Heliyon 7 (2021) e08284

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Monitoring and molecular characterization of bacterial species in heavy metals contaminated roadside soil of selected region along NH 8A, Gujarat

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ARTICLE INFO

Keywords:
Microbial diversity
Heavy metal pollution
16S-rRNA sequencing
Phylogenetic tree

ABSTRACT

Heavy metal contamination is a universal concern due to health risks associated with metal pollution. Soil contamination by heavy metals is known to affect microbial activities at elevated concentrations adversely. However, indigenous soil bacterial populations' response to added heavy metal and metal combinations is poorly understood. Microbes prevailing in the soil are the driving factors. Their properties are recognized as sensitive indicators of soil quality and health. Moreover, these microscopic organisms are accountable for the fertility and aeration of the soil that forms fundamental aspects of soil function. The current study was performed to explore the diversity of bacterial species in heavy metal polluted roadside soil. The roadside soil samples were collected from diverse sites and processed for physicochemical properties, microbial characterization, and heavy metals distribution in the selected locations. Serial dilution and spread plate techniques were used for the isolation of bacterial species. The 16S-rRNA gene sequencing identified bacterial species in roadside soil as *Bacillus drentensis* (MK217088), *Bacillus safensis* (MK774729), *Bacillus haynesii* (MK192808), *Bacillus subtilis* (MK217089), and *Bacillus cereus* (MK801278). In addition, the 16S rRNA sequences of isolated bacterial strains were aligned to generate a phylogenetic tree. Thus, the current research study provides a platform for efficiently investigating roadside soils by microbial profiling that may discover novel microbes of scientific significance and improved potential.

1. Introduction

Soil is an intricate and dynamic biogeochemical framework involving many thousands of bacterial species. The roadside soil acts as a receiver or sink of many pollutants, including heavy metals, hydrocarbons, etc., generated due to vehicular operations. Often, in the roadside soil, a high level of contaminants attributed to motor vehicles is observed (Joshi et al., 2010). Heavy metal pollution in the soil has been specified as one of the most significant unadorned harms (Kumar and Fulekar, 2021). Heavy metal contamination in soil mainly refers to the heavy metal deposition causing elevated concentrations exceeding the background values into the soil (Jin et al., 2018). The accumulation of heavy metals content in the roadside soil is of enormous environmental apprehension as it can cause alteration in the biological activities occurring in the soil system (Singh and Yadav, 2019). The diversity and activity of soil bacterial populations are diminished as a result of the environmental stress instigated by heavy metals, prompting a reduction in the total microbial biomass, a decline in the number of the explicit populace, and a shift in microbial structure (Abd et al., 2013; Wang et al., 2010). Anthropogenic activities, including automobile emissions and the industrial process, cause the raised load of heavy metals in the environment, thus creating pollution (Singh and Yadav, 2019). As metals persist in the ecosystem and cannot be naturally degraded, it accumulates in different food chain levels (Kumar and Fulekar, 2019; Singh et al., 2019). Unlike organic pollutants, it has engendered a grave problem for the safe utilization of soils (Igwe et al., 2005; Tanu and Hoque, 2013).

Elevated levels of heavy metals are widely known to have a qualitative and quantitative influence on the configuration of soil microbial populations and their allied activities, which may directly influence soil fertility (Kumar and Fulekar, 2018). More significant adverse consequences on the activities and diversity of soil microbial biomass can be seen in the presence of various metals together compared to those caused by single metals at high concentrations (Renella et al., 2005).

Few microbes dwell in habitats contaminated with a high level of metals than the unadulterated habitats (Florea and Büsselberg, 2006). The restraint of metabolic procedures and the interaction of metals with proteins (enzymes) might cause the destructive impacts of heavy metals (Gandhi et al. 2015). Most of the heavy metals are lethal to cell growth. In

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https://doi.org/10.1016/j.heliyon.2021.e08284
Received 6 July 2021; Received in revised form 4 October 2021; Accepted 26 October 2021

