



Cadmium and lead removal by new bacterial isolates from coal and aluminum mines

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Abstract

Heavy metals such as cadmium have dangerous effects on ecosystem and human health. In this study, the bacteria diversity of soil samples of coal, salt and aluminum mines and water sample of Mareh wetland of Iran were investigated and their potential to cadmium removal was assessed. Based on partial sequencing of 16S region, the 64 isolates were identified that water sample of Mareh wetland showed high bacterial diversity. Among the isolated bacterial, 11 isolates from 10 different genus including *Leifsonia* sp., *Rhodococcus* sp., *Bacillus* sp., *Microbacterium* sp., *Enterobacter* sp., *Planomicrobium* sp., *Microbacterium* sp., *Thalassospira* sp., *Brevundimonas* sp., *Halomonas* sp. and *Micrococcaceae* sp. (could grow under 50 mg/L CdCl₂) were selected to consider the cadmium bioremediation potential. The *Microbacterium oxydans* CM3 and *Rhodococcus* sp. AM1 as new strains exhibited high ability to removal of cadmium and also degraded 58 and 39% of 400 mg/L lead after 72 h of incubation, respectively. Our result revealed that *M. oxydans* strain CM3 as natural way has a great potential for absorbing and degrading the heavy metal such as cadmium and lead.

Keywords Heavy metals · Bacterial · *Rhodococcus* sp. · *Bacillus* sp. · *Leifsonia* sp. · *M. oxydans* · Bioremediation

Introduction

Industrial activities and the application of various chemicals have contaminated ecosystem and increased the amount of toxic and dangerous substances such as heavy metals (Ayangbenro and Babalola 2017). Cadmium (Cd) is most dangerous and nonessential type of heavy metals that all its compounds are very toxic and non-biodegradable (Rebekic and Lončarić 2016). Cadmium increases the oxidative stress and effects on gene expression patterns (Bernhoft 2013), as well as has negative effects on food safety, plant production and human health (Saifullah et al. 2013; Rebekic and Lončarić 2016; Bernhoft 2013). The conventional methods such as physical (ion exchange, electrochemical treatment

and membrane separation) and chemical (neutralization and oxidation–reduction) methods that were applied to remove heavy metals from contaminated soil and water are not suitable to reduce the cadmium content in large scale and need the expensive equipment (Huang et al. 1988; Bai et al. 2008). The application of microbes such as bacterial and fungi to remove toxic elements from soil, water and air, known as bioremediation, is less expensive and compatible with ecosystem conditions. Recently the different isolates of bacterial species have been identified to use in bioremediation process of cadmium (Peng et al. 2018; Banerjee et al. 2015; Siripornadulsil and Siripornadulsil 2013; Manasi et al. 2014; Lin et al. 2016).

Isolation and identification of microorganisms from extreme environments could introduce isolates that have a high bioremediation potential. Siripornadulsil and Siripornadulsil (2013) identified 24 cadmium-tolerant bacteria isolates from soil samples of rice fields, and four isolates decreased the cadmium concentration in rice and promoted the seedlings growth. Manasi et al. (2014) isolated a new strain of *Halomonas* from electronic industry effluents that could able to absorb cadmium. Recently, Lin et al. (2016) isolated and evaluated three bacteria including *Pseudomonas aeruginosa*, *Stenotrophomonas acidaminiphila* and *Delftia*

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tsuruhatensis that among them, *P. aeruginosa* had high resistant under 2200 mg/L cadmium. Some eukaryotes such as yeasts and fungi have also been considered as cadmium absorption (Gomes et al. 2002; Ahluwalia and Goyal 2007; Mohsenzadeh and Shahrokhi 2014; Fazli et al. 2015). Also, some studies have investigated the cadmium bioremediation mechanisms, for instance Bai et al. (2008) reported that cadmium was removed and transformed on the cell wall in *Rhodobacter sphaeroides*. However, the ATPase pumps are involved in mechanism of cadmium absorption (Guo et al. 2010; Lee et al. 2001).

