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Screening of plant growth promoting and heavy metal tolerance of rhizospheric microorganisms in the mangrove ecosystem

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ABSTRACT

Mangroves create a unique ecological niche with varied environmental conditions and diverse microbial communities, including nitrogen fixers and phosphate solubilizers, which have immense potential as plant growth promoting bacteria (PGPB) including heavy metal tolerance. To address this, the current study was undertaken to screen for the presence of lead and cadmium tolerant microorganisms from the mangrove rhizosphere in Sagar Vihar, Vashi and to evaluate their plant growth promoting (PGP) potential. Isolates were characterized and screened for various PGP properties. Four of the 17 isolates obtained showed the most promising PGP traits, with one isolate exhibiting the maximum PGP traits. Additionally, phyto-remediation of heavy metal by mangrove root or pneumatophores evaluated using Energy Dispersive X-ray Analysis (EDAX) showed absence of metal. The biocompatibility study of these isolates revealed that all lead tolerant isolates were compatible with each other. Through this investigation, we propose to use a consortia based on these microbes as a biofertilizer for the eco-restoration of mangrove forests.

Keywords: Mangrove, Rhizosphere, Heavy metal, PGPB, EDAX, Phytoremediation

INTRODUCTION

Mangroves are common along tropical and subtropical seashores, which are salt-tolerant trees or shrubs. They have unique adaptations that allow them to live in brackish water environments including waterlogged soil and anaerobic conditions (Sakhia *et al.,* 2015). Mangroves are essential in stabilizing shorelines and accelerating soil/sediment accretion, collection of heavy metals and nutrients, leading to better water quality, and barricading against natural disasters in coastal areas. They act as a breeding ground for various types of amphibians, fishes, and crustaceans. They are constantly under threat due to numerous anthropogenic activities leading to rapid industrialization and urbanization which pose a threat of heavy metal (HM) contamination in the mangrove ecosystem. Industrialization has led to the waste dumped directly into waterways without treatment, lowering the quality of water bodies (Luthansa *et al.,* 2021) These industrial wastes may include heavy metals such as Cu, Zn, Cd, Ni, Cr, Mo, Fe, and Mn, which are non-degradable pollutants that exist as free cations and are hazardous to health and the environment at higher concentrations. As a result of which, the coastal environment often receives inputs of heavy metals, due to which the soil around these regions shows significant metal contamination (Kayalvizhi *et al.,* 2019). Heavy metals have profound environmental impacts by altering sediment and water properties, ultimately jeopardizing ecosystem health for plants and animals. This was proven by a study conducted in the Sundarbans mangrove forest in Bangladesh wherein the heavy metals were negatively affecting the growth and survival of mangrove trees leading to significant ecological consequences (Mandal *et al.,* 2019). More than 20 heavy metals with noticeable toxicity are listed by the Agency for Toxic Substances and Disease Registry (ATSDR), but four in particular are cause for concern: arsenic (As), lead (Pb), cadmium (Cd), and mercury (Hg) (González Henao, and Ghneim-Herrera., 2021). Lead is known to degrade the development of the root system whereas cadmium results in inhibition of growth and development, browning of root tips and may even lead to death of the plant at high concentrations; therefore, cadmium and lead are focused in this study. The mangrove rhizosphere contains highly functional, diverse microbial communities that participate in cycling nutrients and maintaining the ecosystem trophic networks (Muñoz-García *et al.,* 2022). Because of their

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capacity to acquire nutrients, solubilize inorganic phosphate, fix nitrogen, produce hydrogen cyanide, ammonia, and indole acetic acid, plant growth-promoting rhizobacteria (PGPR) are extremely significant for stimulating plant growth and development (Ortiz-Ojeda *et al* 2017). Richardson *et al* (2009) found that rhizospheric microorganisms could increase plant uptake of nitrogen by 25%, improving the plant growth. Weller *et al*. (2002) reported suppression of soil-borne pathogens causing plant disease by rhizospheric microorganisms by 50%. Rhizospheric microorganisms show a promising approach for the bioremediation of organic pollutants, offering a cost-effective, eco-friendly, and suitable solution for in situ application. Singh *et al* (2018) reported that the addition of rhizospheric microorganisms significantly enhanced the degradation of pesticides, attributed to the production of enzymes and other metabolites by the microorganisms. Furthermore, the extracellular polymeric substances (EPS) produced by microbial communities in the rhizosphere like polysaccharides, glycoproteins, lipopolysaccharides, and soluble peptides, have significant amounts of anion functional groups and aid in the biosorption of metals from the rhizosphere (Mishra *et al.,* 2017). Owing to such binding ability, the EPS is been recommended as a promising adsorbent for metal contaminants (Mathivanan *et al.,* 2021). As this area has not been screened much, the present study aimed at obtaining isolates of indigenous bacteria from the rhizospheric region of mangroves that have the potential as plant growth promoting bacteria along with heavy metal tolerance to lead and cadmium.

