Plant growth promoting strain *Bacillus cereus* (RCS-4 MZ520573.1) enhances phytoremediation potential of *Cynodon dactylon* L. in distillery sludge

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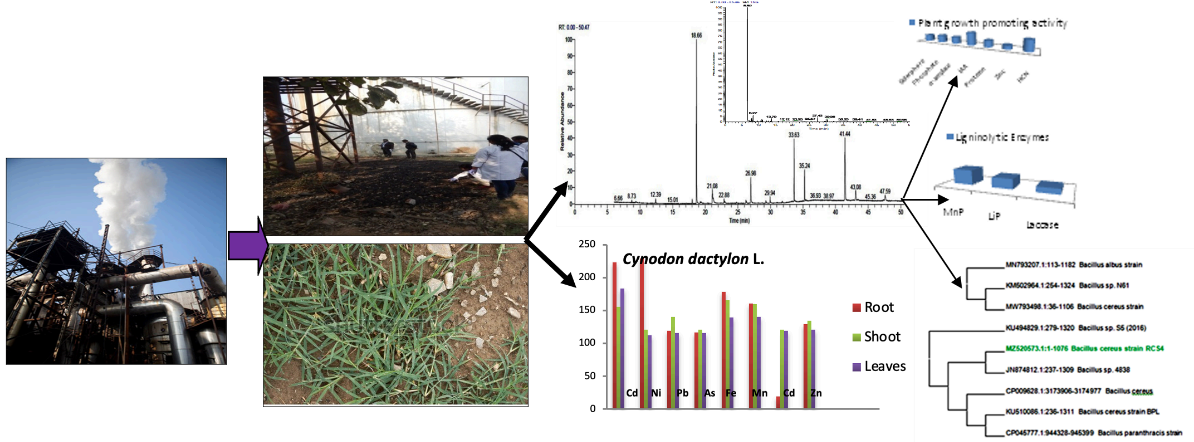
Highlights

- Distillery sludge containing high amount of toxic metals.
- *Cynodon dactylon* L. remediate metals in the sludge in the field by 75%.
- *Bacillus cereus* enhances phytoremediation via high plant growth-promoting activities.
- Invasive plant species can be used to bioremediate distillery waste.

Graphical abstract



Abstract

Elevated levels of physico-chemical pollution including organic pollutants, metals and metalloids were detected in distillery sludges despite of the anaerobic digestion treatment prior to disposal. The concentrations of the metals were (in mg kg^{-1}): Fe (400.98 ± 3.11), Zn (17.21 ± 0.54), Mn (8.32 ± 0.42), Ni (8.00 ± 0.98), Pb (5.09 ± 0.43), Cr (4.00 ± 0.98), and Cu (3.00 ± 0.10). An invasive grass species, *Cynodon dactylon* L., demonstrated its ability to remediate the distillery waste sludge (DWS) in the field study. All the physico-chemical parameters of the sludge significantly improved (up to 70–75%) in the presence of *Cynodon dactylon* L. ($p < 0.001$) than the control with no plant growth. The highest phytoremediation capacity was associated with the uptake of Fe in the root and shoot. Sludge samples collected near the rhizosphere also showed lower amount of organic compounds compared to control sludge samples. Metal resistant *Bacillus cereus* (RCS-4 MZ520573.1) was isolated from the rhizosphere of *Cynodon dactylon* L. and showed potential to enhance the process of phytoremediation via plant growth promoting activities such as production of high level of ligninolytic enzymes: manganese peroxidase (35.98 U), lignin peroxidase (23.98 U) and laccase (12.78 U), indole acetic acid (45.87(mgL^{-1}), phosphatase activity (25.76 mg L^{-1}) and siderophore production (23.09 mg L^{-1}). This study presents information on the performance of *Cynodon dactylon* L., an abundant invasive perennial grass species and its associated plant growth promoting rhizobacteria demonstrated good capacity to remediate and restore contaminated soil contained complex organic and inorganic pollutants, they could be integrated into the disposal system of distillery sludge to improve the treatment efficiency.

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1. Introduction

Worldwide, heavy metals and metalloids have accumulated in soil due to emissions from numerous industries (e.g., distilleries, pulp paper mills, tanneries, the metalliferous surface finishing industry, fertilizer and pesticide industry, agricultural runoff, combustion of fossil fuels, and electroplating faulty waste disposal) (Tripathi et al., 2021c). The discharge of industrial wastes and wastewater contain dangerous amount of heavy metals into the environment can result in harmful consequences on both ecological and human health (Esmaeili and Beni, 2015; Tripathi et al., 2021a & b). This is a particular concern in developing countries where the legislation and treatment practice are less rigorously enforced.

Distillery effluent is a key source of heavy metals resulting from their production process and large volume of this wastewater is being discharged into receiving waters. In India alone, approximately 40.40×10^{10} L of distillery wastewater is discharged annually (Tripathi et al., 2021e). Adsorption methods and membrane filtrations are amongst the most popular means to remove heavy metals (Chandra et al., 2018a, b), but it is challenging to treat large volume of sludge and remove multiple types of ions simultaneously, due to the high retention times and cycling stability of the adsorbents (Teh et al., 2016; Zhang et al., 2020). The prevention of fouling and scaling is also energy-intensive and costly. Electric and photocatalytic techniques are still in their infancy and are not widely used in industry. In contrast, phytoremediation is an eco-friendly, inexpensive, and sustainable technology for the removal of heavy metals in industrial waste contaminated sites. Several studies showed that plant growth-promoting rhizobacteria (PGPR)-assisted phytoremediation were very effective in removing of heavy metal from contaminated soils (Dhiman et al., 2016; Sharma et al., 2021a, b).

The application of PGPR is regarded to be a cost-effective and environmentally friendly treatment to enhance the clean-up process of contaminated soil by plants that are subjected to biotic and abiotic stress (Pandey et al., 2017; Sharma et al., 2020a, b). The rhizosphere is a hotspot for microbial activity in the soil (De et al., 2020; Kalam et al., 2017). PGPR promote plant growth and enhance phytoremediation through a variety of activities, such as synthesis is of phytochromes and plant hormones [e.g., indole acetic acid (IAA)], biocontrol, promote nodulation in legumes, boost seedlingsdevelopment, increase mineral phosphate solubilization, produce siderophores and acetyl-CoA carboxylase (ACC) (Swarnalakshmi et al., 2020; Gopalakrishnan et al., 2015; Tripathi et al., 2021c,d; Khoshru et al., 2020; Ahemad and Kibret, 2014). Through systemic acquired resistance and induced systemic resistance, PGPR also help plants to become more resistant to various plant pathogens and environmental stresses (Parray et al., 2016; Backer et al., 2018; Syed Ab Rahman et al., 2018). Several studies showed that PGPR species including: *Azotobacter, Pseudomonas, Rhodococcus, Methylobacterium, Kluyvera, Ochrobactrum, Variovox, Burkholderia, Bacillus, Brevibacillus, Flavobacterium, Xanthomonas, Azomonas* and *Brevundimonas*, have been successfully used to increase plant growth in phytoremediation (Tarekegn et al., 2020; Delil et al., 2020; Dimkpa et al., 2008; Ankati et al., 2018; Sharma et al., 2020a,b).

