Exploring Nickel-Tolerant PGPR from Industrial Soils of Andhra Pradesh: Isolation, Functional Characterisation, and Molecular Identification.

Swarnalatha Vavilapalli[1]* and G. Sudhakar[2]
1.Research Scholar, Department of Microbiology, Andhra University, Visakhapatnam, Andhra Pradesh, India
2. Professor, Department of Microbiology, Andhra University, Visakhapatnam, Andhra Pradesh, India
[1]*Email: platha122333@gmail.com
[2]Email: profgsudhakar@gmail.com
***Corresponding Author**
Swarnalatha Vavilapalli
Department of Microbiology, Andhra University, Visakhapatnam, Andhra Pradesh, India
Email: platha122333@gmail.com

Abstract
Nickel pollution has emerged as a significant ecological issue, largely caused primarily by swift industrial growth, mining activities, and insufficient waste disposal. The enduring presence and harmful nature of nickel jeopardize ecological health and agricultural yield, requiring effective and lasting cleanup approaches. This research sought to isolate, characterize, and molecularly identify nickel-resistant plant growth-promoting rhizobacteria (PGPR) from soils contaminated by industrial activities. Soil specimens were gathered from five unique industrial sites in Andhra Pradesh and examined for nickel-resistant bacteria. Twelve bacterial isolates were refined and analyzed for their morphological, physiological, and biochemical properties. Among these, seven isolates exhibited notable resilience to nickel levels above 100 ppm. Significantly, isolate ASW-04 demonstrated the greatest nickel tolerance alongside exceptional plant growth-promoting characteristics, such as peak ammonia (4200±79 µg/ml), indole acetic acid (522±25 µg/ml), and gibberellic acid (167±14 µg/ml) synthesis. Molecular characterization utilizing 16S rRNA gene sequencing demonstrated that ASW-04 exhibits significant sequence similarity (94.65%) with *Metapseudomonas otitidis* and is phylogenetically grouped within the Metapseudomonas clade. The results emphasize the promise of indigenous Ni-tolerant PGPR, especially ASW-04, as bioinoculants for bioremediation and as facilitators of plant development in nickel-polluted settings. This research enhances the comprehension of the diversity and roles of Ni-tolerant rhizobacteria, providing an eco-friendly substitute for traditional remediation techniques and bolstering agricultural yield in soils affected by metals. The methodology emphasizes the significance of incorporating microbial assets into sustainable practices for ecological rehabilitation and food security.

Keywords: Nickel-tolerant bacteria, Plant-growth promoting rhizobacteria (PGPR), Bioremediation, 16s rRNA, Molecular phylogeny

Introduction

Heavy metal contamination has surfaced as a significant environmental issue of contemporary times, jeopardizing ecological integrity, agricultural efficiency, and global human health. In contrast to organic pollutants, heavy metals like lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), nickel (Ni), chromium (Cr), and zinc (Zn) are non-biodegradable, noted for their durability and tendency to bioaccumulate via trophic transfer in biological systems (Ali et al., 2019). The ongoing discharge from human activities such as industrialization, mining, urban development, and increased farming has led to their widespread presence in soils, aquatic environments, and the atmosphere, posing significant

ecological and public health risks (Tchounwou et al., 2012). Nickel (Ni), a transition metal, is highly valued in industrial applications due to its robustness, malleability, and resistance to corrosion. Its considerable utilization in metallurgy, electroplating, battery production, and alloys has resulted in significant environmental distribution, especially in areas with inadequate waste management (Das et al., 2020). Natural phenomena, including the weathering of ultramafic rocks and volcanic eruptions, increase the natural levels of nickel; however, anthropogenic actions such as mining, smelting, industrial waste discharge, fossil fuel burning, and inadequate e-waste management are the primary catalysts for modern nickel contamination (Kabata-Pendias, 2011; Chakraborty et al., 2018).

The industrial growth in India has led to substantial nickel contamination, particularly in regions linked to metal plating, tanneries, and mining (Kumar et al., 2020; Subramani et al., 2018). CPCB, (2022) states that industrialization, mining activities, and insufficient regulatory measures drive nickel contamination in India and worldwide. On a worldwide level, unregulated mining and battery manufacturing, particularly in areas such as Indonesia and China, have caused extensive environmental and societal instability (USGS, 2023; Climate Rights International, 2025). Substandard effluent management and the utilization of polluted water for irrigation exacerbate nickel buildup in agricultural produce, compromising food safety. Upon discharge, nickel adheres to soil minerals and organic substances, influencing soil microorganisms, but in aquatic settings it manifests as soluble ions or particulates, posing potential damage to aquatic life (Das et al., 2020). Inhaling atmospheric particulates containing nickel presents respiratory risks (Cempel et al., 2016), and prolonged human exposure through food, water, or occupational interactions has been associated with allergies, respiratory and cardiovascular ailments, as well as carcinogenic consequences (Jaishankar et al., 2014; IARC, 2012). Consequently, efficient remedies necessitate more robust regulatory structures, the implementation of sustainable mining methodologies, and substantial community involvement. The restoration of nickel-contaminated ecosystems utilizes both physicochemical and biological methods. Traditional techniques, including chemical precipitation, ion exchange, and adsorption, tend to be efficient yet costly and produce secondary waste (Fu and Wang, 2011).

Microorganisms have attracted interest in the pursuit of sustainable remediation due to their exceptional ability to detoxify heavy metals through processes such as biosorption, bioaccumulation, biotransformation, and biomineralization (Gupta et al., 2021). Metal-resistant bacteria and fungi acclimatise to hazardous environments using mechanisms such as efflux pumps, enzymatic detoxification, and sequestration employing metallothioneins and extracellular polymers (Nies, 2003). Recent attention has been directed at employing nickel-resistant bacteria and hyperaccumulator flora for bioremediation. These approaches utilise the inherent capabilities of specific bacteria to immobilise, convert, or sequester nickel, thereby diminishing its bioavailability and toxicity (Mishra et al., 2020). The amalgamation of microbial and plant-oriented techniques (phytoremediation) provides an environmentally sustainable and economical approach to rehabilitating polluted soils, especially in areas with limited resources. Nickel-tolerant bacteria (NTBs) are remarkable, as they endure high-metal conditions while aiding in bioremediation and enhancing plant growth under stress (Mishra et al., 2020). The application of bioinoculants sourced from these microorganisms signifies a hopeful, environmentally sustainable substitute for traditional, frequently expensive and contaminating physicochemical remediation methods. Therefore, the current study focused on the isolation, characterisation, and molecular identification of nickel-tolerant plant growth-promoting rhizobacteria by 16S rRNA based phylogenetics.