Figure 1: Sample collection site

MATERIAL AND METHODS

For sample collection, 5 random soil samples were obtained (20 cm depth) in the rhizospheric region of Mangroves from Vashi (19.075435° N to 72.95223°S), Navi Mumbai, Maharashtra, India (Figure 1 and 2) In the month of January 2023. This area is in a tropical zone, therefore the annual temperature ranges between 22°C to 36°C. The mangrove species commonly seen in the area includes *Avicennia marina*, *Acanthus ilicifolius* and *Sonneratia alba.* Plant active root samples (1cm long) were collected from the sampling site to study the metal absorption by the mangrove plants. The collected samples were stored in disinfected zip-lock bags and were used for further studies. Soil sample (1gm) was suspended in 10 mL sterile saline and dispersed by vortexing for 5 mins. Following sedimentation, 1 mL of the clear supernatant was inoculated into 100 mL of modified nutritive broth that contained 2% NaCl (MN) and either 0.1 mM of lead nitrate ($Pb(NO_3)_2$) or Cadmium chloride ($CdCl_2$). Further enrichment at room temperature for 72 hours was carried out by sequentially increasing the metal concentration up to 3mM. After the final enrichment, samples were subjected to 10-fold serial dilution using sterile saline and were spread plated on MN agar plates containing 3 mM of either lead or cadmium (Adal *et al*, 2022). Following an incubation for 48 hours, Pb tolerant (named with Prefix P) and Cd tolerant colonies (with prefix C) were selected based on morphological characteristics like Gram staining, capsule staining and biochemical tests including sugar fermentation, catalase, oxidase, urease and IMViC.

Figure 2: Dense Mangroves at Sagar Vihar, Vashi, Sector-8, Navi Mumbai

For Screening of Plant Growth Promoting properties (PGP) various test was carried out. Indole acetic acid (IAA) production was tested by inoculating a loopful of each culture into sterile Luria Bertani broth, supplemented with tryptophan and the method followed by Radif *et al* (2016). A color change from orange to pink was considered as positive for IAA (Khan *et al.,* 202, Mamarasulov *et al.,* 2022, Panda *et al.,* 2023). Production of ammonia by the isolates was tested using the method described by Saravanan *et al* (2016) and Mir *et al* (2022). To test for hydrogen cyanide (HCN) production by the isolates, a loopful of culture was streaked on sterile nutrient agar plates supplemented with glycine. The plates were incubated upside down for 48 hrs containing Whatman filter paper (no.1) strips impregnated with 2% picric acid placed on the lid. A colour change from yellow to light brown, brown or reddish-brown can be interpreted as HCN producer (Roy *et al.,* 2021, BiBi *et al.,* 2023) Nitrogen fixing activity of the isolates was determined by streaking on sterile nitrogen-free Jensen's agar plates. (Backer Alungal *et al.,* 2021). Phosphate solubilizing ability of the isolates were examined by streaking on sterile Pikovskaya's agar plates and incubated to observe a clear zone around the colony (Lebrazi *et al*, 2020). The isolates were assessed for production of exopolysaccharide using Sudan black screening method. The isolates were grown on nutrient agar supplemented with either Pb or Cd and observed for mucoid colony characteristics. The plates were then flooded with 0.1% Sudan black dye and the excess dye was discarded after 2 minutes of incubation. Exopolysaccharide producing colonies do not retain the dye whereas the non-producers take up the dye and appear black. Biocompatibility studies are essential to understand the potential effectiveness of using multiple isolates in a bioremediation strategy. This was performed using a cross-streak method between the Pb tolerant isolates, Cd tolerant isolates as well as between Pb and Cd tolerant isolates (Kannan *et al.,* 2009). The biocompatibility studies were carried out for Pb tolerant isolates on nutrient agar plates containing 2.5 mM $Pb(NO_3)_2$ and for the Cd tolerant isolates on nutrient agar plates containing 2.5 mM $CdCl_2$.

