Bioaugmentation and Bioaugmentation – Assisted Phytoremediation of Heavy Metals Contaminated Soil By a Synergistic Effect of Cyanobacteria Inoculation, Biochar, and Purtolaca Oleracea

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Research Article

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Abstract

In recent decades, soil contamination with heavy metals has become an environmental crisis due to their long-term stability and adverse biological effects. Therefore, bioremediation is an eco-friendly technology to remediate contaminated soil, that its efficiency requires further research. This study was conducted to comparatively investigate two strategies, including bioaugmentation by using Oscillatoria sp and bioaugmentation assisted phytoremediation by using Oscillatoria sp - portulaca oleracea for the bioremediation of heavy metal (Cr (III), Cr (VI), Fe, Al, and Zn) contaminated soil at 180 days. To facilitate the remediation process, various quantities of biochar (0, 0.5, 2, and 5% (w/w)) were used in the experiments. The results of the bioaugmentation showed a significant improvement in chlorophyll a, nitrogen, organic carbon contents of soil and decrease all heavy metal bioavailability and EC of soil. The remediation efficiency test using plants proved the success of remediation treatments. Moreover, the findings of bioaugmentation-assisted phytoremediation displayed an improvement in soil fertility and a substantial reduction in the bioavailable fraction of heavy metals, especially in soil amended with 5% biochar. Cyanobacteria inoculation and biochar amendment dramatically enhanced the root lengths and shoot heights of portulaca oleracea while it significantly decreased their heavy metal accumulation compared to the control. For all heavy metals, TF and BAC (except Zn) values were found to be less than 1.0 at all treatments, illustrated the successful phytoextraction by the P. In conclusion, cyanobacteria inoculation along with biochar addition enhanced the TI quantities while diminished BAC and BCF values, suggesting the feasibility their applying in heavy metal contaminated soil for the facilitation of phytoremediation and their ability in pollutant immobilization.

Highlights

- Bioaugmentation method displayed an improvement in contaminated soil fertility.
- Bioaugmentation method decreased the bioavailability of heavy metals in soil.
- Cyanobacteria and biochar dramatically enhanced the growth of portulaca oleracea.
- Cyanobacteria and biochar diminished BAC and BCF factors of portulaca oleracea.

1. Introduction

Contamination with heavy metals is a dangerous environmental issue as the mining processing, and use of these elements has enhanced in different world regions (Dhal et al. 2013). Heavy metals are one of the primary pollutants, which exist in the soil for many years. These pollutants are toxic and pose a severe threat to food safety, human and environmental health due to their accumulation in the food chain (Song et al. 2017, Tao et al. 2017). Several physicals, chemical, and biological methods including surface capping, encapsulation, electrokinetic extraction, soil flushing, chemical immobilization, landfilling, soil washing, phytoremediation, bioremediation, solidification, and vitrification have been developed for cleanup and remediation of metal contaminated soils (Liu et al. 2018a).
The biological or bioremediation methods display numerous advantages, e.g. cost-effective (Liu et al. 2018a, Shah & Daverey 2020), environment friendly (Shah & Daverey 2020), high public acceptance (Liu et al. 2018a), simple to implement (Liu et al. 2018a), sustainable (Megharaj & Naidu 2017, Pandey et al. 2016), without disturbing the soil fertility, and biodiversity (Ahmad et al. 2016, Xiao et al. 2019), compared to others. Bioremediation can be classified into phytoremediation, microbial remediation, and animal remediation, based on the organism utilized (Luo et al. 2017). Although the phytoremediation technique is an appropriate option for remediation of heavy metal contaminated soil, it faced some challenges, including the lengthy period for cleanup, the slow growth rate of the plant, hard to mobilize some metals, an application for the site with low pollution, transfer accumulated metals into the food chain (Mahar et al. 2016). Recently, many researchers focused on improving its process efficiency by using amendment materials and microorganisms (Girolkar et al. 2021, Gong et al. 2021, Sharma et al. 2021). For example, several studies proved that microbe-assisted phytoremediation (e.g., *pseudomonas*, *burkholderia*, *bacillus*, *rhizobium*, *enterobacter*, *bradyrhizobium*, *paenibacillus*, *thiobacillus*, *Arbuscular mycorrhizal fungi*, etc.) showed a positive effect on the phytoremediation by enhancement of the growth, health, and establishment of the plant, and sequestration of heavy metal in soil (Harindintwali et al. 2020, Khalid et al. 2021, Lebeau et al. 2008). On the contrary, few research applied soil algae (e.g., cyanobacteria) for soil remediation (Biglari Quchan Atigh et al. 2020b). However, cyanobacteria inoculation experience demonstrated that their species such as *Nostoc*, *Anabaena*, etc have outstanding capabilities for the restoration of degraded soil in the arid area (Chamizo et al. 2018b, Li et al. 2019).

Cyanobacteria are known as blue-green bacteria, belong to the kingdom prokaryotes, which are found in various habitats, even in the desert, salty soil (Li et al. 2019, Rossi et al. 2017). They are considered as ecosystem engineers (Jones et al., 1996), due to their ability to sequestration of carbon, fixation of nitrogen, secretion nutritious, therefore improvement of soil conditions (fertility, microbial community, and productivity) and as results provide an appropriate environment for the existence of micro and macro-organisms (Kheirfam & Roohi 2020, Kheirfam et al. 2017, Mugnai et al. 2018). The previous studies proved that cyanobacteria have the potential to be developed as a bioremediating agent for plant growth promotion and salt-affected soil remediation through most effective mechanisms such as nitrogen fixation, production of extracellular polysaccharides, and growing the organic carbon contents (Li et al. 2019, Muñoz-Rojas et al. 2018). Therefore, cyanobacteria inoculation can be considered a novel technique for remediation of contaminated soil (Biglari Quchan Atigh et al. 2020a).