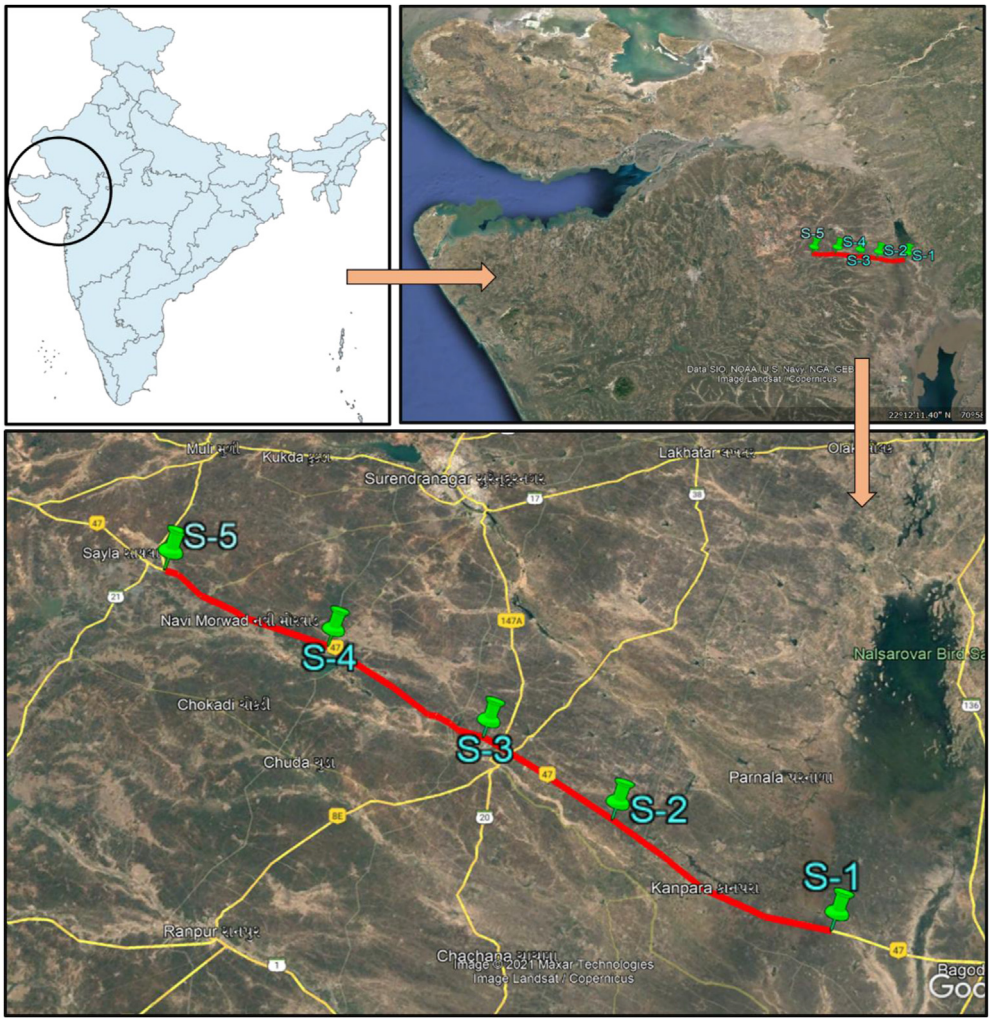

Figure 1. Location map of the sampling sites (NH-8A), Gujarat, India.

Table 1. Geographical coordinates of the sampling locations.

Sampling Sites	Geographical Coordinates	
	Latitude	Longitude
S-1	22°35′48.52″N	72° 5′19.09″E
S-2	22°34′29.98″N	71°54′34.11″E
S-3	22°34′18.15″N	71°47′29.25″E
S-4	22°33′46.38″N	71°38′49.64″E
S-5	22°32′25.82″N	71°29′58.14″E

Table 2. Physicochemical characterization of the roadside soil samples.

Parameters	Site 1	Site 2	Site 3	Site 4	Site 5	Mean ± SD
pH	7.09	7.17	7.47	7.02	7.06	7.16 ± 0.18
EC (μs/cm)	69	255	123.5	451	215	222.70 ± 147.21
Soil moisture (%)	3.25	4.25	5.25	6.25	7.25	5.25 ± 1.58
Bulk density (mg/m^3)	1.05	0.80	1.05	1.43	1.11	1.09 ± 0.23
Organic Carbon (%)	1.68	5.1	3.6	1.32	3.3	3.00 ± 1.53
Organic Matter (%)	2.93	8.88	6.27	2.29	5.74	5.22 ± 2.64

contrast, some microbes can tolerate and accumulate heavy metals through various metabolic activities (Braud et al., 2006). Furthermore, many microbial species, including bacteria present in the soil, has the proficiency to resist heavy metals. Thus, heavy metal resistant bacteria have an influential part in the biogeochemical processes of metal ions (Kumar et al., 2011; Issazadeh et al., 2013).

Therefore, it is worth mentioning that many heavy metals are toxic at higher concentrations, even though they are essential trace elements. However, some microbial species have reformed to put up with the existence of metals. The relationship between the microbial species and the heavy metals has a vital application in bioremediation procedures. Therefore, the bioremediation of metal ions using various microorganisms has drawn significant attention in recent years (Gandhi et al. 2015). The soil microbial population's responses in connection to heavy metal pollution give a pertinent model for ecological studies to evaluate the impact on environmental qualities (Guo et al., 2009). *Acinetobacter, Azospirillum, Achromobacter, Alcaligenes, Bacillus, Psychrobacter, Pseudomonas,* etc. are the highest heavy metal tolerant bacterial species prevailing in soil (Gray and Smith, 2005). Knowing bacteria's biological, genotypic, and phenotypic characteristics is obligatory in distinguishing them from other microorganisms. Several research studies have reported heavy metal-resistant bacterial species isolated from an extensive range of habitats (Adu et al., 2012; Ezaka and Anyanwu, 2011; Mgbemena et al., 2012; Murthy et al., 2014; Shi et al., 2002; Rajaganapathy et al., 2011). On the other hand, there is no existing data on heavy metal resilient bacterial species from roadside soil, Gujarat, India. Therefore,

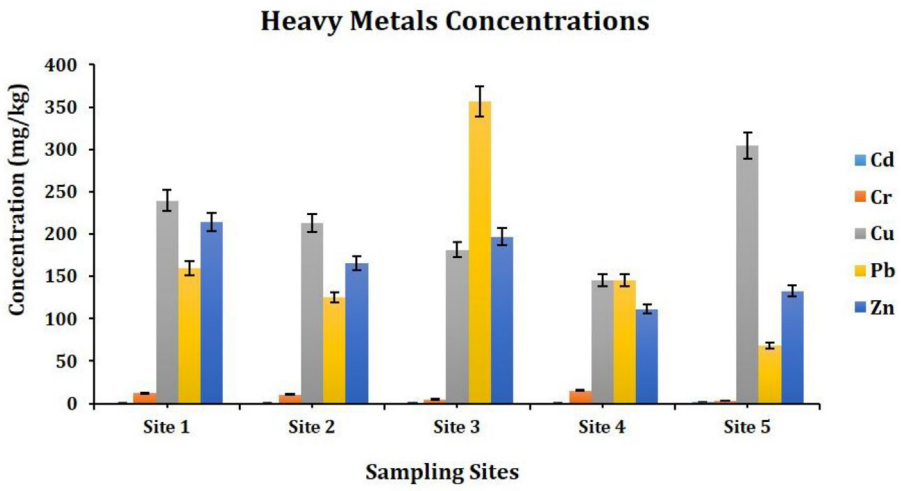

Figure 2. Average concentrations (mg/kg) of different heavy metals.

Table 3. Pearson's correlation coefficient (r) among heavy metals.