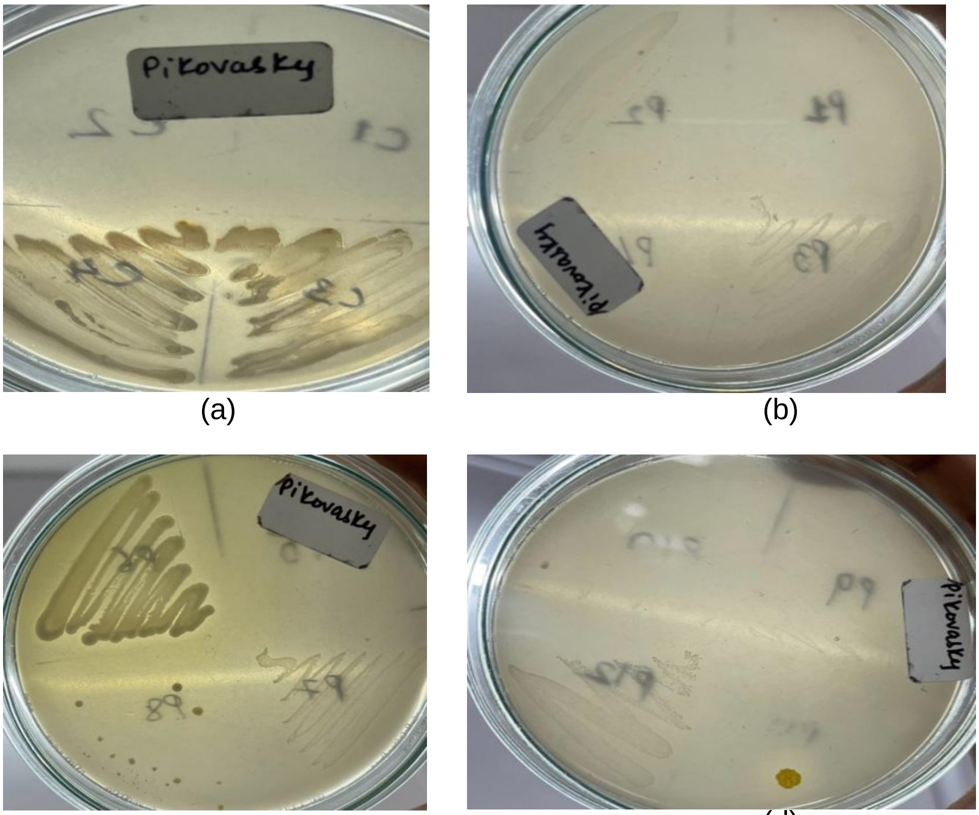
The minimum inhibitory concentration (MIC) was carried out to determine the heavy metal tolerance of the selected PGP isolate. The assay was performed using nutrient broth containing Pb (NO_3) in the range of 5 mM to 100 mM. After incubating the tubes at room temperature for 48 hrs, the tubes were observed for turbidity. For determining the minimum bactericidal concentration (MBC), a loopful of culture was streaked from the MIC tubes showing no turbidity onto sterile nutrient agar plates without heavy metal (Benhalima *et al.,* 2020). The isolates obtained were identified using 16SrRNA sequencing technique using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. BLAST analysis of 16S rRNA gene sequence was performed against the nr database of NCBI GenBank. Using Mega 11, a phylogenetic tree was constructed by aligning the first ten sequences based on the maximum identity score. The collected root samples were analyzed for elemental mapping to determine metal accumulation on the root surface as well as absorption by the roots using Energy Dispersive X-ray Analysis (EDAX) (Evo-18, Zeiss India Ltd). For analyzing the heavy metals, the root samples were washed to remove any potential surface contaminants and cut into 1x1 mm sections followed by dehydration using Quorum K850 (Quorum, USA).

RESULTS AND DISCUSSIONS

Twelve Pb tolerant and five Cd tolerant microorganisms were obtained after enrichment of samples. The morphology of these isolates ranged from cocci to coccobacilli to short rods. All the isolates showed the presence of a capsule except for the isolate P8.

Screening of PGP traits

The phosphate solubilizing properties was seen in 4 of the isolates viz. P6, C3, C4, and C5 (Fig. 3). Behera *et al*. (2016) reported the presence of phosphate solubilizers belonging to the genus *Pseudomonas, Bacillus, Alcaligens, Klebsiella, Serratia, Azotobacter,* and *Micrococcus* from the mangrove soil of Mahanadi River delta, Odisha, India (Behera *et al*. 2016 , Tirry *et al.,* 2018) Similarly, Pallavi *et al* (2023) reported the presence of phosphate solubilizing *Bacillus* spp. in the mangrove region of Sundarbans.



(a) (b)

(c) (d)

Figure 3: HM Tolerant isolates Pikovskaya's agar (a) isolates tolerant to Cadmium, (b) Lead tolerant isolates P1-P4, (c) P5-P8 (d) P9-P12

Rhizobacterial strains indirectly support plant growth by inhibiting the growth of phytopathogenic microbes through production of hydrolytic enzymes and hydrogen cyanide (HCN), thus, minimizing the effects of biotic stresses (Singh *et al* 2020). None of the isolates obtained in this study showed the ability to produce HCN (Table 1). Similar to the current study, Samuel *et al* (2011) reported the absence of HCN producers in the rhizospheric region of rice and mangrove. In addition to HCN, elongation of the plant's roots and shoots has been linked to ammonia synthesis, a PGP characteristic that improves plant productivity and increases resistance to phytoparasitic stresses (Djebaili *et al.,* 2020). From the 17 isolates screened, 11 isolates showed ammonia production (Table 1 and Figure 4). Pallavi *et al* (2023) also reported the presence of several ammonia producing *Bacillus spp.* in the mangrove area of Sundarbans. Similarly, Kumar *et al* (2012) reported the presence of ammonia producers in the rhizospheric region of French bean plants.