The combined utilization of microorganisms, plant, and amendment materials was suggested as an innovative, effective method for remediation of heavy metal contaminated soil. Among various amendments, biochar “a carbon-rich material” was considered as an exciting remediation option for contaminated soil. According to literature (Chen et al., 2018; Lahori et al., 2017; Siedt et al., 2020), it is most effective in increasing the soil water holding capacity, alterations chemical soil properties (e.g., pH, cation exchange capacity, and buffering capability), retaining plant available water, increment nutrient concentration, decreasing the bioavailability of heavy metal, reduce plant uptake, and microbial communities. Furthermore, some studies indicated that the combination of biochar with animal-remediation (such as earthworms, *Eisenia fetida*) or microbial-remediation (such as bacteria,
Pseudomonas sp., Enterobacter sp.) enhanced the soil fertility and metal uptake by the plant due to its synergistic effects (Chen et al. 2019, Sanchez-Hernandez et al. 2019, Tu et al. 2020, Wu et al. 2019, Xiao et al. 2020). However, the combination mechanism between microorganisms and biochar for the transformation or stabilization of heavy metal in the soil is not well understood. There is also a research gap in the effect of biochar on microbial assisted phytoremediation, especially from the aspect of plant growth and heavy metal bioavailability.

In this study, Oscillatoria sp, a filamentous cyanobacteria species, purtolaca oleracea, and biochar were employed as microbial, plant, and material agents to remediate soil contaminated with heavy metals (Cr (III), Cr (VI), Fe, Al, and Zn), respectively. However, to the best of our knowledge, interactions between biochar and cyanobacteria on heavy metal uptake by purtolaca oleracea grown on contaminated soil have not been reported. Hence, the specific aim of this study is to: (1) evaluate the effect of cyanobacteria inoculation accompany with biochar amendment on the properties of soil and heavy metal bioremediation efficiency, (2) explore the influence of Oscillatoria sp - purtolaca oleracea - biochar partnerships on soil properties and heavy metal bioremediation efficiency, (3) assess the heavy metal accumulation in parts of purtolaca oleracea under metal stress and assisted remediated materials.

2. Material And Method

2.1 Chemicals

Chemicals with analytical grade including potassium dichromate (K₂Cr₂O₇), magnesium carbonate (MgCO₃), ethanol 99.8% (C₂H₅OH), acetic acid (CH₃COOH), hydroxylamine hydrochloride (NH₂OH.HCl), hydrogen peroxide 35% (H₂O₂), ammonium acetate (C₂H₃NO₂), nitric acid 65% (HNO₃), hydrochloric acid 37% (HCl), acetone (CH₃O), BG 11 Broth, and diphenylcarbazide (C₁₃H₁₄N₄O) were purchased from Merck Company.

2.2 Contaminated soil description and characterization

The soil was collected from a contaminated industrial area in Mashhad, Iran (36° 29' 92" N, 59° 31'16" E). The sample was gathered from 0 to 40 cm of soil depth and air-dried at room temperature. After homogenizing and sieving (> 2 mm mesh), the soil texture was determined using a hydrometer method (Bouyoucos 1962), which was classified as loam (sand 50%, silt 28%, clay 22%). Soil pH and EC were characterized using pH meter (basic 20, Crison, Spain) and EC meter (4510, Jenway, England) with soil to distilled water ratio of 1: 25. The organic carbon (OC) and total nitrogen (N) contents of soil samples were determined by loss of ignition (Park et al. 2017) and the Kjeldahl method (Kirk 1950), respectively. Some of the important soil properties were as follows: pH; 9.05, EC; 121.8 dsm⁻¹, OC; 0.022 g kg⁻¹, N; 1120 mg kg⁻¹, K; 101.7 mg kg⁻¹, and P; 155 mg kg⁻¹.

The concentration of heavy metals in soil was determined by the aqua regia digestion method (Alghanmi et al. 2015). 1 g of air-dried soil was mixed with 5 ml of HNO3 (65%) and 15 ml of HCl (37%) and stirred
at room temperature for 12 h. After this, the obtained mixture was digested at 130°C for three h and then filtered. The heavy metal content of filtrate was measured by inductively coupled plasma-optical emission spectrometry (76004555, Spectro Arcos, Germany). The concentration of Cr (VI) was determined by using UV-VIS Spectrophotometer (DR5000- Hach, USA). The following results were acquired: Cr (III) ; 65.45 mg kg$^{-1}$, Cr (VI) ; 12.71 mg kg$^{-1}$, Zn; 104.07 mg kg$^{-1}$, Al; 11287 mg kg$^{-1}$, and Fe; 27405 mg kg$^{-1}$.

Metal distribution in the soil samples was determined using the European Bureau of References (BCR) (Peña-Icart et al. 2011, Wuana et al. 2010). Three sequential extraction phases, including extractable (step1), reducible (step 2), organic-bound (step3), and residual (R), were conducted to attain various fractions of heavy metals in soil. According to this method, 1g of dried soil (105 °C for three h) was digested using sequential extractions (4 steps), which its detail as illustrated in Table 1. The heavy metal ions content of each fraction in soil was measured with the ICP instrument.