	Cd	Cr	Cu	Pb	Zn
Cd	1				
Cr	0.99*	1			
Cu	0.74	0.71	1		
Pb	0.94*	0.93*	0.91*	1	
Zn	0.86	0.91*	0.41	0.74	1

Table 4. Morphology of the isolated bacterial strains from the roadside soil.

Strain	Bacteria Name	Morphological Characteristics	Accession Number
SN 1	*Bacillus drentensis*	Gram-positive, spore-forming, cream colour, pleomorphic-shaped, circular, medium, filamentous, motile	MK217088
SN 2	*Bacillus safensis*	Gram-positive, spore-forming, rod-shaped, white colour, round, undulate, opaque	MK774729
SN 3	*Bacillus haynesii*	Gram-positive, facultative anaerobe, motile, and endospore-forming rod-shaped, colonies appear as creamy white, mucoid, translucent, raised, and highly moistened	MK192808
SN 5	*Bacillus subtilis*	Gram-positive and rod-shaped, circular, small, rough, opaque, fuzzy, slightly yellow with jagged edges	MK217089
SN 12	*Bacillus cereus*	Gram-positive, rod-shaped, flat, opaque, slimy, facultative anaerobe, motile, beta-hemolytic, spore-forming, irregular,	MK801278

this research study was intended to sequester and identify heavy metal resilient bacterial species from roadside soil samples along with NH-8A Gujarat.

2. Materials and methods

2.1. Description of the study area

Gujarat has a diverse climate, as it is dry in the northern districts and moist in the southern region. Temperature ranges between 12 °C to 27 °C in winters. In contrast, it varies between 25 °C to 43 °C in summer and has been known to reach as high as 48 °C. Gujarat has an average rainfall of 33–152 cm. Sampling sites were chosen between Ahmedabad to Surendranagar along NH-8A at roughly 20 km each for the sample collection (Figure 1). Soil samples in triplicates were collected from each sampling point to get homogenous samples. A total of 15 soil samples were collected. Each soil sample was carefully obtained from a composite of five sub-samples (0–10 cm) with a stainless steel scoop. The geographical coordinates of the sampling locations have been recorded with the help of GPS and are listed in Table 1.

2.2. Soil sampling and analytical procedure

Soil samples were collected in triplicates at a depth of 0–15 cm from selected sampling locations. The soil samples were kept in zip lock bags with proper labelling, and further analysis was performed. The soil samples were preserved at ambient temperature (4 °C) for microbial characterization to avoid contamination. Numerous physicochemical parameters such as pH, electrical conductivity (EC), soil moisture (SM), bulk density, organic matter, and organic carbon analysis were executed as per the standard methods for the collected soil samples. pH and EC were evaluated (1:2.5 w/v) by a digital pH meter and conductivity meter (Woermann, 1973). Soil moisture content was also determined as per the standard methods (Oven drying method). Finally, the organic carbon content in the soil samples was analyzed using standard procedures by Walkley and Black (1934).

2.3. Heavy metal analysis in roadside soil samples

The soil samples were air-dried to eliminate the moisture content at room temperature and further crushed using mortar-pestle to strained

Table 5. CFU count (cfu/g) of bacterial isolates from soil samples of various sampling sites.

Sampling Sites	*Bacillus drentensis*	*Bacillus safensis*	*Bacillus haynesii*	*Bacillus subtilis*	*Bacillus cereus*	Mean cfu/g
Site 1	6.9×10^6	5.1×10^6	6.9×10^6	6.9×10^6	7.3×10^6	6.62×10^6 cfu/g
Site 2	7.1×10^6	6.1×10^6	5.7×10^6	6.8×10^6	6.7×10^6	6.48×10^6 cfu/g
Site 3	6.8×10^6	6.4×10^6	6.1×10^6	6.1×10^6	7.5×10^6	6.58×10^6 cfu/g
Site 4	6.5×10^6	5.9×10^6	6.8×10^6	5.1×10^6	7.1×10^6	6.28×10^6 cfu/g
Site 5	6.4×10^6	5.4×10^6	5.7×10^6	5.8×10^6	6.9×10^6	6.04×10^6 cfu/g

