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ORIGINAL RESEARCH ARTICLE

Bioremediation of Cadmium, Lead, Zinc and Copper Contaminated Soils obtained from Dutse and Hadejia Regions by *Lysinibacillus sphaericus* Strain FUD-001 and *Stenotrophomonas maltophilia* Strain FUD-002 Immobilized on a Biochar

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ABSTRACT

Toxic heavy metals have a detrimental impact on the human body and trigger acute or chronic effects or lead to cancer and death, gastrointestinal tract disorder, and nervous system breakdown. An efficient approach for the amelioration and restoration of soil heavy metal contamination is through bioremediation using biochar-immobilized bacteria. Soil samples were analyzed for Cadmium, Lead, Zinc, and Copper using Atomic Absorption Spectroscopy (AAS), and the isolates were subjected to heavy metal tolerant tests for Cd, Pb, Zn, and Cu at 50mg/L, 80mg/L, 100mg/L, and 150mg/L. The isolates were immobilized on a biochar produced from orange peels (*Citrus sinensis*) after morphological, microscopic, biochemical, and molecular identification, then subjected to a test for bioremediation potential for Cd, Pb, Zn, and Cu at concentrations of 10mg/L, 25mg/L, and 50mg/L. Biochar-immobilized *Lysinibacillus sphaericus* Cd percentage removal at 10mg/L, 25mg/L, and 50mg/L was 99.3, 99.7, and 99.8, respectively. Pb percentage removal was 100 for all three concentrations. Cd percentage removal for biochar-immobilized *Stenotrophomonas maltophilia* at 10mg/L, 25mg/L, and 50mg/L was 99.3, 99.7, and 99.8, respectively. Pb percentage removal at the same concentrations was 100. Orange peel was found to be a suitable substrate for biochar production and immobilization of bacteria. Chemical activation of the biochar using HNO_3 improved its sorption capacity. Based on the result obtained, the biochar-bacteria complex is a highly remarkable strategy and can be employed in the bioremediation of heavy metal-contaminated soils.

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Immobilization, Bioremediation, Biochar, Heavy-Metals, Atomic Absorption Spectroscopy

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INTRODUCTION

Soil contamination has become a major global concern as a result of increased industrialization and urbanization which has made soil and water contamination a serious phenomenon influencing human health and ecosystem safety. Numerous pollutants, such as heavy metals and organic contaminants, are released into water bodies and soils (Amen *et al.*, 2020). Despite many efforts of agencies such as the Environmental Management Agency (EMA), World Health Organization (WHO) or European Environment Agency (EEA), industrial development leads to rising levels of toxic metals in soil, water, and air, constituting a direct and/or indirect threat for human health (Witkowska *et al.,* 2021). Toxic metals have proven to be a major threat to human health, mostly because of their ability to cause membrane and DNA damage and to perturb protein function and enzyme activity. Some heavy metals stimulate through different pathogenetic links the progression of cancers and reduce their sensitivity to treatment (Romaniuk *et al.*, 2017; Pietrzak *et al.*, 2021). Oxidative stress (rising level of oxidative damage in a cell) caused by these metals destroys lipids, proteins, and DNA molecules and supports carcinogenesis. A wider discussion on this topic can be found in recent reviews (Kim *et al.*, 2015; Carver *et al.*, 2018; Guo *et al.*, 2019). This group comprises toxic metals, including cadmium (Cd), lead (Pb), nickel (Ni), chromium (Cr), mercury (Hg), and metalloids, such as arsenic (As), from both natural sources and industry. It is well known that exposure to xenobiotic metals can cause gastrointestinal, respiratory, cardiovascular, reproductive, renal, hemopoietic, and

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neurological disorders (Gerhardsson and Kazantzis, 2015; Garza-Lombo *et al*., 2018).

