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Isolation and Molecular Identification of Nickel-Adsorbing Bacteria from Industrially Polluted Soils of Visakhapatnam with Emphasis on Bioremediation

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ABSTRACT
The current study for the isolation of Ni tolerant bacterial strains, soil samples polluted by paint industry were used. From these soil samples a total of 8 different discrete bacterial colonies were identified which showed minimum tolerance at 100ppm nickel. The eight bacterial isolates were named as MAB-01, MAB-02, MAB-03, MAB-04, MAB-05, MAB-06, MAB-07, and MAB-08. Out of the isolates, the MAB-06 showed greatest growth at 100-300 ppm nickel. MAB-06 was characterized for nickel bio-adsorption. 16S rRNA gene sequencing identified MAB-06 as closely related to Bacillus benzoevorans (99.05% identity). The sequence had 906 nucleotides with 54% GC content. Phylogenetic analysis, morphology and molecular phylogenetics showed MAB-06 clusters with Bacillus benzoevorans. MAB-06 showed significant nickel adsorption ability using a biofilm on a glass slide. Nickel concentration decreased to 120 ppm (76% removal) with MAB-06 treatment vs. 495 ppm (1% removal) in the control. MAB-06 exhibits prominent nickel adsorption capacities, making it useful for removing nickel from wastewater from industries like pharmaceuticals, painting, and food. The study concluded that MAB-06 can remediate nickel contamination from soils and industrial discharges.
Keywords: *Heavy metals, Nickel, bio-adsorption, bioremediation, Bacillus benzoevorans.*

INTRODUCTION

Heavy metal contamination of soil is a major global environmental issue and it is caused by a variety of metals, including Cadmium (Cd), Copper (Cu), Chromium (Cr), Lead (Pb), Mercury (Hg), Nickel (Ni), Zinc (Zn) [1]. Through emissions from rapidly expanding industrial zones, mine tailings, decomposition of heavy metal effluents, fossil fuel combustion and varnishes, land fertilizers, livestock manure, pesticide residues, sewage irrigation, combustion of coal debris, petrochemical spillage, and atmospheric deposition, soils may progressively become contaminated by the accumulation of heavy metals and metalloids[2]. Based on the solubility and availability to the living cells, only 17 heavy metals are important and essential to the living system[3]. Some heavy metals such as Cu, Mn, Mo, Ni, and Zn are required as micronutrients for microbes, plants, and animals, nevertheless, at high concentrations, all of these metals are poisonous and constitute an environmental pollution[4]. World Health Organization[5], considered lead (Pb), nickel (Ni), chromium (Cr), cadmium (Cd), cobalt (Co), copper (Cu), zinc (Zn) and mercury (Hg) as priority hazardous metals that needs urgent consideration. Metals such as mercury, cadmium, lead are toxic even at low concentration unlike metals such as Nickel and zinc which are essential for living organisms at lower concentration, but toxic at higher concentrations[6].

Nickel is a transition metal (element 28) commonly exists in oxidation state (Ni^{2+}) in the environment and biological systems[7]. Nickel is ferromagnetic and occurs naturally in the Earth's crust, in soil, meteorites, volcano emissions, and seawater (about 8 billion tons). Nickel is a key engineering metal used in alloys, ceramics, and wires for industrial applications. Major sources of nickel in soil include mining, coal-fired power plants, metal/electroplating industries, paint/dye industries, iron/steel production, sewage/waste treatment, and agricultural chemicals[8]. At high concentrations, nickel is toxic to all living systems[9]. Remediation of heavy metal-contaminated soils is challenging due to metal persistence. Conventional chemical/physical methods are costly and can harm soil properties[10 & 11]. Biological remediation using phytoremediation, bioremediation, or plant-microbe interactions is a promising alternative. Phytoremediation is eco-friendly but has drawbacks: slow process and plant vulnerability to high contaminants. Research now focuses on plant-microbe interactions (rhizoremediation) to enhance remediation using rhizosphere bacteria to reduce metal toxicity on plants[12]. Rhizoremediation uses plants

and rhizosphere microbes to reduce metal toxicity. Metal-tolerant rhizobacteria (PGPR) help plants combat stress via IAA, nitrogen fixation etc[13 & 14]. Legumes can remediate heavy metals, restore soil fertility, and boost growth with metal-tolerant rhizobia[15 & 16]. Root nodule endophytic bacteria are advantageous for metal bioremediation[17].

Biological remediation using microbes, plants, or both is eco-friendly and cost-effective. Metal-resistant microbes in polluted sites have bioremediation potential. Isolating and identifying metal-tolerant microbes is a research focus. Optimizing growth conditions enhances metal removal. Plant growth-promoting (PGP) rhizobacteria and endophytic bacteria can boost phytoremediation. In this study, a paint industry effluent contaminated site was selected and an attempt was made to explore the indigenous rhizosphere-associated bacterial population growing in contaminated soils. This study aimed to create a phytoextraction system using a hyperaccumulator and legume with PGP bacteria for nickel remediation in paint industry effluent-contaminated soil. Nickel-adsorbing rhizobacteria were isolated and evaluated for nickel removal.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from paint industry effluent-contaminated sites in Visakhapatnam, Andhra Pradesh, India (17°40'0.44"N, 83°13'58.14"E). The goal was to identify nickel-tolerant bacteria and evaluate their nickel tolerance and nickel bioremediation potential. Samples (approx. 500g each) were collected from 5-10cm depth at three sites using sterile bags and tools. Samples were stored in iceboxes, transported to the lab, and refrigerated at 4°C for further analysis.