Materials and Methods

Sample collection

The industrially polluted soil samples were collected from five distinct locations along the northeast districts of Andhra Pradesh, which covers Visakhapatnam and Srikakulam, India (Table 1). The samples were collected in order to identify nickel tolerant bacteria and to evaluate their nickel adsorption capabilities as well as the beneficial impacts on plant growth under nickel stress. The soil samples were (approx. 500g each) collected from the top 10 to 15 cm depth at 5 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. Sampling sites of soils near different industrial areas of Andhra Pradesh, India.

Station Number	Industry	Area	Coordinates
1	Visakhapatnam steel plant (RINL).	Pedagantyada, Vizag	17º64'35.210" N; 83º20'16.728" E
2	Coromandel Fertilizers complex.	Sriharipuram, Vizag	17º69'66.217" N; 83º26'86.360" E
3	Hindustan Petroleum Corporation Limited (HPCL)	Malkapuram, Vizag	17º69'47.297" N; 83º23'62.590" E
4	Rare Earths India Pvt Ltd.,	Srikakulam	17º52'10.538" N; 82º98'59.163" E
5	NACL Industries Ltd.,	Atchutapuram, Vizag	18º27'29.997" N; 82º79'36.662" E

Isolation and Purification of Nickel tolerant bacteria

All the soil samples were air dried, subjected to sieving via a 2 mm mesh and all the soil samples are mixed in equal quantities to make a single sample. 1 gm of the mixed soil sample was dissolved in 9 ml of sterile deionized water to make a soil suspension. Then serial dilutions were made from the soil suspension as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. To enrich the bacteria, serially diluted soil suspensions were inoculated in Basal Mineral Medium (BMM) mineral salt medium enriched with nickel, with the initial pH adjusted to 7.2 using 0.1 M NaOH (Chen et al., 2013) and kept at 30ºC in orbital shaking incubator for 48 hours at 120 rpm. After incubation, 100 µL of enriched broth was inoculated on the 100-ppm Ni supplemented LB agar plates via the spread plate technique, and the culture plates were incubated for 48 hours at 35°C. Following incubation, the morphological traits of colonies cultivated on each plate were recorded. The majority of the colonies that were cultured using the spread plate approach exhibited consistent morphological features. The morphologically distinct bacterial strains were identified and isolated on nutritional agar media using the repetitive streak plate method. The predominant bacterial colonies exhibiting varied characteristics were selected to evaluate their nickel adsorption capabilities, and the most effective isolates were chosen for subsequent research.

Morphological and Biochemical characterisation of the bacterial isolates

The identification of bacterial isolates was conducted through phenotypic examinations, which included assessments of colony and cell morphology, Gram's reactivity, sporulation, and motility. (Holt et al., 1994). For the bacterial isolates, physiological and biochemical characterization was performed using assays for IMViC (Indole, Methyl-red, Voges-Proskauer, and citrate), oxidase, urease, nitrate reductase, gelatinase, starch hydrolases, and lipid hydrolysis (Harley and Prescott, 2002; Hammes and Hertel, 2009).

Screening of nickel tolerance ability of isolated bacterial colonies

The nickel tolerance of isolated bacterial colonies was determined by re-plating cultures derived from LB agar plates, which were subsequently sub-cultured in LB broth for 24 hours at 37°C. For this experiment, 5 ml of newly sub-cultured 24-hour-old pure bacterial cultures were inoculated in LB broth that had been supplemented with 5 distinct concentrations of nickel, ranging from 10 to 100 ppm. Control was maintained without Ni supplementation and it was referred as control. The cultures were incubated for 48 hours at 37ºC in an orbital shaking incubator at 120 rpm to ensure optimal development. Subsequent to incubation, the bacterial proliferation from the LB broth at various chromium concentrations was quantified by measuring absorbance at 660 nm with a spectrophotometer. The data were presented as optical density (OD) values, reflecting the turbidity of the culture.

Screening of plant growth promoting ability of isolated bacterial colonies

i) Estimation of ammonia liberation ability

The ammonia liberation capabilities of isolated bacterial strains were quantified by using the Nessler's reagent. This experiment was conducted by inoculating 24-hour-old isolated pure cultures in 100 mL Jenson's broth. Then the culture broth was incubated at 30°C for 48 hours in an orbital shaking incubator. After centrifugation, 1 mL of broth from each tube was aseptically transferred to the separate microcentrifuge tubes and centrifuged for 5 min at 10,000 rpm. Subsequently, 100 µL of supernatant from each tube was transferred to clean new microcentrifuge tubes and added with 100 µL of Nessler's reagent, including a blank. The final volume was adjusted to 1000 µL by adding 800 µL of distilled water and then incubated for 30 min at room temperature. Absorbance was measured spectrophotometrically at 450nm.

ii) Estimation of IAA production

The ability of the bacterial isolates to synthesize IAA was measured qualitatively using Yeast Extract Mannitol broth medium. This experiment was carried out by inoculating all pure isolates in triplicate into 250 ml conical flasks that contained 50 ml of YEM broth media and 0.1% L-tryptophan. The cultures were incubated in an orbital shaker at 28ºC and 120 rpm for 7 days. followed by incubation, the culture broths were centrifuged at 12,000 rpm for 5 minutes, and 500 µl of supernatant from the liquid cultures was transferred to a 1.5 ml tube, to which 1 ml of Salkowski reagent was added. The Salkowski reagent was prepared by dissolving 2% of 0.5M $FeCl_3$ in 35% perchloric acid. Then the reaction tubes were incubated for 30 minutes in the dark at room temperature. The emergence of pink coloration signifies the production of IAA, and the colour intensity was measured at 530 nm. IAA quantification was determined utilizing a standard curve containing known IAA concentrations ranging from 1.25 to 100 µg/ml. The amount of IAA concentration produced by the bacterial isolates was determined by projecting the observed OD value onto a standard graph (Dawwam et al., 2013).

