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Microbes from wastewater treated mangrove soil and their heavy metal accumulation and Zn solubilization

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ABSTRACT

The present study isolated and identified six species of bacteria and fungi from the rhizosphere soil of mangroves species (*Avicennia marina*), treated for 60 days with artificial wastewater, and further tested for their potential in heavy metal accumulation and Zn solubilization. The wastewater treated mangrove soil had higher microbial counts, porewater salinity and nutrients (N, P, K, total organic carbon), but lower soil bulk density, pH and temperature. The predominant bacterial strains isolated from the treated soil were identified as *Bacillus altitudinis, B. anthracis* and *B.marisflavi*, and the fungal strains as *Penicillium citrinum, Aspergillus quadrilineatus* and *Gibberella intermedia*. The heavy metal accumulation was determined in those microbial strains cultured with artificial wastewater under shaker culture for seven days. The accumulation was the highest for Zn (70% over control), Cu (62%), Pb (62%) in the bacterium, *B. marisflavi* and for Mn (50%) in the fungus, *P. citrinum*. The *B. marisflavi* was further tested for Zn solublization in terms of pH decline, after 10 days of incubation with the insoluble Zn sources (ZnO, $ZnSO_4$ and $ZnCO_3$). The highest activity of 31.6% pH drop was observed with ZnO. Overall results revealed the potential of *B. marisflavi* in heavy metal removal and Zn solubilization.

1. Introduction

Heavy metal pollution is a global threat since the inception of industrial revolution. Soil is a major sink of heavy metals in the aquatic systems (Jacob et al., 2018). Heavy metals such as Cu, Zn, Ni, Cr, Co, Mo, Fe, and Mn are essential micronutrients for biological species, but at higher concentrations, they are hazardous to health and environment (Jaishankar et al., 2014). Bioremediation by using microorganisms is proved to be efficient method of heavy metal from the contaminated sites (Kuppusamy et al., 2017; Mukherjee et al., 2017).

Among the microbes, bacterial strains of *Bacillus* and *Pseudomonas* are widely used for the removal of heavy metals from wastewater and soil because of their high metal binding affinities (Arivalagan et al., 2014, 2018). A novel haloalkaliphilic bacterium *Cellulosimicrobium funkei* is proved to be efficient in biosorption and biotransformation of Cr(VI) (Karthik et al., 2017a,b). Bacterial functional groups such as hydroxyl, carboxyl, sulfonate, amide and phosphonate groups are mainly involved in the metal uptake process from aqueous solutions (Arivalagan et al., 2014). Fungi are also well known to tolerate and detoxify heavy metal contaminated effluents. The fungal biomass is commonly used as biosorbents as their cell wall polysaccharides enhance heavy metal biosorption (Thatoi et al., 2014; Raja et al., 2015; Saravanakumar and Kathiresan, 2015; Annadurai et al., 2019).

Mangroves are largely located closer to population centers and industrialized areas of coastal environment, and hence the mangrove habitats have often received inputs of heavy metals and the soil may show significant metal contamination (Lacerda, 1998). However, the mangroves may have ability for self-purification, as evident by their luxuriant growth in sewage discharged areas.This is attributed to the abundance of diverse groups of microorganisms and their activities in heavy metal removal (Kathiresan and Bingham, 2001). The mangroves may efficiently trap trace metals in non-bioavailable forms by rapid precipitation of stable metal sulfides under anaerobic condition and by strong binding with organic complexes (Lacerda et al., 1993; Kathiresan and Bingham, 2001). The bacterial strain of *Pseudomonas aeroginosa* isolated from mangrove soil is efficient in Zn solubilization (Beulah et al., 2017). However, studies are far from clear about the microbes in the mangrove root-soil in bioremediation of heavy metals. Hence, the present study was made to identify the predominant microbes present in the rhizosphere mangrove soil and to prove their ability of accumulating heavy metals for Zn, Cu, Pb, Mn and also solubilizing Zn in the soil.