Biochar is a carbon-rich solid produced upon pyrolysis of organic plant and animal materials at high temperatures in a low- or no-oxygen environment. It is widely used in carbon sequestration and soil improvement due to its rich surface micropore structures and the resulting huge surface areas, as well as rich functional groups. Biochar can be used as adsorbents and passivators to remove heavy metals from water and fix heavy metals in soils and for bacterial immobilization (Qiu *et al.,* 2022; Zhou *et al.,* 2019).

Agnello *et al.* (2016) stated that Huguenot D. and a group from France provided a comparative account of bioremediation strategies for contaminated environments. They have established that the collective utilization of plants and bacteria was the most helpful alternative for the management of the polluted soil in comparison to natural attenuation, bioaugmentation, or phytoremediation strategies alone (Agnello *et al.,* 2016). Bioremediation of metal-polluted sites using plants and microbes is one such cost-effective alternative, having the potential to refurbish metal-contaminated environments. Bioremediation is a variety of metabolic activities using organisms to remove contaminants or leave them unharmed, which is a low-cost and environmental-friendly technique. During the remediation process, microorganisms use organic pollutants dominated by hydrocarbons as "carbon sources" for oxidation and decomposition rather than transfer them to different environmental media (Wu *et al.,* 2020). Although the introduction of microorganisms to contaminated soil and water can improve pollutant removal performances, it has some limitations, such as microbial survival, proliferation, mechanical interference, limited available nutrients, poor adaptability, and competition with native microorganisms (Wu *et al.,* 2019). Jae-Seong So and a group from Korea were involved in the bioremediation of heavy metals using isolated bacteria, bacterial mixtures, and microbially induced calcite precipitation methods (Kang *et al*., 2014, 2015, and 2016). Ma *et al.* (2020) explored the bioremediation of Cd-polluted soils using immobilized *Bacillus* sp. TZ5. Li *et al.* (2018) conducted a similar study on the effects of *Pseudomonas chenduensis* and biochar on cadmium availability and microbial community in paddy soil. Moreover, Chen *et al.* 2021 explored the remediation of chromium-contaminated soil based on *Bacillus cereus* WHX-1 immobilized on biochar. Microorganisms immobilized by biochar tend to exhibit higher survival rates and increased resistance to environmental disturbances compared to inoculation as free cells (Qi *et al.,* 2021).

Ahsan and Shimizu, 2021 reported that Some *Lysinibacillus* spp. accumulate or remove toxic metals through the biosorption process. Chen *et al.* 2014 reported the heavy metals bioremediation potential of *Stenotrophomonas maltophilia.*

This study aims to explore the bioremediation potential of orange peel biochar immobilized *L. sphaericus* and *S. maltophilia* on four different heavy metals (Cd, Pb, Zn, and Cu) as an eco-friendly strategy for ameliorating soil heavy metal contamination.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples from Heavy metals contaminated sites of Hadejia and Dutse regions (Makera, Jari Bola, and Zai) were collected at a depth of 5 - 10cm, packed in sterile polyethylene bags, and transported to the laboratory for analysis (Galitskaya et al., 2016).

Physicochemical Characteristics of Soils

The pH, particle size distribution (sand-silt-clay), pore space (PS), organic carbon, and nitrogen were measured to get the physicochemical characteristics of soils obtained from the study area.

Identification and Analysis of Heavy Metals Concentrations in the Soil Samples

The soil samples were transported to Bayero University Kano Central Laboratory for analysis. The concentration of each heavy metal under study (Cu, Pb, Cd, and Zn) was measured using Atomic Absorption Spectroscopy (Mohammed, 2021). Digestion of the soil samples was done according to the procedure of Sani et al. (2022).

The soil samples were analyzed in triplicates with the concentration of the metals present being displayed in milligrams per liter (mg/L) (Ogunnusi and Oyetunji 2017).

Isolation of Bacteria

A serial dilution of the contaminated soils was made up to 10^{-9}. From each of the dilutions of 10^{6}, 1ml was pour plated on Cetrimide agar (Flumedia High Flown - Global Resources India), Sheep Blood agar, and Sorbitol MacConkey agar (Lifesave biotech, - Trade St. San Diego. CA92121 USA), incubated (in IN-SK100) incubator at 37°C for 24h.