iii)Estimation of GA production

The gibberellins were quantitatively assessed calorimetrically using the standard approach (Holbrook et al., 1961) with minor changes. To carry out this experiment, a 48-hour-old bacterial culture was centrifuged at 10,000 rpm for 20 minutes, and supernatant was collected. Then, the pH of the supernatant was adjusted to 2.5 using 3.75 N HCl. Subsequently, 15 mL of supernatant were mixed with 2 mL of zinc acetate reagent (21.9 grams of zinc acetate mixed with 1 mL of glacial acetic acid, and the volume adjusted to 100 mL using distilled water). After 2 minutes, 2 ml of 10.6% potassium ferrocyanide was added and subsequently centrifuged at 2000 rpm for 15 minutes. followed by centrifugation, 5 mL of supernatant was added with 5 mL of 30% HCl, and the mixture was incubated at 20°C for

75 minutes. Simultaneously, blank was maintained by taking 5 mL of 5% HCl. After incubation, the gibberellin concentration was determined by measuring the absorbance at 254 nm and using a standard curve that was prepared with gibberellic acid at concentrations ranging from 100 to 1000 µg/ml.

Molecular Identification of potential bacterial isolate

Genomic DNA isolation and PCR amplification of 16s rRNA

Of all the bacterial isolates, the potential Ni-tolerant bacterial isolates that exhibited superior plant growth promoting abilities was molecularly identified the species by 16S rRNA gene sequencing. The CTAB lysozyme method was used to isolate the genomic DNA of the purified potent bacterial isolate. The quality and purity of the isolated genomic DNA are evaluated using a UV spectrophotometer and 1% agarose gel electrophoresis. PCR amplification and 16S rRNA gene sequencing were conducted as outlined by Li et al., (2010). Two universal primers, 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R (5'CGGTTACCTTGTTACGACTT3'), were employed to amplify 16S rRNA gene. A PCR reaction mixture with a total volume of 50 µL, comprising 25 µL of 2X Taq mixture, 5 µL of 20 pmol forward primer, and reverse primer, 3 µL of template DNA, and 12 µL of molecular grade water. DNA amplification was performed by the following protocol: an initial denaturation at 94°C for 5 min; 40 cycles consisting of 1 minute at 94°C, 1 min at 48°C, and 2 min at 72°C, concluding with a final elongation at 72°C for 10 min, followed by cooling to 4°C. The quality and quantity of the amplified PCR product were assessed by 1.5% agarose electrophoresis. The purified PCR product was subjected to Sanger di-deoxy sequencing in both forward and reverse orientations, utilizing the Big Dye Terminator v3.1 cycle sequencing kit on the ABI Prism 3700 DNA Analyzer (Applied Biosystems Inc., USA).

Sequence analysis and phylogenetic tree construction

The nucleotide composition and GC percentage of the 16s rRNA gene from potent bacterial isolate were determined using the Biologics International Crop server (Muller, 2005). To find homologous sequences, the 16s rRNA gene sequence of potent bacterial isolate was searched using the NCBI server's BLASTn. The selected gene sequences were obtained from the GenBank nucleotide database for evolutionary studies and for phylogenetic tree development. The homology and evolutionary statistics of the potent bacterial isolate 16S rRNA gene sequence with selected sequences were conducted using multiple sequence alignment (MSA) with the strong clustering method UPGMA. A maximum-parsimony phylogenetic tree was constructed using MEGA-X, based on the discrepancies indicated in the distance matrix. The tree's reliability was assessed using the bootstrap method conducted 1000 times.

Results and Discussion

Isolation of Nickel (Ni) tolerant bacteria from soil sample

In this study, for the isolation of Ni tolerant bacterial strains, 5 different industrial polluted soil samples were used. From these five soil samples total 12 different discrete bacterial colonies were identified which were minimum tolerance at 100 ppm nickel. These discrete bacterial colonies were isolated by sub-cultured onto LB agar plates 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 4^{o}C with successive subcultures every two weeks. The twelve bacterial isolates were named as ASW-01, ASW-02, ASW-03, ASW-04, ASW-05, ASW-06, ASW-07, ASW-08, ASW-09, ASW-10, ASW-11, and ASW-12. The isolated twelve different bacterial colonies were are further

identified by morphological, physiological and biochemical characterization. The isolation and purification plates of nickel tolerant bacteria have been shown in Figure 1 and 2.

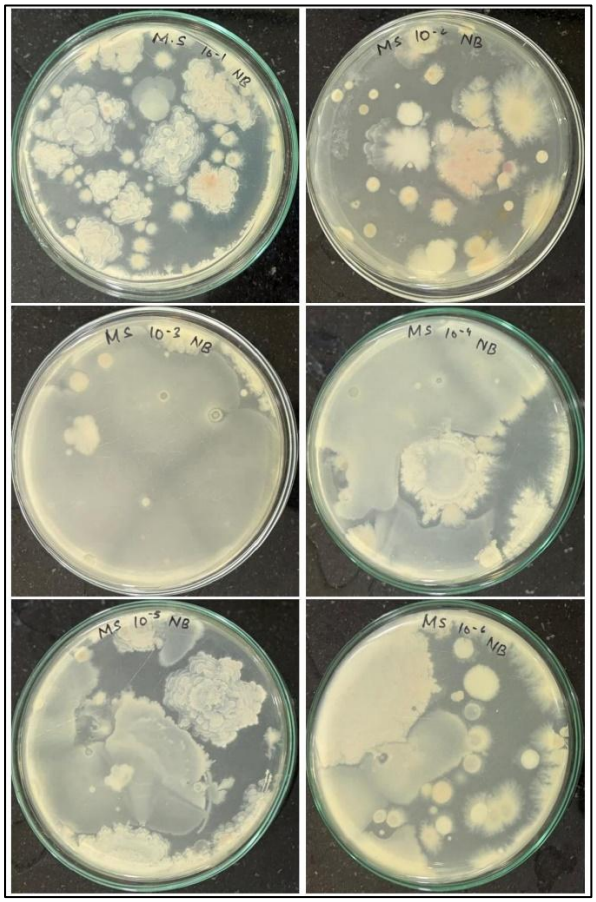

Figure: 1. Isolation of nickel tolerant bacteria from industrial polluted soils by culturing on the 100-ppm nickel supplemented LB agar plates.