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2. Materials and methods

Healthy propagules of *Avicennia marina* were collected from the Vellar area (Lat. 11^0 $25^{'}$ 38.4N; Long. 79^0 47′35.5E) located along the Bay of Bengal on the southeast coast of Tamil Nadu, India. The propagules were raised in cylindrical plastic pots, contained with silt, clay and sand in the ratio of 2:4:94 under greenhouse conditions at temperature of 27 ± 2 °C and light intensity of 800 ± 75 μmol m^{-2} s^{-1} in the nursery of Centre for Advanced Study in Marine Biology, Annamalai University, Parangipettai. One month old seedlings were daily irrigated with artificial wastewater composed of Glucose (0.5),NH_4Cl (0.04), $NaNO_3$(0.01), CH_4N_2O(0.01), KH_2PO_4(0.01), KCl(0.05), $FeCl_3$ (0.03), $CuCl_2$(0.002),$ZnSO_4$(0.005), $Pb(NO_3)_2$(0.002), $K_2Cr_2O_7$(0.001), $MnCl_2$(0.005) prepared in 1 L of 50% seawater. The control was also maintained with irrigation of 50% seawater. After 60 days of the wastewater treatment, the rhizosphere soil samples were collected for analyzing microbial species, and physico-chemical conditions.

Soil samples were collected from the rhizosphere of *Avicennia marina* from the experimental and control pots from 0 to 10 cm depth, and analyzed *in situ* for temperature using a thermometer with 0.5 °C accuracy, pH using a millivoltmeter with plantinum electrode, electrical conductivity using an EC meter, (systronics), and pore-water salinity using hand refractometer (Erma INC, Tokyo). The soil samples were then analyzed in the laboratory for bulk density (g.cm^{-3}) by dividing oven-dry weight and volume, and soil texture by Pipette method. Further the soil samples were analyzed for Total organic carbon (El. Wakeel and Riley,1956), nitrogen by using Kjeldahl method (Subbiah and Asija, 1956), phosphorus by using colorimetric method (Olsen et al., 1954) and potassium by using flame photometer (Guzman and Jimenez, 1992).

The microbes were isolated from rhizosphere soil samples of *Avicennia marina*, by using serial dilution followed by spread plate method. For isolation of bacteria, the Zobel Marine Agar medium was used, and for fungi, the Potato Dextrose Agar medium was used. The isolates were sub-cultured and purified. From the isolates, three fungal and three bacterial species which occurred predominant were selected for sequencing 16sRNA for bacteria and 18SrDNA for fungi and identification.

Six identified microbes were mass cultured individually in appropriate culture media under shaker culture by incubating at 20 °C at 100 rpm for 7 days. The culture media were prepared in the normal dose of wastewater in 50% seawater for the treatment, and that in 50% seawater alone as the control. After seven days of incubation, the microbial biomass was harvested and analyzed for heavy metal accumulation by using the acid digestion method. Microbial biomass weighing at 1 g was digested with Conc. HNO_3 and Conc. $HClO_4$ (4:1) and dried on a hot plate. The ash consequently obtained was made up to 20 ml solution using Milli-QR water with the addition of 20% nitric acid. The mixture was filtered using Whatman filter paper (11 μm) and then the metal concentrations were determined by using the Atomic Absorption Spectrophotometer (Model SL173, ELICO). The level of heavy metals accumulated in wastewater treated microbes and that in untreated microbes was determined for percent of accumulation in treatment over control by using the following formula.

$$\% \text{ over control} = \frac{\text{Value in treated sample}}{\text{Value in control sample}} \text{x } 100 - 100$$

2.1. Zinc tolerance in Bacillus marisflavi

The bacterial strain of *Bacillus marisflavi* was inoculated in the flasks containing 50 ml of sterilized Bunt and Rovira medium containing 0.1% of ZnO, $ZnCO_3$ and $ZnSO_4$ as insoluble sources. An uninoculated sample was also maintained. The samples were drawn on the sixth, eighth and tenth day of incubation. The bacterial cultures were centrifuged at 15,000 rpm for 10 min and filtered using Whatman No. 42 filter paper. The biomass was determined along with measuring the pH and temperature of culture filtrate.

2.2. Statistical analysis

The data were analyzed for mean and standard error and significance at $p < 0.05$ between treated and control.

