ISOLATION AND IDENTIFICATION OF HALOTOLERANT MICROORGANISMS RESISTANT TO HEAVY METALS IN THE CITY OF QOM, IRAN

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ABSTRACT

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Bioremediation is a new and promising technology available for removal and recovery of heavy metals in polluted soil and water. Microorganisms which can resist high concentration of toxic heavy metals are often considered as effective tools of bioremediation from such pollutants. The aim of this study is to screen and characterize the resistance of the halotolerant bacteria to heavy metals from Hoze Sultan lake in Qom. Halophilic and halotolerant microorganisms resistant to heavy metals such as nickel, cadmium, copper and cobalt were isolated from Qom Hoze Sultan lake. Investigating the resistance to heavy metals in the collected samples showed that halotolerant microorganisms exhibited the highest resistance to nickel and cobalt metals and were the most sensitive to cadmium and copper metals.

Keywords: bioremediation, halotolerant, resistant, bacteria, heavy metals

INTRODUCTION

Heavy metals occur naturally in the environment from anthropogenic sources or pedogenetic processes of weathering of parent materials. Research have shown that bioremediation using microorganisms (Mosa *et al*.., 2016) and plants (Rajeendran *et al*., 2003; Dixit *et al*.., 2015) have potential to remove, degrade, or inactivate heavy metals. Bioremediation of metal pollutants from waste water using metal resistant bacteria is a very important aspect of environmental biotechnology. Today, due to industrialization and exploitation of natural resources, soil and water pollution is one of the major global concerns. Bioremediation has been regarded as an environment-friendly, inexpensive and efficient mean of environmental restoration. Since microorganisms constitute a key factor of this technology, knowledge of the nature and molecular mechanisms of their tolerance to increased heavy metal concentrations is essential. Some of these metals are essential to certain levels for microbial growth and higher levels of toxicity to the cells. Cobalt, copper, nickel and zinc plays an important role in regulating gene expression and activity of biomolecules, enzymes or enzyme cofactors (Rajeendran *et al*.., 2003). Bioremediation with the help of microorganisms can effectively reduce heavy metals in the environment (Ahmady *et al*.., 2012). Bioremediation is distinct from other cleaning methods because of the ability of microorganisms to remove heavy metals from aqueous solutions, particularly in the range of less than 1 to 100 mg (Ahluwalia and Goyal, 2007). In the last decade, bioremediation includes a group of applications, which involve the detoxification of hazardous substances instead of transferring them from one medium to another, by means of microbes and plants. This process is characterized as less disruptive and can be often carried out on site, eliminating the need to transport the toxic materials to treatment sites (Mosa *et al.*, 2016). Table 1 shows some microorganisms employed in heavy metal bioremediation in the capital of Qom Province, which is the eighth largest city in Iran. It is located 125 kilometers southwest of Tehran. The aim of this study is the isolation and identification of halotolerant microorganism with resistance to heavy metals.

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Table1. Some microbes that can utilize heavy metals.

Microorganism	Metals	References
Bacillus spp. *Pseudomonas aeruginosa*	Cu, Zn	(Philip et al.., 2000 ; Gunasekara et al.., 2003)
Zooglea spp. *Citrobacter spp.*	U, Cu, Ni Co, Ni, Cd	(Sar and Souza, 2001)
Citrobacter spp. *Chlorella vulgaris*	Cd, U, Pb Au, Cu, Ni, U, Pb, Hg, Zn	(Gunasekara *et al*.., 2003)
Aspergilusniger	Cd, Zn Zn, Ag, Th, U	(Gunasekara *et al*.., 2003)
Pleurotus ostreatus	Cd, Cu, Zn	(Gunasekara *et al*.., 2003)
Rhizopus arrhizus	Ag, Hg, P, Cd, Pb, Ca	(Gunasekara *et al*.., 2003; Favero *et al*..,1994)
Stereum hirsutum	Cd, Co, Cu, Ni	(Gabriel *et al*.., 1994; Gabriel *et al*.., 1996)
Phormidium valderium	Cd, Pb	(Gabriel *et al*.., 1994; Gabriel *et al*.., 1996)
Ganoderma applantus	Cu, Hg, Pb	(Gabriel *et al*.., 1994; Gabriel *et al*.., 1996)

MATERIALS AND METHODS

Sampling

Sampling from different parts of Qom Hoz Sultan Salt Lake, including soil, water and salt was performed. Samples were collected under sterile conditions, coded and transferred to the laboratory, where they were prepared for plating (Figure1).