Table 1: Characteristic parameters of soil samples

Soil Samples	Soil Characteristics			
	pH	Temperature (^{0}C)	DO (mg/l)	Colour
Sample 1	5.8	27.4	5.6	Brownish black
Sample 2	6.1	28.6	5.2	Brownish black
Sample 3	5.1	24.3	6.2	Brownish black

Preparation of soil suspension

All the soil samples were mixed in equal quantities to make a single sample, and the soil sample was powdered finely. Then, 1 gm of mixed soil sample was dissolved in 9 ml of sterile deionised water to prepare a soil suspension. The soil suspension was filtered through the sterile 1 mm mesh to remove the gravel and plant debris. Then serial dilutions were made from the soil suspension as follows: 10-1, 10-2, 10-3, 10-4, and 10-5.

Isolation of nickel tolerant bacteria

The prepared dilutions were inoculated in the nutrient broth and incubated for 48 hours at 35±2°C to enrich the bacterial population from the soil samples. After incubation, the growth of the bacterial culture was measured by taking absorbance at 600 nm using a spectrophotometer and these cultures were used to isolate the nickel tolerant bacteria. The isolation of nickel tolerant bacteria was performed by inoculating the 24 hours old cultures of different dilution were spread on the 100 ppm $NiSO_4$ amended fresh Nutrient agar plates and incubated for 48 hours at 35±2°C. The nutrient agar plates were prepared by dissolving 5.0 gm peptone, 3.0 gm beef extract, 5 gm yeast extract, 8.0 gm NaCl, and 15.0 gm of bacteriological grade agar to 1 litre of distilled water. To this media 100 ppm of $NiSO_4$ was added to enrich the nickel tolerant bacteria growth. Finally, the pH of the media was adjusted to 7.2 by using 0.1 M NaOH. The prepared nutrient agar medium was sterilized in an autoclave at 121°C for 15 minutes. After incubation, the morphological characteristics of colonies grown on each plate were noted. Most of the colonies from the spread plate method showed recurring morphological characteristics. The morphologically discrete bacterial strains were selected and isolated again on 100 ppm $NiSO_4$ supplemented nutrient agar medium by the recurrent streak plate technique.

The dominant bacterial colonies with different colony characteristics were chosen to screen their nickel tolerance potentials and the best isolates were selected for further research.

Screening of nickel tolerant abilities of isolated bacterial colonies

The nickel tolerance of selected bacterial colonies was screened by inoculating of cultures obtained from the nutrient agar plates again sub-cultured in the nutrient broth for 24 hours at 37°C. In this experiment, 5 ml of freshly subcultured 24-hour old isolated pure cultures of bacteria were again allowed to grow in

nutrient broth, which is supplemented with three different concentrations such as 100, 200, and 300 ppm $NiSO_4$. These cultures were incubated at 35±2°C for 24 hours in an orbital shaking incubator at 120 rpm for adequate development. Following incubation, the bacterial growth from the nutrient broth of respective $NiSO_4$ concentrations was measured by taking absorbance at 600 nm using a spectrophotometer. The results were expressed as OD values that indicate the turbidity of the culture. The best culture that shows the highest growth in all the nickel concentrations was selected and stored as a slant culture at 4°C, and this selected bacterial strain was used for the study.

Molecular Identification of MAB-06

Among all the bacterial isolates, MAB-06 exhibit better nickel tolerance ability. Hence it was characterized molecularly to identify the species by using 16s rRNA gene sequencing.

Isolation of Genomic DNA

The CTAB lysozyme method was used to isolate the genomic DNA of the bacterial isolate MAB-06. The isolate MAB-06 was grown on nutrient agar for 2 days at 28°C to study the genomic DNA G+C content of the strain using the thermal denaturation method[18] and for PCR amplification.

Amplification and sequencing of 16S rRNA gene

PCR amplification and 16S rRNA gene sequencing were carried out as described by Li et al.[19]. The 16S rRNA gene of bacterial isolate MAB-06 was amplified by using PCR. Two universal primers 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 907R (5'CCGTCAATTCMTTTRAGTTT3') were used to amplify 16S rRNA genes. PCR reaction mixture of 25 µl total volume, containing 1/10 volume 10× Taq buffer, 2 mm MgCl2, 1-unit Taq DNA polymerase, 0.2 mM dNTP, 20 pmol forward primer, 20 pmol reverse primer and 100 ng DNA. DNA amplification was carried out in a Biorad Mini thermocycler with the following procedure: an initial denaturing step at 94°C for 5 min; 40 cycles for 1 min at 94°C (denature), 1 min at 48°C (annealing), 2 min at 72°C (extension) and a final elongation step at 72°C for 5 min and then cooled to 4°C. Amplified PCR products were separated by electrophoresis on 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide, and photographed. The standard DNA samples (100 bp DNA ladder marker) were used as molecular size marker. The purified PCR products was subjected to Sanger's di-deoxy sequencing, in both forward and reverse direction ns, using Big Dye terminator v3.1 cycle sequencing kit on ABI Prism3700 DNA Analyzer (Applied Biosystems Inc., USA).

Sequence analysis and phylogenetic tree construction

Bacterial isolate MAB-06 16s rRNA gene nucleotide composition was computed by Seqstate V.1.21 server[20]. The molecular identification of bacterial isolate MAB-06 was conducted by constructing a phylogenetic tree with some major bacterial species. Bacterial isolate MAB-06 16S rRNA gene sequence was subjected to the BLASTn in NCBI server to identify the homologous sequence or species and from the NCBI database, 9 species were selected based on their degree of homology with the target 16s rRNA gene sequence. The selected 9 species 16S rRNA gene sequences were derived from the nucleotide NCBI database. Multiple sequence alignment was conducted with UPGMA to search homology of 16S rRNA gene sequences between the MAB-06 and the selected 9 species. Phylogenetic tree was constructed using MEGAX. The accuracy test of the tree was performed by the bootstrap method 1000 times.