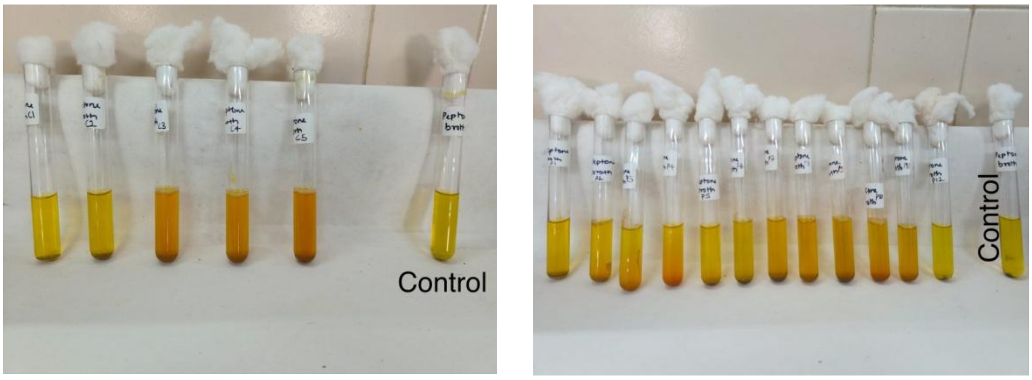


Figure 4: NH_3 production by isolates C1-C5 (left), P1-P12 (right) and control

The bacterial IAA plays a vital role in improving the absorption of minerals and nutrient uptake thereby enhancing the lateral and adventitious rooting and inducing bacterial proliferation on the roots by root exudation (Tirry *et al.,* 2018). Only isolates P6 and P7 were found to produce IAA indicating their potential use as a PGP bacterium (Figure 5).

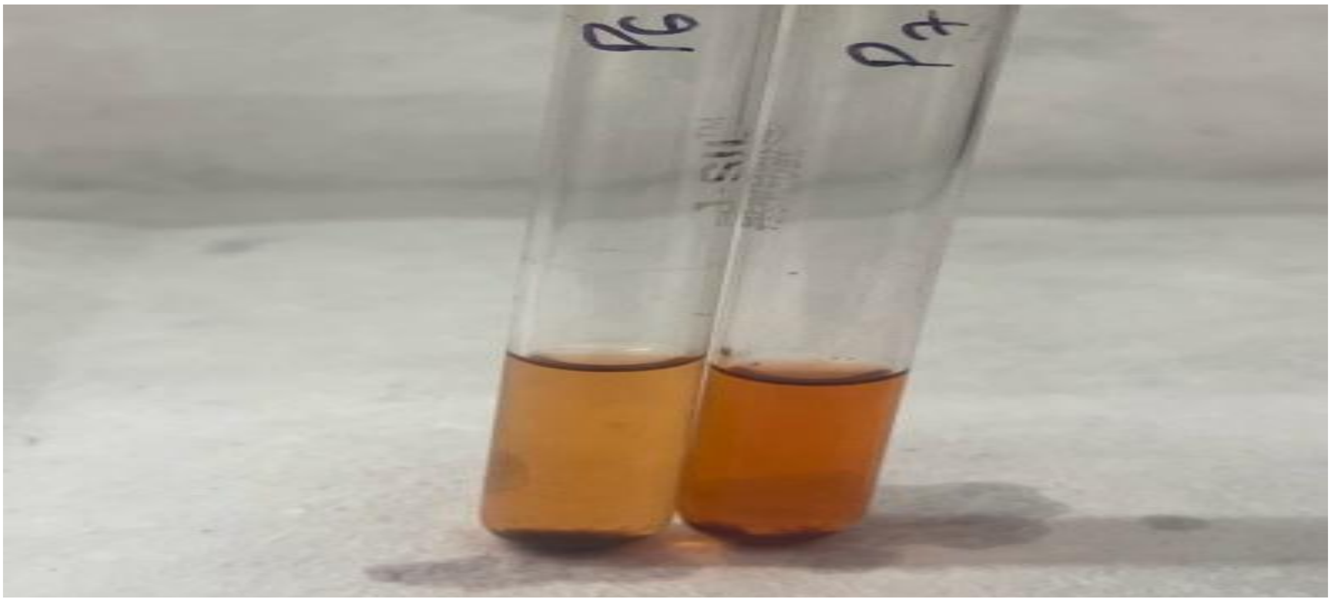


Figure 5: IAA production observed for isolates P6 and P7

Out of all only ten isolates exhibited the ability to grow in complete absence of nitrogen (Figure 6). These organisms could be potentially used as environmentally safe alternative to chemical fertilizers (Chauhan *et al.,* 2017, Nazir *et al.,* 2018).

