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The growth capability of natural and mutated bacteria in a solid nutrient-contaminated medium with lead and cadmium

Huda A. Yaseen Aljanabi[1]*, Meiad M. Al-Jaberi[2]
[1,2]Department of soil and water resources, College of Agriculture, University of Basrah, Iraq; huda.yassen@uobasrah.edu.iq (H.A.Y.A) meiad.naama@uobasrah.edu.iq (M.M.A).

Abstract: This study investigated the growth of natural and mutated bacterial strains (B. subtilis and P. fluorescens) in nutrient agar contaminated with heavy metals (lead and cadmium). The results showed significant differences ($p \leq 0.01$) in bacterial counts for the various treatments. B. subtilis natural outperformed P. fluorescens natural in unpolluted medium, but in lead-contaminated medium, B. subtilis also showed better growth. In contrast, P. fluorescens had a higher count than B. subtilis in cadmium-contaminated medium. Mutagenesis of B. subtilis for 30 minutes led to a significant increase in growth across all media, while 60-minute mutations resulted in reduced growth, particularly under heavy metal stress. For P. fluorescens, 30-minute mutations showed improved growth in lead-contaminated medium compared to the natural strain, while 60-minute mutations also performed well in cadmium-contaminated medium. The findings suggest that mutation can enhance bacterial resistance and tolerance to heavy metals, likely due to changes in DNA and protein structures. These adaptations may involve mechanisms like ion efflux, bioaccumulation, and biotransformation, contributing to the bacteria's ability to survive in contaminated environments. These results highlight the potential of using mutated bacteria for the bioremediation of heavy metal-polluted environments.

Keywords: *B. subtilis, Cadmium, Heavy metals, Lead, Mutation, P. fluorescens, Pollution.*

1. Introduction

Pollution is one of the major and most dangerous problems that humans face it due to the increasing population growth and rapid industrialization and modern agricultural practices to improving living standards, Humans play a clear role in this through their various activities, which have become a threat to human life as well as to other living organisms, This has caused a disruption in the natural balance of the environment and its various living and non-living components, All these developments were not created to serve humanity alone, but also created strong challenges. Therefore, there is a need for combined efforts to treated and reduce them [1, 2].

Heavy metals exist in the soil in various chemical forms, such as water-soluble and exchangeable, depending on their association with specific sites in the organic and inorganic components of primary and secondary elements in agricultural soils, The concentration of heavy metals increases with the development and formation of the soil, and their concentration can be influenced by changes in their movement within the soil due to environmental conditions, land use for agriculture, and climate change, Additionally, the transport of heavy metals largely depends on their chemical form and their absorption in the soil through rapid initial reactions that occur within minutes or hours, This is followed by slow adsorption reactions that take days or even years, with redistribution in different chemical forms through the availability of biodiversity in the soil [3, 4]. Also, as a result of industrial activities, pollution, or natural processes, this may affect the health of the ecosystem and human safety [5].

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* Correspondence: huda.yassen@uobasrah.edu.iq

Heavy metals refer to all elements with a density greater than 5 g/cm^3 and atomic weights ranging from 63.5 to 200.6, with atomic masses greater than 4000 kg/m^3, which is five times greater than water [6, 7]. The ecosystem is more affected in soils contaminated with heavy metals, making environmental pollution a major issue impacting biodiversity, public health, and ecosystems worldwide. The chemical interactions with heavy metals in the soil increase their concentration, in addition to the influence of the heavy metal behavior by the chemical and physical properties of the soil, especially the particle size distribution, density, pH, and the presence of elements within it, which negatively affects the growing microbes [8, 9].

Microorganisms, animals, and plants are affected by heavy metals such as lead and cadmium due to the toxicity of these elements, which lead to the deterioration of metabolic activity and the destruction of microbial communities. Therefore, there is a need for effective methods to remove these elements, with a focus on biological processes as an effective, environmentally friendly, and safe solution compared to costly physical and chemical methods [10, 11]. Lead and cadmium have toxic and inhibitory effects on microorganisms' activities and vital functions, thus reducing soil enzyme activity. The toxicity of heavy metals in the soil is due to the mechanism by which these heavy metals bind to functional groups in enzymes through stable bonds and in the form of complexes, leading to the inhibition of molecules involved in metabolic reactions [12, 13].