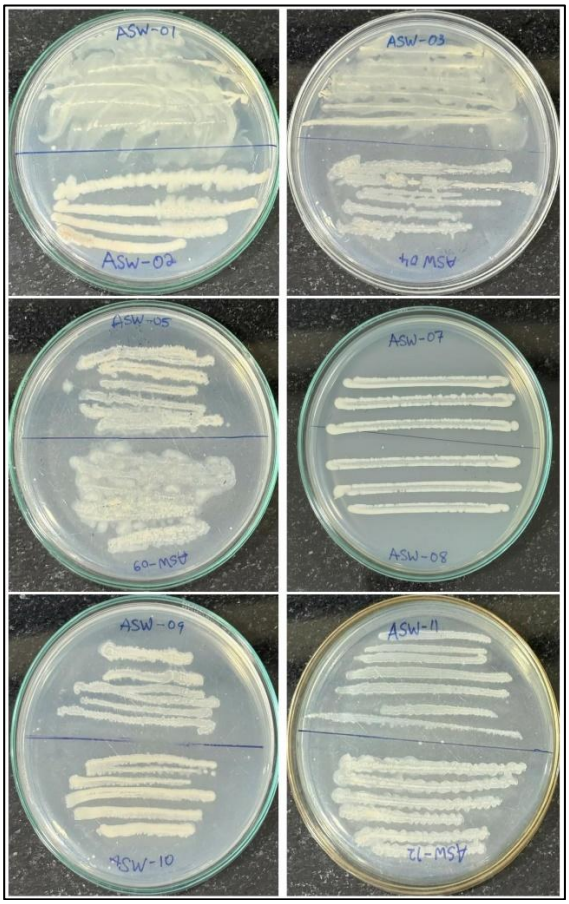

Figure: 2. Pure cultures of isolated nine nickel tolerant bacteria on the 100-ppm nickel supplemented nutrient agar plates.

Microorganisms resistant to heavy metals are believed to exist naturally, particularly in soils that have been contaminated by metals. Research indicates that metals and certain soil physicochemical factors may enhance bacterial resistance to metals (Shi et al., 2013). The emergence of tolerance mechanisms is essential for microorganisms to survive in a stressful environment, particularly when heavy metals are present (Margesin and Schinner, 2001). Bacteria have developed many ways to withstand and ingest heavy metal ions, including the outflow of metal ions from the cell, intracellular accumulation and complexion of metals, and the reduction of heavy metal ions to a less dangerous form (Nies, 1999). Adebusoye et al., (2007) assert that successful bacterial isolation depends on the physicochemical features of contaminated soils, as these factors directly affect the type, quantity, and metabolic activities of bacterial diversity. Moreover, isolation is essential for acquiring novel microorganisms and physiological traits to comprehend their physiological and environmental roles, as well as their potential applications (Vandamme et al., 1996). In earlier research, Spatel et al., (2006) isolated Ni-resistant *Pseudomonas fragi* from industrial effluent comprising varying concentrations of nickel sulfate and it grew effectively in the medium contained up to 2.5 mmol/L of nickel. Similarly, Sevgi et al., (2010) isolated nickel-resistant bacteria (NiRB), which exhibited resistance levels ranging from 2.0 to 4.0 mM of nickel chloride. Rajbanshi, (2008) investigated heavy metal-resistant bacteria in a sewage treatment facility and

successfully isolated nickel-resistant *Staphylococcus* and *Bacillus* species, exhibiting a minimum inhibitory concentration (MIC) between 150 µg/mL. Furthermore, the araciality of efficient nickel-reducing bacteria is essential for the bioremediation of nickel-contaminated soils.

Morphological and phenotypic characterisation of bacterial isolates

The results revealed that out of the 12 isolates, 5 displayed a circular configuration, while 7 exhibited an undulating configuration. Additionally, 4 isolates possess an entire margin, whereas 8 isolates have a diffuse margin. Among the 12 bacterial isolates, 6 exhibited raised elevation, while 6 displayed flattened elevation. The morphological characterisation of 12 isolates by Gram's staining revealed that 9 isolates, such as ASW-01, ASW-02, ASW-03, ASW-04, ASW-6, ASW-7, ASW-8, ASW-11, and ASW-12 exhibit rod shaped morphology, and 3 isolates (ASW-05, ASW-08, and ASW-10) were cocci. Furthermore, 6 of the 12 isolates (ASW-01, ASW-05, ASW-07, ASW-08, ASW-11, and ASW-12) exhibited positive reaction to Gram's staining, while the remaining 6 (ASW-02, ASW-03, ASW-04, ASW-06, ASW-09, and ASW-10) was negative for Gram's staining. The motility was observed in 8 bacterial isolates (ASW-03, ASW-04, ASW-06, ASW-07, ASW-08, ASW-10, ASW-11, and ASW-12), and the remaining 4 (ASW-01, ASW-02, ASW-05, and ASW-09) were nonmotile. The endospores were observed in 8 out of the 12 bacterial isolates such as ASW-03, ASW-04, ASW-06, ASW-07, ASW-08, ASW-10, ASW-11, and ASW-12. The results of morphological and phenotypic characterisation of twelve bacterial isolates are shown in Table 2.

Table: 2. Morphological and phenotypic characterization of the bacterial isolates

Characteristics	**Bacterial isolates**											
	ASW-01	**ASW-02**	**ASW-03**	**ASW-04**	**ASW-05**	**ASW-06**	**ASW-07**	**ASW-08**	**ASW-09**	**ASW-10**	**ASW-11**	**ASW-12**
Configuration	Circular	Circular	undulate	undulate	undulate	undulate	Circular	Circular	undulate	Circular	undulate	undulate
Margin	Diffused	Diffused	Diffused	Diffused	Diffused	Diffused	Entire	Entire	Diffused	Entire	Entire	Diffused
Elevation	Raised	Raised	Flat	Flat	Flat	Flat	Raised	Raised	Flat	Raised	Flat	Raised
Surface	Chalky	Smooth	Smooth	Chalky	Chalky	Chalky	Smooth	Smooth	Chalky	Smooth	Chalky	Chalky
Density	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Transparent	Opaque
Grams Reaction	Positive	Negative	Negative	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Positive	Positive
Shape	Rods	Rods	Rods	Rods	Cocci	Rods	Rods	Rods	Cocci	Cocci	Rods	Rods
Spore	-	-	+	+	-	+	+	+	-	+	+	+
Motility	-	-	-	-	-	+	+	-	-	+	-	+
Colony colour	Cream	Cream	Cream	White	Yellowish cream	White	Cream	Cream	White	Cream	White	Creamy white

