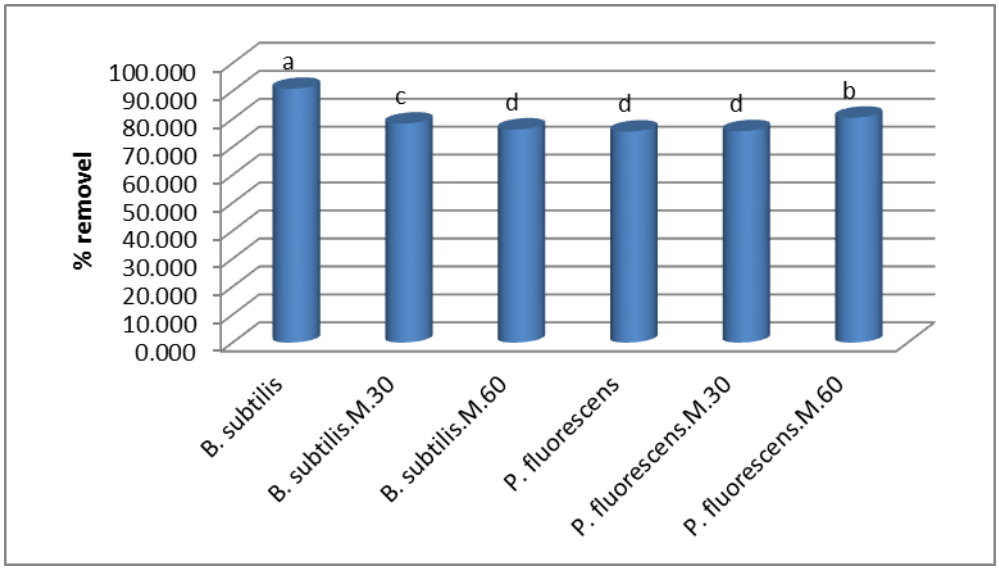

Figure (1) Effect of inoculation with *B. subtilis* and *P. fluorescens* both native and Mutated on the removal percentage of heavy metals (lead and cadmium).

The results showed that there is no significant difference between the removal percentage of the two studied heavy metals (lead and cadmium) which were 80.195% and 78.84% respectively.

Figure 2 illustrates the superior performance of *B. subtilis* (native strain) after 2 and 4 weeks of incubation in removing heavy metals. The removal percentage in the liquid nutrient medium was 91.74% and 89.86%, respectively, compared to *P. fluorescens* (native strain), which achieved removal percentage of 85.60% and 65.43% for the same durations, respectively. A higher removal percentage was observed for the mutated *B. subtilis* for 30 minutes (84.21%) compared to 60 minutes (79.97%) after 2 weeks of incubation. However, no significant difference in removal percentage was observed between the 30 and 60minute irradiation after 4 weeks of incubation (72.53% and 72.56%, respectively). For the mutated *P. fluorescens* at 30 minutes, the removal percentage after 2 weeks of incubation was 85.08%, which did not significantly differ from the native strain for the same period but was higher than the 30-minute mutated strain after 4 weeks of incubation, which achieved a removal percentage of 66.26%. It was also noted that the mutated *P. fluorescens* for 60 minutes showed no significant difference between the two incubation periods (2 and 4 weeks), with removal percentage of 80.63% and 80.31%, respectively. This suggests that the binding sites in the bacteria become saturated after just 2 weeks of incubation, indicating that this time is sufficient for bioremediation by *P. fluorescens*. While when we compare the two types of genetically mutagenic bacteria, we notice that the mutagenic *B. subtilis* bacteria for 30 minutes and *P. fluorescens* bacteria for 60 minutes are superior in the removal percentage . The results agree with (Benjamin *et al.* ,2019) in their study that removing the accumulation of heavy elements with the help of bacteria Especially with the use of genetically modified microorganisms in bioremediation, the strate gic relationship between genetically modified microbes and bioremediation can enhance the effectiveness of pollutant removal, using modern genetic changes to increase the activity of microbes and their role in improving the tolerance of genetically modified microbes to heavy elements and detoxification. Microorganisms such as bacteria need a certain amount of time to grow and reproduce until they reach sufficient numbers to perform their biological functions effectively, such as absorbing heavy elements. A short incubation period may not allow these organisms to grow sufficiently and therefore may be Its ability to remove heavy elements is weak due to toxicity. If the incubation period is short, organisms may not be able to adapt to the presence of these heavy elements in large quantities, which leads to inhibition of their growth or even death compared to the long