Table 1

| Step | Soil phase                           | Reagents                                      | Stirring time and temperature | Separation and storage                  |
|------|--------------------------------------|-----------------------------------------------|--------------------------------|-----------------------------------------|
| S1   | Water- and acid-soluble, exchangeable (extractable) | 40 mL of 0.11 M acetic acid                   | 16 h at room temperature       | Centrifuging at 1500 rpm for 15 min     |
|      |                                      |                                               |                               | store into a polyethylene bottle at 4 °C|
| S2   | Bound to Fe–Mn oxides (reducible)    | 40 mL of 0.5M hydroxylamine hydrochloride     | 16 h at room temperature       |                                          |
| S3   | Bound to organic matter and sulfides (oxidizable) | 10 mL of 8.8 M hydrogen peroxide               | 1h at room temperature, then 85°C for 1h |                                           |
|      |                                      | Then 10mL of 8.8M H$_2$O$_2$                   | 85°C for 1h                    |                                          |
|      |                                      | 50 mL of 1.0 M ammonium acetate               | 16h at room temperature        |                                          |
| R    | Residual                             | 37.5 ml of 12 M HCl and 12.5 ml of 17 M HNO$_3$ (3:1) | digested for 24h at room temperature then, digested at 130°C for 15 min | Filter with filter paper                |

2.3 Production and characterization of biochar

The biochar used in this study was produced from the pyrolysis of three wood wastes, including Eucalyptus, Populus, and Parrotia, at a temperature of 400 °C. The physicochemical properties of the obtained bio-char were determined, which are described in the following. It had a pH of 7.73 and EC of
935 ds m\(^{-1}\) measured in 1:5 suspensions of biochar to distilled water using pH and EC meters, respectively. The total contents of C, N, O, H, and S were measured by the elemental analyzer (FLASH EA 1112 SERIES, Thermo Finnigan, USA). The quantities of C, O, H, N, and S of biochar were 73.41, 23.51, 2.71, 0.37, 0 %, respectively. Besides, the surface functional groups of the bio-char were determined by Fourier to transform infrared spectroscopy (FTIR) (AVATAR 370, Thermo Nicolet, USA). Also, the surface morphology of biochar was analyzed by scanning electron microscopy (SEM) (VP 1450, LEO, Germany).

2.4 Experimental design setup

All experiments were conducted in a greenhouse at the Ferdowsi University of Mashhad, Mashhad, Iran, from July to September 2019. Generally, two experiment series were designated: (1) bioaugmentation by cyanobacteria and (2) bioaugmentation-assisted phytoremediation by cyanobacteria and \textit{purtolaca oleracea}. Biochar was used as an amendment in both series. Table 2 illustrates the experimental design, a 4× 2 factorial design, with three replications with four quantities of biochar (0, 1, 2, 5 % w/w) and two types of treatments (\textit{Osillatoria sp} as microorganism and \textit{purtolaca oleracea} as a plant).

For bioaugmentation testing, the Petri dishes with a diameter of 8 cm and a height of 1 cm, were filled with 80 gr of sterilized contaminated soil, which previously mixed with various amounts (0, 1, 2, 5 % w/w) of biochar. Then, 1.4 g (wet weight) of cyanobacteria separated from the culture medium by centrifugation at 1500 rpm for 15 min, mixed and homogenized with 20 ml of distilled water at room temperature for 24 h, was added to the soil surface using a sterile syringe. The inoculated plates were placed in the greenhouse at 2 ± 27°C under the light intensity of 1600 lux and light to a dark ratio of 16 h: 8 h. They were irrigated with 24 mL of distilled water every 24 hours.

For bioaugmentation-assisted phytoremediation examination, 1 kg of sterilized contaminated soil mixed with different ratios (0, 1, 2, 5 % w/w) of biochar and placed into several polyethylene pots with a total volume of 2 L (area of 133 cm\(^2\) and depth of 10 cm). The drainage holes were embedded in the bottom of the pots to guarantee the health of the roots. The plant seeds were disinfected with ethanol 99.8 % and planted directly in the soils. 0.8 g (wet weight) of the cyanobacteria were added to the surface soil in the pots when the seeds germinated. The pots were placed in the greenhouse at 30 ± 5°C with a light/dark ratio of 16 h: 8 h and irrigated with 50 ml of distilled water every 24 h. After four weeks, the surface soil of each pot was also inoculated with 0.8 g of cyanobacteria mat uniformly. Twelve weeks after planting, the plants were harvested. After washing thoroughly with deionized water, they were dried at 70°C for 24 h and store in a polyethylene bag for future analysis.

Table 2

Design of experiment
| Description                                      | Abbreviation | Treatment        |
|-------------------------------------------------|--------------|------------------|
| Control Soil                                    | S            | Bioaugmentation  |
| Control Soil + Cyanobacteria                    | SC           |                  |
| Control Soil + Cyanobacteria + Bio-char 0.5     | SCB0.5       |                  |
| Control Soil + Cyanobacteria + Bio-char 2       | SCB2         |                  |
| Control Soil + Cyanobacteria + Bio-char 5       | SCB5         |                  |
| Control Soil + Plant                            | SP           | Bioaugmentation  |
| Control Soil + Cyanobacteria + Plant            | SCP          | Phytoremediation |
| Control Soil + Cyanobacteria + Plant + Bio-char 0.5 | SCPB0.5    |                  |
| Control Soil + Cyanobacteria + Plant + Bio-char 2 | SCPB2     |                  |
| Control Soil + Cyanobacteria + Plant + Bio-char 5 | SCPB5     |                  |

### 2.5 Bioremediation efficiency

Lettuce and radish were applied as indicators to evaluate the bioremediation success of tests recommended in prior works (Chen et al. 2018b, Mahbub et al. 2017). 25g of a bio-remediated soil sample by cyanobacteria were placed into small pots (length ×width ×height: 5 ×5 ×10 cm) for planting. Five seeds of each plant (lettuce and radish) were planted in the soil of pots. Plants were irrigated with distilled water every day. After 14 days, the germination, root, hypocotyl, and shoot lengths of the lettuces and radish were measured.