This study aimed to induce physical mutations using ultraviolet radiation in the bacterial species *Bacillus subtilis* and *Pseudomonas fluorescens*, and to compare the natural and mutated bacteria in their growth capabilities in a nutrient medium contaminated with heavy metals such as lead and cadmium.

2. Materials and Methods

Soil samples were collected from various areas of Basrah province. The laboratory experiment was conducted in the laboratories of the Department of Soil Science and Water Resources at the College of Agriculture, University of Basrah. A series of dilutions were prepared for each soil sample by adding 1 g of the soil sample to 9 ml of sterilized water, which was placed in 10 ml tubes and mixed well. A series of decimal dilutions (10^{-1} to 10^{-7}) was then performed by transferring 1 ml of the soil suspension to test tubes containing 9 ml of sterilized water. Subsequently, 1 ml of the 10^{-5} and 10^{-6} soil dilutions prepared above was used to isolate *Bacillus subtilis*, which was spread on a sterile Petri dish and covered with Lauria-Bertani (LB) agar medium according to [14]. Also, *Pseudomonas fluorescens* which was spread on a sterile Petri dish and covered with sterile King's agar medium according to [15].

The Petri dishes were incubated at 30°C ± 2 for 3 days. The dishes were examined by observing the formation of bacterial colonies, which served as an initial indicator of the growth of *Bacillus subtilis* and, *Pseudomonas fluorescens*. Identification of the pure cultures was accomplished by cultural properties, morphological and microscopically characteristics. The colonies were then purified to obtain pure isolates were preserved on slants of solid nutrient medium for daily use.

Subsequently, the process of bacterial mutation was performed using ultraviolet (UV) radiation for different time durations according to [16]. A Yamad UV lamp was used. 1ml of a 48-hour-old bacterial culture was placed in a sterile Petri dish and exposed to UV radiation in the dark at a distance of 30 cm from the radiation source for 30 and 60 minutes, with a wavelength range between 200-280 nanometers. After the irradiation period, the Petri dishes were wrapped in sterile aluminum foil to avoid photo-reactivation and left for 5 minutes. Then, 2 ml of the irradiated suspension was taken and cultured on sterile Petri dishes containing solid nutrient agar medium, and the dishes were incubated at temperature was carried out at 28°C ± 2 for 7 days.

2.1. DNA Extraction, PCR Amplification, and Sequencing

DNA extraction was done according to commercial kits DNA preparation kit (DONGSHENG BIOTECH) protocol. The quality and quantity of extracted DNA were measured by mixing a portion of the extracted DNA with Ethidium Bromide dye on a prepared agarose gel at a concentration of 2% in

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100 ml of TEB Buffer (a mixture of Tris base, boric acid, and EDTA) at a concentration of 100 mM, prepared by (Bio Basic Co.), for the purpose of confirming its purity through electrophoresis. After that, the natural and mutated bacteria were identified using the Polymerase Chain Reaction (PCR) technique.

Table 1.
Some characteristics of the primers used for detecting the DNA sequence of *P. fluorescens* and *B. subtilis* bacteria.

Bacterial type		Nucleotide sequence	Starter name
P. fluorescens	F	GGGCGGTGCGGCGTGCTATAC	16SrRNA
	R	TCAAATCTGTCACCTTAGGCG	
B. subtilis	F	CGCAGTGGCGCAGCTATACATGCAAGT	16SrRNA
	R	ACAAAATTCTGGTCACCTTCGGCGGCT	

The solutions were prepared using lead salts in the form of lead acetate and cadmium salts in the form of cadmium chloride at the critical concentration levels for the elements (100 and 3 ppm, respectively) according to [17]. The growth efficiency of natural and mutated bacterial isolates was then tested in nutrient agar contaminated with heavy metals (Pb and Cd) and sterilized in an autoclave at 121°C and 15 pounds per square inch (psi) for 20 minutes.

