



# Removal of Nickel, Copper, Lead and Cadmium by New Strains of *Sphingomonas melonis* E8 and *Enterobacter hormaechei* WW28

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## Abstract

**Introduction:** Bioremediation as eco-friendly technique has high potential to clean-up the toxicity of heavy metals from contaminated soil and water. In this study, the bioremediation potential of E8 and WW28 strains which had high similarity to *Sphingomonas melonis* and *Enterobacter hormaechei*, respectively have been evaluated under contaminated mediums with lead (Pb), cadmium (Cd), copper (Cu) and nickel (Ni).

**Materials and Methods:** The growth rate and metal removal percentage of isolated strains were investigated at different ranges of pH 4-8, and temperature (25, 30, 35 and 40°C). Also, the bioremediation potential of isolated strains was studied under mixture of metals (50 mg/L of each metal).

**Results:** The highest cell mass of strain E8 was observed after 48 hours at 30°C and pH 5 while strains WW28 showed high growth rate after 72 hours at 25°C and pH 5. Strains E8 and WW28 preferred to more uptake Ni and Cu than Pb and Cd. In addition, Cd appears to show the highest toxicity towards the isolated bacteria. Strain E8 as multi metals-resistance strain could remove 78%, 62% and 56% of Ni, Cu and Cd, respectively from polluted mediums at pH 6 after 48 hours.

**Conclusions:** Overall results revealed that isolated strains as bio-tools have a high potential to be used in bioremediation process of Ni and multi-metals contaminated sites.

**Keywords:** Bioremediation, Heavy Metals, Environmental Pollution, Bacterial Strains

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## Introduction

Heavy metal contamination, as a serious ecological problem, causes many negative effects on human, animal and plant health.<sup>1,2</sup> Cadmium (Cd) and lead (Pb) are examples of heavy metals with harmful effects on all living cells. Cd as non-biodegradable and non-essential type of metal causes many negative effects on the ecosystem and food safety.<sup>3,4</sup> In addition, Pb is a hazardous heavy metal which causes several problems in human beings including renal failure, cancer and impairment of reproductive system.<sup>5</sup>

On other hand, it has industrial applications, including fuels, storage battery, photographic materials and coating.<sup>6</sup> Nickel (Ni) and copper (Cu) are essential elements for microorganisms, animals and plants. It is said that their deficiencies could decrease cell growth.<sup>7</sup> However increasing the Ni and Cu in soil and water are inducing negative effects on cell physiological and biochemical processes and have toxic effects on plant production and human health.<sup>8</sup> Higher concentrations of Ni could cause severe damages to kidneys, renal edema, lungs, skin dermatitis and pulmonary fibrosis.<sup>9</sup> Different methods such as chemical (oxidation-reduction and neutralization), physical (membrane separation,

electrochemical treatment and ion exchange) and biological (phytoremediation and bioremediation) have been developed to remove and reduce the toxicity of heavy metals from contaminated sites.<sup>10-13</sup>

Bioremediation as a cost-effective and green technique uses the microorganism to clean-up the contaminated environments. Microorganisms such as bacteria strains have a great potential to remove and reduce heavy metal from polluted soil and water.<sup>14</sup> However, environmental factors such as temperature, pH, nutritional status and heavy metal concentrations influence the bioremediation efficiency.<sup>15</sup>

Various bacteria species have been identified and their bioremediation potential were evaluated under polluted environments.<sup>16,17</sup> Among bacterial species, *Pseudomonas* sp and *Bacillus* have been widely evaluated to reduce the toxicity of heavy metals from soil and wastewater.<sup>7,18,19</sup> For example, *Bacillus pumilus*, *Pseudomonas aeruginosa* and *Bacillus thuringiensis* showed high ability to remove Pb, Cd and Ni from contaminated sites.<sup>20,21</sup> However other bacteria strains such as *Microbacterium*, *Rhodococcus* sp, *Enterobacter* and *Rhodobacter* were found as heavy metal-tolerant bacteria.<sup>14,22,23</sup> Metal-resistant bacterial strains use different mechanisms

such as biosorption by cell surface, sequestration in the extracellular, and efflux system to reduce negative effects of heavy metals.<sup>24</sup>

Thus, it is important to isolate and identify new appropriate bacterial strains which have a high potential to reduce the toxicity of heavy metals. In this study, two new Ni-resistant strains of *Sphingomonas melonis* and *Enterobacter hormaechei* were isolated from extreme conditions, and the bioremediation potential of the new isolated bacterial strains was evaluated after exposure to different heavy metals including Ni, Cu, Pb, and Cd. Besides, the effects of environmental factors such as temperature, pH, and metal concentration on bacterial growth rate and bioremediation efficiency was investigated.