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Biochemical characterisation of the bacterial isolates

Table 3 presents the biochemical characteristics of twelve isolated bacterial strains obtained from industrially contaminated soil samples. These results demonstrated that twelve isolates exhibited considerable enzymatic diversity. Eight isolates (ASW-02, 03, 04, 07, 08, 10, 11, 12) were indole positive, while seven (ASW-01, 04, 05, 07, 09, 11, 12) tested positive in the methyl red assay, indicating acid production from glucose fermentation. The Voges-Proskauer test identified six positive isolates (ASW-01, 05, 06, 08, 09, 10) for acetoin production, and four (ASW-02, 08, 09, 12) could utilize citrate as a sole carbon source. Catalase activity was observed in seven isolates (ASW-01, 02, 03, 05, 06, 10, 11), while only two (ASW-05, 06) were oxidase positive. Four isolates (ASW-01, 07, 10, 12) showed urease activity, and seven (ASW-01, 02, 03, 04, 08, 09, 11) could reduce nitrate. Gelatinase production was found in seven isolates (ASW-01, 03, 04, 05, 06, 10, 11), with the same number hydrolyzing starch (ASW-01, 03, 04, 05, 07, 08, 11) and lipids (ASW-01, 03, 04, 05, 07, 11, 12). These findings confirm that the isolates possess a broad range of biochemical capabilities, supporting their potential for bioremediation and environmental applications.

Table: 3. Biochemical characterization of the bacterial isolates

Characteristics	**Bacterial isolates**											
	ASW-01	**ASW-02**	**ASW-03**	**ASW-04**	**ASW-05**	**ASW-06**	**ASW-07**	**ASW-08**	**ASW-09**	**ASW-10**	**ASW-11**	**ASW-12**
Indole	-	+	+	+	-	-	+	+	-	+	+	+
Methyl Red	+	-	-	+	+	-	+	-	+	-	+	+
Voges-Proskauer	+	-	-	-	+	+	-	+	+	+	-	-
Citrate	-	+	-	-	-	-	-	+	+	-	-	+
Catalase	+	+	+	-	+	+	-	-	-	+	+	-
Oxidase	-	-	-	-	+	+	-	-	-	-	-	-
Urease	+	-	-	-	-	-	+	-	-	+	-	+
Nitrate reductase	+	+	+	+	-	-	-	+	+	-	+	-
Gelatinase	+	-	+	+	+	+	-	-	-	+	+	-
Starch hydrolysis	+	-	+	+	+	-	+	+	-	-	+	-
Lipid hydrolysis	+	-	+	+	+	-	+	-	-	-	+	+

Microorganisms play crucial geoactive roles in the biosphere, particularly in biotransformation, biogeochemical cycling, and the production of metals, minerals, and soil (Power et al., 2009). Considering the ubiquity and importance of bacteria in the biosphere, geomicrobiology represents a crucial area that need an interdisciplinary approach to delineate

environmental and technological applications. The growth rates of bacterial communities are highly dependent on environmental factors like temperature, salinity, pH, microbial competition, and nutrient availability (Atlas et al., 1991). Moreover, internal variables such as enzymes and metabolites play a significant influence in the biochemical characterization of microorganisms. Microbes, specifically bacteria and fungi, are effective agents for the removal of heavy metals due to their production of metal chelators or enzymes that transform these metals into less hazardous forms. The capacity to endure heavy metals is mostly contingent upon the adaptation of the organism in a contaminated environment or as a result of prolonged exposure to heavy metals (Volesky, 1990). Therefore, microorganisms extracted from soil samples hold substantial importance across diverse domains, including biochemical, medicinal, and environmental biotechnology (Alexander, 1961).

Screening of nickel tolerance ability of isolated bacterial colonies

Table 4 illustrates the nickel tolerance of bacterial isolates with increasing nickel concentration. From these results, the greatest nickel tolerance was found in the ASW-04, ASW-05, and ASW-06 strains. While ASW-07, ASW-08, and ASW-09 have the lowest nickel tolerance. At 100 ppm nickel concentration, the growth of 12 bacterial isolates such as ASW-01, ASW-02, ASW-03, ASW-04, ASW-05, ASW-06, ASW-07, ASW-08, ASW-09, ASW-10, ASW-11, and ASW-12 were observed to be 0.34, 0, 0.41, 0.88, 0.95, 0.38, 0, 0, 0, 0, 0.02, and 0.03 respectively. From the present study, it was observed that, out of 12 bacterial strains 7 bacterial strains including ASW-01, ASW-03, ASW-04, ASW-05, ASW-06, ASW-11, and ASW-12 exhibited nickel tolerance upto the 100 ppm nickel concentrations. These isolates were further characterized to applying as bio-adsorbents of nickel to remove nickel stress from plants which are grown in nickel contaminated soils.

The current results show collinearity with the findings of Virender et al., (2010) who isolated gram-negative Ni-resistant bacterial (NiRB) strains, and all of the isolated showed resistance with maximum tolerated concentrations (MTC) for nickel ranging from 1 to 24 mM. Similarly, Schmidt and Schlegel, (1994) isolated *Alcaligenes xylosoxidans* from a galvanization tank and found that it could tolerate an extremely high concentration of nickel up to 40 mM. Arundhati and Paul, (2010) discovered that *Cupriavidus* sp., a nickel-resistant bacterium, exhibited distinctive inducible nickel resistance ranged from 1.0 to 10 mM. The most well-known mechanisms of nickel resistance are mediated by efflux pumps, including CznABC (Cadmium-Zinc-Nickel) from *Helicobacter pylori*, NccCBA (Nickel-Cobalt-cadmium), and CnrCBA (Cobalt-Nickel resistance) from *Cupriavidus metallidurans* (Salvador et al., 2007).

Table: 4. Nickel tolerance ability of bacterial isolates with increasing nickel concentrations.