incubation period, in which microorganisms have more time to develop mechanisms for biosorption of heavy elements or accumulation inside the cells. Microorganisms may show a greater ability to adapt to the presence of heavy metals during a longer incubation period, and this adaptation occurs more effectively through the development of new tolerance mechanisms or the secretion of enzymes that contribute to the stabilization or transformation of heavy metals into less toxic forms (Marajan *et al.*, 2020; Muzhda and Yahya ,2023).

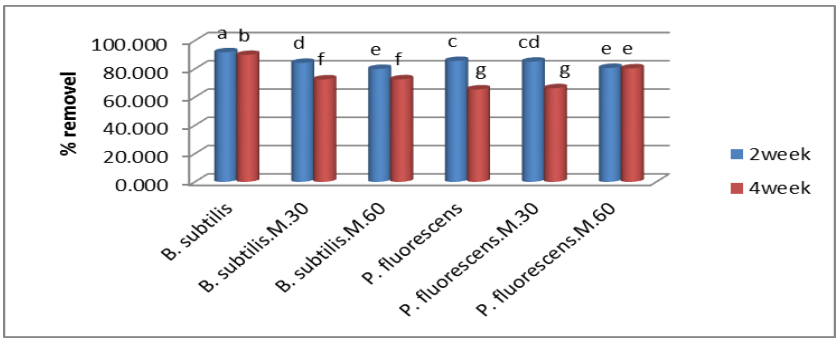

Figure (2) Effect of the interaction between inoculation with natural and genetically mutant *B. subtilis* and *P. fluorescens* bacteria on the removal percentage of heavy elements over different time periods.

Figure 3 shows a significant difference in the effect of the incubation periods (2 and 4)weeks on the removal percentage of heavy elements (lead and cadmium).The removal percentage for lead was 86.45% after 2 weeks of incubation, while the 4-week incubation resulted in a lower removal percentage (71.23%). For cadmium removal, the rate was 82.63% after 2 weeks of incubation, while it was lower after 4 weeks of incubation, reaching 77.76% . This is consistent with the study of Lila *et al.* (2018) which indicated that bacterial isolates are exposed to adverse growth conditions, such as heavy metal stress, which causes damage to microorganisms, leading to reduced cellular activity, decreased growth rate and cell density and hindering cell proliferation, thus reducing the number of bacteria due to the death of some bacteria. This is reflected in reducing the removal percentage of microorganisms and developing different tolerance mechanisms towards heavy elements, including reducing the activity of these organisms and converting them to adapt to live and withstand the stress of heavy elements. Also, the removal percentage vary due to the difference in inoculation with microorganisms and their major role in the process of bioaccumulation and the adsorption of heavy elements by different mechanisms and their transformation into organic complexes within the living cell tissue, as well as the association of heavy element ions with biopolymers, especially in peptidoglycan and the outer cell wall (Yadav *et al.*, 2017).

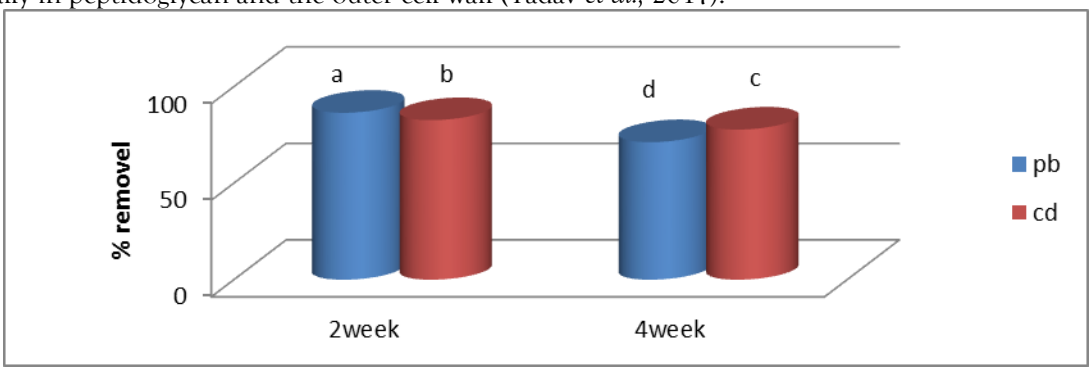

Figure (3) The effect of heavy element pollution and its removal percentage over different time periods

