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Isolation of Extremely Heavy Metal Resistant Strains of Rare Actinomycetes from High Metal Content Soils in Iran

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ABSTRACT: Using of microorganisms to remove industrial pollutions of environment is an efficient method that mimics natural process and decrease remediation cost. In this study, 450 actinomycete strains were isolated from high metal content soils in Iran. Heavy metal salt solutions in different concentrations were used to isolate the resistant strains. 40 resistant actinomycetes which they showed highly resistance to the most applied heavy metals were selected. After examination of these strains in broth media with high levels of metals, 13 resistant isolates were selected as high resistant actinomycetes. Some strains showed resistance to 140 mM $ZnCl_2$, 7 mM $CuSO_4$, 9.2 mM $CdCl_2$, and 60 mM $NiCl_2$. Molecular identification revealed that resistant strains belonged to different actinomycetes genera including *Streptomyces, Nonomuraea, Saccharothrix, Streptosporangium* and *Promicromonospora* which was the first report on occurrence of highly metal resistant strains in *Nonomuraea, Streptosporangium* and *Promicromonospora* genera. Cadmium removal studies by *Promicromonospora* sp. UTMC 2243 indicated 96.5% reduction in cadmium residual concentration, reflecting cadmium removal capacity in *Promicromonospora* sp. UTMC 2243.

Key words: Actinomycetes**,** Heavy metals, Cadmium, Nonomuraea, Promicromonospora, Streptosporangium

INTRODUCTION

Industrialization of urban areas and human activities such as plating, tanneries, battery, mining and using of chemical fertilizers cause the environment to be polluted with heavy metals more than ever (Oztürk *et al*., 2004). Cadmium, lead, mercury, copper, arsenic, zinc and nickel release to the ecosystem plainly, and enter to the food chain. Releasing of these metals to the environment can cause serious health hazards for human and ecosystem (Selvin *et al*., 2009). Although some of these metals like copper, zinc, manganese and cobalt that be denominated trace elements, are necessary and vital in low concentrations for some functions, but in high concentrations have toxic effects for organisms. Some non-essential heavy metals such as mercury, cadmium and arsenic do not have any biological beneficial, and they are utterly toxic (Srivastava & Majumder, 2008). Removal of these pollutants from environment has been became an essential subject in recent decades. Some of microorganisms not only tolerate high concentrations of heavy metals in terrestrial and aquatic ecosystems, but also they can reduce the bioavailability of toxic metals for other organisms. Efflux pumps, reduction, enzymatic detoxification, permeability barriers, and metal sequestration have been found as major resistance mechanisms to heavy metals in microorganisms (Spain, 2003). Bacteria have a high surface to volume ratio and therefore high capacity in bioabsorption. This large contact surface with their environment along with its negative charge enables them to absorb metal cations (Collins & Stotzky, 1992). Actinomycetes, the filamentous bacteria with high G+C content have been known as a considerable group in various environmental habitats (Edwards, 1993). The great potency of actinomycetes in production of several secondary metabolites and enzymes can be considered as the main factors in resistancy to environmental stress. They have a large genome in comparison to the other bacteria which is the origin of their high potentials and application in various aspects of biotechnology. Despite previous reports of metal resistant actinomycetes, strains which resist to more than the reported concentration still are needed in bioremediation industry. Relying on the predicted genomic potential of actinomycetes, attempt for

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finding more resistant strains of actinomycetes is justified. The aim of present study was isolation, screening, and identification of extreme resistant actinomycetes to toxic metals with the potential of bioremediation implementation.

MATERIALS & METHODS

Investigation and recovery of actinomycete strains were carried out on soils which were inherently or artificially had higher metal content based on the geological pattern. Soil samples (30) were collected (5-20 cm depth) in sterile plastic bags from selected sites of industrial and agricultural sewages, mining areas and fields. The soil samples were kept at 4 °C until culturing. The samples were air dried for 72 h, and then were grounded and sieved through a 2-mm sieve (Khan *et al*., 2010).