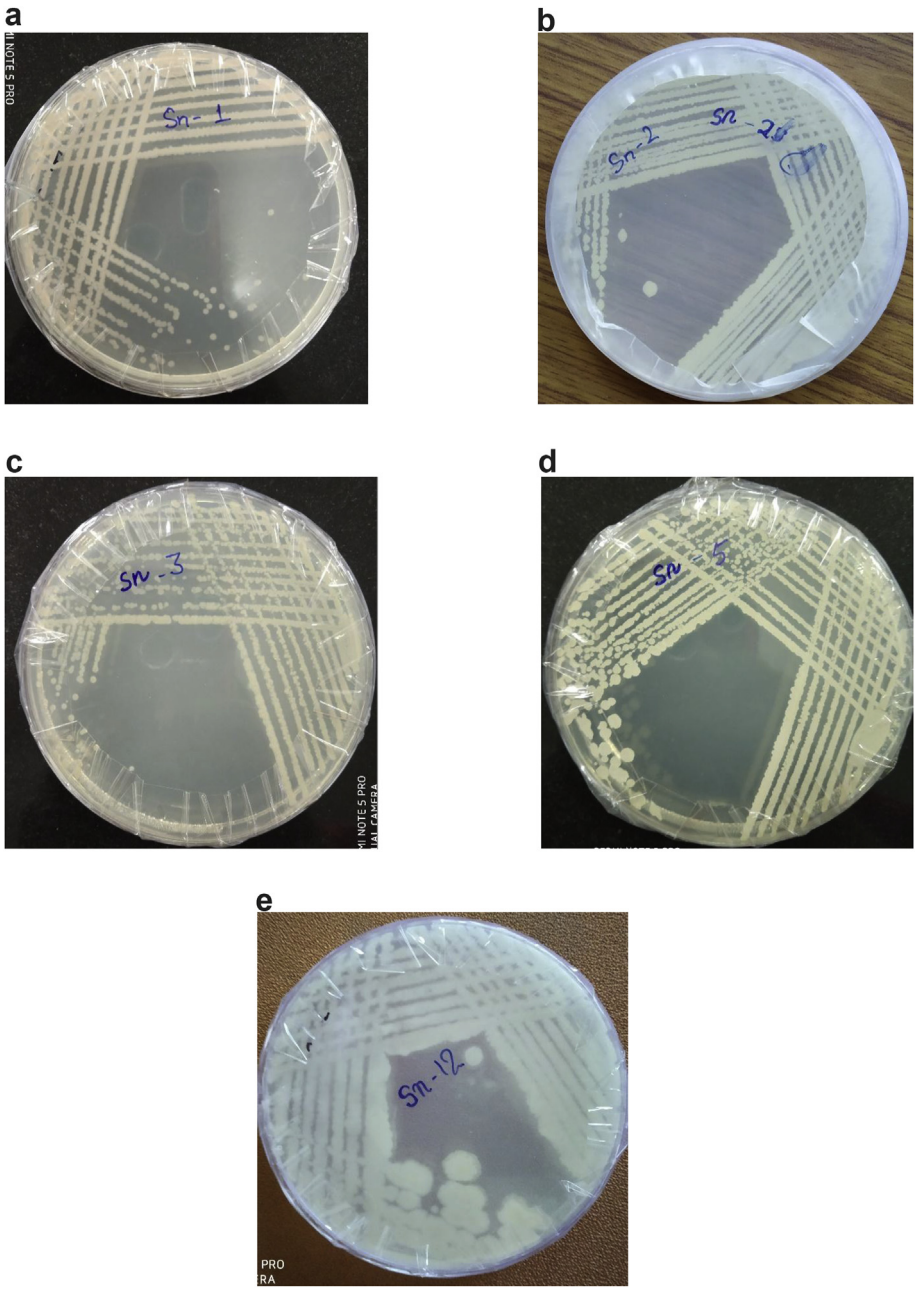

Figure 3. a: *Bacillus drentensis*. b: *Bacillus safensis*. c: *Bacillus haynesii*. d: *Bacillus subtilis*. e: *Bacillus cereus*.

using a 2 mm mesh sieve. Approximately 0.1 gm of the soil samples were weighed and transferred in acid-washed beakers. Next, a 20 mL freshly prepared mixture of HCl and HNO_3 in the ratio of 3:1 (aqua regia) is added into the samples and then digested as per the standard procedure. The mixture was heated at 120 °C for 4 h on a hot plate. After the solution was cool, it was filtered using Whatman filter paper 42 and diluted to 20 ml with distilled water. The contents of Cu, Pb, Cr, Zn, and Cd in the soil samples were investigated by Inductively coupled plasma - optical emission spectrometry (ICP-OES).

2.4. Isolation and screening of bacteria

The serial dilution technique was used for the isolation and screening of bacterial isolates. 1 mg of the soil samples were dissolved adequately in 9 ml sterile water and diluted in the order of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, to 10^{-6}. From these dilutions, 0.1 ml of the solution was used to spread on the prepared plates. From the bacterial colonies that appeared on the agar plates, distinct bacterial isolates were streaked to obtain pure cultures based on the morphology. Inoculated petri dishes were incubated at 37 °C for 24 h. The isolated bacterial species were cultured by repeated streaking and were kept in slants at 4 °C.

2.5. Morphological and molecular identification of isolated bacterial strains

Nutrient agar media was used to grow and isolate the bacterial strains from the soil samples. The morphology of the strains was observed with the help of the microscope after gram staining. Different characteristics such as color, shape, form, texture, and elevation were observed for the morphological identifications. The partial 16S rRNA sequences of all the

