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Research Article

Nickel Bioremediation by Different Wetland Macrophytes Root Associated Bacteria

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Abstract

A study was conducted at ICAR-Indian Agricultural Research Institute, New Delhi (India) to enumerate bacterial load associated with five different macrophytes viz. *Typha latifolia*, *Phragmites karka*, *Acorus calamus*, *Arundo donax* and *Vetiver zizanioides* maintained after monthly applications of metal solution of 5.0 ppm of nickel. The roots of macrophytes supported bacterial associations amounting to 2.25×10^7 cfu/g and of them fifty two bacterial morphotypes were isolated. Mean nickel removal efficiency of the bacterial isolates was 11.06% after 3 days with maximum of 17.98% by the bacteria isolated from *Phragmites*. Most efficient thirteen dominant morphotypes for Ni-bio-accumulation were identified with 16S rRNA sequencing. *Bacillus* were the predominant bacterial genera (53.85%) followed by *Pseudomonas* and *Microbacter* (15.38%) which had shown the nickel removal capacity of 19.78%, 27.66% and 16.90% after 3 days of incubation, respectively. However, Ni removal by *Pseudomonas mendocina* isolated from Vacha root was maximum (34.20%). The overall results showed that macrophytes root associated bacteria may play an important role in plant assisted Nickel bio-remediation.

Keywords: Constructed wetland, Macrophytes, Plant root associated bacteria, Wastewater, Nickel bioremediation

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Introduction

Heavy metals are among the toxic environmental pollutants and unsolved problems worldwide. Due to their toxicity, non-biodegradability, bio-accumulating tendency and extreme persistence in the environment [1] they are considered as priority pollutant by USEPA. Nickel is the most abundant and hazardous heavy metals discharged from industrial processes such as electroplating industry, plastics manufacturing, metal finishing, nickel-cadmium batteries manufacturing, fertilizers, pigments, silver refineries, mining and metallurgical operations. It is dangerous to human health and leading to kidney and lungs damage, gastrointestinal distress, pulmonary fibrosis and skin dermatitis [2]. The maximum permitted discharge level of Ni into surface and potable waters has been set to below 0.2mg/L by the Environmental Protection Agency [3].

Worldwide several physicochemical methods are currently being used to remove heavy metals like nickel from wastewaters viz. adsorption, ion exchange, biological methods, electrocoagulation, electrodialysis, floatation, coagulation, flocculation and membrane separation [4-6]. But most of them are extremely expensive especially for treatment in low concentration range from 1 to 100 mg/L [7]. In developing countries due to lack of proper treatments and strict monitoring, generally industries discharges heavy metals loaded wastewater that along with sewage waters are finally drained into nearby rivers or fresh waterbodies.

Now a day's research on developing low cost and effective decentralized treatment systems is increasing importance in developing countries. Among decentralized treatment systems Constructed wetland system are now a days becoming popular mainly due to its flexibility in the scale of operation, wide range of treatment options, cost-effective and economical nature, green and sustainable, providing reliable treatment and protecting public health and

the environment, and suitable for rural, suburban and urban area [8]. Constructed wetland is an artificially maintained phytoremediation system and a worldwide proved technology for heavy metal remediation and for wastewater treatment. It is built frameworks intended to use characteristic procedures including wetland vegetation, media and the related microbial collections to help with treating wastewaters [9]. Large root biomass of aquatic plants acts as main sequestering organ allowing rhizofilteration of metal contaminants from waste water. In these system micro-organisms are also playing an important role. They are capable of tolerating high concentration of metals and beneficial to both the growing media and the plant. They either increase the entry of heavy metals in to plant by increasing the metal bioavailability and improve the plant uptake [10] or restrict the entry of heavy metals into the plant through bio-sorption (metabolism independent or passive process) and bio-accumulation (metabolism dependent or active process) [11]. However, little is known about the diversity and distribution of plant associated bacteria associated with wetland plants especially in nickel and their potential to enhance phytoremediation and more especially bioremediation.