Both natural and mutated bacterial species of *B. subtilis* and *P. fluorescens*, were inoculated with contaminated and non-contaminated nutrient agar by using 1 ml of a 48-hour-old culture on sterile Petri dishes in Three replicates were used for each treatment, and the plates were incubated at 30°C ± 2, for one week. Afterward, the growing colonies on both the contaminated and non-contaminated nutrient media were counted.

3. Statistical Analysis

Analysis of variance (ANOVA) was used to evaluate the effect of the two factors: efficacy of bacteria (natural and mutated) tolerance and Heavy metals on number of bacteria using SPSS ver. 19.0 program. Means were using Revised Least Significant Differences (RLSD) test at a significance level of 0.01.

4. Results and Discussion

The isolate showed a difference from the reference standard strains at one of the nucleotide base sequence sites. The sequence was sent to the National Center for Biotechnology Information (NCBI) and the nucleotide sequence data of these isolates were registered in the GenBank. The isolate (H230110) was registered in the GenBank with the accession number (PP419989) and showed a 99% similarity to the *B. subtilis* strain, while the isolate (H230110 003) in the GenBank with the accession number (PP556850) showed a 99% similarity to the *P. fluorescens* strain.

Figure 1 show that the natural and mutated bacterial strain, belonging to the species *B. subtilis* and *P. fluorescens*, significantly $p \leq 0.01$ differed in their growth ability in the nutrient agar contaminated with heavy metals. The colony count of *B. subtilis* was higher than that of *P. fluorescens*, with 230×10^6 and 190×10^6 cfu, respectively.

When comparing the natural bacteria with the mutated bacteria after exposure to ultraviolet radiation for 30 and 60 minutes, the growth ability of the genetically mutated B. subtilis for the 30 minute exposure time was significantly $p \leq 0.01$ higher than the natural bacteria, with an average colony count of 290×10^6 cfu, showing increase percentage a 26.08% over the natural bacteria. However, when comparing the mutated B. subtilis after 60 minutes of exposure with the natural bacteria, the count was 130×10^6 cfu, which was decrease percentage a 43.47% compared to the natural bacteria.

In contrast, the growth of mutated *P. fluorescens* after 60 minutes of exposure UV radiation with a bacterial count of 170×10^6 cfu was significantly $p \leq 0.01$ higher than mutated bacteria after 30 minutes, which had a bacterial count of 160×10^6 cfu, showing a decrease percentage 5.88%. Meanwhile, the growth rate of both mutated *P. fluorescens* bacteria after 30 and 60 minutes of exposure decreased by 15.78% and 10.52% percentage, respectively, compared to the natural bacteria.

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When comparing the two bacterial species after exposure to UV radiation, it was observed that the 30-minute mutation duration for *B. subtilis* outperformed than 60 minute. However, the 60 minute for *P. fluorescens* showed better growth efficiency than the 30 minute, but still lower than the number of the natural bacteria. This could be attributed to the fact that genetic mutations can significantly impact the growth efficiency of bacteria, when mutations occur in the bacterial DNA, they can lead to changes in the genes that control growth and reproduction processes. These changes can either increase the rate of bacterial growth or improve their ability to adapt to changing environments, or conversely, have a negative effect [18].