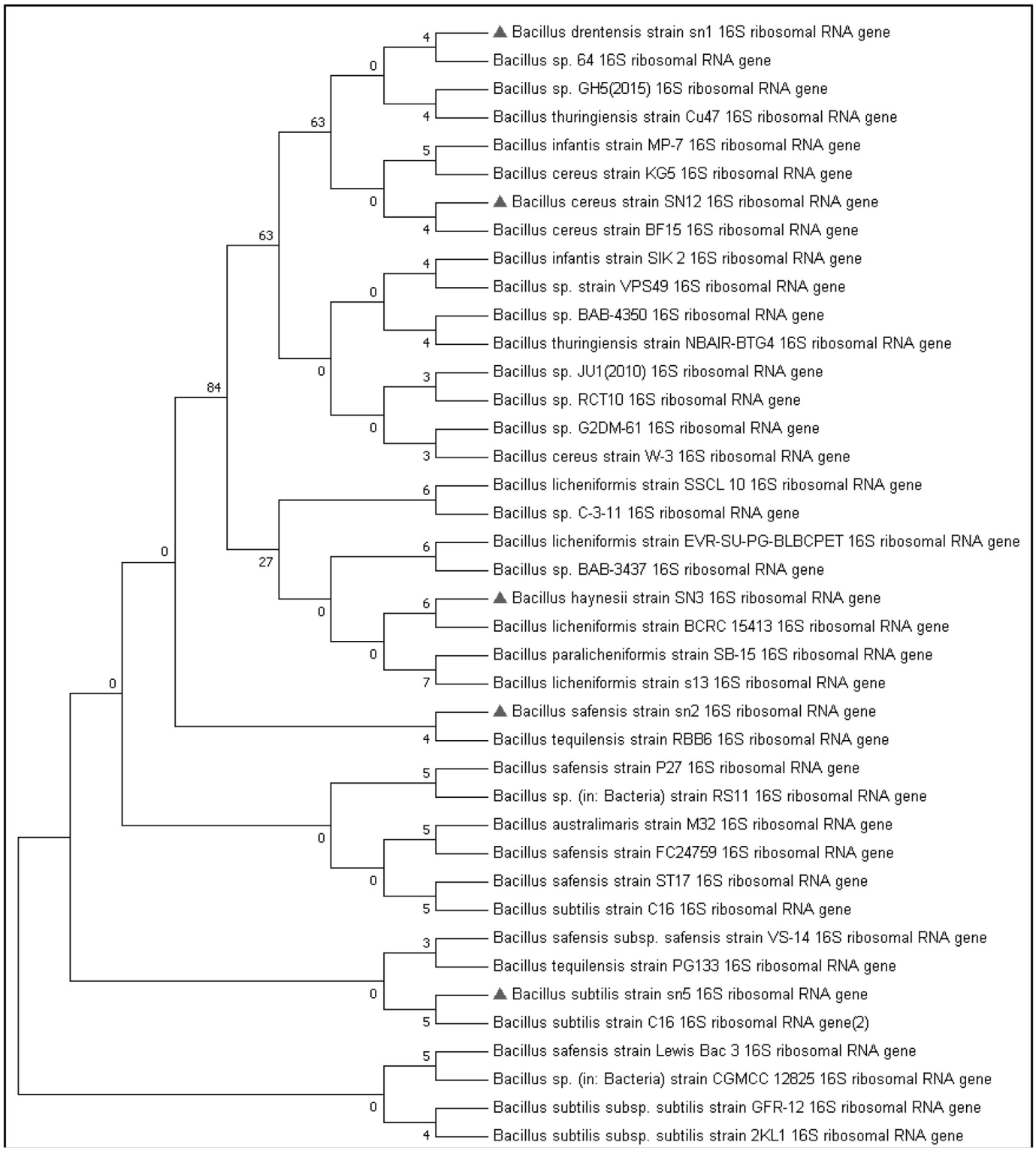

Figure 4. Neighbor-joining phylogenetic tree constructed using 16 S rRNA gene sequences.

bacterial isolates were submitted in the National Center for Biotechnology Information (NCBI) under the accession numbers MK217088, MK774729, MK192808, MK217089, and MK801278, respectively. The 16S rRNA gene sequences of isolated bacterial strains were obtained and allocated accession numbers by Gujarat State Biotechnology Mission, Gandhinagar, Gujarat, India. The isolated bacterial isolates were further sequenced by BLAST using NCBI BLAST tools.

2.6. Construction of phylogenetic tree

A phylogenetic tree was created using 16S rRNA sequences acquired from isolated bacterial strains in FASTA format. The sequences closely related to those with the bacterial species isolated in the current study were attained from the NCBI and aligned using Clustal W. The bootstrap consensus reliability was inferred from 1000 replicates using the

Table 6. 16S rRNA sequencing of different bacteria.

Bacterial Strains	16S rRNA Sequencing
Bacillus drentensis Strain SN-1 MK217088	AACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCG CCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTCCTGACAACCCTAGAGA TAGGGCGTTCCCCTTCGGGGGACAGGATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTAGGTTGGGCACTCTA AGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAGGTTAAGCGAATCCCAT AAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT GTAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA
Bacillus safensis Strain SN-2 MK774729	AGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGAAGAGAGCTTGCTCCCGGAT GTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTT ACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA GGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAACCA GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG GTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGA AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAA GACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGAC AGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGG AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCAAGACCGCAAGGTTTAGCCAATCCCATAAATCTGTTCTCAGTTCGGATCGCAG TCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAGTCGGTGAGGTAAC CTTTATGGAGCCAGCCGCCGAAGGTGGGGCAGATGATTGGGGTGAA
Bacillus haynesii Strain SN-3 MK192808	GACGACGCTGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTA GCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTC AATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCG AATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCT GATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACT CTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATT AAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAAC CCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCA CTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATC CCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG TTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGT
Bacillus subtilis Strain SN-5 MK217089	CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAA ACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATGCGTA GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG ATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA ATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT GTTAGGGGGTTTCCGCCCC
Bacillus cereus Strain SN-12 MK801278	GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAA ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGAC AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGT GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAA CTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTTG GAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTACAA

Table 7. The similarity of the isolates strains based on partial 16S rRNA sequencing.

Strain	Bacterial strain showing maximum homology	Identity (%)	GenBank Accession Numbers
SN-1	*Bacillus drentensis*	99.74	MK217088
SN-2	*Bacillus safensis*	99.93	MK774729
SN-3	*Bacillus haynesii*	99.86	MK192808
SN-5	*Bacillus subtilis*	100	MK217089
SN-12	*Bacillus cereus*	99.86	MK801278

Table 8. Average heavy metal concentrations and microbial population in soil along with roadside sampling sites.

Sampling sites	Metals (mg/kg)					TMC (Cfu/g)
	Cd	Cr	Cu	Pb	Zn	(Mean)
S-1	1.1	12.42	240.47	159.9	214.02	6.62×10^6 cfu/g
S-2	0.76	10.62	213.04	125.32	166.33	6.48×10^6 cfu/g
S-3	0.86	4.86	181.73	356.84	197.05	6.58×10^6 cfu/g
S-4	1.07	15.19	145.37	145.70	111.61	6.28×10^6 cfu/g
S-5	1.64	2.79	304.62	68.48	133.28	6.04×10^6 cfu/g

*TMC = total microbial count; metal concentration in average.

Table 9. Pearson's correlation coefficient (r) among heavy metals and total microbial count.

	Cd	Cr	Cu	Pb	Zn	TMC
Cd	1					
Cr	0.43	1				
Cu	0.68	0.50	1			
Pb	0.56	0.18	0.50	1		
Zn	0.41	0.11	0.23	0.53	1	
TMC	0.80	0.33	0.29	0.64	0.84	1

neighbor-joining distance method by MEGA 7 software (Kumar et al., 2016). From the Gene Bank, a few reference sequences of supreme closely associated strains to the Bacillus sp. were retrieved from NCBI and used to generate the phylogenetic tree.