A long term study is being conducted at ICAR-Indian Agricultural Research Institute, New Delhi, India since Dec. 2013 for the screening of wetland macrophytes namely *Typha latifolia* (Cattail), *Phragmites karka* (Reed), *Acorus calamus* (Vacha), *Arundo donax* (Giant reed) and *Vetiver zizanioides* (Vetiver) for the effective removal of nickel from metal contaminated waste water. The present study was planned to (i) enumerate the bacterial load associated with the macrophytes and (ii) screen dominant bacterial morphotypes for their Ni-bio-accumulation capacity.

Material and Methods

Experimental site and set-up

The study was conducted near sewage treatment plant site of ICAR-Indian Agricultural Research Institute, New Delhi, India (28.08^{o}N and 77.12^{o}E). A microcosm wastewater treatment system was set-up based on vertical sub-surface flow (VSSF) constructed wetland technology. The microcosm tank was assembled using commercially available 100-L capacity plastic tanks measuring 60cm of height and 33 cm of diameter and was attached with one plastic tap 2cm above the bottom. Each tank were filled with bottom layer (30cm) of coarse gravel (approximately 5 cm diameter) and upper layer (20cm) of fine gravel (approximately 1.0 cm diameter) and about 10 cm top space was kept unfilled to occupy irrigation water (**Figure 1**).