3. Results

3.1. Mangrove root-soil conditions as influenced by wastewater treatment

The root soil conditions in experimental mangrove pots treated with or without wastewater are shown in Table 1. In general, the levels of nutrients were higher, but bulk density, temperature and pH were lower in the treated soil samples than the control ones. The mangrove soil treated with wastewater exhibited high N, salinity, P, total organic carbon, and K by 2-fold, 81.5%, 42.1%, 33.3% and 11.8% over control respectively, but reduced bulk density by 28.6%, soil temperature by 14.3% and pH by 2.4% in treated soil.

3.2. Microbes from mangrove rhizosphere soil under wastewater treatment

The bacterial counts were higher than fungal counts in the wastewater treated soil. For instance, the bacterial count was 44.1% higher in wastewater treated soil than that in control, whereas the fungal count was 25% more in the former than that in the latter (Table 1). The phylogenetic relationships of taxa of the identified microbes are shown in Fig. 1. The predominant bacterial strains were identified as *Bacillus altitudinis* (NCBI Acc No:1751823), *B. anthracis* (NCBI Acc No:1751656), *B. marisflavi* (NCBI AccNo:1751664) and the fungi were *Penicillium citrinum* (NCBI Acc no: 1751827), *Aspergillus quadrilineatus*(NCBI Acc no:1752075) and *Gibberella intermedia* (NCBI Acc no: 1751825).

3.3. Microbial accumulation of heavy metals

The six microbial species were tested for accumulation of heavy metals under culture conditions treated with wastewater, in comparison with their respective controls. The results are shown in Table 2. Zn accumulation ranged from 22.4 to 60% in fungi, and 35.3–70.8% in bacteria, over controls, with the maximum Zn accumulation in the bacterium, *B. marisflavi* (70%), followed by *B. altitudinis* (65%). Cu accumulation varied from 28.6 to 60% in fungi and 33.3–62.5% in bacteria, over controls, with the highest Cu accumulation in the bacterium, *B. marisflavi* (62.5%). followed by the fungus *P. citrinum* (60%). Pb accumulation was within a range of 28.6–60% in fungi and 33.3–62.5% in bacteria, over controls, with the maximum Pb accumulation in the bacterium, *B. marisflavi* (62.5%) followed by the fungus *P. citrinum* (60%). Mn accumulation varied from 30 to 50% in fungi and 30–44.4%

Table 1
Root soil conditions of *Avicennia marina* plants treated with or without synthetic wastewater.

Soil condition	Wastewater treated	Untreated (control)
Temperature (°C)	24 ± 1.2^a	28 ± 1.8^b
pH	8.1 ± 0.7^a	8.3 ± 0.8^b
Pour water salinity (ppt)	49 ± 4.2^a	27 ± 3.1^b
Bulk density (g.cm^{-3})	0.5 ± 0.01^a	0.7 ± 0.02^b
Total organic carbon (%)	0.4 ± 0.03^a	0.3 ± 0.02^b
Total Nitrogen (%)	6.0 ± 0.4^a	2.7 ± 0.3^b
P (%)	2.7 ± 0.2^a	1.9 ± 0.2^b
K (%)	1.9 ± 0.1^a	1.7 ± 0.2^b
Bacterial counts (CPU.g^{-1} soil)	28.1 ± 2.3^a	19.5 ± 1.2^b
Fungal counts (CPU. g^{-1} soil)	9.0 ± 1.1^a	7.2 ± 0.4^b
Significance ($p < 0.01$)	**	**

Values not sharing a common superscript differ significantly at $p > 0.05$.