2.7. Statistical analysis

The physicochemical and heavy metals characterization of the soil samples was performed in triplicates. The outcomes were presented as mean with standard deviations (SD). To determine the affiliation among the heavy metals, Pearson's correlation coefficient was analyzed. The correlation coefficient matrix is commonly used to measure the degree of correlation amongst the logarithms of the elemental concentrations (Kumar et al., 2017; Kumar and Fulekar. 2017).

3. Results

3.1. Physicochemical characterization of the soil samples

The soil samples were analyzed for physicochemical characteristics such as pH, electrical conductivity, soil moisture, bulk density, organic carbon, and organic matter (Table 2). The soil pH was on the alkaline side ranging from the lowest 7.02 at site 4 and the highest 7.47 at site 3. The EC varied from 69 to 451 μs/cm, with the lowest at site 1 and highest at site 4, respectively. Soil moisture and bulk density were recorded lowest 3.25 at site 1 and highest 7.25 at site 5, 0.80 at site 2, and 1.43 at site 4, respectively. Organic carbon and organic matter ranged between 1.68 to 5.1 and 2.28 to 8.79, respectively.

3.2. Heavy metal contents in soil samples

The heavy metal contents in the soil samples were observed more than the USEPA screening standards. The results indicated that the collected soil samples were immensely adulterated with the higher concentration of several heavy metals. The primary toxic metals in the contaminated soil samples were copper, lead, chromium, zinc, and cadmium. The average concentrations for Cu (304.62 mg/kg at site S-5 and lowest 145.37 mg/kg at S-4), Pb (highest 356.84 mg/kg at S-3 and lowest 68.48 mg/kg at S-5), Cr (highest 15.19 mg/kg at S-4 and lowest 2.79 mg/kg at S-5), Zn (highest at 214.02 mg/kg at S-1 and lowest at 111.61 mg/kg at S-4) were perceived. In contrast, Cd ranged from 1.64 mg/kg highest at S-5 to lowest 0.76 mg/kg at S-2 (Figure 2).

To analyze the correlation coefficient amid various heavy metals, a two-tailed test of the significance level of ($P < 0.05$) was used (Table 3). Significant positive correlations were witnessed amongst the pairs of certain heavy metals in the polluted soil samples. The correlation investigation discovered that chromium exhibited an extremely positive correlation with cadmium ($r = 0.99$). Lead and cadmium showed a positive correlation ($r = 0.94$), chromium ($r = 0.93$), and copper ($r = 0.91$). A highly positive correlation was observed between zinc and chromium ($r = 0.91$).

3.3. Morphological characteristics of bacterial isolates

Several morphological characteristics, such as shape, size, margin, consistency, pigmentation, surface, and optical characteristics, were perceived and listed in Table 4. The isolates were recognized as *Bacillus*

Table 10. Microbial strains isolated from different polluted soils.

Soil Type	Isolated Microorganisms	References
Coal Mine area	*Enterobacter ludwigii, Klebsiella pneumonia Enterobacter ludwigii, Enterobacter ludwigii Klebsiella oxytoca, Enterobacter cloacae Acinetobacter gyllenbergii, Enterobacter cloacae*	(Gandhi et al., 2015)
Industrial affected soil	*Proteus vulgaris, Bacillus cereus, Bacillus decolorationis, Pseudomonas fluorescence*	(Ahirwar et al., 2016)
Non-sanitary closed landfill	*Cloacibacterium sp, Acidovorax ebreus, Chryseobacterium gleum, Stenotrophomonas acidaminiphilia, Bacillus aryabhattai, Rhodococcus rubber, Brevundimonas diminuta, Bacillus Pumilus, Delftia tsuruhatensis, Bacillus kochii Aeromonas caviae, Ochrobacterium intermedium, Janibacter hoylei, Bacillus cereus Pseudomonas mendocina, Serratia marcescens marcescens, Burkholderia vietnamiensis, Pseudomonas alcaligenes*	(Fauziah et al., 2017)
Soil adjoining Automobile workshop	*Alcaligens faecalis, Entrobacter aerogens Pseudomonas putida, Bacillus licheniformis Citrobacter kosari, Bacillus cereus, Klebsiella pneumonia*	(Akhter et al., 2017)
Heavy Metals Polluted E-waste Dumping Sites	*Bacillus jeotgali, Kocuria turfanensis, Bacillus velezensis, Bacillus haikouensis, Micrococcus aloeverae, Bacillus licheniformis*	(Kumar et al., 2020)
Lead Contaminated Soil	*Achromobacter, Pseudomonas, Alcaligenes, Citrobacter, Corynebacerium, Staphylococcus, Klebsiella, Escherichia, Agrobacterium, Enterobacter, Diplococcus, Bacillus, Proteus,*	(Kazaure, 2018)
Heavy Metals polluted soil	*Bacillus lentus, Escherichia coli, Micrococcus roseus, Enterobacter aerogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus sp.*	(Kazaure, 2018)
Waste Engine Oil Polluted Soil	*Klebsiella pneumoniae, Plesiomonas shigelloide, Acinetobacter species, Bacillus licheniformis, Pseudomonas aeroginosa*	(Samuel et al., 2019)

drentensis, *bacillus safensis*, *Bacillus haynesii*, *Bacillus subtilis*, and *Bacillus cereus*. The 16S rRNA gene sequencing and phylogenetic analysis specified that isolated bacterial species specifically belonged to the *Bacillus* genera.