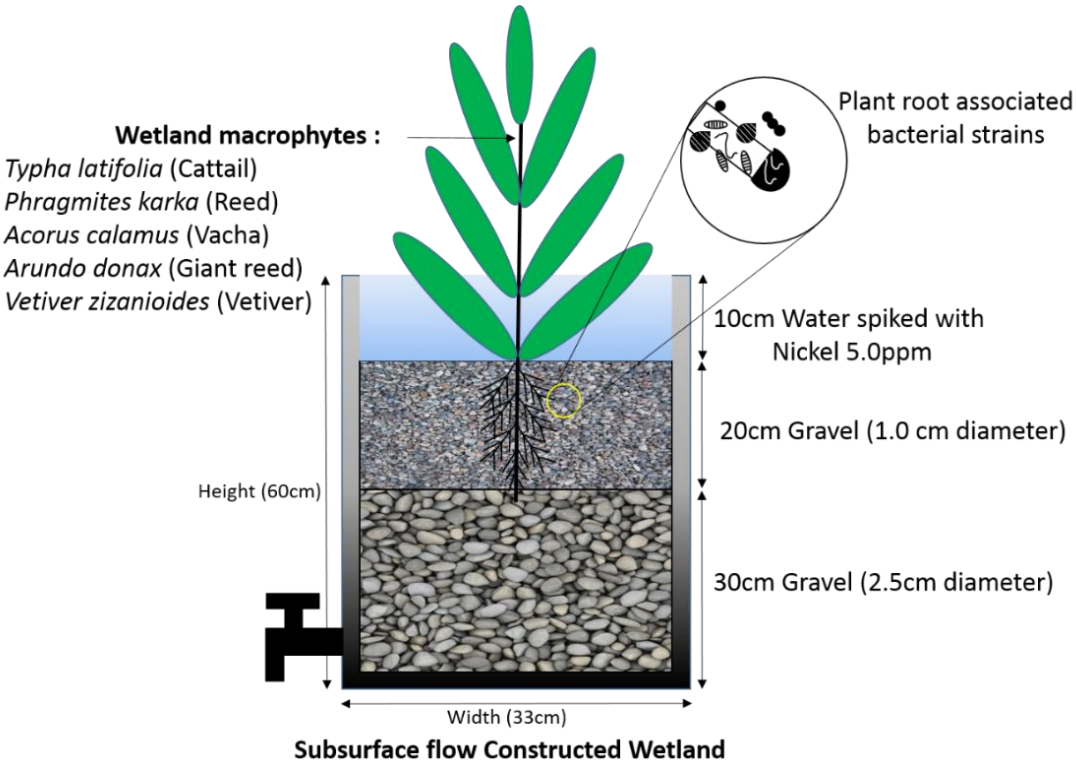

Figure 1 Experimental set-up

Five emergent wetland plants viz. *Typha latifolia* (Cattail), *Phragmites karka* (Reed), *Acorus calamus* (Vacha), *Arundo donax* (Giant reed) and *Vetiver zizanioides* (Vetiver) were selected on the basis of their Nickel accumulation efficiency (earlier report). About 30 days old plants were planted (6 plants in each tank) in triplicate in September 2013 and two months were given for system stabilization. All the tanks were labelled accordingly and distributed randomly. After system stabilization (November 2013) microcosm tanks were manually irrigated with 5ppm Ni solution in the form of nickel nitrate in ground water and about 22 litres of this working solution was used in a month. Monthly inlet and out water were collected and analysed to assess the nickel removal capacity of each macrophyte.

Isolation of macrophytes root associated bacteria

At 12 month stage one plant of different macrophytes viz. Typha, Phragmites, Vacha, Arundo and Vetiver from the respective tanks was removed for sampling of roots. The collected macrophyte root samples were washed gently with distilled water to remove the surface dust and dead plant tissues. Five grams of root sample was crushed in the sterilized pestle and mortar in a laminar airflow cabinet and the extract was transferred to 45 ml of 0.9% saline solution in 100 ml erlenmeyer flask and were kept on shaker for 2 hrs. at 120rpm. Serial dilution of this suspension were plated on nickel (5ppm) primed nutrient agar medium (Sodium Chloride 0.5%, Peptone 0.5%, Beef Extract 0.3%, Bacteriological Agar powder 1.8%, pH 6.8) plates in triplicate [12]. Plates were incubated in BOD incubator at 30°C temperature for 24-72 hrs. Bacterial colonies appearing after incubation on Ni primed plates were counted to enumerate the associated bacterial population. Different bacterial morphotypes were selected on the basis of their morphological characteristics like pigments, colony form, elevation, margin, texture and opacity etc. [13]. Cultures obtained were nickel tolerant (5ppm) and were purified and maintained at 4°C as slant and glycerol stock (20%) at 80°C for further use.

Nickel removal efficiency of the root associated bacteria

Nickel removal efficiency of each bacterial isolate was quantified by inoculating in nutrient broth primed with 5ppm nickel (NBNi). 50 ml of NBNi in 100 ml flask was inoculated with 1ml of culture suspension (10^6 cells). Two set of such flasks for each bacterial isolate were prepared, inoculated and incubated at 30°C in incubator shaker for 3 days. The optical density (OD) was measured by a Double Beam UV visible Spectrophotometer (LMSP-UV 1900S, Lab India) against a media blank at 0 and 3 days. After OD measurement 10ml of bacterial suspension was centrifuged (SIGMA 3-18KS) at 10,000 rotations per minute for 10minutes in 50 ml pre labelled sterilized centrifuge tubes. The supernatant of all the bacterial isolates were dispensed in the sterilized labelled plastic bottles and stored in a refrigerator at 4°C after immediately acidifying by adding 3-4 drops of 1N nitric acid (HNO_3). 10 ml of bacteriological supernatant and the media blank were mixed with 5 ml of concentrated nitric acid for acid digestion in a microwave digester (TITAN MPS^{TM}, Perkin Elmer) at 200°C and 35 bar pressure for 1 hour. Digested samples volume was made up to 50 ml using deionised water in volumetric flask. The diluted samples were filtered using Whatman No. 42 filter paper and then Nickel concentrations was determined through atomic absorption spectrometer (AA8000, Lab India) [14].