Screening of Heavy Metal Resistant Bacteria

Prominent colonies were selected and sub-cultured on a fresh nutrient agar amended with different heavy metal salts cadmium chloride ($CdCl_2$), lead acetate ($Pb(C_2H_3O_2)_2$, copper sulfate ($CuSO_4$) and zinc sulfate ($ZnSO_4$) of analytical grade at concentrations of 50mg/L, 80mg/L 100mg/L and 150mg/L for the test of heavy metal tolerance and incubated for 24h at 37° C (Nath *et al.*, 2019).

Identification of Bacteria

Isolates that resisted all the heavy metals were sub-cultured and identified by colony morphology, Gram staining, and biochemical tests (indole production, methyl red, indole, citrate utilization, catalase, oxidase, etc.)

(Agarwal et al., 2021). Moreover, molecular identification was carried out on the isolates, including Polymerase Chain Reaction (PCR) and 16S rRNA sequencing as employed by Nath *et al.* 2019. The 16S forward primer used was GGACTACAGGGTATCTAAT, and the reverse primer used was AGAGTTTGATCCTGG. The obtained 16s rRNA genes of both species had 789 base pairs.

Production of Biochar

Orange peels were used in the production of biochar. The orange peels were obtained from a local market in Dutse and were first washed with running tap water and then rinsed with distilled water. The peels were air-dried, smashed into smaller pieces, placed in porcelain crucibles, and covered with aluminum foil for pyrolysis. The biochar was produced using a muffle furnace (SX – 2.5 – 10 PEC medical USA) with retort heating. The peak temperature of the process was 300 °C and lasted for 2h (Adeniyi et al., 2020) at the Environmental Management and Toxicology Laboratory at Federal University Dutse. The biochar was removed from the furnace and cooled. The biochar was ground to powder, passed through a 0.5mm sieve , and chemically activated and modified using 2M HNO_3.

Immobilization of Bacteria on Biochar

In order to immobilize the bacterial isolates on the activated biochar, 5g of the activated biochar was weighed and placed in a sterile test tube. Six (6) ml of double distilled water was dropped on the biochar and mixed thoroughly. Bacterial inoculi were mixed with the biochar. It was incubated at 37° C for 8h to allow contact between the bacteria and biochar for adsorption (Chen *et al.*, 2022). The mixture was centrifuged (800-1 Xiangtian centrifuge, max RCF 1790xg) and filtered using Whatman filter paper (41 Ø 150mm) to remove unattached bacteria. The mixture was placed in a sterile petri dish and placed in an oven at 45° C for 3h. The morphological characteristics of the biochar and bacterial adsorption on the biochar were ascertained using Scanning Electron Microscopy.

Bioremediation Activity of Bacteria Immobilized on Biochar

One (1) ml of 10mg/L, 25mg/L, and 50mg/L of heavy metal Cd, Pb, Zn, and Cu solution was suspended in a sterile petri dish. 0.1g of biochar-immobilized bacteria was added, and 0.1g of sterilized soil sample was also added. Molten nutrient agar at 45° C was added and rocked gently to obtain an even mixture. It was incubated at 37° C for 48 h. The samples were digested for AAS to assess the final heavy metal concentration. Scanning Electron Microscopy was conducted to see the bacterial adsorption on the biochar. The bioremediation efficiency of the bacteria was obtained using the method of Naghipour *et al.* (2018) below;

$$\% \text{ Removal} = \frac{C_i - C_t}{C_i} \times 100$$

Where C_i is the initial concentration of heavy metal in the sample.

C_t is the final concentration of heavy metals in the sample.

Data Analysis

All the experiments were conducted in triplicate. Data were presented as means with standard deviations. Analysis of variance (ANOVA) was applied at $p < 0.05$ to evaluate the relationship between the biochar, the isolates, biochar immobilized bacteria, the different heavy metals, and the different concentrations used.