### 2.6 Plant analysis

After three months of planting, first, the *purtolaca oleracea* plant was cut, and its stem heights and root lengths were measured. The plant parts were washed thoroughly with deionized water and then dried at 70°C for 24 h. The concentration of heavy metals (Cr (III), Cr (VI), Zn, Al, and Fe) in the roots and shoots of *purtolaca oleracea* were determined using HNO₃ - H₂O₂ digestion procedure according to Ashrafi et al. [5]. 0.2 g of the sample was digested with 5 mL HNO₃ (65%) at 110°C for two h. Then, after cooling, 1 mL of H₂O₂ (30%) was added and boiled for 1 hour. Finally, the obtained solution was passed through filter paper, and its heavy metal contents were analyzed using the ICP instrument. The capability of the plant to tolerate and accumulate heavy metals was determined by bioconcentration factor (BCF) (Mani et al. 2016), Translocation Factor (TF) (Meeinkuirt et al. 2016), and bioaccumulation coefficient (BAC) (Hamzah et al. 2015), and Tolerance Index (TI) (Ali et al. 2002) which were estimated using the following formula:
2.7 Statistical analysis

The Kolmogorov–Smirnov test was utilized to test data normality and homogeneity of variance. The statistically significant difference between data among control and treatments was determined using one-way analysis of variance (ANOVA) followed by Tukey’s and Duncan’s test. The significant difference ($p$-value < 0.05) among various treatments was displayed by using different letters. Data analysis was performed using IBM SPSS Statistics version 20.

3. Results And Discussion

3.1 Characterization of Biochar

The surface morphology of biochar was observed by SEM analysis (Fig. 1a). From this image, regular pores similar to honeycombs can be seen on its surface, which indicates its ability the adsorption of heavy metals. This honeycomb structure was related to the carbon skeleton of biochar (Ghani et al. 2013). The surface functional groups were determined by FTIR analysis (Fig. 1b). The peak at 3425 cm$^{-1}$ was attributed to the $\text{–OH}$ stretching or phenolic groups (Liu et al. 2018b). The bands located at 3033 and 1591 cm$^{-1}$ were related to aliphatic $\text{–CH}_2$ (Bandara et al. 2017) and $\text{–COO} –$ antisymmetric stretching or aromatic C = C (Yang et al. 2016a), respectively. The peak at about 1431 cm$^{-1}$ corresponds to the stretching vibrations of conjugated C = O bonds in aromatic rings (Meier et al. 2017). The bands presented at about 1376, 751, and 874 cm$^{-1}$ were associated with -COO (Dong et al. 2017) and C-H aromatic groups (Song et al. 2012), respectively. Besides, the peaks at 1230 cm$^{-1}$ were belonged to -CO and OH aromatic groups (Liu et al. 2018b).

3.2 Bioaugmentation

3.2.1 Cyanobacteria inoculation

Figure 2 showed the application Oscillatoria sp. in the heavy metal contaminated soil amended with various biochar. After three months of inoculation, cyanobacteria were progressively distributed and covered on the soil’s surface (Fig. 2 (b)). The SEM image, taken from the surface of inoculated soil, displays mineral particles, and soil fragments were coated by exopolysaccharides (EPS) assumed to
have been secreted by the cyanobacteria strain. Similar results were reported by Maqubela et al. (Maqubela et al. 2010), Issa et al. (Issa et al. 2007), Chamizo et al. (Chamizo et al. 2018a) after cyanobacteria inoculation in soil.

### 3.2.2 Soil physicochemical properties

Table 3 summarized the influence of bioaugmentation on soil physicochemical properties under different treatments. The pH and EC contents of soil were decreased, while the OC, N, and Chlorophyll-a amounts were enhanced in various treatments. Compared with the control soil (S), there were higher soil EC, but lower OC, N, and Chlorophyll-a among treatments with cyanobacteria inoculation and biochar amendment ($p < 0.05$). Previous studies (Biglari Quchan Atigh et al. 2020a, Kheirfam & Roohi 2020, Kheirfam et al. 2017, Nisha et al. 2018, Peng & Bruns 2019) found that the inoculated cyanobacteria into soil reduced the pH and EC, while elevated OC, N, and Chlorophyll-a. It implies that *Oscillatoria sp.* used in this study as a genus of cyanobacteria, with carbon and nitrogen fixation process, which convert atmospheric carbon dioxide and nitrogen to organic compounds and ammonium or nitrate, results in improvements in OC and N. Besides, cyanobacteria reduce pH and EC by producing organic acids and absorbing sodium ions from the soil (Kheirfam & Roohi 2020, Kheirfam et al. 2017, Peng & Bruns 2019). The biochar treatments (SCB0.5, SCB2, and SCB5) had the highest OC, N, and chlorophyll a. The biochar was found to considerably increase the availability of nutrients, water holding capacity, and heavy metals sorption (Siedt et al. 2021, Wang et al. 2021). It also affects the soil microorganisms directly or indirectly by providing C and other nutrients, and space (macro-, meso- and micro-pores) for colonization and changing soil conditions (Siedt et al. 2021). The SCB5 represented the highest OC, N, and chlorophyll contents, mainly attributed to the most significant growth of inoculated cyanobacteria in soil.

| Treatment | pH (±) | EC (ds m$^{-1}$) | OC (g kg$^{-1}$) | N (mg kg$^{-1}$) | Chlo a (mg m$^{-2}$) |
|-----------|--------|-----------------|-----------------|----------------|-------------------|
| S         | 9.05±0.07$^a$ | 121.8±2.88$^a$ | 0.0221±0.007$^b$ | 1120±12.7$^c$ | 0±0               |
| SC        | 9.04±0.02$^a$ | 50.56±2.22$^b$ | 0.059±0.012$^b$ | 1610±62.35$^b$ | 3.5478±0.24$^b$   |
| SCB0.5    | 9.01±0.03$^a$ | 50.1±3.95$^b$  | 0.0601±0.019$^c$ | 1652±30.02$^b$ | 4.3076±0.84$^{ab}$|
| SCB2      | 8.99±0.04$^a$ | 48.03±0.88$^b$ | 0.07495±0.003$^{ab}$ | 1708±12.12$^{ab}$ | 5.1094±0.47$^{ab}$|
| SCB5      | 8.89±0.06$^a$ | 44.56±4.29$^b$ | 0.0955±0.001$^a$ | 1792±17.32$^a$ | 5.7994±0.82$^a$   |