Our aims were the isolation, identification and comparison of new strains of cadmium-resistant bacterial from soil sample of coal, aluminum and salt mines and water of Mareh wetland and investigation of their ability to cadmium bioremediation to apply as bio-absorbing tools. Our results are the first report of bioremediation ability of new isolated bacterial strains. This study was performed in Shahrood University of Technology, Shahrood, Iran, from February 17, 2017, to October 26, 2018.

Materials and methods

Samples collection and bacterial isolation

Bacterial isolates were identified from soil samples at coal mine (36°21'36.6"N, 54°42'28.2"E), aluminum mine (37°03'18.2"N, 56°28'52.5"E), salt mine (35°11'00.8"N, 52°09'27.2"E) and water of Mareh wetland (34°57'46.0"N, 51°18'19.6"E) from Semnan, North Khorasan and Qom Provinces of Iran (Fig. 1). For extracting the cadmium from soil samples, DTPA method (Wang et al. 2019) was used and the cadmium content was measured using atomic absorption spectrometer (GBC sensAA). For isolating bacterial, one gram of soil samples were used to prepare the serial

dilution (0.1, 0.01, 0.001 and 0.0001) in double sterile water and 200 µL of each dilution solution was cultured in LB agar medium (Merck KGaA). The solid medium plates were incubated in an incubator with 30 ± 2 °C for 72 h. According to the morphology and color, different bacterial colonies were selected and subcultured in solid LB agar medium at 30 ± 2 °C for 48 h, and then the pure colonies were used to molecular identification and determination of cadmium absorption.

Molecular identification of isolated bacterial

The genomic DNA of selected colonies was extracted using heat (90 °C for 3 min) and cold (−20 °C for 3 min) cycles (in five cycles). The quality and quantity of extracted DNA were analyzed by Nano Photometer (Implen N50) and agarose gel 1%. The universal primers (F4: 5'-CCGCCTGGG GAGTACG-3' and Rn2: 5'-GACGGGCGGTGTGTAC-3') were used to amplify partial sequence of 16S rRNA region of isolated bacterial. The sequence of amplified DNA fragments was determined by MacroGen Inc., Seoul, South Korea. Then, the BLASTn tool of NCBI databank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was applied to find the related bacterial to our query (DNA sequences). The partial sequences of 16S rRNA region of new strains were submitted and recorded in NCBI databank with sequence IDs: LC427568–LC427573 and LC429388–LC429391. Finally, the evolutionary relationships among isolated bacterial were conducted by a neighbor-joining method using MEGA7 (Kumar et al. 2016) based on the 16S partial sequences of bacterial.

Assay the cadmium bioremediation potential

In order to study the potential of bacterial bioremediation, 500 µL of liquid medium of each isolated bacterial