Genomic characterization of efficient nickel bio-remediating bacterial isolates

Isolation of genomic DNA

Genomic DNA isolation was done using prescribed protocol by Zymo Research isolation kit (ZR Fungal/ Bacterial DNA MiniPrep^{TM}). Genomic DNA quality was checked on 0.8% agarose gel electrophoresis stained with ethidium bromide on Gel Electrophoresis System (40-1214) and the bands were visualized on a gel documentation system (MultiGel-21).

16S rRNA gene amplification

Using universal primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') 16S rRNA gene of selected isolates was amplified through PCR as described by Edwards *et al*. 1989. PCR reaction mixture containing 5 U of *Taq* DNA polymerase, 1X *Taq* buffer, 10 pmol of each primer (pA and pH), 0.2 mM each of dNTP mixture, 1.5 mM $MgCl_2$ and 50-100 ng DNA template was used for amplification. The PCR amplification conditions were: initial denaturation of 1 min at 95°C, followed by 39 cycles consisting of 1 min at 95°C (denaturation), 1 min at 52°C (annealing) and 1.5 minute at 72°C (Primer extension) and a final extension

period of 6 min at 72°C and 20 min on hold at 4°C. The PCR products were resolved in 1.2 % agarose gel in 1X TAE buffer by electrophoresis at 60 V for 1 hour. Gels were visualized on a gel documentation system (MultiGel-21).

16S rRNA gene sequencing and phylogenetic analysis

The amplified PCR products of each isolates were purified with a QIA quick purification kit (Qiagen). PCR products of partial 16S rRNA gene were sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in 3130xl Applied Biosystems ABI prism automated DNA sequencer at SCI Genome Chennai, India. 16S rRNA gene sequences were analysed using Codon Code Aligner v.4.0.4. The 16S rRNA gene sequences were aligned to those of closely related bacterial species available at GenBank database using BLASTn program. Bacterial isolates were identified based on percentage of sequence similarity (≥97%) with that of a prototype strain sequence in the GenBank.

Statistical Analysis

All experiments were carried out in triplicates and statistical analysis of different experimental data was done by SAS software 9.4 (TS1M1) at ICAR-Indian Agricultural Statistics Research Institute (IASRI), New Delhi.

Result and Discussion

Five different macrophytes viz. Typha, Phragmites, Vacha, Arundo and Vetiver grown with single metal solution of nickel (Ni 5.0ppm) were found to harbor various Ni tolerant root associated bacteria in their rhizosphere.

Typha root associated bacterial isolates

Ni tolerant mean bacterial population associated with Typha roots was found to be 3.6×10^6 cfu/g as the selection pressure of Ni was provided in the growth medium. Eighteen morphologically distinct bacterial isolates were purified. Ni removal efficiency by these bacterial isolates and their growth measurement as OD is presented in Figure 3. The overall nickel removal by these isolates was 11.73% after 3 days of incubation. The mean bacterial growth was found to increase 10 folds from 0.03 to 0.29 after 3 days. Bacterial isolates T1(20.59%), T7(20.68%), T13 (16.71%) and T18 (23.26%) showed significant Ni removal that was about 8.86%, 8.95%, 4.98% and 11.53% more than mean removal along with proper bacterial growth, respectively and were selected for further characterization and identification.

Phragmites root associated bacterial isolates

Ni tolerant mean bacterial population associated with Phragmites roots was found to be 3.73×10^7 cfu/g as the selection pressure of Ni was provided in the growth medium. Four morphologically distinct bacterial isolates were purified. Ni removal by these bacterial isolates and their growth measurement as OD is presented in Figure 3. The overall nickel removal by these isolates was 17.98% after 3 days of incubation. The mean bacterial growth was found to increase 12 folds from 0.025 to 0.30 after 3 days. Bacterial isolates P1 (21.11%) and P4 (18.51%) showed significant Ni removal after 3 days that was about 3.13% and 0.52% more than mean removal along with proper bacterial growth, respectively and were selected for further characterization and identification.