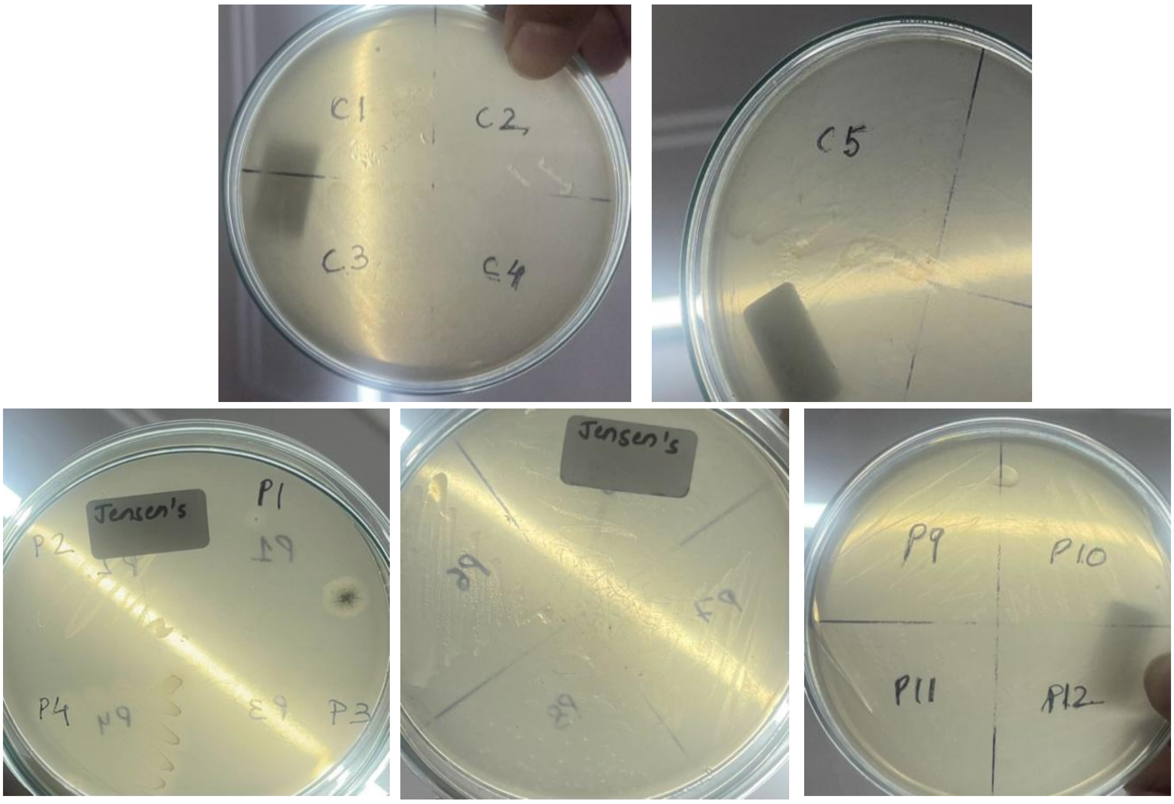


Figure 6: Isolates showing growth on nitrogen free Jensen's agar

In metal-polluted environments, bacterial strains have been found to develop resistance to metal ions by employing various cellular and molecular mechanisms. One such mechanism is the production of exopolymeric substances (EPS) which bind metal ions with the help of negatively charged functional groups. The isolates P1 and P4 were found to produce EPS, while P2 and P6 showed weak positive results (Figure 7).

Table 1: PGP characteristics of Pb & Cd tolerant isolates

Isolates PGP	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	C1	C2	C3	C4	C5
PS	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+
HCN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NH_3	-	+	+	+	-	+	+	/-	+	+	+	-	-	-	+	+	+
IAA	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
NF	-	+	-	+	+	+	-	+	-	-	+	+	-	-	+	+	+

[Key: (+) positive, (-) negative, NF =Nitrogen Fixation, PS =Phosphate Solubilization], Exopolysaccharide production

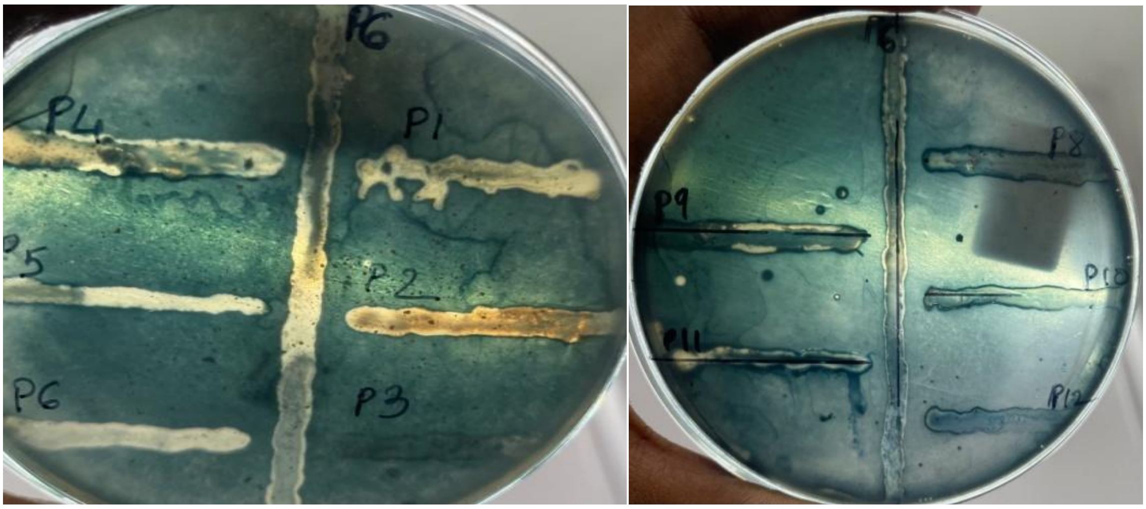


Figure 7: Staining of colonies using Sudan Black to check for EPS-producing bacteria

Biocompatibility Studies and Co-metal Tolerance:

All Pb-tolerant isolates were found to be compatible with each other indicating they can be used in consortium (Figure 8). As for the Cd-tolerant isolates, only C4 and C5 were found to be compatible with each other (Figure 9) indicating that the cooperation between different Cd-tolerant isolates may be limited and that their use in combination may need to be carefully considered.