Primary screening media: Tap water agar (15 g agar, 1000 ml tap water; pH 7.0) (Ara *et al*., 2012), soil extract agar (15 g agar, 1000 ml soil extract; pH 7.0) (Hamaki *et al*., 2005) and glycerol casein agar (glycerol 10g; Casein vitamin free 0.3g; KNO_3 2g; KH_2PO_4 2g; NaCl 2g; $MgSO_4.7H_2O$ 0.05g; $CaCO_3$ 0.02g; $FeSO_4.7H_2O$ 0.01g; agar 15g; pH 7.0) (Malibari, 1991). The media were supplemented with filter sterilized (0.22 µm) heavy metal salt solutions (0.5 mM cadmium chloride, 8.5 mM nickel chloride, 1.5 mM copper sulphate and 20 mM zinc chloride) after autoclave. Purification and maintenance medium: ISP2 medium (containing per liter: glucose 4 g; yeast extract 4 g; malt 10 g; agar 15 g; pH 7.2) was used for purification and preservation of the strains (Shirling & Gottlieb, 1966). Medium for determination of minimum inhibitory concentration: minimal medium (MM) (containing per liter: L-asparagine 0.5 g; K_2HPO_4 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; agar 18 g and glucose 10.0 g) (Yadav *et al*., 2010) supplemented by metal salt solution at concentration of 3 mM $CuSO_4$, 2 mM $CdCl_2$, 10 mM $NiCl_2$, 30 mM $ZnCl_2$.

The treated soil samples were diluted using sterilized normal saline (0.9% NaCl). Each dilution (100 µl) was spread onto the isolation media. The plates were incubated for 14 days at 28 °C and putative actinomycete colonies were purified on ISP2 medium. In order to differentiate resistant actinomycete isolates to high concentration of metals the strains were cultured in MM agar and incubated for 14 days at 28 °C. Resistant actinomycetes obtained on MM agar, were inoculated on MM broth with metals salt solutions in a range of concentrations (3.5, 7, 10.5 and 14 mM of $CuSO_4$, 2.3, 4.6, 6.9 and 9.2 mM of $CdCl_2$, 15, 30, 45, 60 and 120 mM of $NiCl_2$, 35, 70, 105, and 140 mM of $ZnCl_2$) and incubated at 28 °C for 48 h. Minimum metal concentrations that prevent visible growth in liquid medium were reported as MIC of the metals (Lakshmipathy *et al*., 2010).

For determination of cadmium removal ability, spore suspension of the isolate was inoculated into MM broth supplemented with 5.5 mM. The cultures were incubated at 100 rpm, 28 °C for 10 days. The cells were harvested with centrifugation (4000 *g*) for 15 min and the supernatant was analyzed for metal residual content by measuring of the absorbance at 600 nm by atomic absorption spectrophotometry. For growth kinetics studies, selected strains were inoculated into MM and soil extract broths supplemented with high concentrations of cadmium (4.5 and 9.2 mM). During incubation time, the wet and dry weights of biomass were measured and growth kinetics was determined. All tests were performed in triplicates (Yadav *et al*., 2010). For characterization of the isolates, they were grown in ISP2 broth and the cells were collected by centrifugation. Diaminopimelic acid (DAP) analysis was performed by thin-layer chromatography (cellulose TLC plates 20 × 20 cm; Merck) in methanol: distilled water: HCl 6 M: pyridine (80: 17.5: 4.5: 10). The plates were dried, sprayed with ninhydrin reagent (0.1% ninhydrin in acetone) and heated at 100 °C for 2 min (Becker *et al*., 1965). In order to study the morphological features of the aerial mycelia, the slide culture of the isolates was prepared on ISP2 agar and the plates were incubated for 14 days at 28 °C using the cover-slip technique (Kawato & Shinobu, 1959).