Fast community fingerprints of 16S rDNA denatured gradient electrophoresis (DGGE) based on a polymerase chain reaction (PCR) showed bacterial phyla such as Proteobacteria and Firmicutes are commonly found in the rhizospheric zone of plants to remove heavy metals from different industrial contaminants (Tozser et al., 2019; Chauhan et al., 2020; Hejna et al., 2020). The development of mutually beneficial interaction between the host plant and rhizosphere micro-organisms leads to the enhancement of plant growth and resilience (Mhlongo et al., 2018). Hence, the use of appropriate rhizosphere microorganisms can simultaneously increase metal phyto-availability, decrease metal toxicity and stimulate the host plant biomass to accumulate a high level of metals. The enhancement of interactions between plants and rhizosphere bacteria could be a critical component of phytoremediation technology.

In heavy metals contaminated environment, the luxuriant growth of several indigenous plants suggested the phytoextraction capacity of these native plants against the heavy metals (Petelka et al., 2019; Tripathi et al., 2021a, b, c). The use of native plants is strongly recommended, but little work has been carried out regarding using invasive species for phytoremediation. The invasive species often outcompete native plants due to their adaptability and robustness (Lee et al., 2020; Szymura et al., 2018). This study examined *Cynodon dactylon* L., a perennial grass and an invasive species, growing in a site containing contaminated distillery sludge, its ability to phytoremediate heavy metals and its relationship with a rhizospheric bacterial strain *Bacillus cereus* (RCS-4 MZ520573.1). This study gave an unparallel insight into the workings of this partnership in field condition where both the phytobiont and microbiont are subject to authentic environmental stressors. The mechanistic role of PGPRs in enhancing phytoremediation were elucidated, providing vital information for potential scale-up of this technology to protect the environment and restore contaminated sites.

2. Materials and methods

2.1. Site description and sample collection

Clean pre-sterilized polythene bags were used to collect the fresh distillery sludge samples from M/s Unnao Distillery and Breweries Limited, located in Unnao (26°320″ N, 80°30′0″E) Uttar Pradesh, India. Despite the presence of a fully operational activated-sludge treatment plant, the discharged waste did not fulfil statutory pollution requirements, resulting in severe environmental consequences. The distillery waste sludge (DWS) from the secondary treatment has been disposed on the site in large quantities where plants, herbs, and grasses had been observed growing on the dumped distilleries sludge (Sharma et al., 2021a).

Cynodon dactylon L. Grew luxuriantly to a height of 2 m and had a diameter of 3–10 mm, producing large, robust clumps in the discharged sludge. The plants were densely packed and reticulated, with roots reaching a length of 25 cm. Sludge samples were collected round the rhizosphere and area where plants were absent (control) as per the standard methods (APHA, 2012). Before collecting the rhizospheric sludge, a sterile plastic bag was put over the plant roots and shaken vigorously for 10 min to eliminate bulky particles. The sludge adhering to root hairs was collected in a sterilized glass test tube Distillery sludge samples from the control site were collected in triplicate from a depth of 0–20 cm using an auger and kept in pre-sterilized biohazard bags (HiMedia Laboratories Pvt. Ltd., Karnataka, India). The samples were transported under ice-cold (4 °C) conditions to the laboratory for further examination (Chandra et al., 2018a, b).

2.2. Estimation of heavy metals and physico-chemical parameters

Sludge samples were homogenised and air dried at 25 °C for three weeks. The physico-chemical properties of all of the samples were evaluated, as well as the overall pollutant loads from DWS. The biological oxygen demand (BOD), total suspended solids (TSS), total nitrogen (TN), total dissolved solids (TDS), chemical oxygen demand (COD), ions (K^+, Na^+, and Cl^-), and total phenols (TP) were measured using the American Public Health Association standard techniques (APHA, 2012; Tripathi et al., 2021c). The levels of phenols and chlorophenols, pH, electrical conductivity (EC), sulphate, phosphorus, and ammoniacal nitrogen were measured as described previously (Model-960, Thermo Scientific, United States; Model-150, Thermo Scientific, United States) (Chandra et al., 2009; Tripathi et al., 2021a,b). Heavy metals in sludge samples are assessed after digestion with acidified nitric acid and perchloric acid (method no. 3030H) using the AAS technique (ZEEnit 700, Analytic Jena, Germany) (APHA, 2012). Briefly, one gm of dry sludge was digested for 15 min at 95 °C in 10 mL of HNO_3 (1:1, v/w). After cooling, the container was filled with 5 mL concentrated HNO_3 and reflux at 95 °C until it was translucent. Samples were allowed to cool to room temperature and the contents filtered using a No. 1 Whatman filter paper and diluted to a final volume of 100 mL with double deionized water. Each sample and the relevant blanks have been digested three times. AAS was used to determine the total concentrations of heavy metals in the digested solution (i.e., Cd, Mn, Cu, Ni, and Fe, As, Zn, Cr, Hg, and Pb).

2.3. Identification of compound from rhizospheric and non rhizospheric distillery sludge

GC–MS analysis has been carried out to identify organic and inorganic compounds in the sludge. The extracted compounds were derived from trimethylsilyl (TMS), as previously stated by (Tripathi et al., 2021a, b). An aliquot (2.0 µL) of the derivatives was injected using a TriPlus auto sampler paired with TSqQuantumXLS triple quadrupole mass spectrometer (Trace GC Ultra Gas Chromatogram; Thermo Scientific, FL, USA) (Thermo Scientific, FL, USA). In addition, organic chemicals have been separated in the capillary column DB-5MS. The GC oven temperature was set to start at 65 °C (2.0 min holding), increased to 230 °C by 6 °C min^{-1}, and eventually reached 290 °C (20 min holding) at 10 °C min^{-1}. Helium was utilised to carry gas at a 1.1 mL min^{-1} flow rate. In positive electron ionisation mode (+EI) the 70 eV mass spectrums (MS) were operated. Organic compounds from fresh and plant specimens retrieved from the MS Bibliotheque NIST version 1.0.0.12 with a tool were found to be accessible reported by (Tripathi et al., 2021a).

2.4. Plant selection and heavy metal quantification

Cynodon dactylon L. were cleaned using tap water and then washed with deionized water to remove sludge particles that remained on the root surface of the plant. The samples were dried for 7 day at 70 °C before they were put into a silk oven at 460 °C for 6 h (AOAC, 2002). Heavy metal build-up was measured in different sections of plants using Atomic Absorption Spectrometry (AAS-ZEEnit 700, Analytic Jena, Germany).