Vacha root associated bacterial isolates

Ni tolerant mean bacterial population associated with Vacha roots was found to be 2.02×10^7 cfu/g as the selection pressure of Ni was provided in the growth medium. Eleven morphologically distinct bacterial isolates were purified. Ni removal by these bacterial isolates and their growth measurement as OD is presented in Figure 3. The overall nickel removal by these isolates was 11.05% after 3 days of incubation. The mean bacterial growth was found to increase 6 folds from 0.03 to 0.19 after 3 days of incubation. Bacterial isolates V1 (17.61%), V5 (34.20%) and V10 (15.38%) showed significant Ni removal after 3 days that was about 6.56%, 23.16%, 11.02% and 4.33% more than mean removal along with proper bacterial growth, respectively and were selected for further characterization and identification.

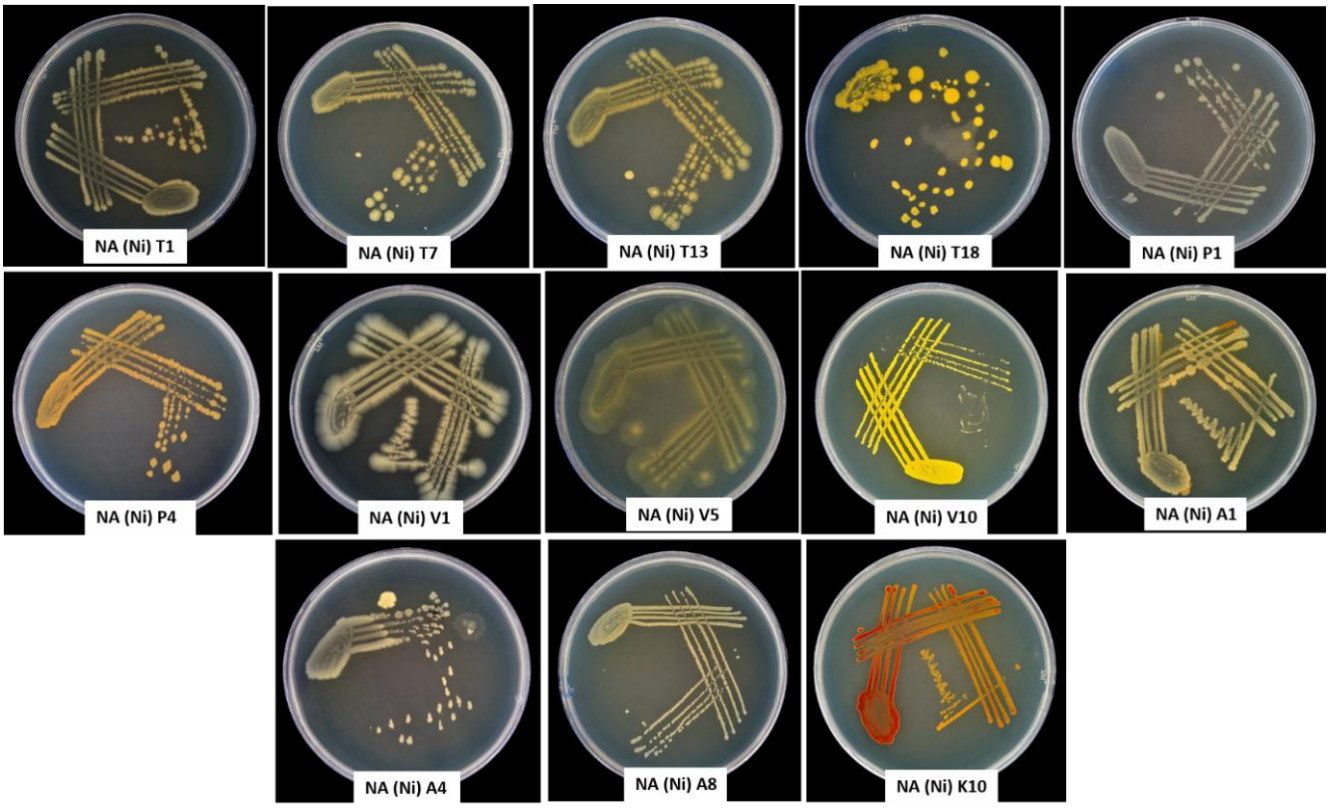

Figure 2 Nickel bio-remediating selected bacterial isolates from different macrophytes

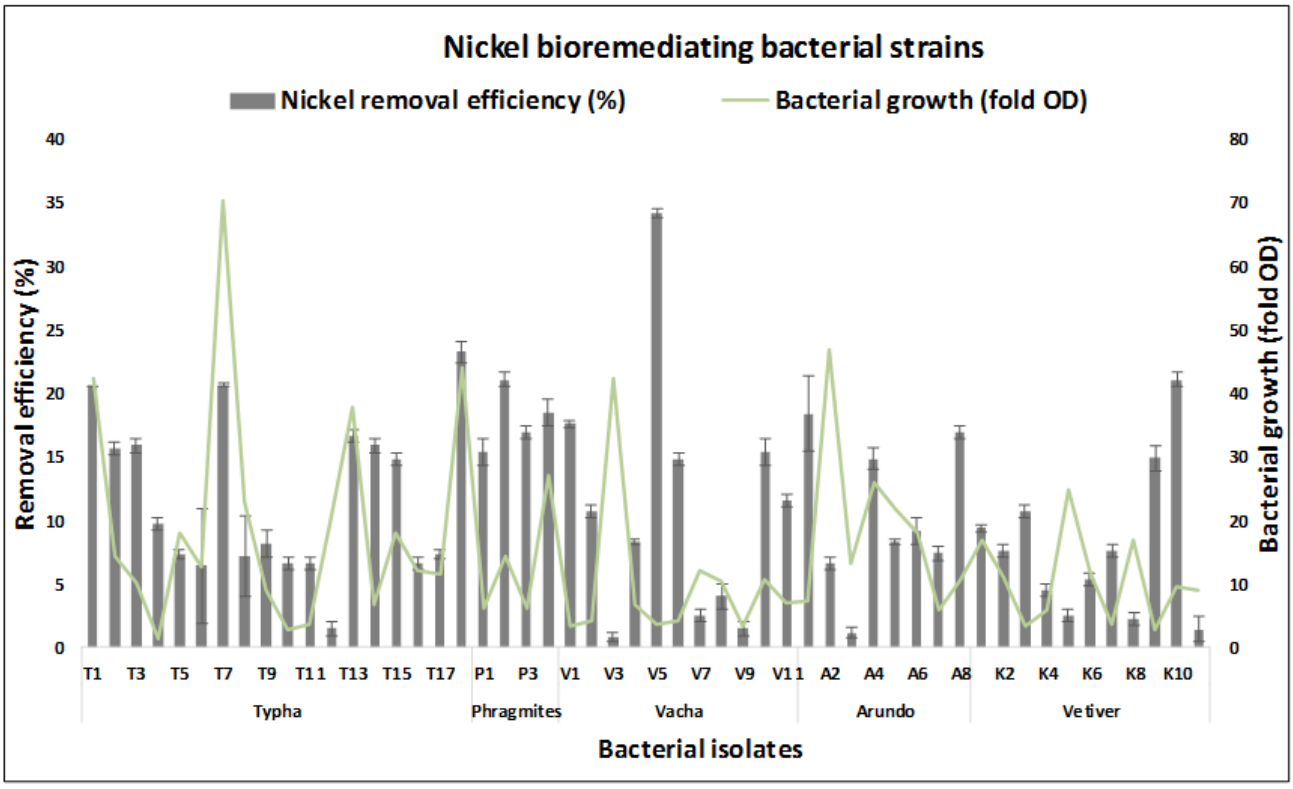

Figure 3 Macrophytes root associated bacterial isolates for nickel removal and their bacterial growth in terms of folds increase in Optical Density