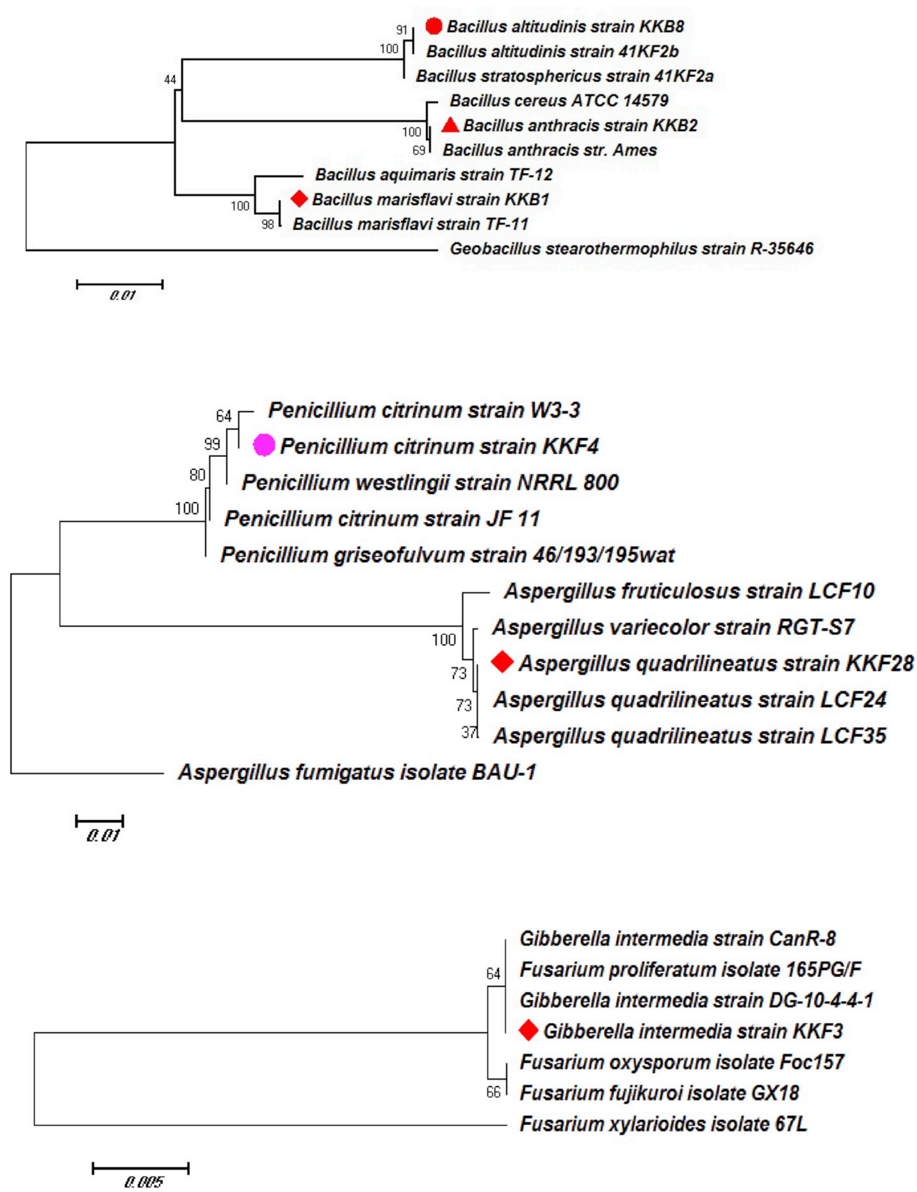

Fig. 1. Phylogenetic relationships of bacterial and fungal taxa.

Table 2
Heavy metal accumulation in bacteria and fungi cultured in synthetic wastewater over their respective controls without wastewater treatment (values in % accumulation over control).

Species	Zn	Cu	Pb	Mn
B. altitudinis	65.2 ± 0.01^{e}	40.0 ± 0.01^{d}	40.0 ± 0.01^{c}	30.0 ± 0.00^{a}
B. anthracis	35.3 ± 0.01^{c}	33.3 ± 0.01^{c}	33.3 ± 0.01^{b}	43.4 ± 0.01^{b}
B. marisflavi	70.8 ± 0.01^{f}	62.5 ± 0.16^{e}	62.5 ± 0.01^{f}	44.4 ± 0.01^{b}
P. citrinum	22.4 ± 0.01^{b}	60.0 ± 0.01^{a}	60.0 ± 0.01^{e}	50.0 ± 0.00^{c}
A. quadrilineatus	60.0 ± 0.01^{d}	28.6 ± 0.01^{b}	28.6 ± 0.01^{a}	30.0 ± 0.00^{a}
G. intermedia	20.0 ± 0.00^{a}	40.0 ± 0.01^{d}	40.0 ± 0.01^{d}	30.0 ± 0.00^{a}
P value	0.000	0.000	0.000	0.000
F value	3.494	2.737	1.179	7.534

Values not sharing a common superscript differ significantly at $p > 0.05$.

in bacteria, over controls, with the highest Mn accumulation in the fungus, *P. citrinum* (50%) followed by the bacterium *B. marisflavi* (44.4%). Thus, microbes especially bacteria played a significant role in heavy metal accumulation.