Isolation of microorganisms

In solid samples, 1 gram of soil was added to 10 ml normal saline. Lake water samples of 1 ml of saline was added to 9 ml of normal saline. Soil, water and salt samples in different serial dilution were incubated at 37°C for two hours. To determine salt tolerance, all isolates were aseptically transferred from nutrient agar vials into sterile nutrient broth tubes with 10% (w/v) NaCl and incubated at 37°C for 24hrs. The exponentially growing broth cultures were subsequently transferred to nutrient broth tubes with different salt concentrations (0%, 3%, 10%, 15% and 25% (w/v) NaCl) with an inoculation loop and incubated at 37°C for 24 hrs.

Chemicals and media

Stock solutions of the heavy metals (1000 mg/L) were prepared. The metal salts used were $CdCl_2$, $Pb(NO3)_2$, and $NiSO_4$ (Merck, Germany). Nutrient agar and Luria Bertani (*LB*) medium were used to grow and isolate microorganisms (Mehrshad *et al*., 2012; KF Owlia *et al.*, 2014). To achieve a complete heterotrophic microorganism, aerobic culture was used in a variety of media such as *MH* (moderate halophilic medium with 12% salt), *SWN* (Sea water nutrient agar with 3% salt) and *MGM* (Modified Growth Medium with 23% salt) (Mehrshad *et al*., 2012).

Figure1. Qom Hovz-e Sultan lake, Iran

Characterization of bacterial isolates

After purification of microorganisms, identification based on macroscopic characteristics, including size, shape, color, colonies surface and margin on solid culture media was conducted. Identification based on morphology (rod, spherical, spiral), size aggregation, and Gram stain reaction was carried out. In addition, mobility, catalase and oxidase tests, some biochemical characteristics of the isolates were also determined.

Determination of minimum inhibitory concentration (MIC)

The bacterial samples tolerance to heavy metals was determined by slowly increasing the concentration of heavy metals, on the nutrient agar plate until the bacterium failed to produce colonies, Minimum inhibitory concentration (MIC) was noted when the bacterial isolate failed to grow on the plate after incubation.

Screening for salt tolerance

To determine halophiles or halotolerant microorganisms, all of the isolates were cultured on medium with varied sodium chloride concentration.

Screening for heavy metal resistance using blots on solid media and micro plate methods

Resistant bacteria to 10%-23% sodium chloride (resulting from separation on the *MGM* and *MH* media) were selected to evaluate resistance to heavy metals $NiSO_4.6H_2O$, $CdCl_2.H_2O$, $CoSO_4.7H_2O$, and $CuSO_4.5H_2O$. To measure metal resistance with blots methods (Abolmaali *et al.*., 2008) in the solid medium, soluble metal salts of nickel, copper, cobalt and cadmium, with concentrations of 0, 0.5, 1, 2, 3, 5, 7 mM in the *MH* medium was prepared, blotting was then performed on solid medium and Petri dishes were incubated for 36 h at 37°C.

In the microplate method (Abolmaali *et al.,* 2008) concentrations of 0.5, 1, 3.12, 6.25, 12.5, 25 and 50 mM of the above mentioned heavy metals were prepared in the micro plate wells in *MH* medium. In this method, metal with non-inoculated bacterial culture medium was used as a negative control and metal-free medium containing the bacterial culture was used as a positive control .Plates were incubated for 36 h at 37°C.

RESULTS

Isolation of microorganisms

Isolation of microorganisms was carried out in different media; 20 isolates in *MGM* medium, 31 in *MH* medium, 70 in *SWN* medium, and 33 in *NA* medium without sodium chloride.

Macroscopic screening of isolates showed that most were primary-colored colonies, some colonies were brownish orange (earth color), some colorless, and few were pinkish red. Red pigments were often observed in isolates obtained from *MGM* medium. The colonies varied in size from very small (with a diameter of about 1 mm) to large (about 1 cm in diameter). Colonies consistency also varied from solid to viscous to watery.