## Materials and Methods

### Isolation of Bacterial Strains

The soil samples of coal mine (36°21'36.6"N, 54°42'28.2"E) and wastewater sample of Mareh wetland (34°57'46.0"N, 51°18'19.6"E) of Semnan and Qom provinces of Iran, were collected to isolate the heavy metal-resistant bacteria. The collected samples were diluted by double sterilized water, and 500 µL of solutions was plated in Luria–Bertani (LB) agar. The plates were incubated at 30 °C ± 2 for 72 hours. Finally, the different colonies were sub-cultured in LB agar medium to obtain pure colonies of isolates.

### Screening Heavy Metal-resistant Bacteria

To select heavy metal-resistant bacteria, the isolated strains were cultured in LB agar plates containing 100 mg/L Ni and Cu. After on, plates were incubated at 30 °C ± 2 for 72 hours. The grown colonies were selected as heavy metal-resistant strains to molecular identification.

### Molecular Identification of Isolated Strains

The DNA genomic of heavy metal-resistant strains was extracted using heat and cold cycle method.<sup>14</sup> The partial fragment of 16S rRNA of isolated strains were amplified by specific primers ((F4: 5'-CCG CCT GGGGAG TACG-3' and Rn2: 5'-GAC GGG CGG TGT GTAC-3') using PCR with following steps: three min at 94 °C (initial denaturation step), followed by 40 cycles under denaturation step at 94 °C for 30 seconds, annealing step at 56 °C for 30 seconds and extension at 72 °C for 50 seconds followed by final extension at 72 °C for 8 minutes. The DNA sequences of amplified samples were determined by Bioneer Inc. (South Korea). The Basic Local Alignment Search Tool (BLAST) software of NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was applied to determine the type of isolated strains. The phylogenetic study of isolated strains was conducted by Mega 7.0 software using the Neighbor-Joining (NJ) method with 500 bootstrap.<sup>25</sup>

### Effects of pH, Temperature and Heavy Metals on the Growth of Isolated Bacteria

To find the best growth conditions, the heavy metal-resistant bacteria were cultured in LB broth medium at different ranges of pH 4-8, and then cultured mediums were incubated at different ranges of temperature (25, 30, 35 and 40 °C) and 160 rpm. The growth rate of isolates was measured by wavelength

spectrophotometer (600 nm) at 12, 24, 48 and 72 hours after incubation. Also, the growth rate of isolated strains was evaluated in LB broth medium containing 50 mg/L of Ni, Cu, Pb and Cd, and a mixture of metals (50 mg/L of Ni, Cu, Pb, Cd) at pH 6 and 35 °C (as optimal conditions) and 160 rpm.

### Bioremediation of Ni, Cu, Pb, and Cd by the Isolates

The bioremediation potential of isolated bacteria was evaluated at pH 6 and 8. In brief, 400 µL of each isolates suspension were cultured in contaminated LB broth mediums with 50, 100 and 200 mg/L of lead (PbNo3), cadmium (CdCl2), copper (CuSo4), nickel (Ni(NO3)2) both individually and with a mixture of metals (50 mg/L of each metal) at 35 °C and 160 rpm. After 48 hours, the concentration of used metals was measured by atomic absorption spectrometer (GBC SensAA). The removal% of metals were calculated using the following equation<sup>26</sup>:

$$\text{Heavy metal removal \%} = [(C_i - C_f) / C_i] \times 100$$

The  $C_i$  and  $C_f$  are the initial and final concentration of metal in liquid mediums, respectively.

### Data Analysis

Statistical analysis was carried out by analysis of variance (ANOVA) in triplicate to determine the significant metal removal percentage ( $P$  value <0.05), using SPSS software version 17 (SPSS 2008). The final results were presented based on mean and SD, and all graphs were drawn using GraphPad Prism software package version 6.0.