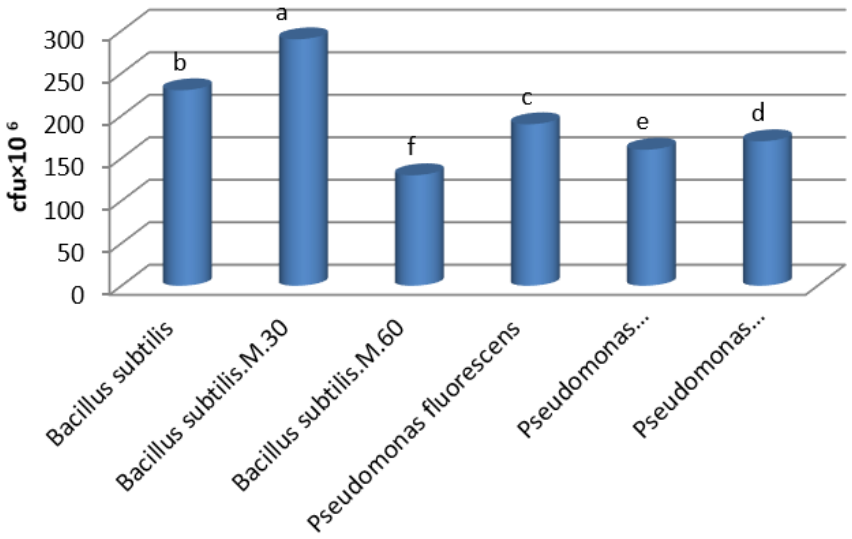

Figure 1.
Growth of *B. subtilis* and *P. fluorescens* (natural and mutated) in solid nutrient agar medium contaminated with heavy metals.

Figure 2 show a significant $p \leq 0.01$ decrease in the growth of both *B. subtilis* and *P. fluorescens* (natural and mutated) in the nutrient agar contaminated with heavy metals. The bacterial count in the contaminated medium with lead and cadmium was 190×10^6 cfu, showing a 5% of reduction compared to the control treatment (200×10^6 cfu). This decrease could be due to the heavy metals (lead and cadmium) causing damage to the cell membranes, altering enzyme functions, disrupting metabolism, hindering protein synthesis, and altering their structure, thus damaging the DNA structure. This occurs by displacing elements from their original binding sites or interacting with bound molecules, leading to disruptions in cellular functions, membrane destruction, enzyme inhibition, and oxidative phosphorylation, which primarily causes toxicity and negatively impacts bacterial growth and numbers [19].

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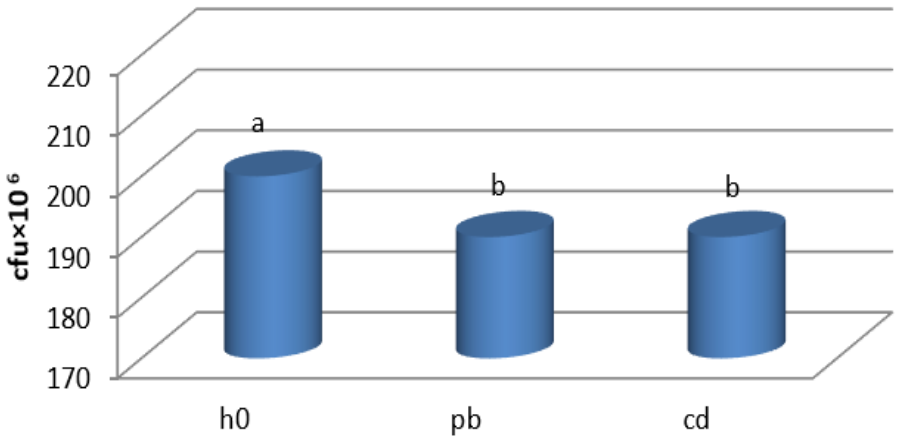

Figure 2.
Effect of heavy metal contamination in the solid nutrient agar medium on the bacterial colony counts of natural and genetically mutated isolates.

The table shows significant p≤0.01 differences in bacterial counts for the studied treatments. The results indicated that *B. subtilis* (natural) outperformed *P. fluorescens* (natural) in the unpolluted nutrient agar, with bacterial counts of 270 × 10^6 and 250 × 10^6 cfu, respectively. In the contaminated medium with lead, *B. subtilis* outperformed *P. fluorescens*, with counts of 260 × 10^6 and 140 × 10^6 cfu, respectively. In the cadmium contaminated medium, *P. fluorescens* outperformed *B. subtilis*, with counts of 170 × 10^6 and 150 × 10^6 cfu, respectively.