Bacillus drentensis is a facultative anaerobe, spore-forming, mesophilic bacterium that forms circular colonies and has a brown pigmentation (Figure 3a). *Bacillus safensis* is a gram-positive, endophytic bacterium found in hydrocarbons contaminated soil and produces biosurfactants (Wu et al., 2019). The elevated, irregular-edged colonies of *Bacillus safensis* were beige, opaque, and nearly spherical (Figure 3b). *Bacillus haynesii* is a gram-positive, thermophilic, motile, and endospore-forming rod-shaped facultative anaerobe. The *Bacillus haynesii* appeared as creamy white, 3–4 mm diameter, mucoid, translucent, raised, and highly moistened colonies (Figure 3c). *Bacillus subtilis* appears to be a rod-shaped, gram-positive, facultative aerobe. It is spore-forming, motile, frequently dwelling in soil and vegetation with an optimal growth temperature from 25-35 °C (Figure 3d). *Bacillus cereus* is observed to be gram-positive aerobic or facultatively anaerobic, rod-shaped, motile, spore-forming bacterium (Figure 3e). *B. cereus* is widely dispersed in the environment, mainly in soil, where spores persist under hostile circumstances. It can grow under an extensive temperature range, but it is not well suited to tolerate low pH values or water content.

The colony-forming unit of the bacterial strains isolated from the five sampling sites was observed and is listed in Table 5. The highest mean cfu count was observed in the soil sample collected from site 1 whereas the lowest count is found to be in the sample of site 5.

3.4. Molecular (16 S rRNA) characterization of isolated bacterial strains

The identified isolates were *Bacillus drentensis, Bacillus safensis, Bacillus haynesii, Bacillus subtilis,* and *Bacillus cereus*. All the gene sequences of the isolated strains were submitted to the National Center for Biotechnology Information (NCBI) under the accession numbers MK217088, MK774729, MK192808, MK217089, and MK801278, respectively. The molecular classification based on the 16S rRNA of isolated bacterial strains is presented in Table 6.

3.5. Identification of the isolates based on partial 16S rRNA sequencing and phylogenetic tree

The gene sequences have been studied to describe the associations between development and nomenclature using the phylogenetic tree. The sequences of the 16S rRNA gene from isolates (SN-1, SN- 2, SN-3, SN-5, and SN-12) were allied to find the closest match using the Basic Local Alignment Search Tool (BLAST). The percentage similarity of isolated bacterial strains to their closest match was described in Table 7. The BLAST analysis revealed that the gene sequences of strain SN-1 were 99.74% similar to *Bacillus drentensis* (MK217088), the sequences of strain SN-2 were 99.93% homologous to *Bacillus safensis* (MK774729), the sequences of strain SN-3 were 99.86% similar to *Bacillus haynesii* (MK192808), the sequences of strain SN-5 were 100% homologous to *Bacillus subtilis* (MK217089), and the gene sequences of strain SN-12 were 99.86% homologous to *Bacillus cereus* (MK801278). A phylogenetic tree was created by aligning the 16S rRNA sequences of different bacterial strains (Figure 4).

To analyze the correlation coefficient amid various heavy metals and the total bacterial count, a two-tailed test of the significance level of ($P < 0.05$) was used (Table 9). Significant positive correlations were witnessed among certain heavy metals pairs with the total bacterial count present in the polluted soil samples. The correlation investigation discovered that chromium exhibited an extremely positive correlation with cadmium ($r = 0.99$). Lead with cadmium, chromium and copper showed a positive correlation ($r = 0.94$), ($r = 0.93$), and ($r = 0.91$) respectively. A highly positive correlation was observed between cadmium and the total bacterial count in the samples ($r = 0.84$) and zinc and the total bacterial count.

4. Discussion

4.1. Soil physico-chemical properties and heavy metal contents

The soil is alkaline as the pH of the soil samples ranged from 7.02 to 7.47 at all the sites. The alkalinity of the soil is attributed majorly as the area is a semiarid region having a high evapotranspiration rate generally exceeding the precipitation rate in the area. According to Adelasoye and Ojo (2014), the microbial population was higher near the roadside sites than further away. The high concentrations of metals are usually known to reduce microbial biomass, resulting in the decreased population size of the microbial communities. Heavy metals in this area mainly originated from the accumulation of vehicular exhaust as there is no other possible source of the contaminants in the area. Heavy metals exhibited a positive affiliation amongst each other. The physical, chemical and biochemical interfaces are accountable for keeping the various soil systems in a dynamic equilibrium. Heavy metal pollution has turned out to be one of the gravest environmental problems due to various factors. As a result of industrial progress, the adulteration of the environment with heavy metals is spreading worldwide. The existence of even trace amounts of heavy metals is toxic and detrimental to all living organisms (Islam et al., 2007; Iwegbue et al., 2010). Moreover, heavy metals cannot be degraded, unlike other pollutants comprising a wide range of polyaromatic hydrocarbons (PAHs), organo-chlorines, organics, dyes, etc.

4.2. Soil bacterial count in the roadside soil

The findings of the current study show that the total bacterial count varied along with the roadside soil. The lowest and the highest numbers were found at S-3 and S-2, respectively. The increase of heavy metals content harmed the bacterial count present in the affected soil. Heavy metals could decrease soil bacterial count, which has been talked about in many previous studies (Nwuche and Ugoji, 2008; Wang et al., 2007). Several researchers also determined that soil physicochemical properties could influence heavy metals toxicity or modulate their bioavailability and toxicity (Kenarova et al., 2014; Ramakrishnan et al., 2011). The population dynamics of soil microorganisms are controlled primarily by soil characteristics along with vegetation. Soil microflora employs a substantial influence on plant growth and soil fertility (Joshi et al., 2010).

In the present study, five bacterial strains were isolated from roadside soil samples along NH 8A, Gujarat, India, and characterized for morphological and molecular characteristics. 16S rRNA gene sequencing identified the isolated bacteria as *Bacillus drentensis* (MK217088), *Bacillus safensis* (MK774729), *Bacillus haynesii* (MK192808), *Bacillus subtilis* (MK217089), *Bacillus cereus* (MK801278).

Bacterial species may tolerate a certain level of heavy metal concentrations that can be potential candidates for eliminating heavy metals from the contaminated habitats. Moreover, heavy metal resistant bacteria play an essential role in the biogeochemical cycling of the metal ions (Kumar et al., 2011; Issazadeh et al., 2013). Several microorganisms have become resistant to heavy metals by utilizing them for detoxification and respiration mechanism. The sequestration of heavy metal-resistant bacteria is noteworthy for its resistance capacity along with its metal accumulation capability (Ezaka and Anyanwu, 2011).