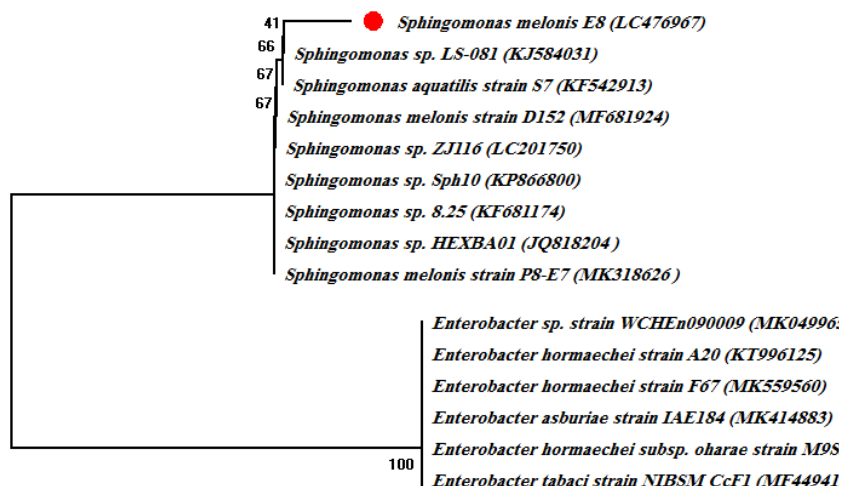
## Results and Discussion

### Evolutionary Analysis of Isolated Bacteria

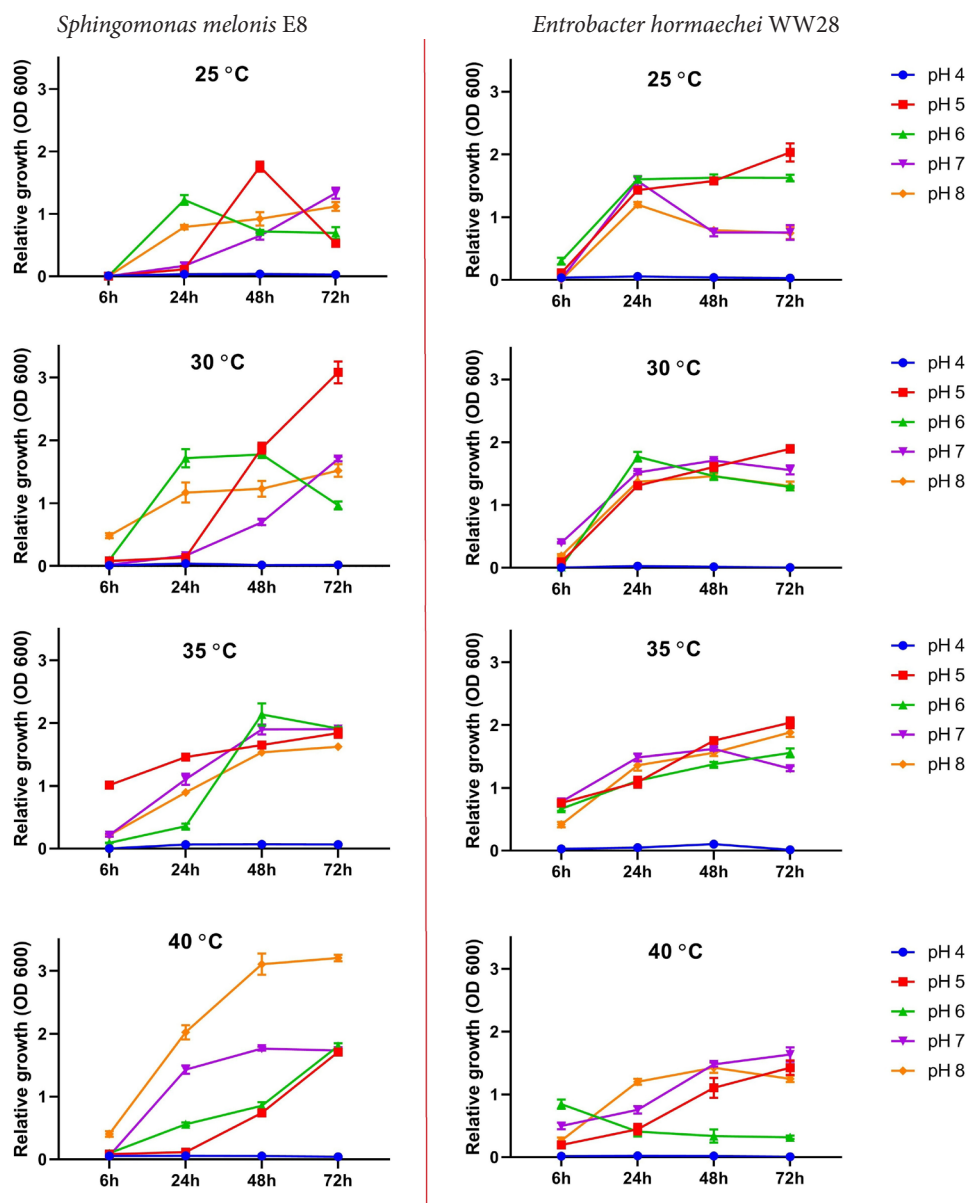
Bioremediation as an ecosystem friendly technique has a great ability to remediate the polluted sites. In this study, strains E8 (isolated from soil of coal mine) and WW28 (isolated from water of wetland) were found as heavy metal resistance among isolated bacteria. According to sequence analysis of 16S rRNA, strains E8 and WW28 showed high similarity (99%) to *S. melonis* and *E. hormaechei* respectively (Figure 1). The 16S rRNA sequences of E8 and WW28 were recorded in GenBank with accession numbers: LC476967 and LC427572 respectively.