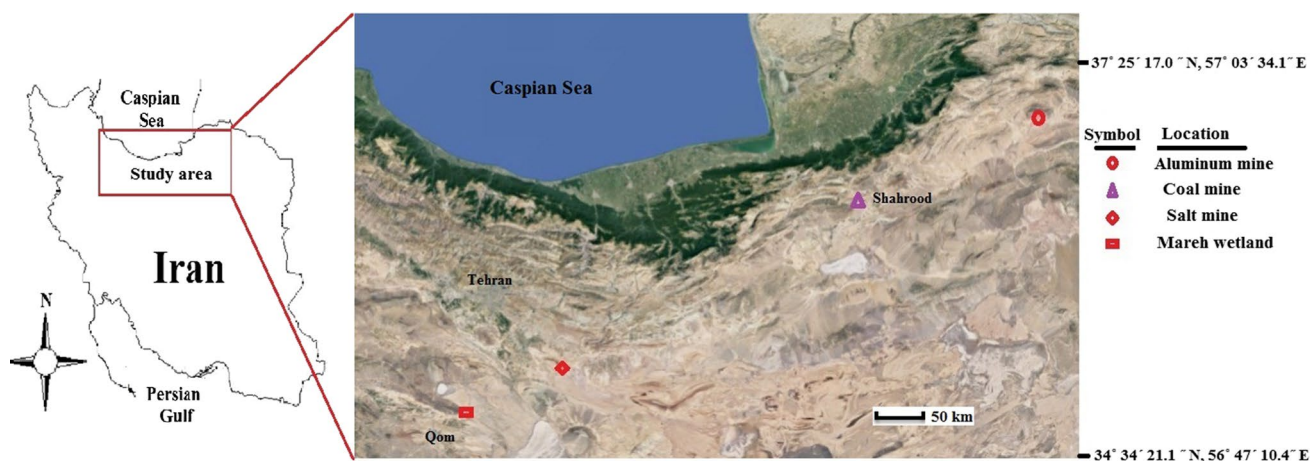


Fig. 1 The location of the collection samples

were separately cultured in 50 mL of LB broth medium that contaminated at 50, 100, 150, 200, 300, 400 and 500 mg/L of cadmium chloride (CdCl_2), and samples were placed in incubator shaker at 30 ± 2 °C and 160 rpm for 72 h, and then the cadmium concentration of each samples and control (bacterial free) was determined using atomic absorption spectrometer. Also, two isolates that showed high cadmium absorption were assessed under 400 mg/L lead at 30 ± 2 °C and 160 rpm. The percentage of removed cadmium and lead by isolated bacterial was calculated by the following equation (Wu et al. 2016):

$$\text{Removal\%} = [(C_i - C_f)/C_i] \times 100$$

that C_i and C_f are the initial and final concentration of cadmium/lead in solution, respectively.

Growth rate of selected isolates

The growth rates of two isolates which showed high cadmium absorption were evaluated in liquid LB medium contaminated with 400 mg/L lead and cadmium separately. The samples were incubated at 30 ± 2 °C and 160 rpm and growth curve was determined at 0, 12, 24, 48 and 72 h after incubation using wavelength spectrophotometer at 600 nm. Also, pH changes of LB medium + bacterial, LB medium + bacterial + 400 mg/L lead and LB medium + bacterial + 400 mg/L cadmium in time series (0, 12, 24, 48 and 72 h after incubation) were examined.

Data analysis

All experiments were run in triplicate, and one way analysis of variance (ANOVA) was used to detect significant bioremediation potential of each isolated strains using SPSS software version 17 (SPSS 2008). The all graphs of cadmium removal percentage for each isolate were drawn using GraphPad Prism software package version 6.0, according to values of mean and standard division.

Results and discussion

Physicochemical properties of studied samples

The physicochemical properties of soil and water samples demonstrated that the water samples of Mareh wetland had the highest pH and EC values (Table 1). Cadmium concentration range of studied samples varied from < 1 to 32 mg/L that soil sample of inside coal mine showed high concentration of cadmium. The most bacterial diversity was observed at water samples of Mareh wetland (17 isolates of 6 genus), while it was the minimum (five isolates of two genus) at soil samples from inside coal mine. The soil microorganisms such as bacterial have critical roles in promoting plant health and regulating soil fertility that diversity of bacterial is influenced by physicochemical properties of soil and water such as organic matter (Boopathy 2000; Faoro et al. 2010; Lauber et al. 2008). Our results indicated that bacterial diversity is impressed by environmental characteristics.