The determination of nickel remediation potential of isolated bacterial strain was evaluated by preparing the biofilm of MAB-06 and screening of Ni remediation potential by atomic adsorption spectrophotometry.

Preparation of Biofilm

Biofilm development was performed by using the methodology of O'Toole and Kolter[21] with slight modifications. The glass slide (25 mm in width, 75 mm in height and 1mm in thickness) were used for the biofilm formation. The glass slides were precleaned with 1N HCL and treated with sodium hypochlorite solution of 10 mg/L for 24 hours and rinsed with sterile distilled water before the experimental setup. Then the slides were placed in PVC chamber at 21oC and covered by aluminium foil. After drying of slides, 24 hours old fresh MAB-06 culture was poured at flow velocity of 10 to 20 drops/minute on the glass slide. Then, the slides were placed in the PVC chamber and allowed to incubate at 37°C for 2 days for the formation of biofilm[22]. Fresh medium was added when necessary to prevent drying of isolates. After incubation, the plate was inverted, tapped vigorously on paper towels, and airdried. The ability to form biofilm was scored visually by comparing the thickness of the biofilm formed. The negative control was prepared by using only medium without MAB-06.

Determination of Ni remediation potential

The nickel remediation potential of MAB-06 was determined by using NiSO4. In this experiment, the known concentration of nickel solution (500ppm) was prepared by dissolving 0.05gm of NiSO4 in 1 litre of HPLC grade deionised water. After preparation of nickel solution, the prepared MAB-06 biofilm was placed in a beaker which contain 200 mL of 500ppm nickel solution and it allowed to incubate in an orbital shaking incubator for 2 days with 50rpm at 35°C. After incubation, the biofilm slide was removes and the Ni solution was centrifuged at 1000 rpm for 5 minutes. The supernatant was collected from test and negative controls. Finally, the concentration of Ni in the NiSO4 solutions from MAB-06 treated and negative control were measured by using an atomic absorption spectroscopy. Working parameters of atomic adsorption spectrophotometer such as wave length and band width for Ni estimation were adjusted as 324.8 and 0.7 respectively.

$$\text{Percentage of Ni remediation} = \frac{\text{Conc. of Ni in std.} - \text{Conc. of Ni after treatment}}{\text{Conc. of Ni in std.}} \times 100$$

Figure 1: Preparation MAB-06 Biofilm on the glass slide.

RESULTS AND DISCUSSION

Soil pollution from heavy metal contamination is a serious issue due to industrial releases, chemical fertilizer overuse, and poor irrigation management. Nickel (atomic number 28) is widely used in electroplating, alloys, ceramics, and wires. Major sources of nickel in soil include mining, power plants, metal industries, and agricultural chemicals. Elevated nickel levels can cause health issues (nausea, organ failure, death) in humans and reduce plant growth/seed germination[23 & 24]. This study investigated bacterial diversity in paint industry effluents in Visakhapatnam to isolate nickel-tolerant bacteria for potential nickel bioremediation.

In this study, for the isolation of Ni tolerant bacterial strains, paint industrial polluted soil samples were used. From these soil samples total 8 different discrete bacterial colonies were identified which were minimum tolerance at 100 ppm nickel. These discrete bacterial colonies were isolated by sub-cultured onto nutrient agar plates supplemented with 100 ppm nickel by streaking to obtain pure cultures. Thus, colonies that were single and no crossed were picked and again streaked onto Nutrient agar plates, incubated at 37°C for 24 hrs, and stored at 4°C until further use. The purified strains were maintained on nutrients agar slants and stored at 4oC with successive subcultures every two weeks. The eight bacterial isolates were named as MAB-01, MAB-02, MAB-03, MAB-04, MAB-05, MAB-06, MAB-07, and MAB-08. The isolated 8 different bacterial colonies where are further identified by morphological, physiological and biochemical characterization. The isolation and purification plates of nickel tolerant bacteria has been shown in figure 2 and 3.

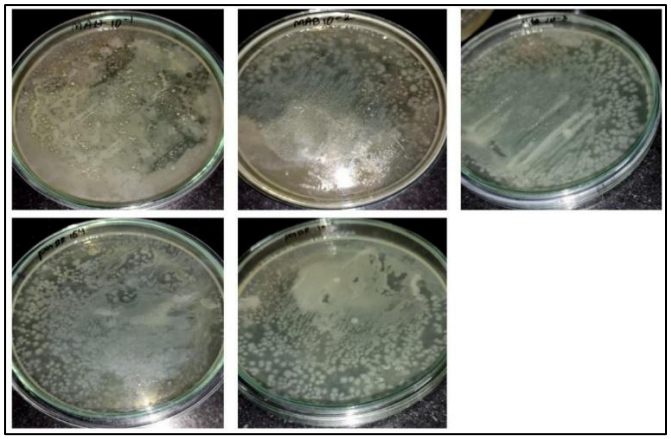

Figure 2: Isolation of nickel tolerant bacteria from paint industrial polluted soils by culturing on the 100-ppm nickel amended fresh nutrient agar plates.