When comparing both natural and mutated bacteria of each species, a significant p≤0.01 increase in *B. subtilis* mutated for 30 minutes was observed, with bacterial counts of 280 × 10^6, 300 × 10^6, and 290 × 10^6 cfu respectively in the unpolluted, lead contaminated, and cadmium contaminated medium , However, for *B. subtilis* mutated for 60 minutes, the bacterial count decreased compared to the natural bacteria and the 30-minute mutated bacteria, with counts of 100 × 10^6 in the unpolluted and contaminated medium with lead and cadmium. 150 × 10^6 and 130 × 10^6 cfu, respectively. the decrease in microbial growth due to stress caused by heavy metals might be due to the fact that microorganisms need to redirect their energy from growth to maintaining cellular functions in order to tolerate heavy metal toxicity [20].

On the other hand, when comparing *P. fluorescens* (natural) and mutated strains, the 30 minute mutated bacteria significantly p≤0.01outperformed the natural bacteria in growth efficiency in the lead-contaminated medium, with counts of 160 × 10^6 cfu compared to the natural bacteria at 140 × 10^6 cfu, and the 60 minute mutated bacteria at 150 × 10^6 cfu. This indicates that mutagenesis of these bacteria for 30 minutes increased its resistance and tolerance to contamination. Many studies have pointed out that exposure of microorganisms to factors that cause mutations due to changes in the nitrogenous bases of their DNA might affect their ability to tolerate heavy metal contamination [21].

Meanwhile, the growth rate of bacteria in the cadmium contaminated medium showed that *P. fluorescens* (natural) outperformed with 170 × 10^6 cfu compared to *B. subtilis* (natural) with 150 × 10^6 cfu. However, the bacteria mutated for 60 minutes showed significant p≤0.01 superiority with a count of 200 × 10^6 cfu over the bacteria mutated for 30 minutes, where their count in the non-contaminated nutrient medium was 120 × 10^6 cfu. No significant differences were observed in the counts of *P. fluorescens* (natural and mutated for 60 minutes) in the medium contaminated with cadmium, where the count was 170 × 10^6 cfu. When comparing between the bacterial species and the mutated strains, an increase in the growth rate was observed for *B. subtilis* mutated for 30 minutes compared to mutated for 60 minutes and *P. fluorescens* mutated for 30 and 60 minutes in nutrient agar contaminated with heavy metals.

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Table 2.
Shows the effect of the interaction between contamination of heavy metal and bacterial species (*B. subtilis* and *P. fluorescens*) in the nutrient agar on bacterial counts (cfu × 10^6).

Bacteria	Bacterial counts (cfu × 10^6)			Mean
	H_o	Pb	Cd	
Bacillus subtilis	270b	260b	150d	230
B.subtilisM.30	280a	300a	290a	290
B.subtilis.M.60	100f	150d	130e	130
P.fluorescens	250c	140e	170c	190
P.fluorescens.M.30	120e	160c	210b	160
P.fluorescens.M.60	200d	150d	170c	170
Mean	200	190	190	200

This is consistent with Jarosławiecka and Piotrowska-Seget [22] who pointed out that the toxicity of heavy metals plays a role in inhibiting vital activities by causing changes in the structure of nucleic acids and proteins in microorganisms. Additionally, the process of mutation alters the structural and biochemical characteristics of bacteria, their growth patterns, and the genes responsible for producing proteins that contribute to the formation of cell wall components. This enhances their adaptation and resistance to the toxic effects of heavy metals. This occurs through several mechanisms, including efflux, where proteins pump ions out through ion transporters, bioaccumulation on the cell surface, and the formation of complexes between metal ions and cellular proteins, as well as biotransformation into a less toxic form of heavy metals [16].

5. Conclusion

This study highlighted the significant impact of mutagenesis on bacterial growth in heavy metal-contaminated media. *B. subtilis* showed better growth than *P. fluorescens* in unpolluted and lead-contaminated media, while *P. fluorescens* outperformed *B. subtilis* in cadmium-contaminated media. Mutagenesis for 30 minutes improved bacterial resistance to heavy metals, particularly in *B. subtilis*, while longer exposure (60 minutes) reduced growth. These findings suggest that mutated bacterial strains can enhance tolerance to heavy metals, offering potential for bioremediation. However, further studies are needed to explore the underlying mechanisms and optimize their use in environmental cleanup.

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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