Figure 4 shows the ability of both bacterial species to remove heavy elements. The results showed that *B. subtilis* bacteria significantly outperformed the efficiency of removing lead from the culture medium, as the removal percentage reached 84.21% compared to With *P. fluorescens* bacteria, the removal percentage of lead reached 73.47%, while the results showed that *P.fluorescens* bacteria was the most efficient in removing cadmium, which reached 80.97%. Compared to Bacillus subtilis, which had the lowest removal percentage of cadmium, reaching 79.42%, this indicates that each of the microorganisms has different mechanisms that enable it to tolerate heavy element pollution and the ability to remove one heavy element without others. This is what studies have indicated that different bacterial species differ in their ability to remove heavy elements according to the type of bacteria, as some species have more efficient mechanisms to tolerate or convert one of the Heavy elements and not others, and have effective tolerance systems against heavy elements, or the presence of resistant genes, as some bacteria contain special genes in plasmids or chromosomes that enable them to resist heavy elements, These genes may give bacteria the ability to tolerate high concentrations of toxic elements or even convert them into less toxic forms. The levels of heavy metal pollution in the environment also affect the ability of bacteria to deal with those elements. Bacteria living in environments containing high concentrations of heavy elements may develop stronger resistance strategies, While other bacteria may be less adaptable depending on the type and concentration of the element (Debora *et al.*, 2024 and Al Jaberi, 2024).

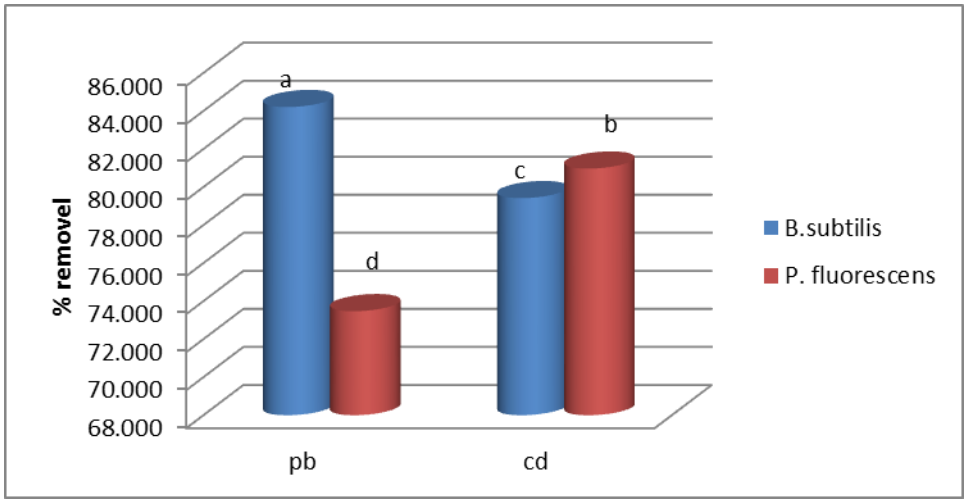

Figure (4) Effect of bacterial species on the percentage of heavy element pollution removal

Table 2 shows significant differences in the triple interaction between the studied treatments in the percentage of heavy metal removal. We note that the genus and type of bacteria have a significant effect on the percentage of removal. The native bacteria *B. subtilis* had the best percentage of lead removal for 2 weeks of incubation, which reached 96.77%, and for cadmium 93.48% for 4 weeks of incubation in the liquid nutrient medium. As for *P. fluorescens* , it was better in removing lead in 2 weeks of incubation, reaching 95.29% .However, for cadmium, *P. fluorescens* achieved a removal the percentage of 79.46% , after 4 weeks of incubation. For genetically mutated B. subtilis strains, the one exposed to UV radiation for 30 minutes showed the highest removal percentage for lead at 2 weeks of incubation, with 84.98%. The UV-mutated *B. subtilis* strain exposed for 60 minutes achieved 87.93% removal of lead after 4 weeks of incubation. In contrast, for cadmium, the genetically mutated *B. subtilis* strain exposed for 60 minutes showed the best removal percentage of 90.75% after 2 weeks of incubation, compared to 83.45% for the strain exposed for 30 minutes. Regarding *P. fluorescens* (genetically mutated for 30 and 60 minutes), For lead, the best removal percentage was observed at 30 minutes of UV irradiation after 2