Evolutionary analysis of isolated bacterial

The PCR products of 64 isolated bacterial were sequenced and analyzed against the identified genome of bacterial using BLASTn tool (Data not shown). The 10 of 11 tested bacterial had 99% similarity of known bacterial that they were recorded as new strains in NCBI database, and 10 different genus of bacterial were identified based on BLAST result (Table 2). An isolate (SM22) which was isolated from soil samples around salt mine had 88% genetic relationship similarity to *Micrococcaceae* bacterium. It could be introduced as new species, although additional testing is required. The 11 bacterial isolates that were tested in cadmium bioremediation were analyzed to estimate the relationships and diversity based on partial sequences of 16S region (Fig. 2). Phylogenetic tree analysis revealed that isolated bacterial had high diversity that it is possible to select the appropriate bacterial to practical application such as heavy metals absorption (Paul et al. 2005). The studied isolates were clustered into two main groups that isolates from second group

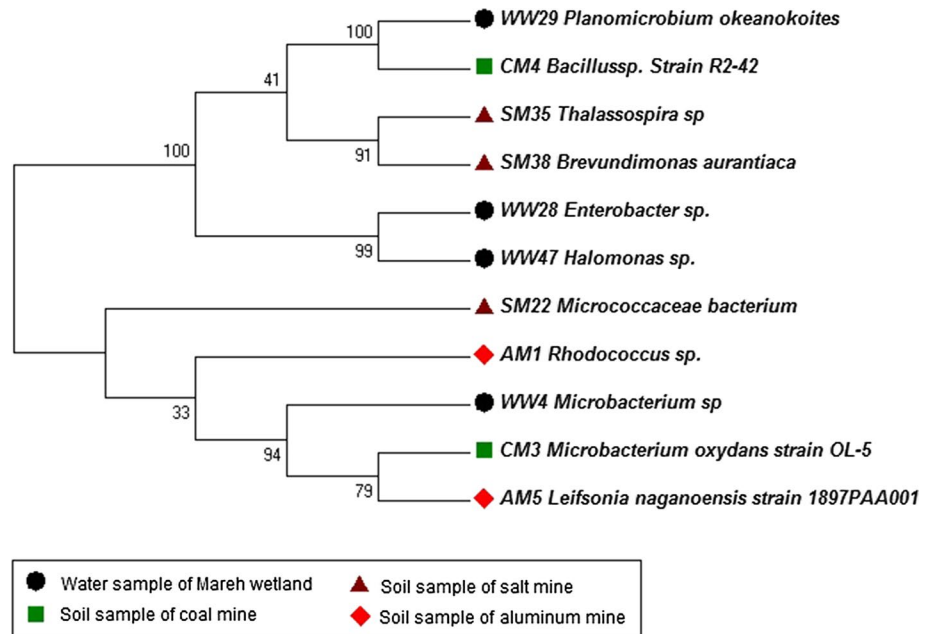
Table 1 Physicochemical properties of studied soil and water samples

Studied samples	Clay%	Silt%	Sand%	EC dS/m-1	pH	Cd ($\mu\text{g}/\text{mg}$)	Number of identified isolates
Inside aluminum mine	19.50	12.00	68.50	2.57 ± 0.06	7.97 ± 0.15	0.021	7 (3 genus)
Outside aluminum mine	2.00	14.00	84.00	1.00 ± 0.10	9.80 ± 0.10	<0.001	13 (5 genus)
Inside coal mine	2.00	2.00	96.00	3.27 ± 0.40	5.83 ± 0.21	0.032	5 (2 genus)
Outside coal mine	7.50	21.00	71.50	2.27 ± 0.12	7.87 ± 0.15	0.016	14 (5 genus)
Outside salt mine	1.50	2.00	96.50	9.80 ± 0.13	10.20 ± 0.15	0.011	8 (4 genus)
Water of Mareh wetland	–	–	–	10.20 ± 0.11	10.50 ± 0.18	0.025	17 (6 genus)



Table 2 Molecular identification of cadmium-resistant isolates based on result of BLASTn tool