### 3.2.3 Metal speciation in the contaminated soil

Figure 3 displays the speciation of heavy metals including Cr (III), Cr (VI), Al, Zn, and Fe during various treatments. As shown in Fig. 3, with cyanobacteria inoculation and biochar addition into the soil, the S1
fractions of Cr (III), Cr (VI), Zn, Al, and Fe declined by 15–38 %, 1.7–87 %, 45–59 %, 5–47 %, and 1.6–58 %, relative to control, respectively. In other words, the fractions of heavy metals in soil were noticeably influenced by the treatments. A significant decrease in the S2 form (Fe- and Mn-bound) of Cr (VI) was observed by 49–72 % following the treatments. In contrast, a sharp increment in the S2 fraction of Cr (III), Fe, Al, and Zn was obtained after treatments. The fraction bound to organic matters and sulfides (S3) of Cr (III) and Fe increased by 8.9–324 and 133–219 while that of Al, Zn, and Cr (VI) decreased by 83–91 %, 18–66 % and 13–67 %, respectively. The previous researches pointed that the application of biochar in soil notably enhanced the proportion of organic matter bond various metals (Park et al. 2011, Rinklebe & Shaheen 2015). Similarly, the cyanobacteria inoculation and the addition of biochar also grew the residual forms (S4) of Cr (III), Al, and Fe by 291–439 %, 82–207 %, 133–219 %, respectively. The Cr (VI) and Zn indicated the reduction trend in the S4 forms. The exchangeable bound form displays the labile (easily bioavailable) fraction, while the Fe- and Mn, organic matter bound, and residual fractions are related to the recalcitrant (less bioavailable) portion. The effectiveness of immobilization assesses by both the labile and recalcitrant fractions. Nie et al. (Nie et al. 2018) and Tu et al. (Tu et al. 2020) found that the easily bioavailable fraction of Cd and Cu in soil reduced biochar addition. In the present study, a similar trend was observed.

3.2.4 Evaluation of the efficacy of soil bioaugmentation

Prior works (Aparicio et al. 2015, Chen et al. 2018a, Wang et al. 2014) proved that radish and lettuce could be used as a biomarker for assessing bioremediation efficiency. Figure 4 displays the root and hypocotyls lengths and the vigour index (VI) of the radish and lettuce among various treatments. As shown in this figure, the root and hypocotyls lengths and VI of lettuces and radish in control soil were significantly smaller (p < 0.05) than those in soil with treatments, indicating that the bioremediation treatments improved plant growth by the diminution of heavy metals stress in soil. Furthermore, the soil inoculation with cyanobacteria and 5 % biochar (SCB5) supported the most development of longer root and hypocotyls lengths and higher VI. Similar results were observed by Mahbub et al. (Mahbub et al. 2017), who confirmed that the root lengths of lettuce were a good indicator for evaluating the remediation effectiveness of mercury-contaminated soil. This also supported the findings of Aparicio et al. (Aparicio et al. 2015). They utilized the roots and hypocotyls lengths and the VI of lettuce as a biomarker for bioremediation efficiency of soil contaminated with Cr (VI) and lindane. In general, our finding confirms that the bioremediation effectiveness of cyanobacteria and biochar amendment.

3.3 Bioaugmentation-assisted phytoremediation

3.3.1 Influence of treatments on Portulaca oleracea growth

The effect of cyanobacteria inoculation and biochar amendment on Portulaca oleracea growth was measured (Table 4). By cyanobacteria inoculation in soils, the shoot and root lengths increased from 12.73 and 1.67 cm to 18.87 and 3.07 cm, respectively. This was attributed to the enhancement of nitrogen and organic carbon content of the soil in the presence of cyanobacteria, which promote plant growth (Rossi et al. 2017). Besides, with an increment of biochar dose from 0 to 5 percent in the soils, the
shoot and root lengths grew 4.6 and 3 fold, respectively. This may be due to the decreased availability of heavy metals and improvement in nitrogen and organic carbon content (Table 3) in the soil by adding biochar (Puga et al. 2015). The weight of dry plant biomass after all treatments increased significantly by 25 fold as compared to control. This is in good agreement with Puga et al. (Puga et al. 2015), Kiran and Prasad (Kiran & Prasad 2019), and Qi et al. (Qi et al. 2020). They observed that a significant enhancement in plant growth factors in soil applied with biochar.

Table 4
The effect of various treatments on growth performances of Portulaca oleracea cultivated in heavy metal contaminated soil

| Treatments | The dry weight of shoot (g) | The dry weight of root (g) | The dry weight of the whole plant (g) | Root length(cm) | shoot height(cm) |
|------------|-----------------------------|---------------------------|-------------------------------------|-----------------|-----------------|
| SP         | 0.0211 ± 0.013\(^b\)        | 0.0012 ± 0.001\(^b\)      | 0.0223 ± 0.014\(^b\)               | 1.67 ± 0.08\(^b\) | 12.73 ± 5.79\(^b\) |
| SCP        | 0.0644 ± 0.014\(^{ab}\)     | 0.0040 ± 0.001\(^{ab}\)   | 0.0685 ± 0.015\(^{ab}\)            | 3.07 ± 0.63\(^{ab}\) | 18.87 ± 5.55\(^b\) |
| SCPB0.5    | 0.0928 ± 0.019\(^{ab}\)     | 0.0051 ± 0.002\(^{ab}\)   | 0.079 ± 0.021\(^{ab}\)             | 3.33 ± 1.16\(^{ab}\) | 22.67 ± 8.20\(^b\) |
| SCPB2      | 0.3820 ± 0.142\(^a\)        | 0.0138 ± 0.007\(^a\)      | 0.3958 ± 0.149\(^a\)               | 4.47 ± 0.95\(^a\) | 49.77 ± 5.21\(^a\) |
| SCPB5      | 0.4657 ± 0.093\(^a\)        | 0.0343 ± 0.10\(^a\)       | 0.5 ± 0.193\(^a\)                  | 5 ± 0.28\(^a\)  | 58.5 ± 7.94\(^a\)  |