Morphological characteristics of the isolates

Around 90% of all isolates were gram-positive bacilli and the rest were gram negative bacilli, and a few were actinomycetes. Microscopic observations showed that around 5% of all isolates contained spores. 20 % of the isolates from the *MGM* culture medium were catalase-negative and 80% were catalase-positive. All microorganisms isolated from the *MH* medium were catalase-positive. 10% of the isolates in the *MGM* medium were oxidase-positive. In the *MH* medium, 42% of the isolates were oxidase-negative, 6% showed strong oxidase reaction and 52% were oxidase positive (Table2).

Table 2. Biochemical and morphological characterization of the metal–resistant bacterial isolates collected from the city of Qom, Iran.

Features	Bacterial isolates				
	J1	**J2**	**J3**	**J4**	**J5**
	Bacillus cereus	***Bacillus amyloliquefaciens***	***Bacillus subtilis***	***Bacillus aerius***	***Pseudomonas aeuginosa***
Morphological	(+)	(+)	(+)	(+)	
Gram stain	White	White	Cream	White	
Color	Rods	Rods	Rods	Rods	(–)
Cell shape	(+)	(+)	(+)	(+)	Bluish green
Motility	(+)	(+)	(+)	(+)	Rods
Endospore	Irregular	Irregular	Irregular	Irregular	(+)
Colony					(–)
margin					Irregular
Biochemical					
Catalase	(+)	(+)	(+)	(+)	(+)
Oxidase	(–)	(+)	(–)	(+)	(+)
Citrate	(+)	(–)	(+)	(+)	(+)

Resistance to metals

To measure metal resistance, isolates were evaluated in *MGM* medium containing 23% NaCl using the micro plate method. Isolates showed more resistance to nickel and were most sensitive to cadmium. The highest concentration of microorganisms growth was at the concentration of 6 mM for nickel, and at 3.0, 0.5 and 0.5 mM concentration for cobalt, cadmium and copper metals, respectively (Figure2).

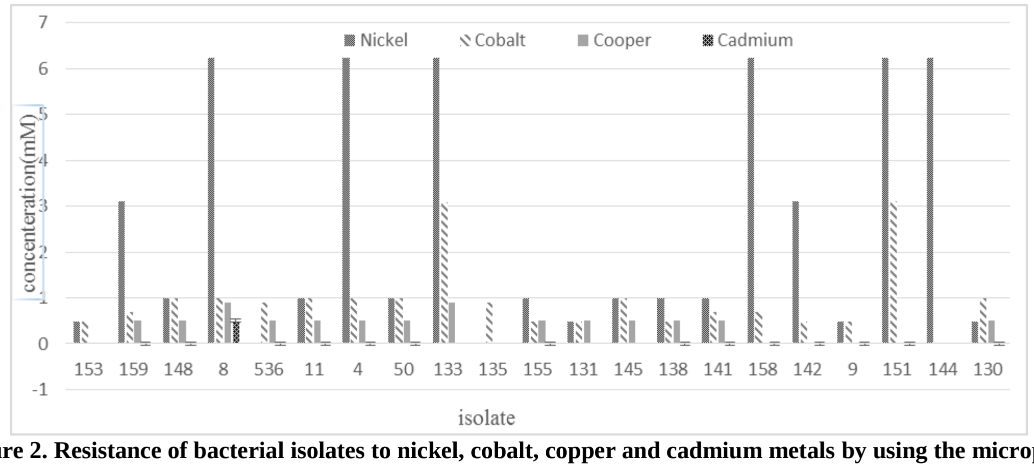

Figure 2. Resistance of bacterial isolates to nickel, cobalt, copper and cadmium metals by using the microplate method.

Results obtained by using the blot method indicated that 10% of all isolates tolerated 3 mM nickel. 30% of isolates tolerated 2 mM, 5% of all isolates tolerated 1 mM, 5% of all isolates tolerated 5.0 mM nickel and 20% of all isolates did not tolerate any concentration of the metal. Figure 3 shows the results obtained with this method for a strain resistant to nickel, cobalt, copper, cadmium (DDCB151) and a strain sensitive to the metals cobalt, copper, cadmium and nickel (DDCB11).