4.3. Impacts of heavy metal pollution on bacterial diversity

In the soil environment, the heavy metal pollutants are eventually dumped in the form of low solubility compounds, such as pyrite or sorbed on surface-reactive phases, like Fe and Mn oxides. While this phenomenon restrains the mobilization of the contaminants, preventing their influences on biota and human health, it also brings metal ions in intimate interaction with the soil microbial community.

In the present study, the effect of heavy metal concentration on the bacterial count has been observed and is presented in Table 8. The

observed results might be due to the effect of metal concentration at these sites. Microbes are the principal living organisms that experience direct and indirect influences of heavy metals. In low concentrations, certain metals, viz., Fe, Zn, Cu, Ni, and Co, are vital for numerous microbial activities. These elements are often involved in the metabolism and redox processes. The adverse effects of metals on the soil biota causes decreased diversity, reduction in the putrefaction of organic matter, soil respiration, and declined activity of several soil enzymes (Tyler, 1974). Microbial populations in metal-polluted environments are adapted to different heavy metal concentrations and thus become metal resistant (Prasenjit and Sumathi, 2005). In a natural environment, the consequences of organic compounds and heavy metals are very convoluted on the bacterial community. The soil microbial activity and conformation are meticulously connected to soil efficacy and ecological features. The bacterial community configuration is mostly exaggerated by heavy metals toxicity and different PAH levels (Kumar et al., 2020; Zhang et al., 2010). The movement of the heavy metals might be altered by soil pH and organic matter content present in the soil (Pakzad et al., 2016; Calugaru et al., 2016), which may interrupt the microbial community.

Thus it is noteworthy that even though some heavy metals are crucial trace elements, they can be noxious at higher concentrations to microbes. In contrast, certain microbes have been reformed to endure the metals at higher concentrations and use them for their growth. These interactions between the metals and the microbes have some significant environmental implications, basically in bioremediation.

Various decontaminating mechanisms, for instance, binding of metal in bacterial cell envelopes, complexation by exopolysaccharides, metal reduction, and metal efflux or using them as terminal electron acceptors in anaerobic respiration, can be attributed for the microbial tolerance to heavy metals (Haferburg and Kothe, 2010). Tiku et al. (2016) described a higher number of bacterial strains isolated from the petroleum contaminated soils were capable of tolerating seven heavy metals (Ni, Pb, Cd, Cr, Co, Cu, and V). Thus, they can be utilized for the bioremediation of the environment polluted with hydrocarbon and heavy metals.

Congeevaram et al. (2007) experimented with studying the bioaccumulation of Cr (VI) and Ni (II) by heavy metal resistant fungi and bacteria from the soil samples adjoining the electroplating industry. Sevgi et al. (2010) isolated heavy metal tolerating bacterial strains from the contaminated soil of the industrial area in Kazanh, Mersin, Turkey, and documented 272 *Pseudomonas* sp 161 *Bacillus* sp. strains. The resistance of the bacterial species to the heavy metals might be chromosomal or plasmid-mediated (Silver and Walderhaug, 1992). Zolgharnein et al. (2007) reported that plasmids in heavy metal resistant bacteria were higher than present in common bacteria. Alam and Ahmad (2011) studied chromium removal from tannery effluents contaminated soil through biosorption and bioaccumulation using bacterial species. The studied isolates were *Exiguobacterium* sp., *Stenotrophomonas maltophilia*, *Aeromonas* sp., and *Pantoea* sp.

Adulteration of soil with heavy metals released from automobile sources has become a critical environmental issue. These heavy metals are discharged during various road transportation operations, including component wear, combustion of fuels, fluid leakage, and metals corrosion. Zinc, along with lead, copper, cadmium, is the chief metal pollutants present in the roadside environments (Dolan et al., 2006).

Microorganisms consume chemical contaminants as a source of energy during the microbiological processes. However, microbial inhibition is caused due to the extreme quantities of inorganic nutrients in the soil (Ahirwar et al., 2016). Specifically, the microorganisms can degrade, detoxify, and even mount up harmful organic and inorganic compounds. Therefore, heavy metals impact soil microbes in many ways, mainly the effects on soil microbial activity, soil enzyme activity, and the composition of the soil microbial community (Chu, 2018). A comparative analysis of the present finding with the findings of previous research studies is presented in Table 10.

5. Conclusion

Soil is a complex and dynamic biogeochemical system comprising tens of thousands to millions of microbes. However, environmental stress may reduce bacterial diversity in the soil. Therefore, microbes capable to thrive in high concentrations of heavy metals are of pronounced importance as bioremediation agents since they can attain diverse transformations and immobilization practices. Five heavy metal resistant bacteria were isolated and characterized in the current research study from the roadside soil sample at NH 8A, Gujarat, India. 16S rRNA gene sequencing helped to identify the isolates as; *Bacillus drentensis*, *Bacillus safensis*, *Bacillus haynesii*, *Bacillus subtilis*, and *Bacillus cereus*. The present study revealed that these microbes could persist in the heavy metals polluted areas and utilized metal constituents for their growth. Therefore, these heavy metals tolerant bacterial species can be exploited to clean up metal-contaminated sites.

Animal studies

This article does not contain any data based on the experiments performed on animals.

Declarations

Author contribution statement

Snigdha Singh: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

R. Y. Hiranmai: Conceived and designed the experiments; Reviewed the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data associated with this study has been deposited at National Center for Biotechnology Information (NCBI) under the accession numbers MK217088, MK774729, MK192808, MK217089, and MK801278.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors are also thankful to the Central University of Gujarat for providing the instrumentation facilities. The authors are also grateful to the Gujarat State Biotechnology Mission (GSBTM), Department of Science and Technology (DST), Gandhinagar, Government of Gujarat, India, for bacterial identification.

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