During the past decades extreme environments have been extensively explored, and the nickel tolerant bacteria has been found in a wide range of industrially polluted soils especially in the paint and alloy industries. Visakhapatnam is one of the largest industrially developed coastal city in India, and hence there is a tremendous possibility for bacterial diversity to remediate nickel. The present study demonstrated that the polluted soils of paint industries in the coastal region of Visakhapatnam are a potent source for nickel tolerant bacteria due large microbial diversity. The successful bacterial isolation depends on the physico-chemical properties of polluted soils, because these factors can directly influence the type, number, and metabolic activities of the bacterial diversity[25]. Furthermore, isolation is a necessary approach to obtain novel microbes and physiological characteristics for under-standing their physiological and environmental functions and for their application potentials[26]. Previous studies[27 & 28]reported that many microbial species such as Pseudomonas, Enterobacter, Bacillus, and Shewanella can significantly mitigate the nickel toxicity by adsorbing nickel by secreting certain soluble enzymes. Moreover, the rarity of effective nickel reducing bacteria is a critical need for bioremediation of nickel contaminated soils.

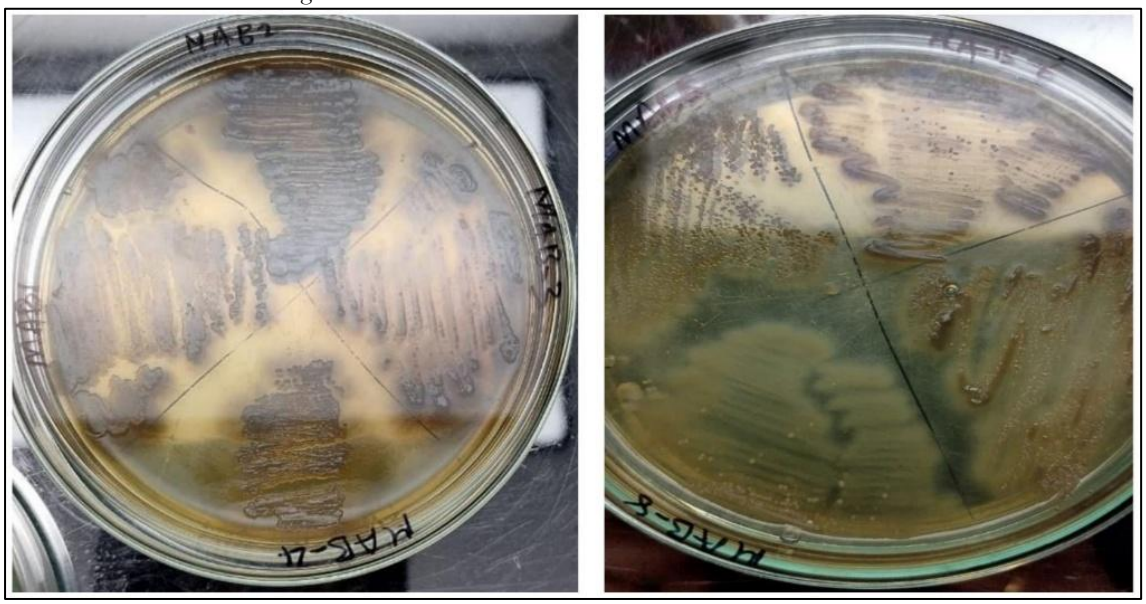

Figure 3: Pure cultures of isolated eight nickel tolerant bacteria on 100 ppm nickel amended fresh nutrient agar plates.

Nickel tolerant ability of bacterial isolates

The major attribute studied was effect of nickel on the isolated bacteria and to identify the high nickel tolerant bacteria. In this study we have studied at 100, 200, and 300 ppm nickel concentrations and the OD was observed at 660 nm for the bacterial growth. Isolates were found to have varying tolerance levels at maximum nickel concentration for growth. All the eight isolates have shown tolerance at 100 ppm nickel concentration. Whereas, with increasing concentration of nickel, all the isolates exhibit decreased

growth rates. however, among all the isolates, only one isolate (MAB-06) exhibits higher growth rates at all tested nickel concentrations. The results of nickel tolerance of the eight isolates were shown in table 2.

These results indicated that MAB-06 can significantly tolerate nickel. Hence, the MAB06 isolate was further characterized to applying as bio-adsorbents of nickel to remove nickel from industrially contaminated soils. The present results exhibit collinearity with the reports[29] that rates of bacterial growth decreased with increasing nickel concentrations in the range of 3.3% to 28.4% and attributed the results to a general reduction in microbial metabolic rates. A high concentration of nickel in the medium may decrease the water activity of the surrounding environment, which can disrupt the normal cellular activities and the growth of bacteria[30]. The present work is evident by previous works[31], who have isolated the nickel tolerant bacteria from tannery effluent and shown that it can exhibit growth with more than 100 ppm of nickel concentration. As well as, nickel is effectively removed from the waste water through an extracellular process by using the bacterial species Shewanella loihica[32].

Table 2: Effect of increasing Ni concentrations on the growth of eight bacterial isolates.

S. No	Bacterial Isolates	OD at 660 nm		
		100 ppm $NiSO_4$	200 ppm $NiSO_4$	300 ppm $NiSO_4$
1	MAB-01	0.151	0.526	0.409
2	MAB-02	0.303	0.373	0.436
3	MAB-03	0.734	0.520	0.301
4	MAB-04	0.503	0.486	0.342
5	MAB-05	0.672	0.450	0.333
6	**MAB-06**	**0.948**	**0.430**	**0.370**
7	MAB-07	0.436	0.438	0.343
8	MAB-08	0.464	0.810	0.319

Molecular and Phylogenetic Characterization of Selected bacterial Isolate MAB-06

Molecular approaches which involve 16s rRNA isolation and sequencing techniques have been developed for identifying microbial populations occurring in nature. In nature, microbial populations are much more diverse, with various abilities. Hence, these techniques have been proven to be powerful tools to identify bacterial species in microbiological studies. The present study applies the 16s rRNA approach by PCR amplification to identify the isolated bacteria (MAB-06) from polluted soils of brass industries. Molecular identification can be useful for the characterization of evolutionary relationships between MAB-06 and its related groups. The evolutionary distances were computed using the Maximum Composite Likelihood method[33].