2.5. Bioaccumulation factor and translocation factor

To measure the plant's ability to extract heavy metals from sludge, the bioaccumulation factor (BAF) and translocation factor (TF) of *Cynodon dactylon* L. were determined (Yoon et al., 2006; Tripathi et al., 2021c) as follows:

$$\text{Bioaccumulation factor} = \frac{[HM]_{root}}{[HM]_{sludge}}$$

where, HM= Heavy metals (mg kg^{-1}).

$$Translocation\ factor = \frac{\{HM\}_{shoot}}{[HM]_{root}}$$

where, HM= Heavy metals (mg kg^{-1}).

A TF value less than one (<1) indicates the plant has a special ability to retain heavy metals in its root tissues, whereas a TF value larger than one (>1) implies the plant tends to transfer metals in its harvestable parts (Yoon et al., 2006; Tripathi et al., 2021c). Furthermore, a higher TF value indicates that the plant's metal accumulation efficiency is higher when exposed to high levels of metals contamination.

2.6. Rhizospheric bacterial isolation and ligninolytic enzyme activity

To remove adherent sludge from the roots of *Cynodon dactylon* L., the roots were gently shaken in sterile saline solution (0.85 wt percent) for 15 min at 4 °C. Sludge samples (10 g) from the rhizosphere of *Cynodon dactylon* L. were used for serial dilution, with each sample being applied to three nutrient agar plates. Serial dilutions were performed, and the dilutions 10^{-6}, 10^{-7}, and 10^{-8} were inoculated in a nutrient agar medium (NAM) (Chandra et al., 2018a, b). The inoculated plates were incubated at 30 ± 1 °C for 24–48 h. This was able to obtain rough and abundant colonies with waxy growth (1–4 mm diam) and an irregular spreading edge. After conducting preliminary tests, one isolates (RCS4) was chosen and cultured on NAM slants at 4 °C in preparation for further analysis (Gamalero et al., 2009). To measure the ligninolytic activity such as manganese peroxidase (MnP), lignin peroxidase (Lip), and laccase from isolated bacterial strains phenol red (Lobachemie, Mumbai, India), Azure B, and guaiacol were used as substrates, respectively (Sigma-Aldrich, St. Louis, MO, USA) (Arora et al., 2002). One IU equalling the amount of enzyme needed to produce 1 mol of product per minute under standard conditions.

2.7. Plant growth-promoting traits in bacteria

2.7.1. IAA estimation and nutrient solubilization

The production of IAA by the bacterial isolates was determined using the methods Gordon and Weber (1951). The bacterial isolates were grown on Luria Bertani (LB) broth (HiMedia Pvt Ltd) and incubated at 28 °C for 24 h at 120 rpm. Exponentially grown culture (10^8 cells mL^{-1}) was centrifuged at 10,000×*g* for 15 min at 4 °C to collect the supernatant. The presence of IAA was indicated by the appearance of pink color when two drops of O-phosphoric acid was added to two mL of supernatant. The cultures were inoculated onto Pikovskaya's agar and mineral salts agar (MSA) (HiMedia Pvt Ltd) for phosphate and zinc solubility tests, respectively (Hu et al., 2006). A loop full of the 24 h culture was spread on to the agar plate and incubated it at 28 ± 1 °C for 96 h to determine the bacteria's phosphate and zinc solubilization activity (Venkatakrishnan et al., 2003).

2.7.2. Hydrolytic enzymes and production of siderophore

One loopful of the bacterial cell culture was streaked over a starch agar plate, and skim milk agar plate (HiMedia Pvt Ltd) to determine the production of α-amylase and protease enzymes activity, respectively. The plates were examined for the presence of a clear zone surrounding after 48 h of incubation at 28 °C. The synthesis of siderophores on Chrome-azurol S (CAS) medium was determined using a modified Schwann and Neilands (1987) method. The bacterial strains (24 h old cultures) were spotted separately on CAS medium and incubated at 28 ± 1 °C for 48–72 h. Observation of orange to yellow halo surrounding the colonies showed the presence of siderophore production (Tripathi et al., 2021c).

2.8. Bacterial 16S rRNA sequencing and phylogenetic analysis

The isolated bacterial strains were cultured overnight in Luria Bertani broth for 16S rRNA sequencing, and total DNA was extracted using a commercial kit (real Biotech Corporation). Universal primers sets16sf (5′ CAGCAGCCGCGGTAATAC 3′) and 16Sr (5′ TACGGCTACCTTGTTACG 3′) were used to amplify the 16S rRNA gene. An agarose gel electrophoresis confirmed the PCR amplified product of the 16S rRNA gene of bacteria. In addition, the QIA gel extraction kit was used to purify the PCR product in the gel, which was processed for sequencing. After sequencing, a phylogenetic tree was constructed using the MEGA- 7.0 software (Tamura et al., 2013). After retrieval, the query sequences and other homologous sequences were saved in a single FASTA file format in the National Centre for Biotechnology Information (NCBI) nucleotide database. *2.9. Statistical analysis*

The data was presented as the average of three different observations. The mean concentration of various physico-chemical parameters of rhizospheric and non-rhizospheric were measured to confirm the variability of the data collected and the validity of the results. The mean of physico-chemical parameters as well as heavy metal analysis on *Cynodon dactylon* L. were calculated (MS Office; Microsoft, USA). Comparison of the mean of rhizospheric and non-rhizospheric was carried out using student t-test (SPSS, v. 19.0 SPSS, USA).

3. Results and discussion

3.1. Physico-chemical characteristics and reduction of heavy metals concentration

The physico-chemical parameters of distillery industry sludge discharged after secondary treatment is shown in Table 1. All physico- chemical parameters were found to be higher than the permissible level (CPCB, 2010; EPA, 2002). A considerable amount of pollution indicators was found in the distillery sludge, posing a health hazard to both individuals and the environment. High BOD (4841.21 ± 56.88 mg L^{-1}), COD (12789.88 ± 298.11 mg L^{-1}), EC (2085.52 ± 21.09 mhos cm^{-1}), total dissolve solid (9812.12 ± 211.11 mg L^{-1}), total nitrogen (197.45 ± 1.54 mg L^{-1}), phenol (6000 ± 23.10 mg L^{-1}), sulphate (13212.43 ± 231.12 mg L^{-1}), phosphate (43.11 ± 0.90 mg L^{-1}), Na (312.11 ± 2.12 mg L^{-1}) and K (211.21 ± 08.21 mg L^{-1}) values were observed. One of the most important sources of environmental pollution is the discharge of distillery industrial wastes sludge produced during the fortification process (Tripathi et al., 2021d, e). This is due to the combination of carbonic acid, magnesium hydroxide ions, bicarbonate ions, and sodium ion, which are all utilised to modulate the pH throughout the production process (Tripathi et al., 2021d,e). Bacterial association with plants in the rhizosphere can also increase metal accessibility by sequestering secondary metabolites, amino acids, enzymes, and protons, lowering the pH of sewage and promoting plant development in a highly contaminated environment. Organic substances, organic acids, and other co-pollutants in the root zone also influenced metal bioavailability (Pinto et al., 2015; Tripathi et al., 2021c). The COD, BOD and TDS are essential assessment criteria widely used to analyse pollutants in released wastewater. The experiments demonstrate that *Cynodon dactylon* L. enhanced the quality of the sludge, most likely because metals were bioavailable in the rhizosphere at acidic pH.