Strain	Accession number	Location	Similarly	Identity%	E value
AM5	LC427571	Aluminum mine	<i>Leifsonia naganoensis</i>	99	0.000
AM1	LC427570	Aluminum mine	<i>Rhodococcus</i> sp.	99	0.000
CM4	LC427569	Coal mine	<i>Bacillus</i> sp.	99	0.000
CM3	LC427568	Coal mine	<i>M. oxydans</i>	99	0.000
WW28	LC427572	Mareh wetland	<i>Enterobacter</i> sp.	99	0.000
WW29	LC427573	Mareh wetland	<i>Planomicrobium okeanokoites</i>	99	0.000
WW4	LC429388	Mareh wetland	<i>Microbacterium</i> sp.	99	0.000
SM35	LC429389	Salt mine	<i>Thalassospira</i> sp.	99	0.000
SM38	LC429390	Salt mine	<i>Brevundimonas aurantiaca</i>	99	0.000
WW47	LC429391	Mareh wetland	<i>Halomonas</i> sp.	99	0.000
SM22	–	Salt mine	<i>Micrococcaceae bacterium</i>	88	0.000

Fig. 2 Phylogenetic analysis of isolated bacterial based on partial sequence of 16S region by neighbor-joining method with 1000 bootstrap using MEGA 7 software

had more diversity and they also showed high cadmium bioremediation potential.

The bioremediation potential of isolated bacterial

Among the isolated bacterial (64 isolates), 11 isolates could grow in contaminated medium with 50 mg/L of CdCl₂ and they were selected to assay the bioremediation potential in different concentrations of cadmium (100, 150, 200, 300, 400 and 500 mg/L). The results of ANOVA revealed significant differences ($P \leq 0.05$) between isolated strains in different cadmium concentrations (data are not shown). The result revealed that an isolate (CM3) which had the tight genetic relationship with *Microbacterium oxydans* showed a high potential to cadmium absorption in 100 and 150 mg/L of CdCl₂ (Fig. 3). The isolated bacterial from soil samples of salt mine (SM22, 35 and 38) and an isolate from Mareh

wetland (WW47) could not grow under 150 mg/L of CdCl₂. The *M. oxydans* CM3 could remove 43 and 29% of cadmium after 72 h from LB broth medium contaminated with 100 and 150 mg/L of CdCl₂, respectively.

All bacterial isolates from soil sample of salt mine and water sample of Mareh wetland could not grow at high concentrations of cadmium (200, 300, 400 and 500 mg/L). According to Hacıoglu and Tosunoglu (2014), heavy metals may be caused mutations and changed the function and structure of microbial population that affected on growth and adaptability or reason the death of microorganisms. The *M. oxydans* CM3, *Leifsonia naganoensis* AM5, *Rhodococcus* sp. AM1 and *Bacillus* sp. CM4 were tested under cadmium concentrations > 200 mg/L. The *M. oxydans* CM3 could reduce cadmium ion more than other isolates and it could remove 15% of 500 mg/L cadmium after 72 h (Fig. 4). The high bioremediation potential of *Microbacterium* sp.



Fig. 3 The removal% of CdCl_2 from liquid medium contaminated with 100 and 150 mg/L cadmium using isolated bacterial

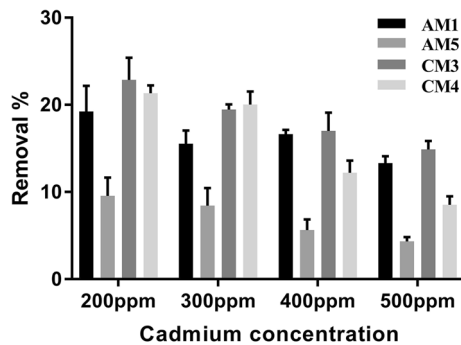
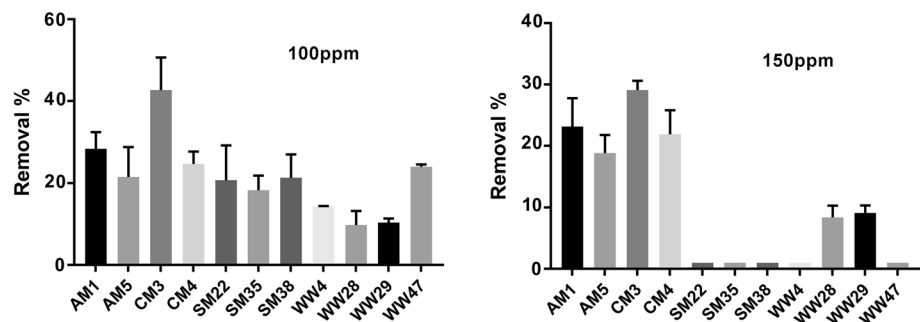