Total DNA quantification Genomic

DNA was isolated from the bacterial strain MAB-06 and the total DNA content was found as 579±23 µg/g wet weight. Absorbance ratio of isolated DNA at A260/280 was in the range of 1.88–1.91 respectively. The results were given as Mean ± Standard Deviation (SD) obtained from three independent experiments. The mol% G + C content of DNA of bacterial strain MAB-06 was calculated[18]. The Tm value for DNA of bacterial strain MAB-06 was recorded as 86°C. The thermal denaturation midpoint (Tm) of DNA increases with increase in the mol% G + C. Whereas, 69.3 is the value from the Marmur equation, obtained by extrapolating to 100 mol% of A + T intercept on the temperature axis at 69.3°C and 0.41 is the slope of the line. On 1% agarose gel the isolated genomic DNA was appeared as prominent band. Figure 4 showed the genomic DNA band in 1% agarose gel.

$$\text{Mol \% G + C} = \frac{\text{Tm} - 69.3}{0.41}$$

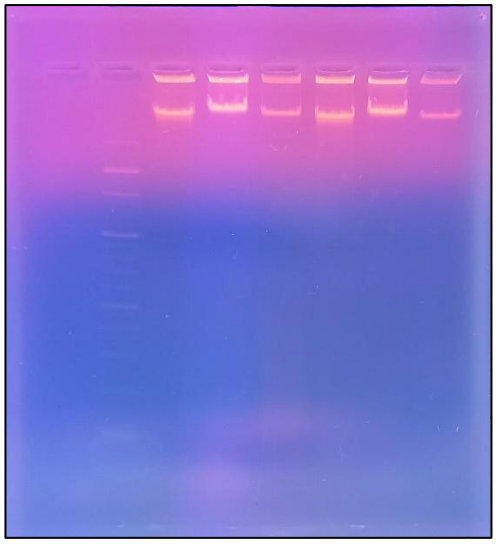

Figure 4: Photograph showing bacterial isolate MAB-06 genomic DNA in 1% agarose gel.

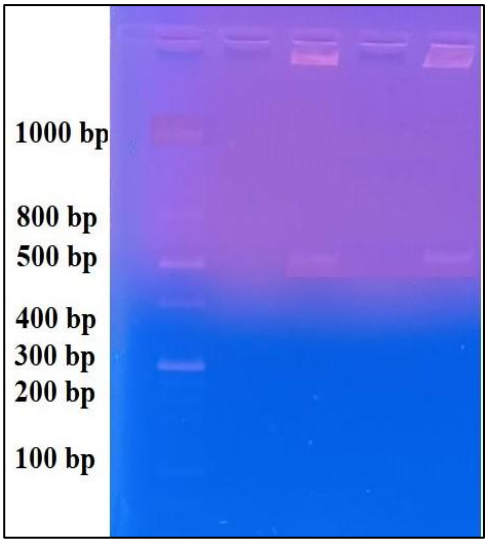

Figure 5: Photograph showing the PCR amplified 16S rRNA gene band in 1.5% agarose gel.

PCR amplification and sequencing of 16s rRNA gene

Microbiology develops by identifying and characterizing microbes for phylogenetic relationships. Traditional bacterial identification can be tough due to slow growth or non-culturable bacteria. So, 16S rRNA gene sequencing is used for rapid and accurate identification[34 & 35]. The 16S rRNA gene is ideal for phylogenetic studies because it's easily retrievable, highly conserved, and useful for species ID. It helps identify uncultivable bacteria and has led to reclassification of some species[36].

Hence, the DNA extracted from the bacterial isolate MAB-06 was used in the PCR experiments to amplify 16S rRNA gene sequence using the two universal primers, 27F - 5'AGAGTTTGATCMTGGCTCAG3' and 907R- 5'CCGTCAATTCMTTTRAGTTT3'. Due to the high primer universality and discriminatory power, the 16S rRNA gene has been routinely used for phylogenetic studies[37]. The selected primers for the 16S rRNA regions of genomic DNA of bacterial isolate MAB-06 yielded clear single and good quality bands. In agarose gel electrophoresis the 1st well shows marker DNA and the second wells shows amplified gene product with approximately 900 bp length. The PCR amplified fragment was purified for further analysis such as nucleotide sequencing and phylogenetic analysis. Figure 5 showed the PCR amplified DNA band in 1.5% agarose gel.

Sequence analysis

A sequence characteristic of 16S rRNA gene was calculated by seqstate v.1.21[20]. The sequence of PCR amplified bacterial isolate MAB-06 16S rRNA gene have 906 nucleotides showed in figure 6. The sequence of 16S rRNA gene have 236 bp of Adenine(A), 178 bp of Thymine (T), 291 bp of Guanine (G) and 201 bp of Cytosine (C). The % of GC was calculated as 54. The results were showed in table 3.

GC content in organisms is highly variable. In bacteria, GC content ranges from 25-75%[38] or even 13-75%[39]. Bacterial isolate MAB-06's 16S rRNA had high GC content. Reasons for GC content differences are unclear but likely involve evolution and environment[40]. High GC content means fewer A/T nucleotides. Microbes in the same environment tend to have similar %GC[41]. GC-rich codons may contribute to evolutionary rates[42]. The GC distribution over the amplified 16S rRNA gene sequence of MAB-06 is shown at figure 7.