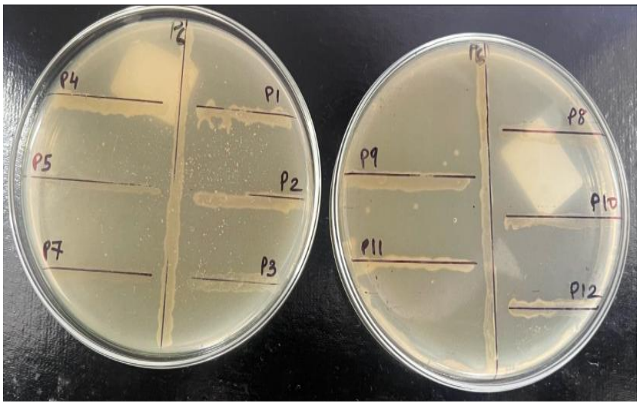


Figure 8: Biocompatibility between Pb-Pb tolerant isolates

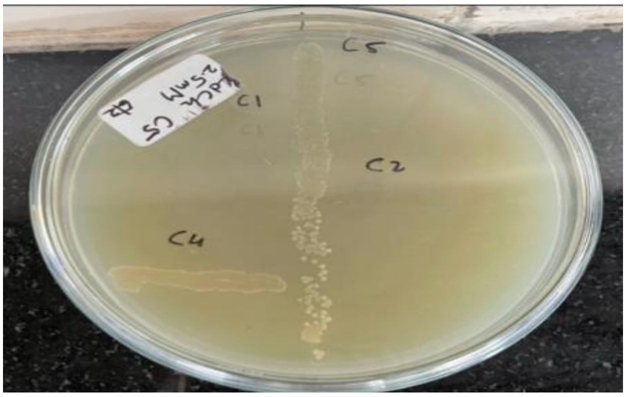


Figure 9: Biocompatibility between Cd-Cd tolerant isolates

Multiple heavy metals co-exist in contaminated soils, which emphasizes the need to select multiple heavy metal tolerant PGPB. The biocompatibility between Pb and Cd tolerant isolates and their co-metal tolerance was also studied. Except for the bacterial isolate P12, all Pb tolerant isolates were also found to tolerate Cd up to 1 mM concentration (Figure 10). Additionally, the isolate P7 showed highest Cd tolerance up to 3 mM concentration.

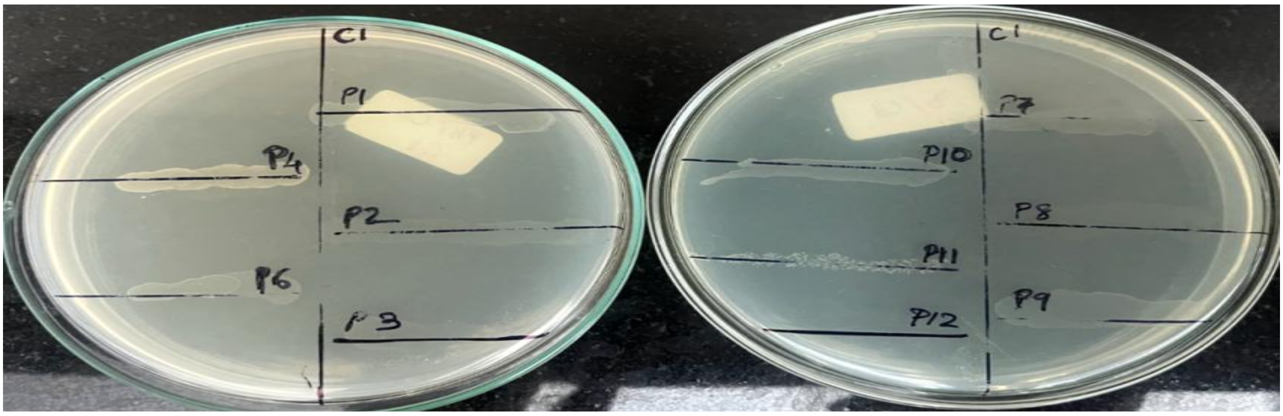


Figure 10: Co-metal tolerance between Pb-Cd isolates

As per the PGP properties summarized in Table 1, the isolate P6 showed the most promising results to act as PGP bacteria because of its ability to solubilize phosphates, fix atmospheric nitrogen and produce IAA as well as NH_3.The organism was also shown to resist Cd metal and weak EPS production. The MIC of the isolate P6 was also performed to check for its ability to tolerate the highest concentration of Pb $(NO_3)_2$. The MIC and MBC were found to be 10 mM indicating that it can resist a maximum concentration of up to 5mM and gets inhibited at 10mM and beyond.