RESULTS AND DISCUSSION

Physical Properties of Soil

The physicochemical characteristics of the soil samples were analyzed to ascertain the conditions at which the soil microorganisms were thriving in order to match such conditions in the laboratory for an optimal result. The results of the physicochemical analysis on the soils obtained from Zai Dutse, Jari-bola, and Makera Hadejia are depicted in Table 1. The soils have 6% silt, while the percentage of clay and sandy differs significantly, with 39%, 14%, and 10% for Jari Bola, Makera, and Zai, respectively. The percentage of sand distribution is 64%, 80%, and 84% for Jari Bola, Makera, and Zai, respectively, with the latter having the highest sand distribution. The pH of the soils is alkaline, with 8.5 for Jari-Bola, 8 for Makera, and 8.4 for Zai, meaning that the optimal pH at which bacteria from the soil survive is alkaline. The soil from Jari Bola has the least nitrogen (0.0008), while the soil sample from Zai has (0.00652) and Makera has (0.001). Soil from Makera has the highest percentage of organic matter (1.5%), followed by Jari Bola (0.6%), with the least from Zai having (0.3%). The discrepancies in nitrogen, PSD, and organic carbon could be due to the topography of the places.

Heavy Metal Concentration of the Soil samples

The heavy metals concentrations in the soil samples were analyzed using AAS. All digestions of samples prior to AAS were carried out using the procedure of Sani *et al.* 2022. The result is shown in Table 2 below.

Table 2 shows the result of the Atomic Absorption Spectroscopy of the soils obtained from Dutse and Hadejia for the analysis. Zai has 0.017mg/L cadmium, 0.273mg/L lead, 0.888 mg/L zinc, and 0.063mg/L copper. Jari Bola has 0.036mg/L cadmium, lead 0.873mg/L, Zinc 5.757mg/L and Copper 0.653mg/L. Makera has 0.019mg/L cadmium, 0.271mg/L lead, 0.780mg/L zinc, and 0.078mg/L copper. These concentrations are higher than the standard limits of heavy metals in the soil.

Isolation of Bacteria

Following serial dilution, 1ml from each 10^{-6} was pour plated on a nutrient agar, Sorbitol MacConkey

agar, Sheep blood agar, and Cetrimide agar. After incubation for 24h at 37°C, the isolates were subjected to heavy metals tolerance test. Morphological/Colonial characteristics and biochemical tests, which include catalase, oxidase, citrate, methyl red, and indole, were carried out.

Screening of Heavy Metal Resistant Bacteria

To screen for heavy metals-resistant bacteria, four concentrations, 50mg/L, 80mg/L, 100mg/L, and 150mg/L of Cd, Pb, Zn, and Cu, were used (Nath *et al.,* 2019). Prominent colonies isolated after serial dilution of the contaminated soil samples were selected for the screening and named X1, X2, X3, X4, and X5. X1 and X2 were able to grow in all the increasing varying concentrations of Cd, Pb, Zn, and Cu. However, all isolates were able to grow at 50mg/L. Isolate X3 tolerated 80mg/L Cd and Cu but inhibited at 100 and 150mg/L. Isolate X4 tolerated Cd at 50, 80, and 100 mg/L but inhibited at 150mg/L. It also tolerated Pb and Cu at 50 and 80 mg/L but inhibited at 100 and 150mg/L. However, it was able to tolerate Zn at all the concentrations. Lastly, X5 tolerated Cd and Zn at 50 and 80 mg/L and inhibited at 100 and 150mg/L. It was able to tolerate Pb and Cu only at 50mg/L , as shown in Table 3. Isolates X1 and X2 were selected due to their outstanding heavy metal tolerance.

Soil incorporated into the media

Soil particles were incorporated into the media during analysis to enrich the media and to mimic the natural habitat of the bacteria (soil). 0.1g of washed, dried and autoclaved soil sample was added to the nutrient agar media. The soil was subjected to AAS to assess the concentrations of Cd, Pb, Zn, and Cu, and the result is portrayed in Table 4.