weeks of incubation, reaching 88.98%. The 60-minute UV irradiation duration showed the best removal percentage for cadmium after 4 weeks of incubation, with a removal percentage of 94.06 %. When comparing the two bacterial genera (native and genetically mutated) in the liquid growth medium, the results showed a significant effect for the genetically mutated *B. subtilis*. The highest removal percentage was achieved with 60 minutes of UV irradiation for 4 weeks of incubation, with a removal percentage of 87.93% for lead, compared to 30 minutes of irradiation for the same period. However, for cadmium, the 60-minute UV irradiation duration for 2 weeks of incubation had a significant effect on the removal percentage , but after 4 weeks of incubation, the removal ability decreased to 57.20%, compared to 64.92% for the 30-minute irradiation period.

For *P. fluorescens*, the highest removal percentage for lead was observed after 30 minutes of UV irradiation for 2 weeks of incubation, with a removal percentage of 88.98%, and for cadmium, 81.19%. Meanwhile, the 60-minute UV irradiation duration after 2 weeks of incubation showed a better removal percentage for lead, with 83.51%, followed by the same duration at 4 weeks of incubation, with 66.56%. As for cadmium, the 60-minute UV irradiation duration was more effective at 4 weeks, with a removal percentage of 94.06%.

This may be due to the fact that genetically mutated bacterial isolates, free of plasmids, are more resistant to heavy metals. In the natural bacterial isolate, the heavy element enters the cell through a common active transport system, while in the mutant, the active transport of the heavy element is significantly reduced compared to the natural bacterial strain, and its tolerance to heavy elements is achieved by using various genetic techniques to be useful in the mechanism of tolerance of heavy elements in the environment (Scales *et al.*, 2014). Some bacteria also contain special genes in their plasmids or chromosomes that enable them to resist heavy metals. These genes may give bacteria the ability to tolerate high concentrations of toxic metals or even convert them into less toxic forms (Debora *et al.*, 2024).

Table 2 Effect of triple interaction between inoculation with native and genetically mutated *B.subtilis*, *P. fluorescens* and heavy metals on the removal percentage over different time periods

Heavy metals	pb		cd		mean
Bacteria	2week	4week	2week	4week	
B. subtilis	96.77 ^{a}	86.24 ^{gh}	86.70 ^{fg}	93.48 ^{c}	90.80
B.subtilis.M.30	84.98 ^{hi}	80.14 ^{kl}	83.45 ^{j}	64.92 ^{q}	78.37
B. subtilis.M.60	69.19 ^{o}	87.93^{ef}	90.75 ^{d}	57.20 ^{r}	76.27
P. fluorescens	95.29 ^{b}	51.40^{t}	75.91 ^{n}	79.46 ^{l}	75.51
P. fluorescens.M.30	88.98 ^{e}	55.09 ^{s}	81.19 ^{k}	77.44 m	75.67
P. fluorescens.M.60	83.51 ^{ij}	66.56 ^{p}	77.76 ^{m}	94.06 ^{bc}	80.47
mean	86.45	71.23	82.63	77.76	79.52

CONCLUSION:

This study highlights the role of bacteria in reducing elements from contaminated culture media. B. subtilis was more effective in removing lead, while *P.fluorescens* was superior in removing cadmium. Experiments of genetic mutation by UV radiation for 30 minutes of *B. subtilis* bacteria showed that it enhanced its ability to remove heavy elements, but when the exposure period increased to 60 minutes, its ability to remove the process decreased. In contrast, *P. fluorescens* exposed to UV for 60 minutes outperformed the strain exposed to a shorter period of 30 minutes. These results suggest that mutagenesis can improve the ability of bacterial strains to remove Heavy metals such as lead and cadmium, offering

potential for bioremediation and use in sustainable agriculture and pollution reduction. Further research is needed to understand the mechanisms and improve their use in ecosystems.

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