Arundo root associated bacterial isolates

Ni tolerant mean bacterial population associated with Arundo roots was found to be 5.11 $\times 10^7$ cfu/g as the selection pressure of Ni was provided in the growth medium. Eight morphologically distinct bacterial isolates were purified. Ni removal by these bacterial isolates and their growth measurement as OD is presented in Figure 3. The overall nickel removal by these isolates was 10.37% after 3 days of incubation. The mean bacterial growth was found to increase 20 folds from 0.01 to 0.20 after 3 days of incubation. Bacterial isolates A1 (18.42%), A4 (14.86%) and A8 (16.94%) showed significant Ni removal after 3 days that was about 8.05%, 4.49% and 6.57% more than mean removal along with proper bacterial growth, respectively and were selected for further characterization and identification.

Vetiver root associated bacterial isolates

Ni tolerant mean bacterial population associated with Vetiver roots was found to be 3.0 $\times 10^5$ cfu/g as the selection pressure of Ni was provided in the growth medium. Eleven morphologically distinct bacterial isolates were purified. Ni removal by these bacterial isolates and their growth measurement as OD is presented in Figure 3. The overall nickel removal by these isolates was 7.96% after 3 days of incubation. The mean bacterial growth was found to increase 7 folds from 0.03 to 0.21 after 3 days. Bacterial isolates K10 (21.11%) shown significant Ni removal after 3 days that was about 13.15% more than mean removal along with proper bacterial growth and were selected for further characterization and identification.

Genomic characterization of nickel remediating bacterial isolates

Thirteen bacterial strains were selected on the basis of their metal removal/ bio-accumulation ability. Morphological features observed are indicated in Figure 2 and Table 1. 16S rRNA gene sequencing and phylogenetic analysis of representative isolates from each macrophyte using BLAST of the nearest match from GenBank data revealed that all the isolates showed >99 to 100% similarity with the sequences within the GenBank which led to the identification of 13 distinct bacteria with assigned NCBI accession numbers (Table 1). Sequences were deposited in the GenBank. Among all the selected bacterial isolates from different macrophytes *Bacillus* was the major bacterial genera (53.85%) followed by *Pseudomonas* and *Microbacter* (15.38%). All the effective Ni bio-accumulating bacteria isolated from Typha, Phragmites and 33% of Vacha was *Bacillus* sp. Among different bacterial isolates from *Bacillus* genera *Bacillus sonoresis, Bacillus aryabhattai, Bacillus megaterium, Bacillus cereus* and *Bacillus thuringinesis* was the major one. While among Pseudomonas and Microbacter genera *Pseudomonas mendocina* and *Microbacter chocolatum* was the major one. Gram staining of the selected bacterial isolates showed that *Bacillus sp., Microbacter sp. and Lysinibacillus sp.* was Gram positive while *Pseudomonas sp., and Halomonas sp.* were Gram negative bacteria.

In constructed wetland *Bacillus* sp. [15], *Pseudomonas* sp. and *Enterobacter cloacae* [16] was also reported earlier. While about Ni tolerance and bioremediation capacity of *Bacillus* was also confirmed by [17], Ni tolerance up to 0.6mM/ml of *Bacillus cereus* and *Bacillus subtilis* isolated from ash dyke sample of thermal power plants of Chhattisgarh by [18], *Bacillus megaterium* isolated as from a nickel rich serpentine soil grown plants *Brassica juncea, Luffa cylindrica* and *Sorghum halepense*) by [19]. Similarly confirmation about *Pseudomonas* sp. for nickel resistant (up to 2.5 mmol/L) as well as bioremediation was reported [20].