Table 4 shows the heavy metal concentrations of the soil incorporated into the media during the analysis. This was done in an attempt to enrich the media, matching the natural environment of the isolates.

Immobilization of Bacterial Cells on Biochar

Scanning Electron Microscopy (SEM) was performed in order to visualize the attached bacteria and the morphology of the biochar. The adsorption capacity of the orange peels was increased to several folds after activation. Peng *et al.* (2016), Zhou *et al.* (2017), Yang *et al.* (2019) supported that the use of HNO_3 for chemical activation of biochar effectively enhances its pore spaces and greatly increases its sorption capacity.

Several researches have shown that biochar contains nutrient sources that contribute to the growth and reproduction of microorganisms (Qi *et al.*, 2021). The orange peel biochar has high carbon content, wide surface area, and enhanced porosity, which make it ideal for bacterial immobilization and use as a proper feedstock for biochar production. (Adeniyi *et al.*, 2020). Biochar can also serve as a nutrient source and provide a protective environment for the bacteria (Chen *et al.*, 2021).

The enhanced immobilization of heavy metals like Cd and Cu by bacteria-loaded biochar has been reported earlier (Chen *et al.,* 2019; Tu *et al.*, 2020). However, orange peel was not used as an agent of immobilization despite several studies highlighting its suitability as a proper feedstock for biochar production. Our result demonstrated that orange peel biochar can serve as an excellent carrier for bacterial immobilization.

Heavy Metals Percentage Removal by Biochar Immobilized X1- (*L. sphaericus strain FUD-001)* and X2-(*S. maltophilia strain FUD-002)*

Biochar immobilized *L. sphaericus* was subjected to a test for bioremediation on three different concentrations: 10, 25, and 50 mg/L of Cd, Pb, Zn, and Cu. The result of the percentage removal efficiency is shown in Table 5.

The percentage removal of Cd, Pb, Zn, and Cu by BX1 – Biochar Immobilized *(L. sphaericus* strain FUD-001*)* for 10mg/L has 100% for Pb as the highest and 98.0% for Cu as the lowest. For 25mg/L, the highest percentage of removal is 100% for Pb and 98.8% for Cu as the lowest, while for 50mg/L, the highest percentage of removal is 100% for Pb and 98.0% for Cu as the lowest, as depicted in Table 5. Wang *et al.* (2021) reported *Bacillus sp.* K1 bioremediation of Cd (II) was always restricted in high concentration (>20 mg L−1) due to the inhibition of cell growth and cell detoxification mechanism (i.e., efflux of Cd (II) from intracellular). Thus, biochar sheltered *Bacillus sp.* K1 from heavy metal stress and improved heavy metal removal.

Table 1: Physicochemical Characteristics of the Contaminated Soils.

I.D	PSD	%	Texture	pH	Organic Carbon%	Organic Matter%	Nitrogen
J/Bola	Silt	6	Sandy – Loam	8.5	0.6	1	0.0008
	Clay	39					
	Sand	64					
Makera	Silt	14	Loamy-sand	8.1	1.5	2.6	0.001
	Clay	6					
	Sand	80					
Zai Dutse	Silt	10	Loamy-sand	8.4	0.3	0.4	0.00652
	Clay	6					
	Sand	84					

Table 2: Heavy Metals Concentration of the Soil Samples

Sample Analyte	Mean Conc. ± S.D mg/L	%RSD
Zai - Dutse		
Cd	0.017 ± 0.0005	3.26
Pb	0.273 ± 0.0157	5.76
Zn	0.888 ± 0.0026	0.30
Cu	0.063 ± 0.0024	3.91
Jari - Bola		
Cd	0.036 ± 0.0014	4.01`
Pb	0.873 ± 0.0675	7.74
Zn	5.757 ± 0.3192	5.54
Cu	0.653 ± 0.0023	0.35
Makera		
Cd	0.019 ± 0.0012	6.45
Pb	0.271 ± 0.0675	24.94
Zn	0.780 ± 0.0015	0.19
Cu	0.078 ± 0.0136	17.48

*Mean Conc. = Mean Concentration, S.D = Standard Deviation, %RSD = Percentage Relative Standard Deviation, mg/L = Milligram per Liter.