### Optimization Growth Conditions

The growth rate of bacteria strains has been influenced by concentration of metal ions, temperature and pH in culture media. In this study, the relative growth rate of isolated bacteria were evaluated at different ranges of pH 4-8 and temperature 25-40 °C in LB broth mediums without any metal cotamination (Figure 2). The results of this study revealed that pH and temperature had significant effects ( $P < 0.05$ ) on the growth rate of bacteria (data not shown). At temperature < 35 °C (25, 30 and 35 °C), pH 5 and 6 were found as optimum pH while at 40 °C, isolated bacteria showed high growth rate at pH 8. The highest cell mass of strain E8 was observed after 48 hours while strains WW28 showed high growth rate after 24 hours. Temperature and pH play critical roles in the solubility and adsorption of heavy metals.<sup>27</sup> The availability and membrane-binding affinity of heavy metals to bacteria cell wall increase



**Figure 1.** Phylogenetic Analysis of Isolated Bacterial Based Partial Sequence of 16S Region Using Neighbor-Joining Method.



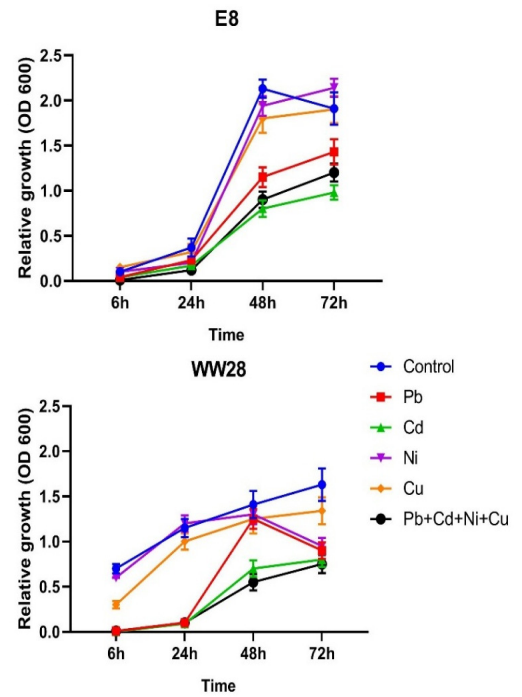
**Figure 2.** The Growth Rate of Isolated Bacteria at Different Ranges of pH and Temperature.

with increasing temperature.<sup>15</sup> However high temperature can degrade bacterial cell wall and also will adsorb heavy metals. The pH influences the absorption of heavy metal by bacteria that at acidic pH (less than 5), the competition between cations and protons to bind adsorbent surface (bacteria cell wall) reduces the metal absorption.<sup>6</sup>

In this study, the growth rate patterns of isolates were measured under cotaminated mediums (polluted with Ni, Cu, Pb and Cd) at pH 6 and 35°C (Figure 3). The results indicated that heavy metal reduce growth rate of isolated bacteria. Strains E8 and WW28 showed the highest growth rate under Ni and Cu stresses while Cd and a mixture of metals (combination of Ni, Cu, Pb and Cd) had the most inhibitory effects on the growth rate of isolated strains. Heavy metals from modulation in DNA inhibit some bacteria-metabolic functions and influence the bacteria growth.<sup>7</sup> It seems that Cd has more negative effects on the growth rate of isolated strains. It was stated that Cd causes oxidative stress and reduces the activity of enzymes and bacterial metabolism, and ultimately reduces growth rate.<sup>15,28</sup>