Table 1 Physico-chemical characteristics of discharged sludge collected from M/s Unnao distillery industry Ltd. Unnao Uttar Pradesh, India.

Parameters	Values (Mean ± SD) Non-Rhizospheric Distillery waste sludge	Values (Mean ± SD) Rhizospheric Distillery waste sludge	CPCB (2017)
pH	8.02 ± 0.26	7.05 ± 0.18^{a}	7.54 ± 0.01
Biological oxygen demand (BOD)	4841.21 ± 56.88	2200.43 ± 98.70^{a}	47.00 ± 0.00
Chemical oxygen demand (COD)	12789.88 ± 298.11	6011.40 ± 111.18^{a}	79.00 ± 0.01
Electrical conductivity	2085.52 ± 21.09	981.40 ± 30.10^{a}	950
Total dissolved solid	9812.12 ± 211.11	5111.11 ± 111.11^{a}	70 ± 0.00
Chloride	2712.12 ± 67.45	999.54 ± 89.01^{b}	11.82 ± 0.01
Total nitrogen	197.45 ± 1.54	120.11 ± 3.90^{a}	9.90 ± 0.00
Phenol	6000 ± 23.10	135.02 ± 12.40^{c}	–
Sulfate	13212.43 ± 231.12	6732.09 ± 70.12^{b}	
Phosphate	43.11 ± 0.90	18.32 ± 0.12^{b}	3.40 ± 0.01
Na	312.11 ± 2.12	62.12 ± 7.8^{NS}	0.01
K	211.21 ± 08.21	110.90 ± 0.12	0.02
Heavy metals (mg kg^{-1})			
Mn	8.32 ± 0.42	1.79 ± 0.76^{b}	0.15
Cr	4.00 ± 0.98	1.06 ± 0.57^{c}	0.01
Zn	17.21 ± 0.54	9.11 ± 0.34	1.28
Cu	3.00 ± 0.10	0.90 ± 0.05^{NS}	0.19
Fe	400.98 ± 3.11	100.90 ± 2.27	1.45
Pb	5.09 ± 0.43	0.90 ± 0.23^{c}	0.02
Ni	8.00 ± 0.98	3.10 ± 0.90^{a}	0.04

All the values are Mean ± SE. (n = 3); Unit of all parameters is in (mg L^{-1}) except for pH, color (Co–Pt. Unit), EC (μmhoscm^{-1}), and heavy metals (mg kg^{-1}); Students *t*-test (two tailed as compared to pre-treated sludge); ^{a}Highly significant at $p < 0.001$; ^{b}Significant at $p < 0.01$; ^{c}Less significant at $p < 0.05$; ^{NS}Non-significant at $p > 0.05$, Central pollution control board (CPCB).

Similar contaminants have also been observed in the inflow of waste sludge discharge from distillery industry (Tripathi et al., 2021c). The decrease in EC can be explained by the presence of rhizospheric bacteria and soluble salt absorption. In the rhizosphere of plant roots, a reduction in EC has been demonstrated to enhance the spread of microbial activity. This means that the interaction between the rhizospheric bacteria and *Cynodon dactylon* L. caused the reduction in magnitude of the physico-chemical parameters. The microbial communities helped to degrade, transform or detoxify contaminants and improved the bioavailability of the metals to plants. Physico-chemical analysis of fresh distillery sludge showed that elevated heavy metal concentrations in non-rhizospheric (control) sludge (in mg kg^{-1}) i.e., Fe (400.98 ± 3.11) followed by Zn (17.21 ± 0.54), Mn (8.32 ± 0.42), Ni (8.00 ± 0.98), Pb (5.09 ± 0.43), Cr (4.00 ± 0.98), and Cu (3.00 ± 0.10). A significant decrease in the non rhizospheric sample for Fe (100.90 ± 2.27) followed by Zn (9.11 ± 0.34), Ni (3.10 ± 0.90), Mn (1.79 ± 0.76), Cr (1.06 ± 0.57), Pb (0.90 ± 0.23), and Cu (0.90 ± 0.05) was noted due to the accumulation of these metals in different parts of plant (Table 1). The presence of *Cynodon dactylon* L. is likely to enhanced bioavailability of metal and acidic pH around root hair leading to greater absorption of heavy metals (Sharma et al., 2021a, b). Previous investigations demonstrated that acidic conditions may be created by the root releasing many acidic chemicals (Rajkumar et al., 2012; Sessitsch et al., 2013). Significantly, it was observed that there was a reduction of the quantity of dangerous metals in the sludge (between 50% and 70.5%) in this study. These results showed the potentiality of in situ phytoremediation to lower pollutants levels.

Metals have excellent binding affinities with cationic compounds like lignocellulosic materials, result in complex molecules formation. Various heavy metals induce oxidative stress and cancerous consequences in both fish and people through the food chain (Farag et al., 2006). Since low pH enhanced solubility and the metals uptake by plants; phytoextraction will be a strong candidate for heavy metals removal from sludge. Most study on the effects of different heavy metals on living plants has been carried out using plant seedlings or matured plants (HE et al., 2008; Sayyad et al., 2010; Delgardo-Cabellero et al., 2017). The excessive levels of pollution demonstrated the eco-toxicological and hazardous nature of the sludge. This is a useful indicator of total salt content or salinity, and the presence of bicarbonates, carbonates, and hydroxides in sludge may result in the highest values of EC. TDS levels were high in the control; however, they were reduced (5111.11 mg L^{-1}) after phytoremediation (Table 1). Microbial strains have likely developed resistance mechanisms that allow organisms to consume or detoxify pollutants due to the wide range of nutritional activity. Bacteria can produce detoxifying enzymes for adaptation in the presence of lignocellulosic wastes at low pH, and thus can survive in the acidic environmental of distillery sludge (Narayanan et al., 2016; Singh et al., 2021).

Results in Table 1 indicated that the sludge generated by distillery industries contained high levels of metal that persist in the environment. Moreover, during chemical treatment, phenols and chlorophenols were removed directly from plant material and dissolved in sludge at high pH, contributing to sludge toxicity (Tripathi et al., 2021d, 2021e). Chlorophenols have a high potential for soil absorption due to their lipophilic properties (Li and Dzenis, 2011). All the physico-chemical parameters of rhizospheric were found significantly better ($p < 0.001$) than the non-rhizospheric control ($p > 0.05$).