3.3.2 Metal speciation in the contaminated soil

To explore the influence of the cyanobacteria inoculation and biochar percentage on heavy metal speciation, the BCR method was carried out after bioremediation experiments. Their results for Cr (III), Cr (VI), Fe, Zn, and Al illustrates in Fig. 5. As shown in Fig. 5 (a), the concentration of Cr (III) in S1 fraction (water- and acid-soluble and exchangeable) reduced from 3.5 % in the sample with cyanobacteria inoculation (SCP) and to 90 % in the sample with the highest value of biochar (SCP5). Besides, the S1 fraction of Cr (VI), Zn, Al, and Fe in contaminated soil decreased by 22 – 13, 2–68, 9–38 %, and 43–93 %, with the addition of biochar and cyanobacteria. The largest decrease in solubility of heavy metal was obtained for the samples were treated with 5 % biochar. The metals with the ionic form or bond to carbonate mainly refer to the acid-soluble and are recognized as the exchangeable fraction. This soil phase can be easily bioaccumulated in living organisms (Gong et al. 2019). Lu et al (Lu et al. 2017) observed a similar reduction in the acid-soluble fraction of Cd, Cu, Pb, and Zn using bamboo and rice straw biochars amendments in soil. Méndez et al. (Méndez et al. 2012) showed that the sewage sludge-derived biochar significantly decreases the extractable fraction of Cd, Pb, and Zn in agricultural soil. Gong
et al (Gong et al. 2019), Beesley et al. (Beesley et al. 2010) and Bashir et al. (Bashir et al. 2018a) also reported similar declining trends for Cd in contaminated soil with various biochar. Cao et al. (Cao et al. 2011) reported that the reduction of the extractable Pb in the soil was increased with the increasing biochar rates. This phenomenon was ascribed to the immobilization of metal by biochar (Gong et al. 2019, Lu et al. 2017). The specific properties of some biochars, including micro-porous structure, high pH, surface oxygenated-containing functional groups, and large surface area causes, the solubility of heavy metal reduced through adsorption, complexation, and precipitation (Chan & Xu 2009, Gong et al. 2019, Yuan et al. 2011, Zhang et al. 2013).

The S2 fraction (reducible) indicates the metal bound to Fe–Mn oxides or hydroxides. From Fig. 5 (b and c), an increase in S2 fraction of Al and Zn by 24–75 % and 10–49 % can be observed, while Fe displayed a declining trend from 47–83 %. The reducible portion of Cr (III) was dropped by 14–46 %, while it increased for Cr (VI) by 7–64 %, except for SCP5. Jiang et al. (Jiang et al. 2012) found that the reducible fraction of Cu, Cd, and Pb rose in contaminated soil with biochar. Bashir et al. (Bashir et al. 2018a) reported that the reducible form of Cd grew in the soil with an increment application rate of biochar. The increase in reduction portions of metals might be due to (1) the precipitation of metal with iron, oxygen, hydrogen, and manganese presents on the biochar surface (Bashir et al. 2018a) and (2) surface complexation creation because of high organic matter the presence of iron and manganese oxides in the soil (Bashir et al. 2018b).

The incorporation of biochar and cyanobacteria increased the S3 fraction (bound to organic matter and sulfides) of all heavy metals. S3 portion of Cr (III), Cr (VI), Zn, Al, and Fe climbed sharply by 290–566 %, 1.5–33 %, 73–188 %, 294–326 %, and 323–1137 %, with cyanobacteria inoculation and biochar amendment. The elevated S3 fraction might be primarily ascribed to high organic matter value in the contaminated soil due to cyanobacteria growth and biochar amendment, which inhibit the bioavailability of heavy metals. This is consistent with Liang et al. ‘s results (Liang et al. 2017). They found that the organic portion of Cu rose by utilizing biochar. Chen et al. (Chen et al. 2020) observed that the application of biochars along with ferrous sulfate and pig manure S3 form of Cd in the contaminated soil. Su et al. (Su et al. 2016) reported that biochar-supported zero-valent iron nanoparticles increased the conversion of more easily available Cr ( exchangeable) to the less available forms ( organic matter- bound). Bashir et al. (Bashir et al. 2018a) found that the S3 forms of Cd enhanced in the contaminated soil with an increment application rate of various biochar types.

S4 fraction (Residual) indicates the main primary and secondary minerals that block the metals within their crystal structures (Tokalioğlu et al. 2006). The S2 portion of Cr (VI), Al, and Fe fell by 20 %, 18 %, and 5% with the application of biochar and cyanobacteria. The Cr (III) and Zn display an increasing trend. Similar results were reported by Liang et al. (Liang et al. 2017). They reported that the residual portion of Zn increased while that of Cu was reduced, with the addition of biochar into contaminated soil. Besides, our results have some similarities with Chen et al. ‘s (Chen et al. 2020), Bashir et al. ‘s (Bashir et al. 2018a), and Chen et al. ‘s (Chen et al. 2020) findings.