S. No.	Bacterial Isolate	OD at 660 nm					
		$NiSO_4$ concentration (ppm)					
		0 (Control)	10	25	50	75	100
1	**ASW-01**	1.75	1.71	1.64	1.21	0.71	0.34
2	**ASW-02**	1.23	1.21	1.07	0.73	0.32	-
3	**ASW-03**	1.74	1.75	1.73	1.54	0.84	0.41
4	**ASW-04**	1.86	1.88	1.74	1.67	1.02	0.88
5	**ASW-05**	1.87	1.86	1.72	1.48	1.27	0.95

6	**ASW-06**	1.96	1.91	1.52	1.14	0.79	0.38
7	**ASW-07**	0.85	0.77	0.52	0.31	0.09	-
8	**ASW-08**	0.67	0.56	0.48	0.22	-	-
9	**ASW-09**	0.79	0.63	0.45	0.19	-	-
10	**ASW-10**	1.21	1.07	0.72	0.48	0.18	-
11	**ASW-11**	1.77	1.56	1.09	0.53	0.24	0.02
12	**ASW-12**	1.67	1.48	0.92	0.56	0.29	0.03

Plant growth promoting ability of bacterial isolates

The results of plant growth promoting activities have been shown in Table 5. From these results, it was observed that the isolate ASW-04 showed highest ammonia production (4200±79 µg/ml) whereas, the lowest ammonia production was exhibited by the isolate ASW-08 and the ammonia production among all the bacterial isolates ranges between 426±43 to 4200±43 µg/mL. Furthermore, the isolate ASW-04 showed highest IAA (522±25 µg/ml), and GA (167±14 µg/ml) production whereas, the lowest IAA and GA production was exhibited by the isolates ASW-08 (44±9 µg/ml) and ASW-06 (64±12 µg/ml). These results indicated that the bacterial isolate ASW-04 exhibit better Ni tolerance and plant growth promoting activities.

Several researchers have documented the plant growth-promoting capabilities of nitrogen-fixing PGPR species, including *Azospirillum amazonense* (Magalhaes et al., 1983), *Azospirillum brasilense* (Tarrand et al., 1978), *Azospirillum halopraeference* (Reinhold et al., 1987), and *Azospirillum irakense* (Khammas et al., 1989). Okon and Labandera-Gonzalez, (1994) discovered diazotrophic nitrogen-fixing bacteria, including *Acetobacter diazotrophicus*, *Herbaspirillus seropedieae*, and *Azoarcus* sp., which convert nitrogen into ammonia through aerobic processes.

Indole acetic acid is an auxin that significantly influences root development by promoting cellular division. Certain microbes synthesize IAA, which enhances root development and elongation, leading to an increased root surface area that facilitates more nutrient absorption from the soil (Boiero et al., 2007). The present study is corroborated by previous reports from several researchers who found multiple soil bacterial species that synthesize IAA. For instance, Rajput et al., (2013) discovered that the soil bacterium *Planococcus rifietoensis* enhances the development of *Triticum aestivum* by synthesizing IAA. Similarly, Siddikee et al., (2010) indicated that many bacterial species, including *Oceanobacillus*, *Halomonas*, *Exiguobacterium*, *Zhihengliuella*, and *Bacillus* demonstrate plant growth-promoting capabilities through the production of IAA.

Gibberellins in plants participate in various developmental processes, including the termination of flower dormancy, fruit senescence, seed dormancy, stem elongation, and sex expression (Kang et al., 2014). Mansour et al., (2014) revealed that various bacterial species, including *Acetobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, and *Pseudomonas*, generate gibberellins in substantial numbers. Piccoli et al., (1997) noted that plant growth-promoting rhizobacteria (PGPR) augment the development and yield of many agricultural plants through the production of gibberellins. The current results indicated that the bacterial isolate ASW-04 exhibit better plant growth promoting activities such as liberation ammonia, IAA, and GA production.

Table: 5. Plant growth promoting ability of bacterial isolates

S. No	Bacterial Isolate	Ammonia (µg/ml)	Indole Acetic Acid (µg/ml)	Gibberellic acid (µg/ml)
1	ASW-01	2200±57	210±18	127±8
2	ASW-02	980±24	368±16	154±15
3	ASW-03	3456±56	498±22	99±11
4	ASW-04	4200±79	522±25	167±14
5	ASW-05	675±42	211±32	78±16
6	ASW-06	524±28	108±16	64±12
7	ASW-07	475±32	92±11	84±9
8	ASW-08	426±43	44±9	69±6
9	ASW-09	672±54	57±8	72±8
10	ASW-10	544±18	222±14	145±12
11	ASW-11	3271±92	208±16	150±14
12	ASW-12	3568±87	314±21	79±6

Molecular identification of Ni-tolerant bacterial Isolate ASW-04

Quality assessment of genomic DNA and PCR amplified 16s rRNA

Genomic DNA was effectively isolated from the bacterial isolate ASW-04, with a total DNA content quantified as 654±14 µg/g. The absorbance ratio of isolated DNA at A260/280 is 1.85. The extracted genomic DNA emerged as a conspicuous band on a 1% agarose gel. Figure 3 illustrates the genomic DNA band on a 1% agarose gel. The primers utilized to amplify the 16s rRNA of bacterial isolate ASW-04 provide distinct single bands of high quality. Figure 4 illustrates the PCR-amplified 16S rRNA gene band on a 1.5% agarose gel. In a 1.5% agarose gel, the very first well contains marker DNA, and the subsequent well has an amplified gene product approximately 1500 base pairs in length. The amplified 16s rRNA gene from ASW-04 bacterial isolate was subjected to Sanger's di-deoxy sequencing, and the sequence was deposited to NCBI database with an accession number of QB005887.

Figure 3. Photograph showing ASW-04 genomic DNA in 1% agarose gel.

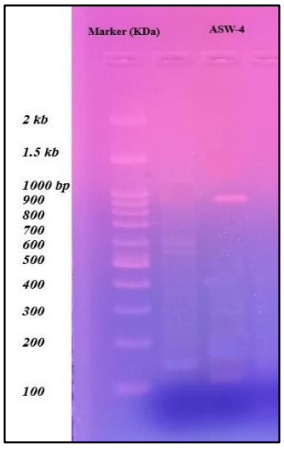

Figure: 4. Photograph showing the PCR amplified 16S rRNA gene band in 1.5% agarose gel.