Thus, on this nickel grown macrophytes root the mean bacteria population was found 2.25 $\times$ 10^7 cfu/g which had shown average nickel removal capacity of about 11.06±0.73% after 3 days of incubation. However, among all the five macrophytes, the mean nickel removal was found maximum by the bacteria isolated from Phragmites (17.98±0.78%). Out of fifty two isolated bacterial morphotytes thirteen were found effective for nickel removal/ bio-accumulation (14.86% to 34.20%). Among all thirteen selected bacterial isolates *Bacillus* was the major bacterial genera (53.85%) followed by *Pseudomonas* and *Microbacter* (15.38%) which had shown the mean Ni bio-accumulation of 19.78±0.48%, 27.66±0.46% and 16.90±2.03% after 3 days of incubation, respectively. However, among all bacterial isolates *Pseudomonas mendocina* isolated from Vacha root had shown maximum Ni bio-accumulation (34.20%).

Table 1 Morphological features and gram staining of selected Ni-bioremediating bacterial isolates

Host Plant	Colony Index	Bacterial Isolates	NCBI accession number	Shape & size	Colour	Form	Margin	Elevation	Density	Gram staining
Typha	**T1**	*Bacillus sonoresis*	KX570922	Medium spherical	Rough creamy	Circular	Serrate	Flat	Opaque	+
	T7	*Bacillus aryabhattai*	KX570914	Medium spherical	Glistering slight yellow	Circular	Serrate	Flat	Opaque	+
	T13	*Bacillus* sp.	KX570915	Large	Slight yellow	Irregular	Smooth	Flat	Opaque	+
	T18	*Bacillus megaterium*	KX570916	Medium spherical	Yellow	Circular	Entire	Flat	Opaque	+
Phragmites	**P1**	*Bacillus cereus*	KX570921	Large spherical	Rough creamy	Circular	Entire	Flat	Opaque	+
	P4	*Bacillus* sp.	KY419157	Large	Slight yellow	Irregular	Smooth	Flat	Opaque	+
Vacha	**V1**	*Bacillus thuringinesis*	KX570926	Large spherical	Rough creamy	Circular	Entire	Flat	Opaque	+
	V5	*Pseudomonas mendocina*	KX570924	Submerged	Slightly yellowish	Irregular	Smooth	Flat	Transparent	+
	V10	*Microbacter chocolatum*	KX570925	Tiny pin pointed	Brownish	Circular	Entire	Raised	Opaque	-
Arundo	**A1**	*Microbacter chocolatum*	KY419148	Tiny pin pointed	Dark brown	Circular	Entire	Flat	Opaque	+
	A4	*Halomonas venusta*	KY419151	Medium spherical	Glistering creamy	Circular	Entire	Flat	Opaque	-
	A8	*Lysinibacillus sphaericus*	KY419149	Medium spherical	Light yellow	Circular	Entire	Flat	Opaque	+
Vetiver	**K10**	*Pseudomonas mendocina*	KX570928	Small	Slight yellow	Regular	Entire	Flat	Opaque	-

Conclusion

Macrophytes root associated bacteria play important role in plant assisted Nickel bio-remediation. Through bio-accumulation these bacteria reduce the nickel stress in the rhizosphere and support the plants. Among the five used wetland macrophytes viz. *Typha latifolia, Phragmites karka, Acorus calamus, Arundo donax and Vetiver zizanioides Bacillus*, *Pseudomonas* and *Microbacter* had played major role in Ni bio-remediation and even bio-accumulate up to 19.78±0.48%, 27.66±0.46% and 16.90±2.03%of applied 5.0ppm of nickel, respectively in 3 days and maximum 34.20% by *Pseudomonas mendocina* isolated from Vacha. However, among all the five macrophytes, the mean nickel removal was found maximum by the bacteria isolated from Phragmites (17.98±0.78%).

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