3.3.3 Heavy metal concentration in plant
Figure 6 shows the concentrations of Cr (VI), Cr (III), Zn, Al, and Fe in shoots and roots of *Portulaca oleracea* depending on the treatment under different treatments. As shown in Fig. 6, the heavy metals concentrations in shoots and roots of *Portulaca oleracea* growing in the remediated soil were significantly (*p* < 0.05) decreased relative to the control. The metals accumulation in the root of *Portulaca oleracea* was higher than the shoot. In other words, the application of biochar and cyanobacteria inoculation significantly reduced the above-mentioned heavy metals concentrations in *portulaca oleracea*. The minimum concentrations of the shoot and the root metals were obtained when the 5 % biochar accompany with cyanobacteria (SCPB5) were used in the pots. Moreover, the highest decrease of -88.7 % and −94.6 % for Cr (III), -93.15 % and −97 % for Cr (VI), -96.6 % and −96.5 % for Zn, -92.7 % and −96 % for Fe, and −93.7 % and −90 % for Al (III) were obtained in shoot and root, respectively. The results showed that the addition of biochar and cyanobacteria to the soil led to a decrease in the heavy metal concentration of *portulaca oleracea*. These findings are consistent with literature results concerning the influences of biochar on the accumulation of Zn (II)(Al-Wabel et al. 2015, Martins et al. 2018), Cr (VI) (Lyu et al. 2018), Fe (Al-Wabel et al. 2015), and Al (Qian et al. 2018) in the plants. The biochar, due to its characteristics (e.g., large surface area and various functional groups), immobilized the heavy metals in the soils through two approaches: (1) direct adsorption of metals via physical adsorption, electrostatic attraction, ion exchange, and complexation; (2) improving indirect adsorption via soil particles due to altering its physicochemical properties such as cation exchange capacity and organic matter content (He et al. 2019a, He et al. 2019b, Wang et al. 2020). Therefore, the immobilization of metals in the soil reduces their phytoavailability and phytotoxicity.

Besides, the parts of *portulaca oleracea* grown in the soils inoculated with *Oscillatoria sp.* (SCP) have also indicated a decreased levels of above heavy metals. Although the concentration of all desired metals in roots and shoots decreased, the highest percentages of reduction were obtained for Zn (II, > 85 %) and Cr (III, > 76 %). The prior studies reported that cyanobacteria have great potential for salt-affected soil remediation (Chamizo et al. 2018b, Li et al. 2019). Cyanobacteria can produce organic carbon and extracellular polymeric materials, including exopolysaccharides (EPS) and extracellular proteins in the soil (Joshi et al. 2020, Li et al. 2019). By biosorption via exocellular polysaccharides and organic matters, they reduce heavy metal mobility and toxicity (Joshi et al. 2020).

### 3.3.4 Phytoremediation potential

The BCF, BAC, and TF were estimated for various treatments and were shown in Fig. 7. BCF indicates a plant's ability to accumulate heavy metals from soil, and TF shows a plant's ability to translate heavy metal from the roots to the shoots. The translocation of heavy metals from soil into the above-ground parts of the plant, such as the shoot, is called the phytoextraction process (Garbisu & Alkorta 2001). This process occurred in the plant when the BCF and TF > 1 (Nazir et al. 2011). In this study, for all heavy metals, TF values were found to be less than 1.0 at all treatments (Fig. 7). It has been proved that plants with TF more than 1 accumulate the metals in the aerial part while that with TF lower than 1 shows the lower ability for transferring the heavy metal from root to shoot (Arán et al. 2017, Chen et al. 2015, Prasad et al. 2001). This demonstrated that *Portulaca oleracea* removed the heavy metals from contaminated
soil and had a lower capability for Translocation them from root to shoot. The highest TF quantities for Cr (III), Cr (VI), and Fe were obtained in planted amendment with 5 % biochar. For Al, the TF values reduced with increasing biochar percentage into the soil. There was no significant difference between the SP, SCP, and SCP 0.5. Besides, it was not observed any significant difference between control and SCP5. This result is also confirmed by the work of Chuaphasuk and Prapagdee (Chuaphasuk &Prapagdee 2019), who reported that the addition of biochar into cadmium contaminated soil did not significantly change the TF of Chlorophytum laxum. Also, similar findings were observed by Chen et al. (Chen et al. 2020), which reported that the influence of biochar on TF of cadmium using wheat.

The stabilization of heavy metal within the plant ‘s root decreases their mobility or bioavailability called phytostabilization (Bolan et al. 2011). It was determined in a plant when the BCF value > 1 and TF value < 1 (Sudmoon et al. 2015, Yoon et al. 2006). As shown in Fig. 7, Portulaca oleracea has the potential for phytostabilization of Cr (III), Cr (VI), and Zn (II). The order of TF was Fe > Al (III) > Zn (II) > Cr (VI) > Cr (III). The highest BCF (122.7) was obtained for Zn (II), and the lowest BCF (0.07) occurred for Fe. The intensity of plant roots for metal absorption was classified into five groups: (1) intensive 100 > BCF > 10; (2) strong 10 > BCF > 1; (3); intermediate 1 > BCF > 0.1; (4) weak 0.1 > BCF > 0.01; and (5) 0.01 > BAC > 0.001 (Nagaraju &Karimulla 2002). Regarding the above classification for BCF, the Portulaca oleracea showed the intensive tendency for Zn (II) and Cr (III) uptake, a strong tendency for Cr (VI) absorption, and a weak tendency for Al (III) and Fe.

Tolerant plants impede soil to root and root to shoot transfers, while hyperaccumulators translocate metals into aerial parts of the plant. BAC factor, which indicates the absorption and accumulation of the metals in the shoot, is another effective tool to assess the phytoremediation ability in polluted soil (Hamzah et al. 2015). It was ranked as (1) excluder BAC < 1; (2) accumulator 1 < BAC < 10; and (3) hyperaccumulator BAC > 10 (Baker 1981). For Zn, the BAC was higher than 1 illustrates that the Portulaca oleracea has the potential to be an accumulator. For other metals, it was excluder due to having a BAC of less than 1.