Table 3: Heavy Metals Tolerance Test Performed on the Isolates

Isolates	Heavy metals	Concentrations (Mg/L)			
		50	80	100	150
X1	Cd	+	+	+	+
	Pb	+	+	+	+
	Zn	+	+	+	+
	Cu	+	+	+	+
X2	Cd	+	+	+	+
	Pb	+	+	+	+
	Zn	+	+	+	+
	Cu	+	+	+	+
X3	Cd	+	+	-	-
	Pb	+	-	-	-
	Zn	+	-	-	-
	Cu	+	+	-	-
X4	Cd	+	+	+	-
	Pb	+	+	-	-
	Zn	+	+	+	+
	Cu	+	+	+	-
X5	Cd	+	+	-	-
	Pb	+	-	-	-
	Zn	+	+	+	-
	Cu	+	+	-	-

Table 4: Heavy Metals Concentrations of the Soil Incorporated into the Media During Analysis

Sample analyte	Mean conc. ± S.D (mg/L)	%RSD
Cd	0.0014±0.0004	3.24
Pb	0.084±0.0215	25.70
Zn	0.494±0.0039	0.80
Cu	0.133±0.0019	1.39

*Mg/L=Milligram per liter, S. D=Standard deviation, %RSD=Percentage standard deviation.

Table 5: Heavy Metals Percentage Removal by Biochar Immobilized X1- (*L. sphaericus strain FUD-001)*

Sample analyte	Conc. (mg/L)	Mean conc. ± S.D (mg/L)	%RSD	%Removal
Cd	10	0.061±0.0018	2.90	99.3
Cd	25	0.064±0.0016	2.50	99.7
Cd	50	0.066±0.0024	3.58	99.8
Pb	10	-0.042±0.0472	112.04	100
Pb	25	-0.020±0.0204	100.17	100

To be continued next page

Table 5 continued

Sample analyte	Conc. (mg/L)	Mean conc. ± S.D (mg/L)	%RSD	%Removal
Pb	50	-0.067±0.0285	42.88	100
Zn	10	0.059±0.0016	2.73	99.4
Zn	25	0.038±0.0018	4.78	99.8
Zn	50	0.041±0.0012	2.80	99.9
Cu	10	0.194±0.0032	1.65	98.0
Cu	25	0.292±0.0016	0.53	98.8
Cu	50	0.957±0.0033	0.35	98.0

*Mg/L=Milligram per liter, S.D=Standard deviation, %RSD=Percentage standard deviation, %Removal=Percentage removal