The selection of plants for phytoremediation is frequently drawn from a well-established but conservative list, for example, *Typha* and *Phragmites* sp. This study presents novel information on the performance of *Cynodon dactylon* L., an abundant invasive perennial grass species in the field and their ability to increase the quality of discharged sludge. The use of invasive species which could extend the choice of phytoremediation candidate.

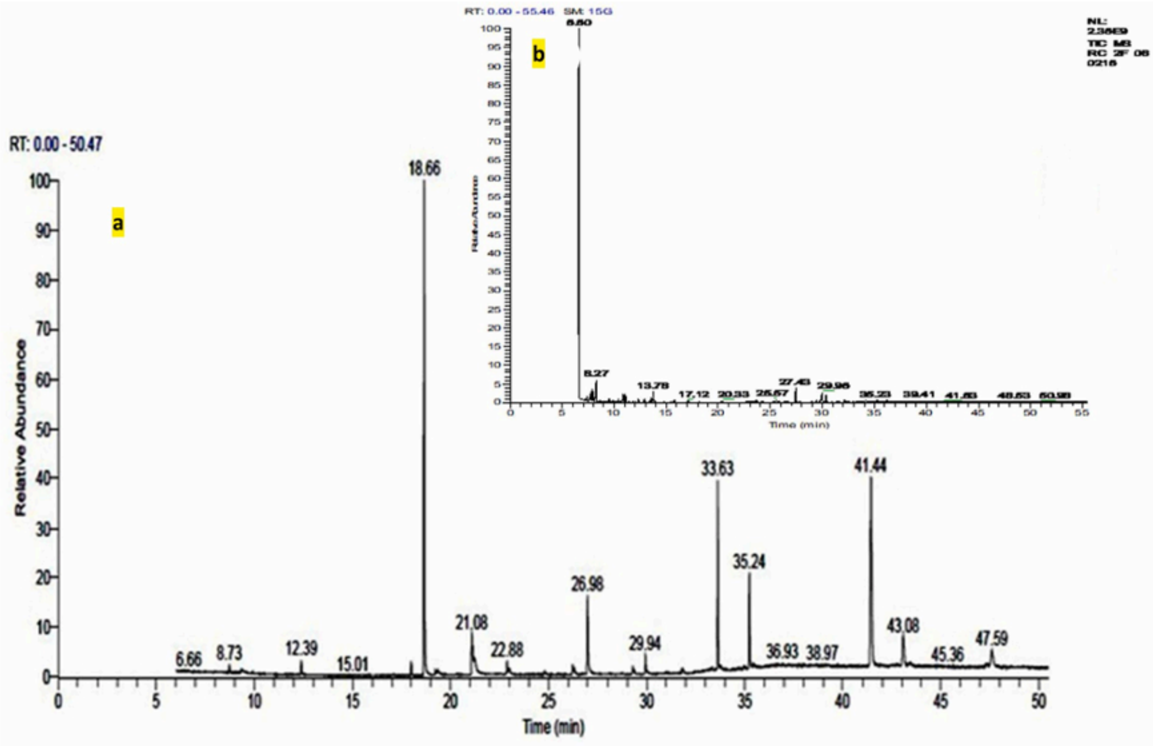

Fig. 1 Gas chromatography mass spectroscopy analysis of distillery industry sludge contaminated site: (a) non-Rhizospheric (b) after Rhizofiltration.

3.2. Identification of organic pollutants

The major peaks of extracted sample in rhizospheric and non rhizospheric samples were observed at RT: 12.39, 15.01, 18.66, 21.08, 9.18, 9.80, 10.08, 16.48, 22.89, 23.75, 25.91, 26.99, and 38.97 as shown in Fig. 1. These compounds were characterized as: Z)-5-Heptenenitrile, Ethanimidic acid, N-(trimethylsilyl)-, Pent-2-yne-1,5- diol, Dibenzoxazabicycloundecane, 7-Aminocholesterol, Ethyl 3-Anilinobutanoate, 1,5-Bis (diethoxyphosphinyl) pentane, 1-Heptadecanol (CAS), 2,6-Dibromo-4-hexoxymethylpyridine, Allyl Cyclohexyl carbonate, Hexadecanoic acid, trimethylsilyl ester, 2,3′-Dimethoxy-1,1′-binaphthyl as per NIST library available with the instrument. The detection of rhizospheric and non-rhizospheric distillery sludge organic compounds were investigated and presented in Table 2. Sludge samples collected near the rhizosphere showed lower amount of organic compounds compared to the control sludge samples. Moreover, the presence of several fatty acids in the sludge could retard the rate of metal accumulation processes and biotransformation of complex organic compounds by *Cynodon dactylon* L. The bulk of these identified chemicals have a negative impact on soil flora, animals and the aquatic ecosystem. The result showed that original organic compounds present in distillery sludge were either degraded or biotransformed by *Cynodon dactylon* L. into new compounds as shown in Table 2. Similar observation has been reported using native herbs and welland plants to reduce organic and metal pollutants in distillery waste sludge (Yadav and Chandra, 2011; Chandra et al., 2018), suggesting *Cynodon dactylon* L. is as good a phytoremediation candidate as native plants to remove recalcitrate organic pollutant which support utilising invasive species to restore contaminated sites.

Table 2 Compound analysis of distillery industry sludge contaminated site: (a) non-Rhizospheric (b) after Rhizofiltration.

Compound analysis of distillery industry sludge contaminated site: (a) non-Rhizospheric (b) after Rhizofiltration.