Another factor was the tolerance index (TI), which analyzed the difference between the plant roots at the control and various treatments (Fig. 7). As shown in this figure, for all heavy metals, TI values were increased significantly in treatments compared to control soil. Besides, in the higher dose of biochar, the roots were more developed and distributed. Previous research (Brennan et al. 2014, Lu et al. 2015, Puga et al. 2015) confirmed that the biochar addition to the contaminated soil enhanced the plant roots.

According to Fig. 7, for all heavy metal uptake by portulaca oleracea, the mean values of BCF, BAC, and TI in control were higher than those of remediated soil. In other words, the inoculation of cyanobacteria into soil and biochar amendment significantly reduced the phytostabilization potential of this plant. For example, the treatments were reduced the BCF of Portulaca oleracea by more than 77 %, 8 %, 85 %, 33 %, and 69 % for Cr (III), Cr (VI), Zn (II), Al (III), and Fe, respectively. Besides, the quantities of BAC in the remediated samples were decreased by more than 80 %, 38 %, 85 %, 44 %, and 71 % for Cr (III), Cr (VI), Zn (II), Al (III), and Fe, respectively. It demonstrated that biochar and cyanobacteria reduced the migration
ability of heavy metals from soil to plant roots and shoots. It proved the effectiveness of biochar amendment and cyanobacteria inoculation for the remediation of heavy metal contaminated soil. This result was in agreement with the research of Puga et al. (Yang et al. 2016b). They found that biochar amendment effectively decreases the accumulation of Pb (II) in cabbage mustard. Also, similar findings were observed by Chen et al. (Chen et al. 2020). They found that the bio-concentration factor including BCF and BAC values of wheat, was reduced by biochar amendments.

Our findings indicated that inoculation of cyanobacteria, although promoted plant growth but it significantly decreased the bioconcentration and bioaccumulation factors (BAC and BCF). It is good to mention that inoculation of some microorganisms, including Arthrobacter sp. (Rojjanateeranaj et al. 2017), Micrococcus sp.(Chuaphasuk &Prapagdee 2019, Rojjanateeranaj et al. 2017), Pseudomonas koreensis (Babu et al. 2015), and Pseudomonas sp. (Rojjanateeranaj et al. 2017), and into soil enhanced the heavy metal accumulation in the shoots and roots. The bioavailability and phytoavailability of heavy metals in the contaminated soil have a crucial effect on absorption or uptake by microorganisms and plants. However, soil chemistry (i.e., pH and organic matters) and plant-microbe interaction are played an essential role in bioavailability and phytoavailability (Shah &Daverey 2020).

4. Conclusion

In this study, two approaches, including bioaugmentation and bioaugmentation-assisted phytoremediation, were utilized for remediation of heavy metals (Fe, Cr (III), Cr (VI), Al, and Zn) contaminated soil. In bioaugmentation experiments, cyanobacteria inoculation accompany with an increment of biochar dose ( from 0 to 5 %) resulted in enhancement of the soil chlorophyll a, nitrogen, and organic carbon contents, reduction of the pH and EC, and also a significant decrease in the bioavailability of all heavy metals. Besides, evaluation of remediation efficiency with the help of lettuce and radish plants, after three months of treatment, confirmed the improvement of soil conditions. The results of bioaugmentation-assisted phytoremediation using biochar- cyanobacteria - Portulaca oleracea showed a substantial improvement in the above – mentioned properties of soil and a notable reduction in the bioavailability of all heavy metals, especially at a 5 % dose of biochar. Moreover, the Portulaca oleracea displays low translocation (TF < 1) for all heavy metals, immobilized, and accumulates the metals in root than shoot, which show their phytostabilization potential. According to the BCF values, the Portulaca oleracea showed an intensive tendency for Zn and Cr (III) uptake, a strong tendency for Cr (VI) absorption, and a weak tendency for Al and Fe. Although inoculation of cyanobacteria and the addition of biochar into contaminated soil promoted plant growth, it significantly reduced the phytostabilization potential of this plant. It can be concluded that the combined use of biochar and bioaugmentation with cyanobacteria is a successful technique for immobilization of heavy metal in soil and mitigate their toxic impacts on the plant.

Declarations

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**Authors’ contributions:**

Fahimeh Zanganeh: Investigation, Project administration, Resources, Validation Writing – original draft, Software, Formal analysis, funding acquisition.

Ava Heidari: Supervision, Visualization, Writing – review & editing, Conceptualization, Data curation, Methodology, Project administration, Funding acquisition.

Adel Sepehr: Funding acquisition, Supervision.

Abbas Rohani: Software, Writing – review & editing.

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Figures

(a) [Image of a micrograph showing a porous structure]

(b) [Image of a graph showing spectral data with peaks at specific wavelengths]

Figure 1
The SEM image (a) and FTIR spectra (b) of biochar.

Figure 2

Images of control soil (a), Oscillatoria sp (b), inoculated soil (c), and surface soil after inoculation with Oscillatoria sp.
Figure 3

Heavy metals fractions changes in the soil bioaugmentation treatments: (S1, exchangeable; S2, Fe-Mn oxide bound; S3, organic matter bound; S4, residual).
Figure 4

Root and shoot lengths and VI of lettuces and radishes in the heavy metals contaminated soils under different treatments
Figure 5

Heavy metals fractions changes in the soil bioaugmentation-assisted phytoremediation treatments: (S1, exchangeable; S2, Fe-Mn oxide bound; S3, organic matter bound; S4, residual).
Figure 6

the concentrations of Cr (VI), Cr (III), Zn, Al, and Fe in shoots and roots of portulaca oleracea.
Figure 7

the estimated BCF, BAC, and TF of Portulaca oleracea grown on heavy metal contaminated soil under various treatments.

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