Microbiology seeks rapid progress by identifying and characterizing microbial species to establish a precise phylogenetic relationship. The 16s rRNA gene serve as a crucial evolutionary marker, enabling the assessment of the three domains of life due to its functional stability and significant conservation (Woese et al., 1990). Sabat et al., (2017) indicated that the 16s rRNA is a highly conserved gene, approximately 1.5 kilobase pairs in length, which serves as a principal focus for phylogenetic study due to its ubiquitous presence in all bacteria, either as a single copy or in multiple copies. Woo et al., (2008) indicated that the

identification and evolutionary relationships of uncultivable bacteria can be elucidated by 16S rRNA sequencing. Tazi et al., (2014) indicated that direct sequencing of 16S rRNA genes from environmental samples has emerged as a prevalent and efficient method for analyzing microbial community prevalence, organization, and function. The present work utilized the 16S rRNA gene, which significantly impacts phylogenetic analyses by providing reliable classification of taxa into groups and genera.

Sequence analysis

The nucleotide composition of 16S rRNA gene was calculated by Biologics International Crop server. The sequence of PCR amplified Ni-tolerant bacterial isolate ASW-04 16S rRNA gene have 814 nucleotides. The sequence of 16S rRNA gene have 221 bases of Adenine(A), 171 bases of Thymine (T), 245 bases of Guanine (G) and 177 bases of Cytosine (C). The % of GC was calculated as 51.84. The results were showed in Table: 6.

The percentage of GC present in any organism is a highly variable characteristic. Lynch, (2007) states that the GC concentration in bacterial species can vary from 25% to 75%. The current results indicate that the 16S rRNA from the bacterial strain ASW-04 exhibits a comparably high GC content, consistent with other investigations. Hildebrand et al., (2010) revealed significant variability in GC content among bacterial species, indicating that the GC content in distinct bacterial species ranges from 13% to over 75%. The findings of Zhang and Gao, (2017) indicate that linear regression analyses reveal that the proportion of amino acids encoded by GC-rich codons positively influences evolutionary rates, while amino acids are preferentially acquired in GC-rich species. Foerstner et al., (2005) showed that microbial communities cohabiting the same habitats tend to exhibit similar %GC regardless of taxonomy.

Table: 6. Nucleotide composition of ASW-04 16S rRNA gene

S. No	Parameter	Number of nucleotides	% of nucleotides
1	Total bases	814	-
2	Adenine	221	27
3	Thymine	171	21
4	Guanine	245	30
5	Cytosine	177	22
6	G+C	422	51.84

Multiple sequence alignment

Through a BLASTn search of the GenBank database, the 814 bp 16S rRNA sequence of ASW-04 was shown to exhibit homology to several *Bacillus* and *Metapseudomonas* species. The BLASTn results indicated that the 16S rRNA gene sequence of bacterial isolate ASW-04 exhibited greater (94.65 %) identity to strain *Metapseudomonas otitidis* strain CEMTC_1357, with an E-value of 0.0. A total of 11 sequences, belonging to *Metapseudomonas and Bacillus* genera, were selected from the NCBI database for multiple sequence alignment (Table 7). The MSA of ASW-04 with the selected species indicates the phylogenetic parameters for the 16s rRNA as follows: conserved sites 1335, variable sites 162, parsimony sites 19, and singleton sites 142, with an overall mean distance of 0.02 (Table 3). The evolutionary difference between ASW-04 and related sequences at the species level ranges from 0.0798 to 0.0986 (Table 9). Bacterial isolate ASW-04 has the greatest evolutionary difference from PX125560.1 *Pseudomonas guezennei* strain AS-14 (0.0986) and the least divergence from *Bacillus subtilis* strain DJK73 (0.0798). The multiple sequence alignment of the 16s rRNA gene sequences demonstrated that the gene sequence is substantially conserved among prokaryotes. Notredame, (2007) asserts that multiple sequence alignments provide greater

insights than pairwise alignments, as they uncover structurally and functionally significant regions within a family.

Table: 7. List of selected species sequences used for phylogenetic study.

Species	Max Score	Total Score	Query Cover	E value	Per. Ident	Len (AA)
PZ293845.1 *Metapseudomonas otitidis* strain CEMTC_1357	1186	1186	94%	0.0	94.65%	1063
LC875691.1 *Metapseudomonas otitidis* 30-39	1186	1186	94%	0.0	94.65%	1483
KU551193.1 *Metapseudomonas otitidis strain* M61	1186	1186	94%	0.0	94.65%	1497
AB698739.1 *Pseudomonas otitidis* strain 81f	1186	1186	94%	0.0	94.65%	1497
HE612877.1 *Bacillus subtilis*	1186	1186	94%	0.0	94.65%	1330
KX016579.1 *Metapseudomonas otitidis* strain SN5	1184	1184	94%	0.0	94.64%	1070
KC113129.1 *Bacillus badius* strain KS11261	1184	1184	94%	0.0	94.64%	1391
OR780603.1 *Pseudomonas otitidis* strain SM41	1182	1182	94%	0.0	94.52%	1337
LN558646.1 *Pseudomonas otitidis* strain RS4	1182	1182	94%	0.0	94.63%	1418
KX016596.1 *Bacillus subtilis* strain DJK73	1182	1182	94%	0.0	94.63%	998
PX125560.1 *Pseudomonas guezennei* strain AS-14	1181	1181	94%	0.0	94.52%	981

Table: 8. Phylogenetic parameters of ASW-04 with its homologous species

S. No.	Parameter	No. of Sites	% of sites
1	Conserved sites	1335/1502	88.88
2	Variable sites	162/1502	10.79
3	Parsimony sites	19/1502	1.27
4	Singleton sites	142/1502	9.45
5	Overall mean distance	0.02	-

Table: 9. Estimates of evolutionary distances for collected PGPR isolate ASW-04 with its homologous species

Species Accession	PGPR ASW-04	PZ293845.1	LC875691.1	KU551193.1	AB698739.1	HE612877.1	KX016579.1	KC113129.1	OR780603.1	LN558646.1	KX016596.1	PX125560.1	PGPR ASW-04
PGPR ASW-04													
PZ293845.1	0.095477												
LC875691.1	0.096770	0.000000											
KU551193.1	0.096770	0.000000	0.000675										
AB698739.1	0.096770	0.000000	0.000000	0.001337									
HE612877.1	0.080	0.0009	0.0007	0.0015	0.0007								