S.No.	RT	Non rhizospheric Compound Name	Nature of Compound	% Similarity (NIST Library)	Abundance (%)
	6.66	3-Fluoroquinoline	Alkane Nature	97.13	76
	8.73	6-Phenylcyclohex-1-ene-1-methanol	Fatty acid	92.05	68
	12.39	(S) and (R)-2,7-Dimethyl-2H-azepine	Sulfonic benzoic	74.35	70
	15.01	(Z)-5-Heptenenitrile	Fatty acid	34.54	52
	18.66	Ethanimidic acid, N-(trimethylsilyl)-,	Fatty acid	95.14	45
	21.08	Pent-2-yne-1,5-diol	Fatty acid	71.23	61
	9.18	Dibenzoxazabicycloundecane	Metabolic product	67.98	98
	9.80	7-Aminocholesterol	Alkane Nature	97.24	59
	10.08	Ethyl 3-Anilinobutanoate	Fatty acid	93.11	73
	10.37	Acetamide,2,2,2-trifluoro-N,N-bis(TMS)-	Saturated FA	89.74	68
	10.65	1H-Dicyclohepta[b,d]pyrrole	Organic Nature	97.13	94
	11.68	5,10,15-tris(hydroxymethyl)-truxen	Saturated FA	92.05	93
	12.26	Cholest-2-en-19-ol, (5à)- (CAS)	Organic Nature	74.35	80
	13.62	2-hydroxygarvin B	Alkane Nature	34.54	81
	16.48	1,5-Bis(diethoxyphosphinyl)pentane	Fatty acid	87.18	31
	22.89	1-Heptadecanol (CAS)	Organic Nature	78.01	54
	23.75	2,6-Dibromo-4-hexoxymethylpyridine	Fatty acid	93.11	68
	25.91	Allyl Cyclohexyl carbonate	Alkane Nature	93.12	42
	26.99	Hexadecanoic acid, trimethylsilyl ester	Alkane Nature	57.01	76
	38.97	2,3′-Dimethoxy-1,1′-binaphthyl	Saturated FA	98.23	68
	41.44	(trifluoroacetyl)-T-2-toxin	Saturated FA	90.02	70
	43.08	Octadecanoic acid, trimethylsilyl ester	Alkane Nature	87.18	76
	47.59	Pallidine	Acyclic Nature	78.01	68
	8.27	3-nitrophenyl)-2-furyl)-2 pyrazoline	Alkane Nature	85.18	98
	13.78	6-Chloro-2-methyl-1,2- -3-carbaldehyde	Fatty acid	88.01	76
	17.12	(+)-Tetraponerine-2	Saturated FA	78.11	68
	20.33	2-O-methylbaeomycesic acid	Organic Nature	93.12	79
	25.57	Ketoprofen methyl ester	Saturated FA	57.01	68
	29.96	phenyl Trifluoromethanesulfonate	Organic Nature	98.23	69
	35.23	1 -2-methoxy-d3-3,5-dichlorobenzene	Alkane Nature	90.02	79
	39.41	L-tartaric acid	Fatty acid	87.18	58
	41.53	2-(1-Methyl-1H-2-pyrrolyl)quinoline	Alkane Nature	78.01	65
	48.53	1-Bromo-2- 3,4,6- methylbenzaldehyde	Saturated FA	93.11	76
	50.28	PENTASILOXANE,	Acyclic Nature	93.12	98

3.3. Heavy metal accumulation in *Cynodon dactylon* L.

To determine the role of plants in the rehabilitation of contaminated environments, it is important to examine the distribution and accumulation of metals in various plant components (Shahid et al., 2015). *Cynodon dactylon* L have the ability to accumulate multiple metals in a diverse pattern. *Cynodon dactylon* L exhibited a higher accumulation of Pb, As, Cd and Zn in its shoot portions (Fig. 2). However, the highest accumulation of Cu, Ni, Fe and Mn was found in the root, which was followed by the shoot. The accumulation of metals in *Cynodon dactylon* L. was noted i.e., Cu (561.28 ± 7.65) >Fe (482.83± 9.11) >Ni (461.83± 4.98)>Mn (459.73 ± 8.42) > Zn (384.23± 6.54), followed by Pb (374.84± 4.67), As (352.7±4.35), Cr (4.00 ± 0.98) and mg kg $^{-1}$). During the accumulation process, the diffusion of cell sap plays an important role in the accumulation of metals in different parts of the plant. Soil pH also plays an important role in bioavailability of metals. The bioavailability of trace metals in plants has been linked to low pH values. It has been shown that root exudates can modify the pH of sludge, increasing the mobility of heavy metals in soil. Furthermore, bacterial-plant interactions in the rhizosphere also increase metal bioavailability in roots (Sessitsch et al., 2013; Rajkumar and Freitas, 2008). Redox potential can influence the bioavailability of metals, while heterogeneity of metals also affects the redox potential. Metal concentration in the root, shoot and leaves of the plant showed the plant's potential for application in phytoremediation of industrially contaminated areas.

3.4. Bioaccumulation and translocation factor

To measure the potential of plants to absorb heavy metals from polluted environments by measuring the BAF and the TF for each metal separately. Results showed that BAF values for Cu (25.98), Ni (48.89), Pb (20.10), As (15.08), Fe (92.99), Mn (2.00), Cd (1.91), and Zn (5.00 mg kg $^{-1}$) in *Cynodon dactylon* L were higher than one (>1) (Table 3).

Results showed that heavy metals were transferred from distillery sludge to roots of *Cynodon dactylon*L. This work corroborated the finding of several researchers (Chandra and Yadav, 2011; Wei et al., 2010; Yoon et al., 2006). Although the physico-chemical properties of the sludge have revealed high pH as well other pollution parameters (TS, TSS, TDS, BOD, COD, lignin and chlorolignin), the secretion of organic compounds by *Cynodon dactylon* L and the rhizospheric bacteria could lower the sludge pH and enhance the metal accumulation. Given the high BAF results, it seems that *Cynodon dactylon* L. had the potential to accumulate hazardous compounds in the above-ground portion of the experiment and therefore more appropriate for phytoextraction technologies than phytostabilization technologies to clean up polluted surroundings (Li et al., 2007). The plant's metabolism, photosynthetic activity, metal concentration and bioavailability, organic matter and evapotranspiration rates can also affect the metal accumulation (Rajkumar et al., 2013).

Table 3 Bioconcentrationfactor (BCF) and Translocation factor (TF) of hyperaccumulator plant species growing at contaminated site of distillery waste sludge site.

	Cu	Ni	Pb	As	Fe	Mn	Cd	Zn
Bioconcentrationfactor (BCF)	25.98	48.89	20.10	15.08	92.99	2.00	1.91	5.00
Translocation factor (TF)	0.55	0.99	1.00	1.78	0.055	0.68	1.00	1.00