Fig. 4 The removal% of CdCl_2 from liquid medium contaminated with 200, 300, 400 and 500 mg/L cadmium using isolated bacterial

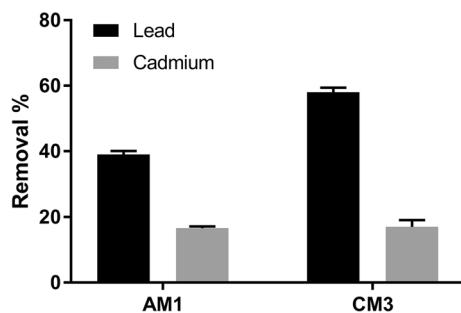


Fig. 5 The removal% of cadmium and lead from liquid medium contaminated with 400 mg/L using *M. oxydans* CM3 and *Rhodococcus* sp. AM1

has been reported under different heavy metals. Wang et al. (2016) reported that *M. oxydans* has the high phenol-degrading ability and it could degrade > 95% of phenol in 500 mg/L concentration after 88 h. Also, three strains of isolated *M. oxydans* from vicinity of a radioactive waste depository showed equal resistance to uranium, copper, chromium, lead and silver (Nedelkova et al. 2007).

The strains of *Rhodococcus* sp. have high potential to degrade the hazardous materials such petroleum products (Quek et al. 2006), *p*-nitrophenol (Zhang et al. 2009) and heavy metals including cadmium and lead (Figs. 4, 5).

Rhodococcus sp. strain AM1 showed the absorption ability of cadmium, and it could remove 13% of 500 mg/L concentration after 72 h. *Bacillus* sp. CM4 also exhibited the high reduction (> 20%) of cadmium in 300 mg/L concentration; however, it had lower efficiency in 400 and 500 mg/L concentrations (Fig. 4). Wu et al. (2016) identified the cadmium-resistant bacterial strains that had high similarity with *Bacillus cereus* and they reported that *B. cereus* S5 has high absorption capacity of cadmium ion. Also the strains of *Bacillus* sp., which were isolated from a stream in Manaus-Amazon (Igarape do Quarenta) of Brazil, exhibited the reduction (> 50%) in hexavalent chromium after 72 h of incubation (Teles et al. 2018).

Assay the lead bioremediation

Microbacterium oxydans CM3 and *Rhodococcus* sp. AM1 were also evaluated on 400 mg/L of lead (Pb(II)) and the results revealed that *Rhodococcus* sp. AM1 and *M. oxydans* CM3 decreased 39% and 58% of lead after 72 h, respectively (Figs. 5, 6). The genus microbacterium and rhodococcus are Gram-positive bacterial which contain peptidoglycan, teichoic and teichuronic acids in their cell wall where the lead can be linked there (Beveridge and Fyfe 1985). The compositions of cell wall as well as phosphate, hydroxyl, carboxyl and sulfate groups play critical role affecting on bacterial biosorbing and biosorption properties (Vijayaraghavan and Yun 2008; Jarosławiecka and Piotrowska-Seget 2014). Two selected isolates (CM3 and AM1) were more resistance to Pb(II) than cadmium under same conditions. Bacterial have different mechanisms such as extracellular and intracellular precipitation, confiscation as insoluble phosphates, ion efflux (including *P*-type ATPase) and transport systems (*PbrA*, *CadA* and *ZntA* pumps) to withstand the lead effects (Jarosławiecka and Piotrowska-Seget 2014; Lee et al. 2001), that these mechanisms cause different responses. Bacterial strains may precipitate Pb(II) on the cell surface as phosphate crystals (Levinson et al. 1996); however, someone such as *Staphylococcus aureus* could precipitate Pb(II) as phosphate $\text{Pb}_3(\text{PO}_4)_2$ on inside cell (Levinson and Mahler 1998). *P*-type ATPase pumps