3.4. Influence of zinc solubilizing organisms on pH of the growth medium

Bacillus marisflavi that exhibited better heavy metal accumulation was tested for zinc solubilization in the culture medium incorporated with different Zn substrates (ZnO, $ZnCO_3$, $ZnSO_4$) by analyzing the pH of culture filtrates after 6,8 and 10 days of incubation. The results are shown in Table 3. The pH of culture filtrates reduced with increasing period of incubation. After 10 days of incubation, the pH was reduced from 5.7 to 3.9 in ZnO, 5.9 to 4.3 in $ZnCO_3$ and 6.1 to 5.2 in $ZnSO_4$; on

Table 3
Zn solubilization in terms of pH reduction by *Bacillus marisflavi* under different inorganic source of Zn.

Zinc source in the medium	pH of growth medium			% of reduction
	6th Day	8th Day	10th Day	
ZnO	5.7 ± 0.7^{a}	4.8 ± 0.6^{b}	3.9 ± 0.4^{c}	−31.6
$ZnCO_3$	5.9 ± 0.6^{a}	4.9 ± 0.6^{b}	4.3 ± 0.5^{c}	−27.1
$ZnSO_4$	6.1 ± 0.6^{a}	5.4 ± 0.7^{b}	5.2 ± 0.5^{c}	−14.8
Without zinc (control)	5.2 ± 0.4^{a}	5.4 ± 0.7^{b}	8.0 ± 0.9^{c}	+53.9

Values not sharing a common superscript differ significantly at $p > 0.05$.

the contrary, the pH increased from 5.2 to 8.0 in control during the period. In other words, the pH reduction was 31.6%, 27.1%, and 14.8% with ZnO,$ZnCO_3$ and $ZnSO_4$ respectively, as against the pH increase of 53.9% in control.

4. Discussion

Mangrove soil provides a unique ecological environment for diverse bacterial and fungal communities, and the soil microbes present in root regions are important for mangrove plant growth and health (Kathiresan and Bingham, 2001). The present work also recorded more counts of microbes in the wastewater treated mangrove soil (Fig. 1). This is in accordance with Agnes Bouchez et al. (2013) who have found that the sewage treatment enhances microbial densities and diversity. Similarly Tam (1998) has recorded that total counts of both aerobic and anaerobic heterotrophic bacteria, nitrifiers and denitrifiers in mangrove soils receiving wastewater are significantly higher than those found in the control soils. This higher microbial count could be attributed to the availability of higher levels of nutrients in the wastewater treated soil (Table 1). However, the wastewater treated soil had higher pore water salinity; even then it had higher microbial counts, indicating the presence of salt tolerant microbes. The wastewater treated soil had lower bulk density than the control did (Table 1). This lower bulk density is due to the higher the total organic carbon present in the waste water treated soil. In addition to this, the mangrove roots are likely to increase soil porosity and water holding capacity, and to reduce soil compaction, as a result of biological activity (Bhomia et al., 2016), favoured by high nutrients and organic matter deposited in the mangrove soil treated with waste water (Table 1). The abundance and activities of microbes are controlled by physical and chemical conditions of the mangrove soil environment (Saravanakumar et al., 2018).

Heterotrophic bacteria and saprophytic fungi are very important in mangrove habitats as they decompose the mangrove litter and recycle the nutrients (Kathiresan, 2000; Rajendran and Kathiresan, 2007). However, the present work recorded higher bacterial counts than fungal counts, as a result of wastewater treatment (Fig. 1). In general, the mangrove soil supports more diverse bacterial population than fungi. This is also evident by the fact that the number of bacteria recorded is 52 as against only 23 species of fungi recorded in the study area of mangroves (Kathiresan, 2000). However, wastewater-borne heavy metals are reported to be toxic to the mangrove soil microbes and their activities (Yim and Tam, 1999). This is not true with the present work, which used only normal concentration of artificial wastewater rather than higher concentrations.