The selected isolates were grown in LB broth for 3 days and the cell mass were collected with centrifugation and washed twice with distilled water. Extraction of DNA was carried out using chloroform-phenol methode and the DNA samples were kept at -20 °C (Sambrook *et al*., 1989). Bacterial 16S rRNA gene was amplified using the eubacterial universal 16S rRNA gene primers (forward primer: 5'-AAGAGTTT-GATCATGGCTCAG-'3 and reverse primer: 5'-AGGAGGTGATCCAACCGCA-'3) (Kumar *et al*., 2010). PCR products were purified using the Expin combo GP, (Geneall, 112-1150) kit and were run using 0.8% agarose gel. After staining by ethidium bromide, they were visualized using an Image Analyzer Gel Doc.

Multiple sequence alignment was performed using CLUSTAL X program, and phylogenetic tree was constructed using MEGA version 5 software package. Genetic distance analysis was determined with the maximum-likelihood method and Bootstrap values were based on 1000 replications.

RESULTS & DISCUSSION

From various soil samples collected from different ecosystems, including heavy metal polluted area, 450 morphologically different actinomycete isolates were obtained after primary screening on media containing heavy metals (Fig. 1). Forty isolates were able to grow

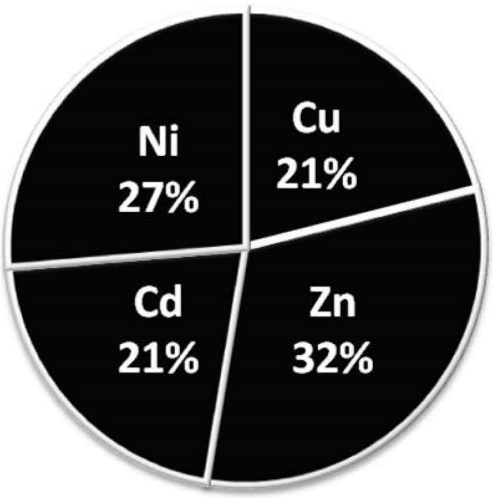

Fig. 1. Overall resistance ratio of isolated actinomycetes on metal containing media to 3.5 mM Cu, 2.3 mM Cd, 35 mM Zn and 15 mM Ni.

on MM agar containing higher levels of metal salt solutions than concentrations which were applied in primary screening. Thirteen strains were selected for determination of minimum inhibitory concentration due to their higher resistivity to tested metals among 40 resistant strains (Table 1). Resistant actinomycete strains up to 140 mM $ZnCl_2$, 9.2 mM $CdCl_2$, 7 mM $CuSO_4$, and 60 mM $NiCl_2$ along with the report of extreme heavy metal resistance in new taxa including *Saccharothrix*, *Promicromonospora* and *Nonomuraea* genera comprised the peculiar outcome of the current study.

According to the cell wall amino acids analyzes and aerial mycelium properties, between the 13 selected isolates (Table 1), eight strains were identified as the member of the *Streptomyces* genus and the remnants of strains were belonged to rare actinomycete genera. The resistant rare actinomycetes isolated in this study are accessible in Gene bank according to the following accession numbers. *Saccharothrix* sp. UTMC 2163 (KF432135), *Saccharothrix* sp. UTMC 2185 (KF660595), *Streptosporangium* sp. UTMC 2188 (KF577727), *Promicromonospora* sp. UTMC 2243 (KF432136) and *Nonomuraea* sp. UTMC 2237 (KF601692). Due to resistance to a broad range of metals and high resistance to cadmium, *Streptomyces* sp. UTMC 2241 and *Promicromonospora* sp. UTMC 2243 strains were selected for subjecting to growth kinetic investigation. Cadmium was selected for metal removal study since it was the most toxic metal amongst the surveyed metals in this study.