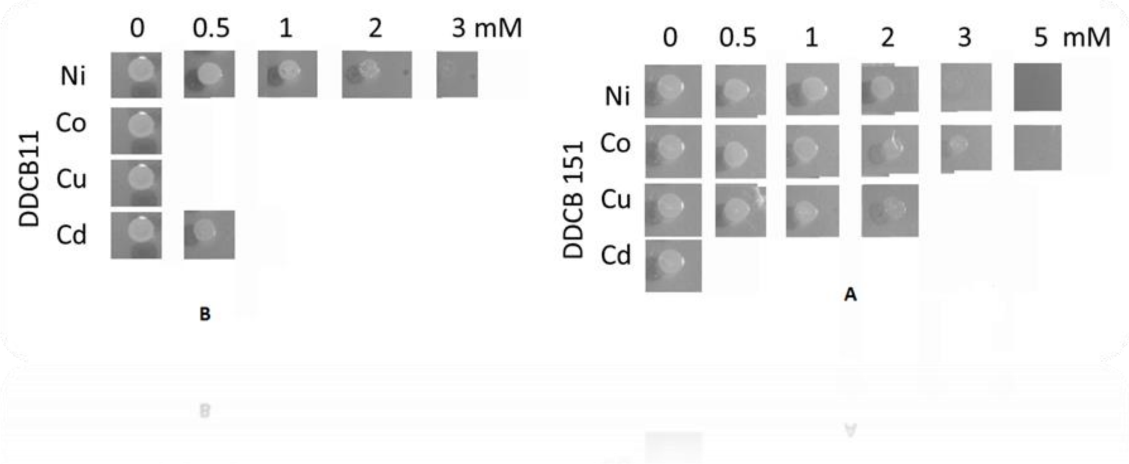

Figure 3. Results of screening bacterial isolates to heavy metals using the blotting method. (A) Shows the reaction of an isolate resistant to nickel, cobalt and copper metals, (B) shows the reaction of an isolate sensitive to nickel, cobalt, copper and cadmium metals.

DISCUSSION

Bioremediation procedures may be conducted by native microorganisms, which naturally inhabit the soil/water environment undergoing filtration, or by other microorganisms isolated from different environments. There are a number of microorganisms that can be used to remove metal from the environment, such as bacteria, fungi, yeast and algae (Vieira and Volesky, 2000). Bioremediation provides a tool for cleaning up polluted areas by enhancing the natural biodegradation processes.

Developing an understanding of microbial communities and their response to the natural environment and pollutants, expanding the knowledge of microbial genetics to increase capabilities to degrade pollutants, conducting field trials of new bioremediation techniques which are cost effective, and dedicating sites which are set aside for long term research purpose, offer potential for significant advances. According to Sharm and Rehman (2009), heavy metals are normally regarded as metals with an atomic number 22 to 92 in all groups from period 3 to 7 in the periodic table (Sharma and Rehman, 2009). Some of the metals such as Pb, Fe, Cr, Cu, Zn, Cd, Co, Ni, Mn, Mo, V, and Se are essential in trace quantities for the general wellbeing of living organism, whereas an excess of these metals can be lethal. Costa and Duta (2001) reported that heavy metals, such as copper, cadmium, lead, chromium and mercury are important environmental pollutants (Costa and Duta, 2001).

Zaki and Farag isolated *Entrobacter sp., Chryseobacterium sp.* and *Stenotrophomonas sp.* as the most resistant strains (Xie *et al.*., 2010). Testosteroni reported *Ralstonia pickettii* and *Sphingomonas sp.* to be resistant to high level of Zn, Ni, Pb and Cu from contaminated soil (Stanilan *et al.*., 2010). In examining the biodiversity of halophiles and halotolerant microorganisms by Amoozegar, many gram-positive, gram-negative bacilli and gram-positive cocci were identified (Mehrshad *et al.*., 2012; Khdabakhsh *et al.*., 2011). In this study, five bacterial isolates that were highly resistant to the heavy metals cadmium, lead, and nickel were identified. No microorganisms exhibited multiple resistances to two or more metals. The results showed that the most common microorganisms observed in the agar culture medium were gram-positive bacteria and fungi, and the microorganisms that had the highest resistance to nickel had the highest sensitivity to cadmium metal. It can be concluded that microorganisms isolated from the MH medium were the most resistant to nickel and cobalt. Most sensitivity was found to cadmium and copper metals. Accordingly, halotolerant microorganisms isolated from the environment in media containing 10% and 20% salt can show better resistance to heavy metals. Microorganisms isolated from the environment in media containing 2% or less salt are not likely to show such feature.

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