Mangrove soil generally has a capacity for absorbing and holding heavy metals and nutrients (Lacerda, 1998). The present work showed that the microbes accumulated heavy metals in response to wastewater treatment (Table 2). This finds support of the findings of Tam and Wong (1996, 1997). These researchers have irrigated mangrove soil samples with metal-laden artificial wastewater and found that the upper layer of the soils binds Cu, Cd, Mn and Zn. However, there are also higher concentrations of Mn, Zn, Cd in the water-soluble, exchangeable fraction of the treated soils than in the untreated soil. The mangrove soils are capable of retaining nutrients and heavy metals from wastewater, and that the degree of retention was related to the salinity of wastewater (Tam, 1998).

Microbial sorbents are efficient in wastewater treatment due to their adaptation to varied growth conditions. Among the microbes, bacteria are often used for metal biosorption (Masood and Malik, 2011). *Bacillus* species can efficiently remove Cu and Cd (Arivalagan et al., 2014, 2018). Similar to these earlier findings, the present study also demonstrated that *Bacillus marisflavi* exhibited better heavy metal accumulation for Zn, Cu and Pb than other bacteria and fungi studied (Table 2). Constructed mangrove wetland treatment systems are proved to be promising to effectively treat municipal wastewater (Wu et al., 2008).

Zinc is one of the essential micronutrients required for plant growth, but the Zn is mostly unavailable to assimilate by the plants, wherein the role of soil microbes in Zn solubilization is important to make the Zn biologically available (Beulah et al., 2017). The microbial solubilization of insoluble zinc compounds ($ZnCO_3$ and ZnO) is confirmed using radiotracer (http://www.ncbi.nlm.nih.gov/pubmed/?term=Sarathambal%20C%5Bauth%5D, Sarathambal et al., 2010). The present study tested the Zn solubilization of the bacterial strain *Bacillus marisflavi* isolated from wastewater treated mangrove soil. Among the inorganic Zn compounds, ZnO showed maximum activity (27.1%) as evident by decline in pH. This drop in the pH of the culture medium with insoluble Zn compounds is due to organic acid production such as 2-ketogluconic acid and gluconic acid and subsequent acidification of the medium (Fasim et al., 2002). Similar to the present work, the potential of *Bacillus* spp., has already been documented for Zn solubilization. For example (Ramesh et al., 2014), have evaluated Zn solubilization ability of *Bacillus aryabhattai* strains on trisminimal medium amended with different Zn compounds. Gandhi and Muralidharan (2016) have also reported the highest zinc solubilization in ZnO amended medium with *Acinetobacter* sp. This is further substantiated by our earlier finding that *Pseudomonas aeroginosa* isolated from mangrove root soil is efficient in solubilizing ZnO (Beulah et al., 2017).

5. Conclusions

This work identified six predominant species of bacteria and fungi from the rhizosphere soil of mangroves species (*Avicennia marina*), treated for 60 days with artificial wastewater. These were bacterial strains of *Bacillus altitudinis, B. anthracis* and *B.marisflavi*, and the fungal strains of *Penicillium citrinum, Aspergillus quadrilineatus* and *Gibberella intermedia*. The wastewater treated mangrove soil was found to have higher microbial counts, porewater salinity and nutrients (N, P, K, total organic carbon), but lower soil bulk density, pH and temperature. The heavy metal accumulation was determined in those microbial strains cultured with artificial wastewater under shaker culture for seven days. The accumulation was the highest in the bacterial strain of *B. marisflavi* for Zn followed by Cu and Pb. Hence, *B.marisflavi* was further tested for Zn solublization in terms of pH decline, after 10 days of incubation with the insoluble Zn sources (ZnO, $ZnSO_4$ and $ZnCO_3$). The Zn solubilization was the highest with ZnO. The work suggested the potential of *B. marisflavi* in heavy metal removal and Zn solubilization in the mangrove soil, and its utility in bioremediation of heavy metals. Further studies are required to demonstrate the potential of the bacterial strain in the field.

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