	920	56	52	05	52								
KX016579.1	0.081243	0.000959	0.000936	0.000936	0.000936	0.001872							
KC113129.1	0.098654	0.013403	0.028264	0.029018	0.028264	0.000753	0.001872						
OR780603.1	0.096902	0.000000	0.011658	0.016291	0.015509	0.011966	0.000944	0.023144					
LN558646.1	0.092771	0.005702	0.043027	0.043027	0.043027	0.021691	0.000936	0.046559	0.027394				
KX016596.1	0.079826	0.001002	0.001002	0.001002	0.001002	0.000000	0.001002	0.000000	0.001010	0.001002			
PX125560.1	0.098691	0.007210	0.008243	0.008243	0.008243	0.002083	0.003135	0.014568	0.008294	0.011403	0.002089		

Phylogenetic tree analysis

The findings indicated that ASW-04 exhibited the closest clustering with strains belonging to the genus *Metapseudomonas*, including *Metapseudomonas otitidis* strain CEMTC 1357 (PZ293845.1), *Metapseudomonas otitidis* 30-39 (LC875691.1), and *Metapseudomonas otitidis* strain M61 (KU551193.1). The phylogenetic distances, evidenced by minimal genetic divergence values (ranging from 0.0000 to 0.0013 across various *Metapseudomonas* and *Pseudomonas* strains), reveal a significant level of similarity and imply that ASW-04 is affiliated with or closely associated with this group. Conversely, significant distances were noted with *Bacillus subtilis* (HE612877.1, KX016596.1) and *Bacillus badius* strain KSI1261 (KC113129.1), exhibiting divergence values between 0.0118 and 0.0335, so affirming that ASW-04 is phylogenetically separate from these *Bacillus* species. The examination also encompassed certain anomalies, like *Pseudomonas guezennei* strain AS-14 (PX125560.1), which exhibited moderate deviation from ASW-04. The phylogenetic tree robustly endorses the positioning of PGPRI isolate ASW-04 inside or near the *Metapseudomonas otitidis* clade, highlighting its close evolutionary ties to other *Pseudomonas* and *Metapseudomonas* strains while distinctly separating it from *Bacillus* species. These results establish a solid molecular foundation for the precise categorization of ASW-04 and shed light on its evolutionary history within plant growth-promoting rhizobacteria. The maximum-parsimony phylogenetic tree was showed in Figure 5.

Analysis of DNA sequences across many species yields significant insights into their taxonomy, genetic composition, and applications. DeGroot et al., (2011) indicated that generic level identification was considered effective when a single genus was present in all instances, with maximum percent identification scores above 95%. Species identification was considered effective only when a single species had a percentage identity score exceeding 95%. The phylogenetic tree organizes clades primarily through the amalgamation of several species and strains. Consequently, the creation of a local barcode database is essential for several ecological applications, including the development of community phylogenetics (Kress et al., 2009).

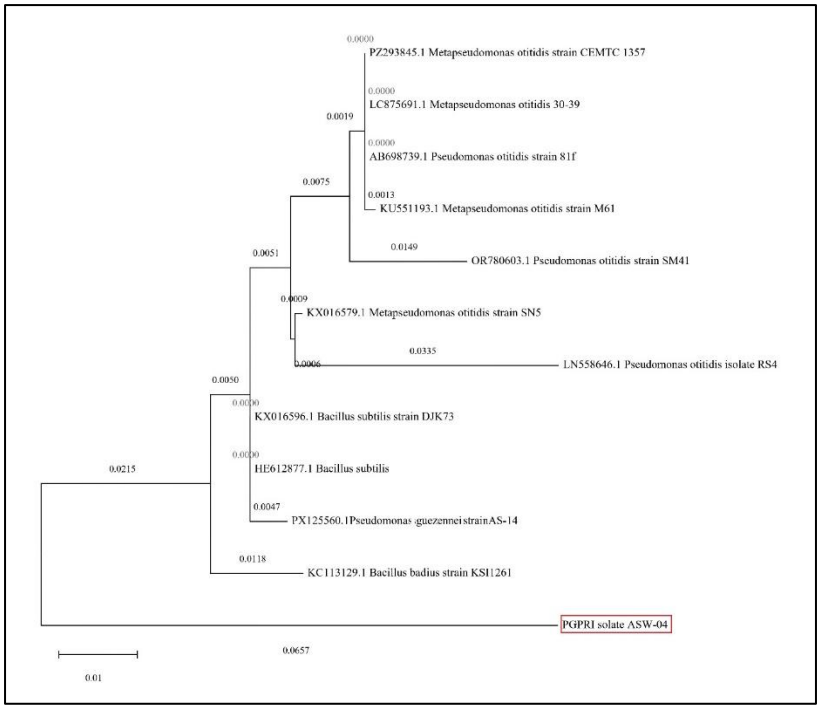

Figure: 5. Maximum Parsimony tree of nickel tolerant bacterial isolate ASW-04 and other relative species based on the 16S rRNA gene.

Conclusion

This research demonstrates the effective extraction and analysis of nickel-resistant plant growth-promoting rhizobacteria from soils contaminated by industrial activities in Andhra Pradesh, India. Among the twelve isolates, strain ASW-04 demonstrated exceptional nickel tolerance and characteristics that promote plant growth, such as elevated production of ammonia, indole acetic acid, and gibberellic acid. Molecular characterization indicated its strong relationship with *Metapseudomonas otitidis*, underscoring its promise as a bioinoculant for bioremediation. The results underscore the importance of native microbial resources in creating sustainable and environmentally acceptable methods for heavy metal cleanup and enhancing agricultural yield in polluted settings. Employing these bacterial strains can mitigate the environmental and health hazards linked to nickel contamination while promoting agricultural sustainability. Subsequent investigations ought to concentrate on field experiments and the formulation of microbial consortia to enhance remediation efficacy, thus promoting the practical utilization of Ni-tolerant PGPR in sustainability and food safety.

Conflict of Interest

All the authors declare that they have no conflict of interest

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