Table 3: Nucleotide composition of MAB-06 16S rRNA gene

S. No	Parameter	Number of nucleotides	% of nucleotides
1	Total bases	906	-
2	Adenine	236	26
3	Thymine	178	20
4	Guanine	291	32
5	Cytosine	201	22
6	G+C	492	54

TGCAAGTCGAGCGGACTTAAAAAGCTTGCTTTTTAAGTTAGCGGCGGACGGGTG
AGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGGAG
CTAATACCGGATAATCCTTTCCTACTCATGTAGGAAAGCTGAAAGACGGTTTACG
CTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAA
AACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGG
TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTT
TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG
GGACTTGAGTGCAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACG
CTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCA
AACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATG

Figure 6: The nucleotide composition of MAB-06 16S rRNA gene (906 bp).

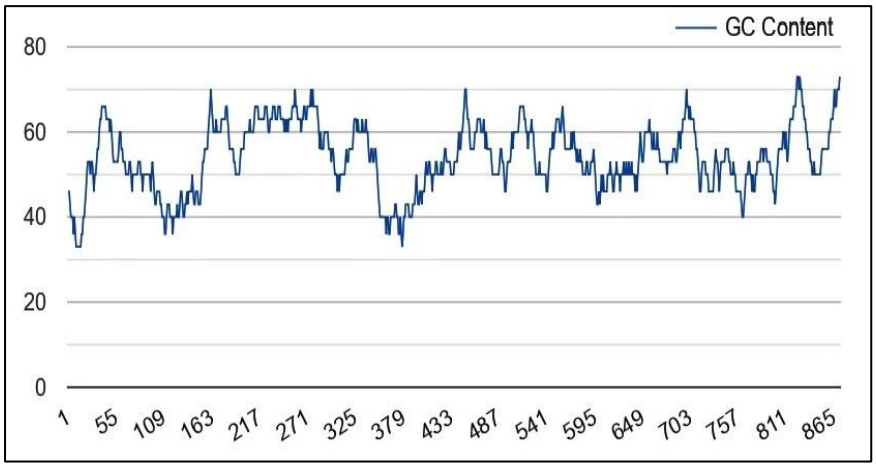

Figure 7: GC distribution over the amplified 16S rRNA gene sequence of MAB-06

Multiple sequence alignment

Multiple sequence alignment analyzes DNA, RNA, and protein sequences to find closely related sequences. It accounts for evolutionary events like mutations, insertions, deletions, and rearrangements[43]. Multiple alignments reveal more info than pairwise alignments about structural and functional areas in a sequence family[44]. The 16S rRNA gene (1-906 bp) sequence of bacterial isolate MAB-06 was used as a query against the nucleotide sequence database. The BLASTn search of the Gen Bank database using the 906 bp 16S rRNA gene sequence of MAB-06 showed its similarity to many species of the Bacillus genus. From the BLASTn results, it was observed that the 16S rRNA gene sequence of bacterial isolate MAB-06 showed the highest similarity to strain Bacillus benzoevorans, with an identity of 99.05% and an E-value of 0.0. From the hits, a total of 9 sequences which belongs to the Bacillus genus were selected from the NCBI data base, and the selected sequences were used for multiple sequence alignment. The selected sequences for the multiple sequence alignment and phylogenetic tree construction were listed in table 4. Multiple sequence alignments of the 16S rRNA regions were obtained using the CLUSTAL program[45].

Table 4: List of selected species sequences used for phylogenetic study.

Species	Max Score	Total Score	Query Cover	E value	Per. Ident	Len (bp)
JN872340.1 Bacillus sp. TISAE2B	1659	1659	99%	0	99%	1004

AB637141.1 Uncultured bacteria	1653	1653	99%	0	98.65%	1294
KC200015.1 Bacillus sp. No.16	1664	1664	100%	0	98.78%	1447
KJ7333936.1 Bacillus sp. KT67	1657	1657	100%	0	98.99%	1428
KJ733966.1 Bacillus sp. KT57	1657	1656	100%	0	98.99%	1432
DQ346732.1 Bacillus benzoevorans	1664	1664	100%	0	99.05%	901
DQ346731.1 Bacillus benzoevorans	1664	1664	100%	0	99.05%	906
DQ346733.1 Bacillus benzoevorans	1665	1665	100%	0	99%	896
KF984419.1 Bacillus sp. BAB-3119	1655	1655	99%	0	98.67%	1495

Phylogenetic tree analysis

The selected homology sequences for the target 16S rRNA gene were extracted from the NCBI database for constructing a phylogenetic tree. All the selected nine species belong to the genus Bacillus and the differences among the selected sequences were determined by calculating the distance matrix. Depending on the expressed differences in the distance matrix, the maximum-parsimony phylogenetic tree was built using MEGAX and the evaluation of phylogenetic tree topologies were done by the bootstrap method with 1000 replicates for all nodes[46]. The evolutionary divergence of nickel tolerant bacterial isolate MAB-06 with its relative members was determined. A satisfactory result was established by the use of 16S rRNA gene as a marker to evaluate the phylogenetic relationship.

In the phylogenetic tree, there were 4 main clades. The first main clade consists of two subclades. The first subclade of main clade 1 was composed of nickel tolerant bacterial isolate MAB-06 and Bacillus benzoevorans (DQ346732.1). The second subclade of the main clade 1 was composed with the Bacillus benzoevorans (DQ346731.1), and Bacillus benzoevorans (DQ346733.1). The second main clades consist of Bacillus sp. KT67 (KJ7333936.1), and Bacillus sp. BAB-3119 (KF984419.1).