Table 1. MICs of the most resistant isolates. The minimum inhibitory concentrations of thirteen resistant isolates in MM broth. Strains UTMC 2241 and UTMC 2243 showed the highest level of resistant to Cd, Zn and Cu

Strain	MICs (mM)			
	Ni	Cd	Zn	Cu
Streptomyces sp. UTMC 2179	45	4.6	140	<3.5
Streptomyces sp.UTMC 2241	45	9.2	70	7
Saccharothrix sp.UTMC 2163	<15	4.6	70	3.5
Saccharothrix sp. UTMC 2185	60	<2.3	105	7
Streptomyces sp.UTMC 2195	60	6.9	70	3.5
Streptomyces sp.UTMC 2200	60	<2.3	140	7
Streptomyces sp.UTMC 2236	30	<2.3	70	<3.5
Streptomyces sp.UTMC 2207	30	6.9	<35	<3.5
Streptomyces sp.UTMC 2190	<15	<2.3	140	<3.5
Promicromonospora sp.UTMC 2243	<15	9.2	140	7
Streptosporangium sp. UTMC 2188	45	<2.3	105	<3.5
Streptomyces sp.UTMC 2244	<15	2.3	70	3.5
Nonomuraea sp.UTMC 2237	60	<2.3	<35	<3.5

Table 2. Morphological properties of *Promicromonospora* sp. UTMC 2243 on ISP media

Medium	Growth	Mass Spore production	Aerial mycelium	Substrate mycelium
Yeast extract/malt extract (ISP 2)	Good	+	White	White
Oatmeal agar (ISP 3)	Moderate	-	White	White
Inorganic salts/starch agar (ISP 4)	Good	+	Cream	Cream
Glycerol/asparagine agar (ISP 5)	Good	+	White	Cream
Peptone-yeast extract-iron agar (ISP 6)	Poor	-	Cream	Cream
Tyrosine agar (ISP 7)	Good	+	Light yellow	Yellow

Table 3. Comparison of resistivity level to heavy metals between previous studies and current study

	Maximum resistivity obtained in this study		Maximum resistivity reported in the literature		
	Concentration (mM)	Strain	Concentration (mM)	Strain	Reference
Zn	140	*Streptomyces* sp. UTMC 2179 *Streptomyces* sp.UTMC 2200 *Streptomyces* sp.UTMC 2190	100	*Streptomyces mirabilis*	Haferburg *et al*., 2009
Cd	9.2	*Promicromonospora* sp.UTMC 2243 *Promicromonospora* sp.UTMC 2243 *Streptomyces* sp.UTMC 2241	0.54	*Streptomyces* sp.	Sineriz *et al*., 2009
Cu	7	*Streptomyces* sp.UTMC 2241 *Saccharothrix* sp. UTMC 2185 *Streptomyces* sp.UTMC 2200	6.25	*Streptomyces* sp.	Albarracin *et al*., 2005
Ni	60	*Promicromonospora* sp.UTMC 2243 *Saccharothrix* sp. UTMC 2185 *Streptomyces* sp.UTMC 2195 *Streptomyces* sp.UTMC 2200 *Nonomuraea* sp.UTMC 2237	100	*Streptomyces mirabilis*	Haferburg *et al*., 2009