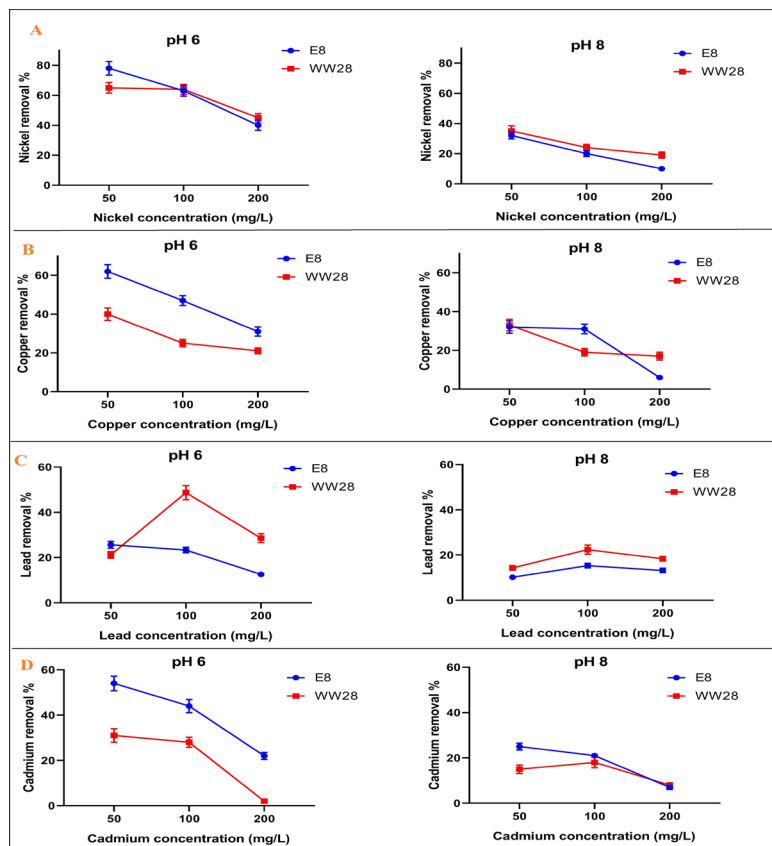
**Heavy Metals Remediation**

Heavy metal-resistant bacteria strains remediate metals at specific pH and temperature ranges. According to results of growth rate, the bioremediation potential of isolated strains was investigated at pH 6 and 8, and 35°C. The study found that the remediation potential of isolated bacteria varied between 2% and 78% at pH 6 and 8 (Figure 4). Strains E8 and WW28 showed the highest remediation at pH 6, and Ni was more



**Figure 3.** The Growth Rate of Isolated Bacterial LB Broth Containing 100 mg/L of Ni, Cu, Pb and Cd.

remediated compared to other metals. Strain E8 could remove 78% Ni after 48 hours from medium containing 50 mg/L Ni at pH 6 while 63% of Ni was removed by strain WW28 (Figure 4A). Also, the strain E8 could remove 62% and 56% of Cu and Cd from mediums containing Cu and Cd respectively



**Figure 4.** The Percent Removal of Ni (A), Cu (B), Pb (C) and Cd (D) After 48 Hours at pH 6 and 8 Using Strains E8 and WW28.