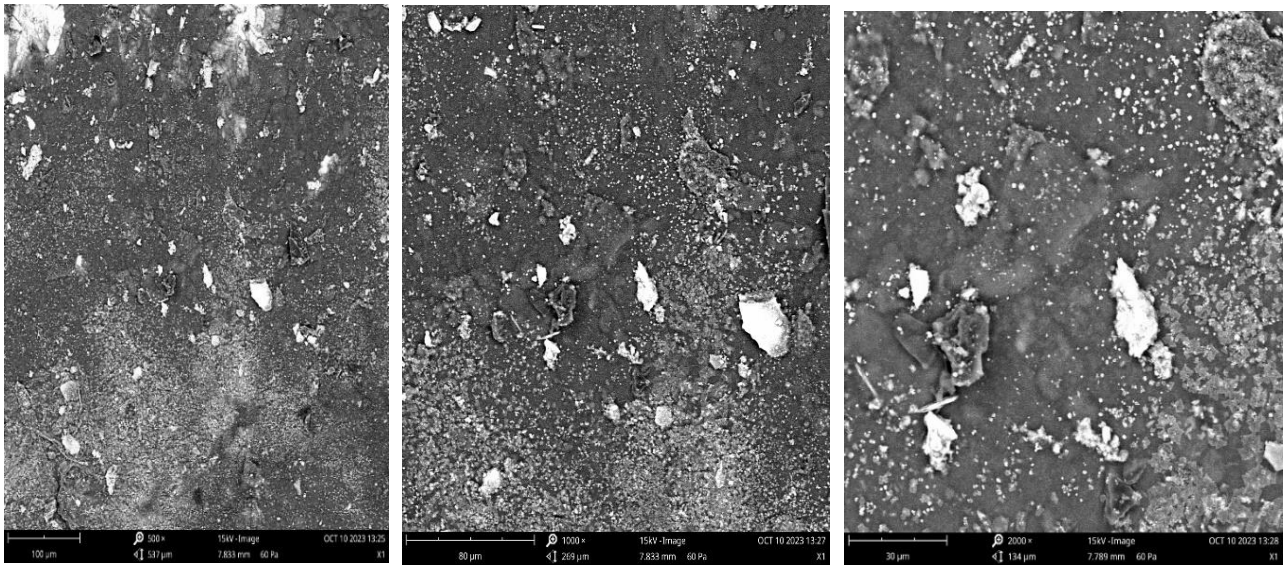


Figure 1: SEM images of biochar immobilized X1 – *L. sphaericus strain FUD-001* with magnification ×500, ×1000 and ×2000

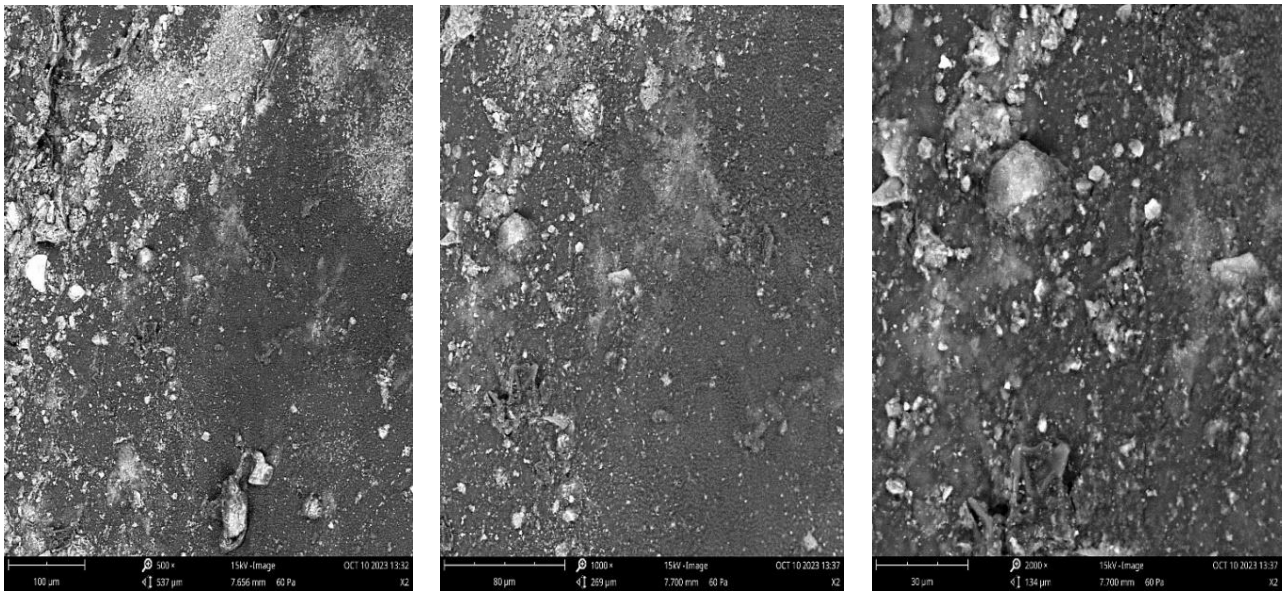


Figure 2: SEM images of biochar immobilized X2 – *S. maltophilia* strain FUD-002 with magnification ×500, ×1000 and ×2000.