Identification of isolates by 16SrRNA sequencing:

The three isolates, viz. C4, C5 and P6 were identified by the 16S rDNA sequencing and the evolutionary analyses were conducted using Mega11. The 16S rDNA sequence alignment revealed that the isolates C4, C5 and P6 exhibited more than 99% homology to *Bacillus cereus* (Genbank Accession No: OR924281.1), *Staphylococcus hominis* (Genbank Accession No: OR924300.1) and *Bacillus altitudinis*, respectively (Figure11). The biochemical characteristics of the obtained isolates corresponded to the respective identified organisms. Many studies have reported the presence of Bacillus sp in the rhizospheric area of mangrove (Ma *et al.,* 2018, Yatmeen and Sharrah 2011, Deepila *et al.,* 2023). Similar to isolate C4, Zhang *et al.* (2015) also reported the presence of phosphate solubilizing *B. cereus* in the mangrove sediment of Shenzhen Bay, Guangdong, China. Deepika *et al* (2023) identified salt tolerant endophytic *B.altitudinis* from Corangi sanctuary mangrove ecosystem, Andhra Pradesh with IAA production abilities. Current study also found P6, *B. altitudinis*, to be an IAA producer, which is in accordance with the previous study conducted. *S. hominis* has been isolated from rhizospheric soil in various studies (Cheng *et al.,* 2021, Rios *et al* 2020). Rahman *et al* (2019) and Bakhtiyarifar *et al* (2021) have also reported a heavy metal tolerant and non-rhizobial endophytic bacteria. Contrary to these studies, the identified isolate is a nitrogen fixer lacking the ability to produce IAA.

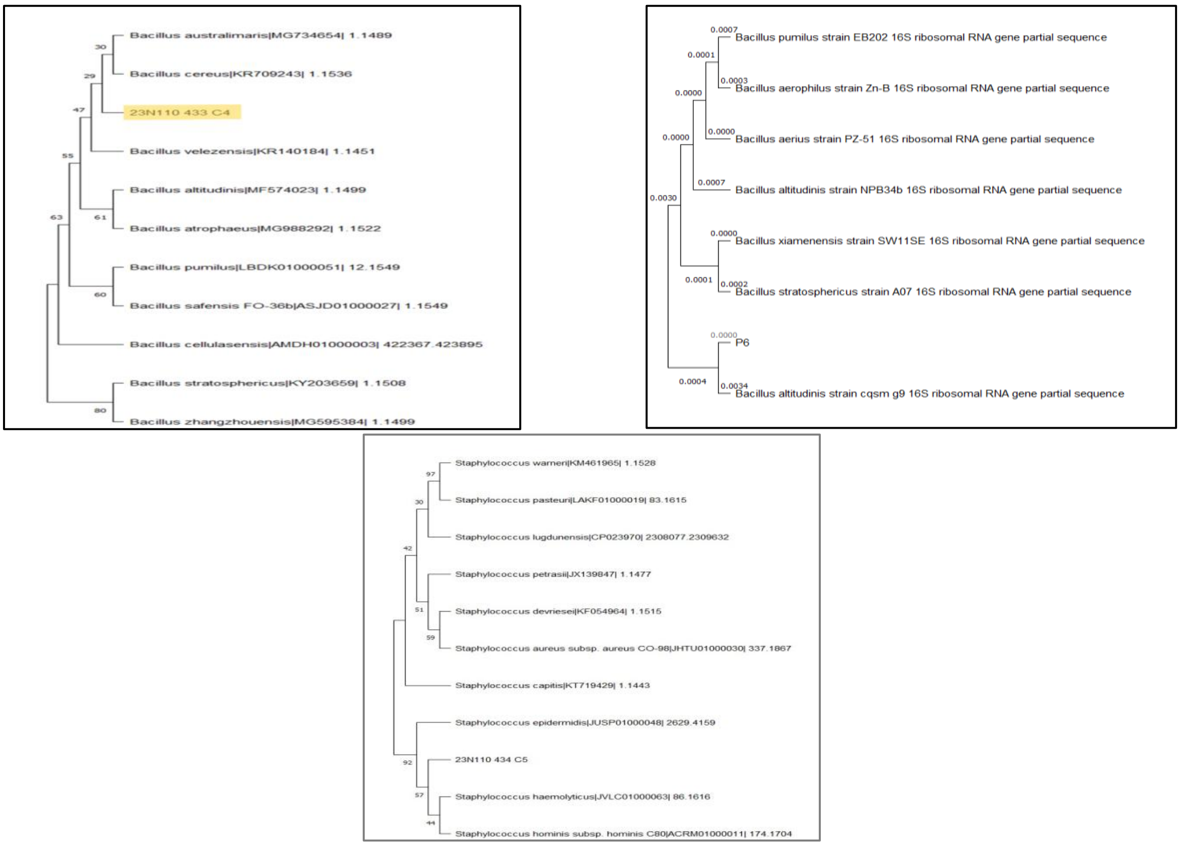
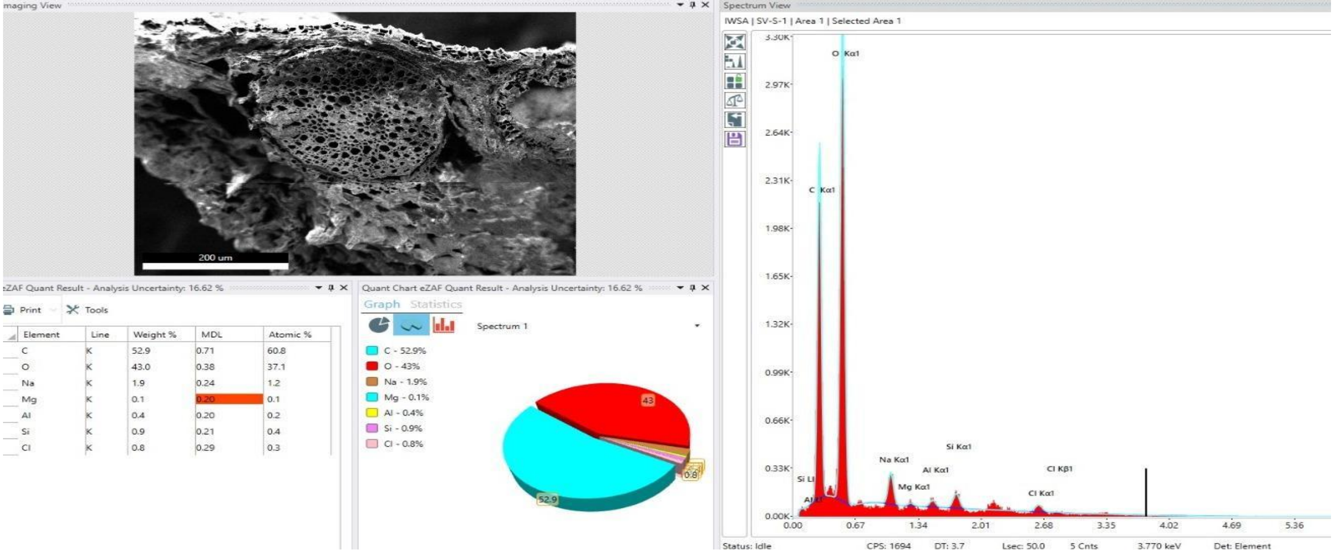