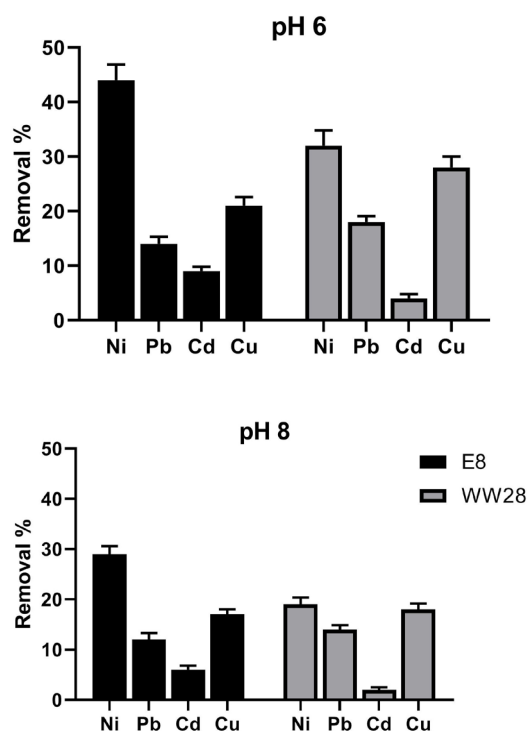
(Figure 4B and D). However, the highest Pb removal (48%) was observed by strain WW28 at pH 6 (Figure 4C). The study clearly indicates that *S. melonis* strain E8 and *E. hormaechei* strain WW28 have high bioremediation potential to use in environments which are polluted by multi metals. Previous results have also revealed that strains of *Sphingomonas* genus could remediate dangerous components<sup>29</sup> such as pentachlorophenol<sup>30</sup> and Cd.<sup>31</sup> Also, previous studies stated that bacterial isolates belong to genus of *Enterobacter* have high potential to remediate the heavy metals.<sup>23,32,33</sup> However, the heavy metal-resistant bacteria use various mechanisms to reduce the toxicity of metals. The components of bacteria cell wall play key role to uptake heavy metals.<sup>34</sup> The *Sphingomonas* and *Enterobacter* are gram-negative bacteria having lipopolysaccharides and lipoproteins in their cell wall which could bind metals. The components of cell wall of both gram-negative and gram-positive bacteria strains regulate transformation of heavy metals across the cell membrane.<sup>35</sup> In gram-negative, the phosphate groups play a critical role to metal binding.<sup>36</sup> Phosphate groups are found to participate in binding Pb and Ni to cell wall of gram-negative bacteria such as *Pseudomonas aeruginosa* ASU6a.<sup>37</sup> The remediation of all used-metal decreased at pH 8. These results revealed the fact that pH plays a key role in bioremediation efficiency. Actually, pH could have an effect on the solubility and remediation capacity of heavy metals.<sup>34,38</sup> Under high concentrations of hydrogen (acidic pH), the binding metal to cell surface is decreased, however when pH rises to 6 the cell surface can be negatively charged and can enhance the uptake metals.<sup>39</sup> Also, the composition of the bacterial cell wall such as phosphate and carboxyl play key roles for binding metal to cell surface.<sup>40</sup> At pH 8, some heavy metals such as Cd and Pb will be more precipitated in media and as a result their biosorption is decreased.<sup>18,41</sup> Choińska-Pulit et al<sup>42</sup> also observed that Pb, Cu and Cd biosorption highly remediated at pH 5-6. It seems that pH 6 has a positive effect on the interaction between isolated strains and used metals, and the bioavailability and mobility of metals are increased under pH 6.

### Bioremediation of Multi-metals Contamination

Under real conditions, the environment was polluted by multi heavy metals. In this study, the mediums containing a mixture of Ni, Cu, Pb and Cd were used to study the remediation potential of isolated strains under multi metal stress. In mixture metals condition, the percentage removal of Cd was decreased 96% and 87% by E8 and WW28 respectively (Figure 5). The results of the present study exhibited that under multi metals contamination, isolated strains do not prefer to uptake Cd, however the percentage removal of other metals also reduced under a combination of metals stress. Decrease in metals remediation can be due to the negative effects of multi metals on bacteria growth.<sup>21</sup>

### Conclusions

In this study, two new heavy metal-resistant bacteria strains (E8 and WW28) were isolated from extreme environments which were similar to *S. melonis* and *E. hormaechei*. The



**Figure 5.** The Percent Removal of Used Metals From Mediums Containing Ni, Cu, Pb and Cd

growth rate and bioremediation potential of isolated strains were evaluated under Ni, Cu, Pb and Cd mediums and a mixture of four metals. Our results revealed that the isolated strains as novel bacterial strains had a high growth rate at pH 5 and 6. Also, it has been demonstrated that Cd can have more toxic effects compared to Pb, Ni and Cu on the growth rate of tested bacteria. In addition, the highest metal remediation from contaminated mediums was observed at pH 6 and 35°C after 48 hours. The results of the present study revealed that there is an interaction between heavy metals that reduces the efficiency of bioremediation in environments contaminated with several heavy metals. This study wishes to introduce *S. melonis* strain E8 as a bio-absorbing tool to remove multi metals from polluted sites; however its mechanism should be determined.

### Authors' Contributions

All authors conceived the experiments. Also, all authors conducted the experimental work. PH analyzed data and wrote the manuscript. All authors approved the final version.

### Conflict of Interest Disclosures

The authors declare that they have no conflict of interests regarding the publication of this manuscript.

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