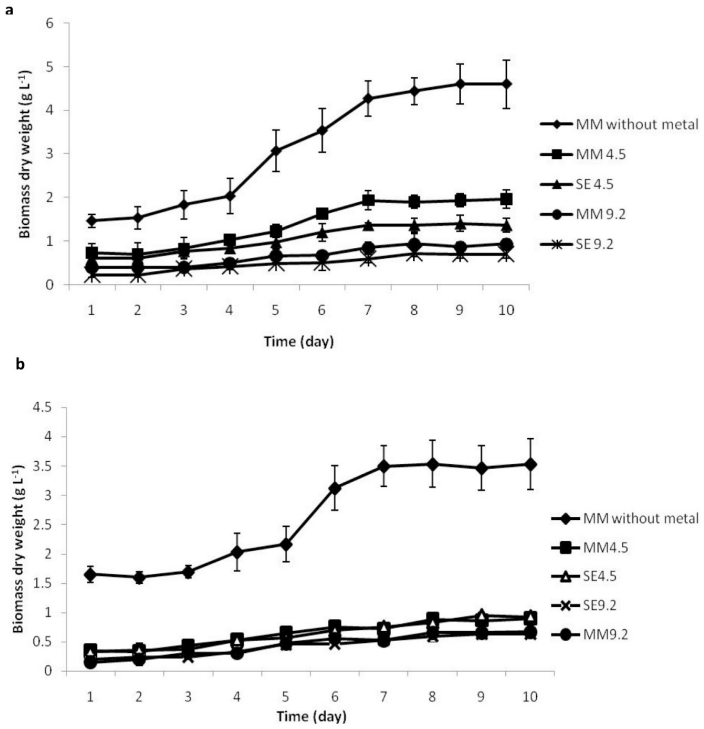

Fig. 2. The growth curves of UTMC 2243(a) and UTMC 2241(b). Growth kinetic of the isolates UTMC 2243(a) and UTMC 2241(b) that have been grown in minimal medium (MM) and soil extract broth (SE) in presence and absence of cadmium 4.5 and 9.2 mM after 10 days incubation. (MM 4.5= minimal medium with cadmium 4.5 mM, MM 9.2 = minimal medium with cadmium 9.2 mM, SE 4.5= Soil extract with cadmium 4.5 mM, SE 9.2 = Soil extract with cadmium 9.2 mM)

It is important to note that both strains showed good growth in soil extract broth and minimal medium as media which are oligotrophic and nutrient deficient media close to the natural environmental condition. *Promicromonospora* sp. UTMC 2243 represented the higher growth rate compared to *Streptomyces* sp. UTMC 2241 which justified its implementation for monitoring its ability in removal of cadmium in the low nutrient medium. The specific growth rate (μ) of *Promicromonospora* sp. UTMC 2243 and *Streptomyces* sp. UTMC 2241 in MM broth containing 4.5 mM $CdCl_2$ were recorded as 0.01 and 0.09 during ten days, respectively. As it is shown in Fig. 3, *Promicromonospora sp.* UTMC 2243 revealed a great ability in removal of cadmium. The percentage of removal was 57.23% to 96.50% during the 10 days. The phylogenetic relationships between strain UTMC 2243 and other members of the genus *Promicromonospora* were shown in Fig. 4. Growth occurs at 10–28 °C and pH 7-12. The morphological properties of isolate UTMC 2243 have been listed in Table 2. According to the results

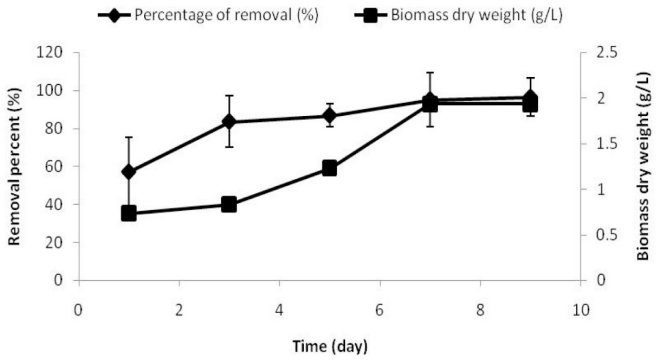

Fig. 3. Cadmium removal percentage by UTMC 2243. (Initial Cd (II) concentration=4.5 mM, temperature=28 °C)

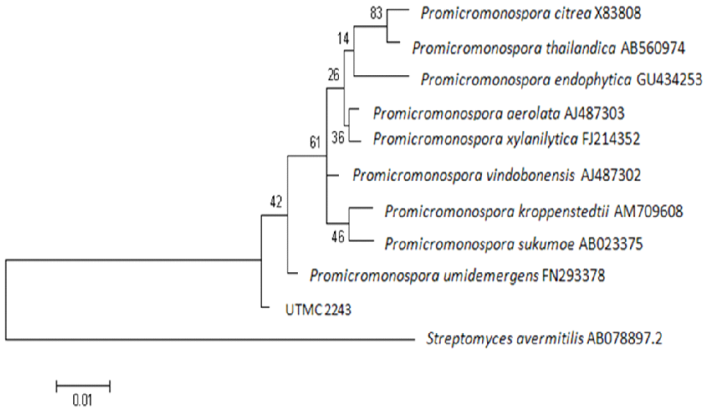

Fig. 4. Maximum likelihood phylogenetic tree based on nearly complete 16S rRNA gene sequences between strain UTMC 2243 and its phylogenetic neighbours