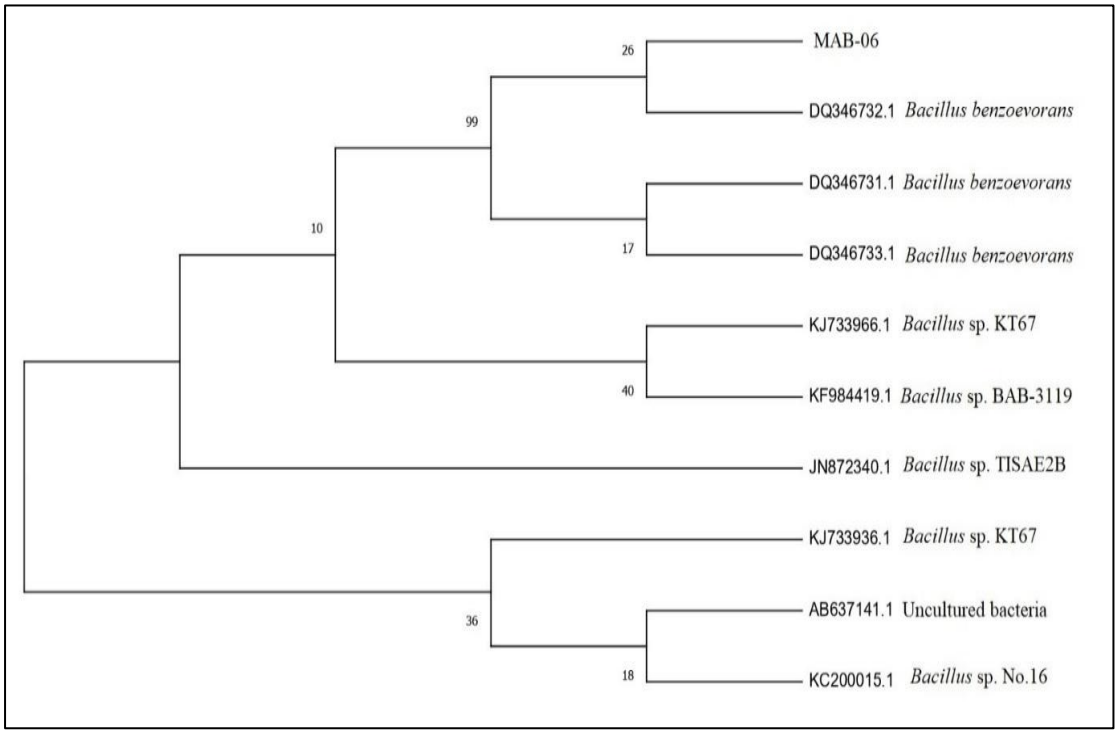

Figure: 8: Maximum Parsimony tree of nickel tolerant bacterial isolate MAB-06 and other relative species based on the 16S rRNA gene.

The third main clade only consist Bacillus sp. TISAE2B (JN872340.1). The fourth main clades consist of two subclades. The first subclade of main clade 4 was composed only with Bacillus sp. KT67 (KJ7333936.1) whereas, the second subclade of the main clade 4 was composed with the Uncultured bacteria (AB637141.1), and Bacillus sp. No.16 (KC200015.1). The phylogenetic tree indicated that the nickel tolerant bacterial isolate MAB-06 has a close branch with Bacillus benzoevorans (GenBank Accession No. DQ346732.1). On the basis of morphological studies, the molecular phylogenetics, it is revealed that the bacterial isolate MAB-06 is belongs to Bacillus benzoevorans species. The maximum parsimony phylogenetic tree was showed in figure 8. DNA sequence analysis in several species provides valuable information about their taxonomy, gene makeup, and utilizations. The generic level identification was deemed effective when a single genus was involved in all hits with maximum percent identification scores are greater than 95%[47]. While species identification was deemed effective only when a single species was included with the highest percent identity score of greater than 95%. In the phylogenetic tree, the clades are organised mostly with the combination of several species and strains. Therefore, generating a local barcode database is necessary for a wide range of ecological applications, including the construction of community phylogenetics[48].

Determination of nickel adsorption ability of MAB-06

The nickel removal potency of MAB-06 was determined by preparing the biofilm on glass slide. In the present study performed as two experimental groups, in the first experimental group the 500ppm nickel containing water was treated with the MAB-06 biofilm. In the second group that is control group, 500ppm nickel containing water treated with the biofilm that only contain nutrient broth without MAB-06. After the treatment, the nickel concentration from both the water samples were measured by using atomic adsorption spectroscopy. All the results expressed as mean value ± standard deviation of three replicates. The data were assessed by one-way analysis of variance (ANOVA) and $P<0.05$ was considered as significant difference.

The results of nickel adsorption ability by MAB-06, and control groups were shown in Figure: 9. from these results, the MAB-06 culture exhibit significant nickel adsorption ability, whereas, in the control group, the nickel concentration remains same as before treatment. The nickel concentrations in the water sample treated with MAB-06 and Control groups were observed as 120 and 495ppm respectively. The nickel removal percentage of MAB-06 was reported as 76%, whereas the nickel removal percentage of control biofilm was reported as 1%. In the present results (figure 10 &11), it was observed that the bacterial isolate MAB-06 exhibit significant (<.0005) nickel adsorption abilities. According to these results, the prepared MAB-06 biofilm exhibits prominent nickel adsorption capacities, hence, they can be used to remove nickel contamination in waste water which is discharged from the pharmaceutical, painting, and other food related industries. Waste water is water affected in quality from various standard parameters set by anthropogenic influences such as discharges of waste from domestic, industrial, agricultural and related sectors containing various organic and inorganic pollutants[49].