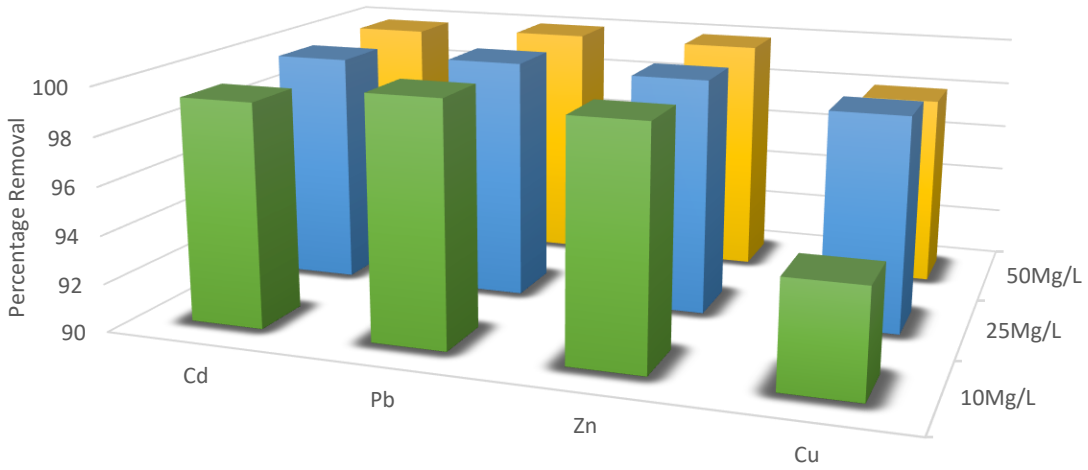
It has been reported that biochar influences bacterial metabolism (Adeniyi *et al.,* 2020). The percentage removal of Cd, Pb, Zn, and Cu by BX2 – Biochar Immobilized *(S. maltophilia* strain FUD-002*)* depicted in Figure 3 shows the percentage removal for 10mg/L has 100% for Pb as the highest and 94.4% for Cu being the lowest. For 25mg/L, the highest percentage of removal is 100% for Pb , while 98.9% for Cu is the lowest. Lastly, for 50mg/L, the highest percentage of removal is 100% for Pb, and 98.0% for Cu is the lowest. The result of a similar study conducted by Manikandan and Nair, 2022 showed

Pseudomonas stutzeri not immobilized on a biochar percentage removal of Ni was only 83%.

Statistical Analysis

Analysis of Variance (ANOVA) was used as employed by Mohammed *et al.* (2021) in order to establish statistical significance between the biochar, isolates, biochar immobilized bacteria, the different heavy metals and different concentrations used. The ANOVA was used to test whether or not the different heavy metals and different concentrations have any impact on the bioremediation efficiency of the biochar, isolates, and biochar immobilized isolates.

The result of statistical analysis showed that the varying concentrations at 95% confidence interval ($P < 0.05$) (with significance Sig.: 0.976), the heavy metals (with Significance Sig: 0.579) had no significant impact on the bioremediation efficiency of the biochar, bacterial strain (*L. sphaericus* and *S. maltophilia*) and the biochar immobilized bacteria (with Significance Sig: 0.993).



	Cd	Pb	Zn	Cu
10Mg/L	99.3	100	99.7	94.4
25Mg/L	99.7	100	99.8	98.9
50Mg/L	99.8	100	99.9	98

Figure 3: Heavy Metal percentage Removal by Biochar immobilized *S. maltophilia strain FUD-002*

CONCLUSION

L. sphaericus Strain FUD-001 and *S. maltophilia* Strain FUD-002 immobilized on orange peel biochar demonstrate high potential in bioremediating heavy metals contaminated soil, particularly Cd, Pb, Zn, and Cu. The biochar's porous structure enhances adsorption and immobilization efficiency. By retaining bacteria, the biochar facilitates sorption, accelerating bioremediation and improving strain robustness and tolerance to high pollutant concentrations.

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