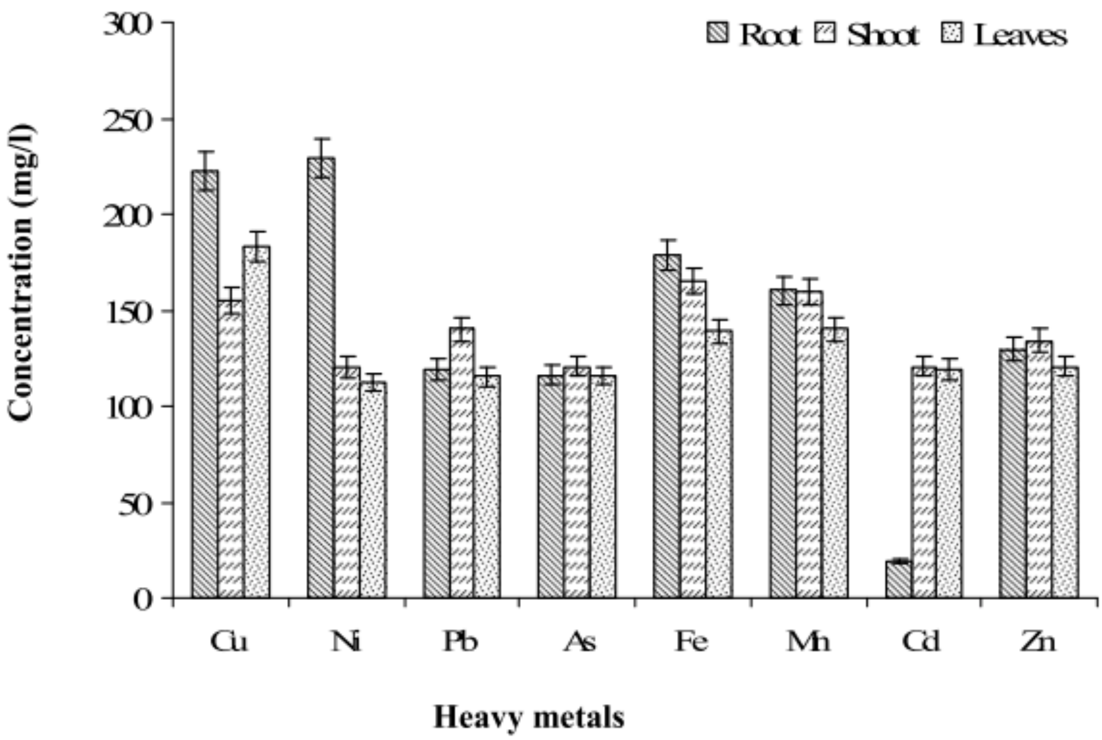

Fig. 2 Accumulation pattern of various heavy metals in Leaves, root and shoot, of *Cynodon dactylon* L. grown on distillery industry sludge contaminated site.

3.5. Assessment of ligninolytic enzyme activity

The capacity of bacteria to release extracellular enzymes (i.e., manganese peroxidase MnP, lignin peroxidase LiP and Laccase) via lignin complex breakdown was shown in Fig. 3. The figure shows the presence of MnP (35.98 U), LiP (23.98 U) and laccase (12.78U) production by the *Bacillus cereus* (RCS-4 MZ520573.1). The result provides an understanding of the mechanistic role of the PGPR in supporting phytoremediation of both the organic pollutants. The presence of ligninolytic enzymes activity in microbes enhances the degradation of recalcitrant pollutants (Chandra et al., 2011). Several lignin monomeric compounds had been produced or identified during the early phases of alcohol production. After three days of development, guaiacol and 4- ethyl guaiacol were generated by the bacteria, suggesting the dissolution of lignin. In the case of a variety of substrate compounds for which nutrients are required, the presence of all three extracellular ligninolytic enzymes in the *Bacillus cereus* (RCS-4 MZ520573.1) is essential; the enzymes oxidise the organic molecules' single-electron for free radical production. Laccase, which has many copper cofactors, generates a brown zone when guaiacol is present (Ladole et al., 2020; Kumar and Chandra, 2020; Tripathi et al. d & e). The PGPR was able to degrade the lignin to enable further microbial activities to take place to support the plant growth.

3.6. Plant growth-promoting traits of isolates

Plant growth-promoting microbes colonised on the surface or inner layer of the roots play a crucial role in directly or indirectly influencing plant growth and development. The PGPR isolated from *Cynodon dactylon* L. clearly played an important part relationship that enabled the invasive species to thrive in the contaminated environment. The mechanisms involved are highlighted below.

3.6.1. Production of IAA and hydrolytic enzymes

The most important auxin is IAA, which requires L-tryptophan as a substrate and regulates a variety of morphological and physiological plant functions (Glick, 2012; Singh et al., 2020). The maximum production capacity of IAA (45.87 mg L^{-1}) by *Bacillus cereus* (RCS-4 MZ520573.1) could be an indirect outcome of the extra plasmid-encoded genes, as the level of stress in the bacterium increases with the metabolic load (Fig. 3). In the medium, IAA production was confirmed by the pink color. Thus, *Bacillus cereus* (RCS-4 MZ520573.1) was a producer of IAA. *Bacillus cereus* as PGPR has been reported in wheat and potato (Rana et al., 2011; Kumar and Gera, 2014). Hydrolytic enzymes produce an increased amount of lytic enzymes in the cell wall; they function as agents for plant disease prevention by inducing lysis of microbial diseases in close proximity to the plant. The presence of halo zones around the colonies confirmed the production of hydrolytic enzymes by *Bacillus cereus* (RCS-4 MZ520573.1). The results showed that bacterial isolates producing hydrolytic enzymes and interacting with *Cynodon dactylon*L. rhizospheric sludge that could increase plant growth and N_2 fixation activity.

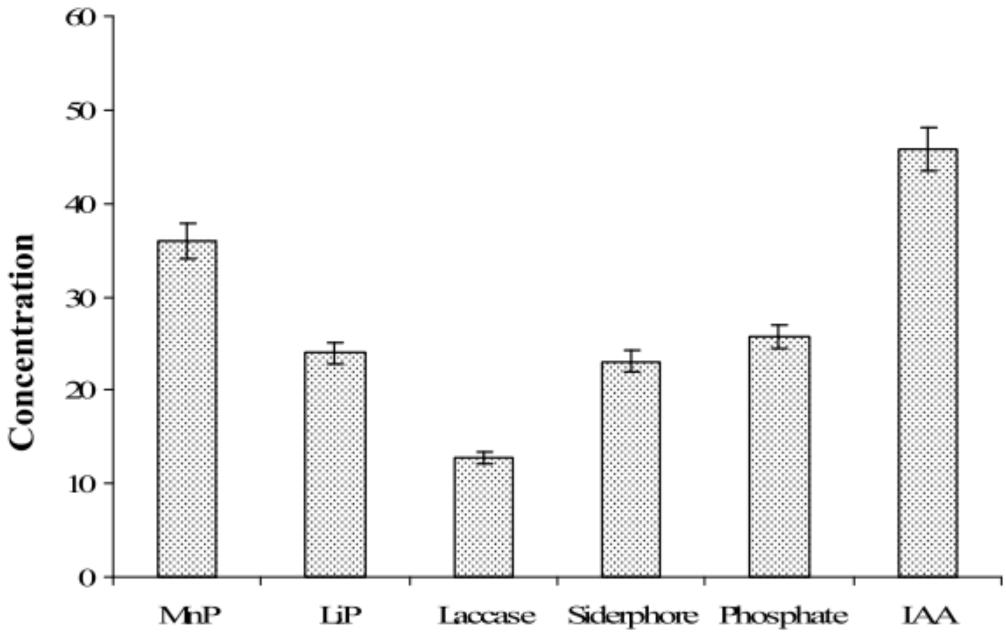

Fig. 3 Production of ligninolytic enzymes (U) and plant growth promoting activity (mg/L) by Bacillus cereus strain (RCS-4 MZ520573.1) isolated from Cynodon dactylon L. root of Contaminated site sludge. Lip = Lignin peroxidase; MnP = Magnese peroxidase.