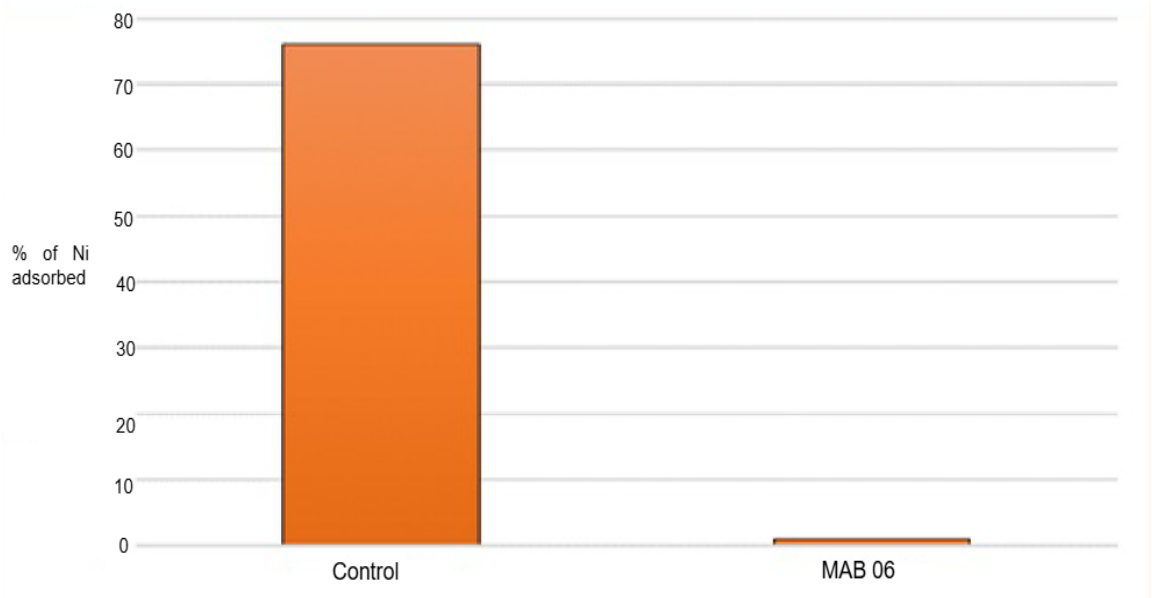

Figure 9: Nickel adsorption abilities of bacterial isolate MAB-06 and control group.

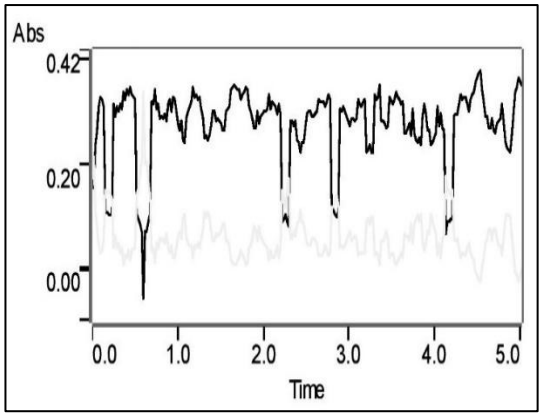

Figure 10: Atomic absorption spectrum of nickel from the control group.

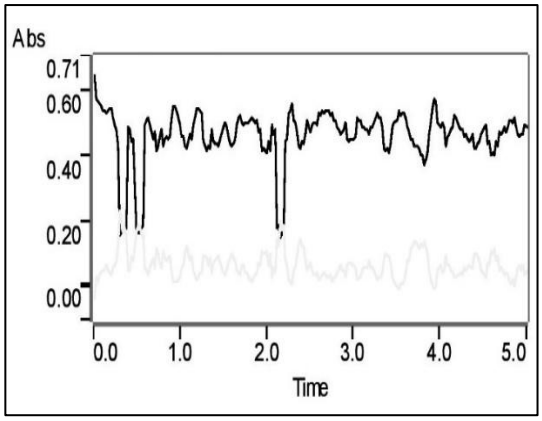

Figure 11: Atomic absorption spectrum of nickel from the MAB-06 treated group.

Heavy metals like lead, zinc, nickel, arsenic, cadmium, chromium, nickel, and mercury from industries are toxic to humans and living species[50 & 51]. Some heavy metals are essential in trace amounts but toxic at higher concentrations. Methods to remove heavy metals from wastewater include chemical precipitation, ion exchange, and adsorption. Adsorption is efficient, reversible, and allows adsorbent regeneration[52]. Biosorption using low-cost, eco-friendly bioadsorbents from biological materials is effective for removing heavy metals from surface water.

CONCLUSION

Heavy metal contamination (Cd, Cu, Cr, Pb, Hg, Ni, Zn) is a major global environmental issue caused by industrial, urban, and agricultural activities. Some heavy metals are micronutrients but toxic at high levels. Nickel is a common heavy metal pollutant due to its toxicity and accumulation in plants. This study focused on nickel remediation using a bacterial isolate from paint industry-polluted soil in Visakhapatnam. Eight nickel-tolerant bacteria (MAB-01 to MAB-08) were isolated; MAB-06 (Bacillus benzoevorans) showed greatest nickel tolerance and adsorption. MAB-06 removed 76% of nickel (from 495 ppm to 120 ppm) using a biofilm. The isolate can potentially remediate nickel contamination in wastewater from industries like pharmaceuticals, painting, and food.

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Conflict of interest

The authors declare that there is no conflict of interest.

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