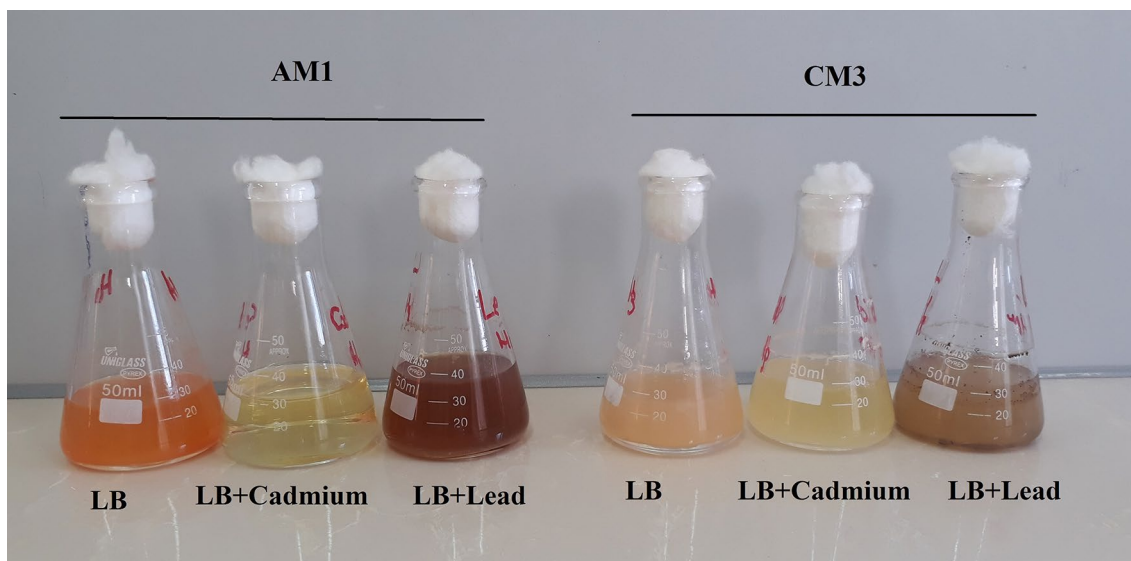


Fig. 6 The comparison of colony color between *Rhodococcus* sp. AM1 and *M. oxydans* CM3 in 400 mg/L of cadmium/lead

are the most effective mechanisms which provide resistance to the toxicity of heavy metals including cadmium and lead (Jarosławiecka and Piotrowska-Seget 2014). It seems that *M. oxydans* CM3 has mechanisms to reduce the lead harmful effects. Our results exhibited that *M. oxydans* CM3 as a new bacterial strain could grow under strict conditions and as a natural way has a high potential for using in bioremediation processes.

Growth rates of *M. oxydans* CM3 and *Rhodococcus* sp. AM1

In order to determine the growth rate, *M. oxydans* CM3 and *Rhodococcus* sp. AM1 were cultured in normal medium (only LB broth) and contaminated with 400 mg/L of

cadmium/lead. *Microbacterium oxydans* CM3 and *Rhodococcus* sp. AM1 showed maximum growth rate after 12 h in normal medium (Fig. 7). When the isolates exposed to cadmium and lead, they displayed high growth rate after 48 h. It seems that the cadmium and lead delay the growth rate of selected isolates, although cadmium was more effective. Previously study revealed that the bacterial isolates are adaptable in contaminated medium during the first 24 h and their growth is lower than normal conditions (Teles et al. 2018).