acquired in this study, the highest levels of metal resistivity (mM) in resistant isolates were 140 for Zn, 9.2 for Cd, 7 for Cu and 60 for Ni that in the cases of cadmium, zinc, and copper resistivity were higher than the reported tolerance of actinomycete strains in literature (Table 3). Despite high toxicity of cadmium for microorganisms, as shown in Table 1 among thirteen resistant isolates, *Streptomyces* sp. UTMC 2241 and *Promicromonospora* sp. UTMC 2243 showed high resistance to cadmium (9.2 mM Cd^{+2}). As these two strains presented the highest resistant capability and spectrum to the tested toxic metal, they were subjected to the further investigation regarding their resistance to cadmium as a model of toxic metals. The *Promicromonospora* sp. UTMC 2243 was resistant to high concentrations of Zn^{+2} (140 mM) and Cu^{+2} (7 mM). Yadav *et al*., (2009) have already reported *S. fradiae* as a copper resistant *Streptomyces* with resistance to 3 mM Cu^{+2}. Also, they reported that two actinomycetes belonging to *Streptomyces* sp. showed resistance up to 3.14 mM copper and *Streptomyces hygroscopicus* was resistant to 1.88 mM Cu^{+2} (Yadav *et al*., 2010). Abbas and Edwards (1990) reported for *S. coelicolor* with a growth inhibition of 50% in medium with 0.02 mM of Cu^{2+}. Additionally, metal resistance has reported in several genera of actinomycetes such as *Streptomyces*, *Amycolatopsis* (Albarracin *et al*., 2010), *Nocardia*, *Micromonospora* (Ali *et al*., 2012), and *Frankia* (Richards *et al*., 2004). Strain UTMC 2243 was closely related to *Promicromonospora vindobonensis* (99.5% similarity) and metal resistivity in this genus is reported for first time in this study. Most of researches have been reporting the metal resistant *Streptomyces*, the results acquired in this study showed the metal resistance to heavy metals in *Streptomyces*, *Saccharothrix*, *Promicromonospora*, *Streptosporangium* and *Nonomuraea* genera. Notably, it is the first report of the metal resistivity in *Saccharothrix*, *Promicromonospora* and *Nonomuraea* genera that can be considered as new candidates for bioremediation. In this work we isolated highly resistant actinomycete strain, which showed resistance to high concentrations of all tested metals and in the case of Cd and Cu, MIC values (9.2 mM Cd^{2+} and 7 mM Cu^{2+}) were significantly more than what is reported before in actinomycetes. Growth in high concentration of metals and having a board resistance spectrum introduced *Promicromonospora* sp. UTMC 2243 and *Streptomyces* sp. UTMC 2241 as candidates for toxic metal diminution agents in contaminated

environments. Besides production of spores that make long-term revival of these two strains in polluted area, due to the higher rate of biomass production in a certain span of time, *Promicromonospora* sp. UTMC 2243 can be regarded for further field work trials in metal contaminated sites. Kinetic of the cadmium survey of this strain also revealed that despite ceasing the growth in stationary phase (day 7 till day 10), rate of cadmium detoxification had an ascending trend during this period.

CONCLUSIONS

This study reported the highly heavy metals resistant actinomycete strains that amongst them *Promicromonospora* sp. UTMC 2243 was capable of removing cadmium as high as 96.50%. Additionally, it worth noting that this is the first report of the metal resistivity in *Promicromonospora* genus. Based on data obtained, *Promicromonospora* sp. UTMC 2243 could be an effective agent for removal of cadmium from polluted wastes.

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