Figure 11: Phylogenetic analysis of selected isolates by 16SrRNA gene sequencing

Analysis of phytoremediation using EDAX

Several heavy metal tolerant PGP bacteria have been reported to facilitate metal uptake and translocation, improving the plant growth and metal tolerance (Fan *et al.*, 2018). In the present study, the ability of mangrove root tissues and pneumatophores in the phytoremediation of heavy metals was analyzed using Scanning Electron Microscopy (SEM) and Energy dispersive X-ray analysis (EDAX). The EDAX report of frozen dried sections of root tissues and pneumatophores is shown in Figure 12.



zAF Quant Result - Analysis Uncertainty: 16.62 %

Element	Line	Weight %	MDL	Atomic %
C	K	52.9	0.71	60.8
O	K	43.0	0.38	37.1
Na	K	1.9	0.24	1.2
Mg	K	0.1	0.20	0.1
Al	K	0.4	0.20	0.2
Si	K	0.9	0.21	0.4
Cl	K	0.8	0.29	0.3

Status: Idle CPS: 1694 DT: 3.7 Lsec: 50.0 5 Cnts 3.770 keV Det: Element

Figure 12: Results of EDAX analysis of mangrove root samples

The results indicate that apart from the essential metal constituents no heavy metal was present in the sample. This could mean that the plant is not able to uptake heavy metals. However, it is important to note that the absence of heavy metals in the plant sample does not necessarily imply the absence of heavy metal in the mangrove soil.

CONCLUSIONS

These findings collectively underscore the potential of mangrove rhizosphere microbiota as valuable contributors to enhancing plant growth and ecosystem restoration efforts. Among these isolates, *B. altitudinis S. hominis* and *B.cereus* demonstrated the most robust PGP properties. Furthermore, the compatibility observed among these isolates suggests the feasibility of creating a potent microbial consortium. While no heavy metal contamination was detected in the root tissue samples through EDAX analysis, further examination of soil and water samples from the same region using Atomic Absorption Spectroscopy will help in to assess heavy metal contamination. Notably, the high tolerance of the *B. altitudinis* isolate to lead contamination, makes it as a promising candidate for phytoremediation endeavours. In summary, this study's outcomes deepen our understanding of the vital role microorganisms play in ecosystems, offer insights into bioremediation strategies, bolster mangrove ecosystem restoration initiatives, and hold potential for advancing agricultural practices.

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