3.6.2. Production of siderophore and nutrient solubilization activity

Bacillus cereus (RCS-4 MZ520573.1) influenced plant growth by the synthesis of siderophore. After 36 hours of incubation, the highest production of siderophores was detected, and subsequently production began to fall until it reached equilibrium state. Additionally, compared to $FeCl_3$, siderophores have a decreased affinity for metal complexes. Metals including Cd, Hg, and Co impaired productivity and growth of siderophores, possibly due to competitive metal binding, which enhanced cell death in bacterial cells. Siderophore-producing bacteria have been found to improve stomatal conductance and plant development in polluted environments by enhancing the absorption of iron from a pool of imported heavy metals. *Bacillus cereus* (RCS-4 MZ520573.1) also able to convert insoluble mineral phosphate such as tricalcium phosphate or hydroxyapatite into a soluble form. Around the inoculation of the spot, the isolated bacterial strain developed clear halo zone. Due to a decrease in the pH of Pikovskaya's medium containing bromothymol blue, the colour changed from blue to yellow during metabolite activity.

3.7. Bacterial 16S rRNA sequencing

The isolated bacterial strain was identified using 16S rRNA sequencing data was submitted to the NCBI using BLAST, a phylogenetic tree was constructed (Fig. 4). Moreover, 16S rRNA sequencing revealed that the isolated strain is related to *Bacillus cereus (RCS-4 MZ520573.1),* and the bacterial genomes were submitted to the GenBank public database under the accession number RCS-4 MZ520573.1. *Bacillus* sp. and *Pseudomonas* sp. are by far the most frequently reported genera of rhizobacteria that promote plant development (Zahid, 2015). The ability of *Stenotrophomonas*, *Pseudomonas,* and *Bacillus* sp strains to regulate pathogenic microorganisms and increase plant growth was shown.

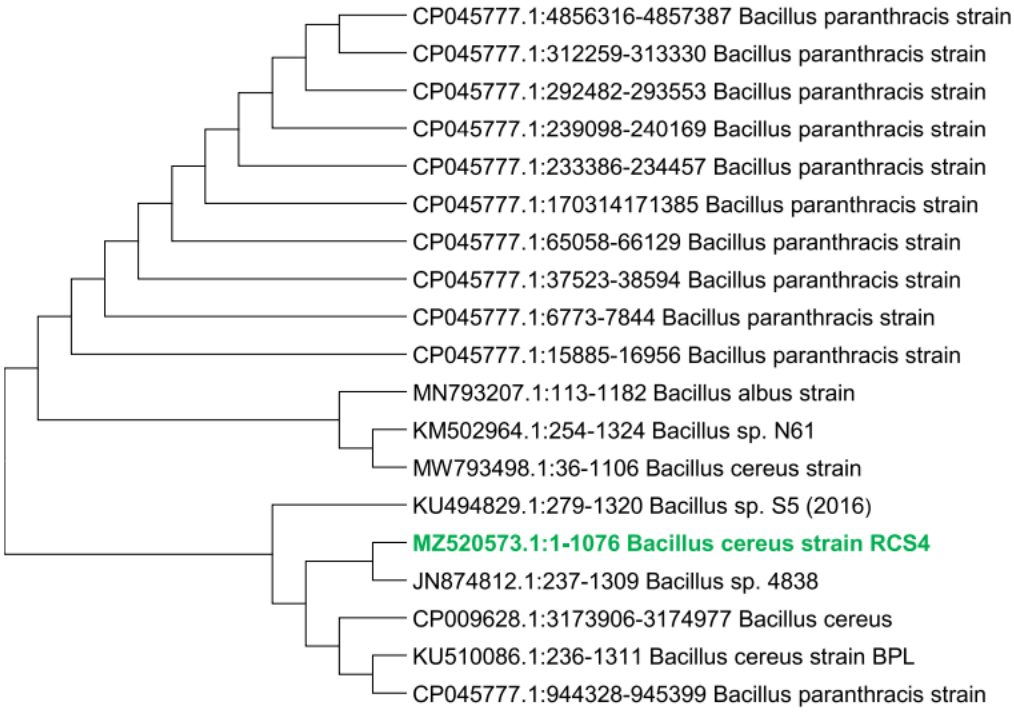

Fig. 4 Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd + Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 386 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

4. Significance of this study

The use of invasive species and their PGPR to phytoremediate contaminated land has received very little attention, despite this group of plants that often outcompete the native species. They are often adapted to hostile environment and has faster growth rate. They produced higher biomass, could either accumulate higher levels of pollutants. In this study we showed that the invasive species *Cynodon dactylon* L. is a hyper-accumulate and its microbiont, *Bacillus cereus* acted as PGPR to support the plant growth. The PGPR ability to produce extracellular ligninolytic enzymes, plant hormones, siderophores clearly enhanced the nutrient uptake by the phytobiont. Together, this partnership can be explored to produce a fast and sustainable way to remediate and restore contaminated sites. Given invasive plant species are global phenomenon, this study provided evidence that this much overlooked group and their microbionts could be utilised to bring benefit to the environment. Further work in the field is needed to fully quantify the efficacy of these plant species and their microbionts in comparison to phytoremediation using conventional established plants.

5. Conclusion

Sludge released from alcohol production processes after secondary treatment has become a significant environmental problem. *Cynodon dactylon* L. showed considerable in-situ phytoremediation capability, decreasing sludge parameters such as BOD, COD, TS, TSS, and TDS by more than 70%. The total phosphorus and nitrogen content dropped by 70% and 75%, respectively. However, the phenol and chlorophenol level was found to increase by 50% and 60%, respectively. The elimination of heavy metal resulted in decreases ranging from 40% to 70%. The plant's capacity to accumulate metals was aided by its high bioconcentration and translocation factor, and they all turned out to be hyperaccumulator plants. *Bacillus cereus* (RCS-4 MZ520573.1) produces PGPR activity such as IAA, ligninolytic and hydrolytic enzymes, siderophore, and nutrients, which is one of the qualities where the symbiotic microbe donates additional arsenals to the host to resist unfavourable impacts in stress environment. As a conclusion, the invasive plant species *Cynodon dactylon* L. can be employed on a field scale to restore the polluted site's ecosystem. More research is needed, however, to quantity and improves their effectiveness and integrate them with the present sludge treatment process. Additional, research of the detailed mechanisms involved in microbe-assisted phytoremediation is also required for the development of successful phytoremediation techniques.

Credit author contribution statement

Sonam Tripathi: Experiment designed, Writing – original draft, Writing – review & editing, Formal analysis. Sangeeta Yadav, Pooja Sharma, Diane Purchase and Asad Syed: Formal analysis, update, writing and review manuscript. Ram Chandra: Conceptualization, experiment design, Visualization, Project administration, Funding acquisition, reviewing of the manuscript.

Author statements

There are no conflicts in any authors. The corresponding author has all the authority regarding the manuscript behalf of all authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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