The physicochemical factors such as pH influence the microbe's bioremediation (Srivastava et al. 2014). Investigation of pH change in different mediums which were treated with 400 mg/L cadmium or lead revealed that the pH value was increased during the growth of *M. oxydans* CM3 and *Rhodococcus* sp. AM1 (Table 3). The microorganisms

Fig. 7 Growth profile of *Rhodococcus* sp. AM1 and *M. oxydans* CM3 under control (LB broth) and contaminated LB broth medium with 400 mg/L of cadmium/lead

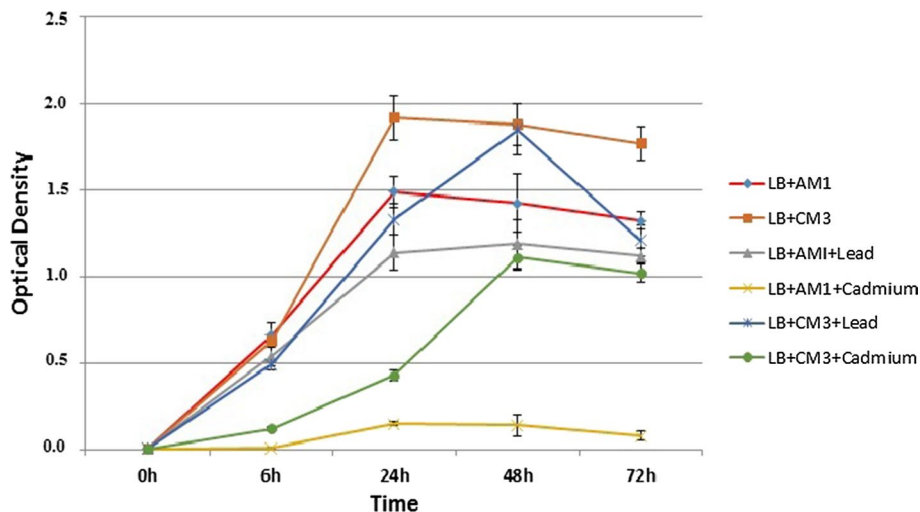


Table 3 pH modification in different mediums

Conditions	Time				
	0 h	6 h	24 h	48 h	72 h
LB + AM1	7.65	7.89	8.21	8.32	8.34
LB + CM3	7.61	7.87	8.33	8.32	8.46
LB + AM1 + Lead	7.72	7.90	8.20	8.49	8.44
LB + AM1 + Cadmium	7.85	7.90	7.95	8.00	8.08
LB + CM3 + Lead	7.59	7.85	8.11	8.44	8.38
LB + CM3 + Cadmium	7.72	7.94	8.12	8.34	8.34

such as bacterial could change the medium condition for more compatibility (Somenahally et al. 2013). The most pH change observed in the first 24 and 48 h after culturing the *M. oxydans* CM3 in normal LB and LB contaminated with 400 mg/L cadmium, respectively. Interestingly, when *M. oxydans* CM3 had high growth rate, the pH was more changed.

Conclusion

Here, the bacterial diversity of different regions was assessed, and 64 isolates with high variation were identified. The ability of cadmium bioremediation of isolated bacterial was investigated, and from among them, 11 isolates were selected as cadmium resistance. Among cadmium-resistant bacterial, *M. oxydans* CM3 and *Rhodococcus* sp. AM1 exhibited high ability to removal of cadmium. Also, *M. oxydans* CM3 was able to degrade the lead and reduced 59% of 400 mg/L lead after 72 h. Our results enclosed that *M. oxydans* CM3 and *Rhodococcus* sp. AM1 as new bacterial strains have high bioremediation potential to reduce the toxicity of heavy metals such as cadmium and lead. The cadmium and lead bioremediation mechanisms of new isolated strains should be further studied in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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