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ORIGINAL ARTICLE

High potential application in bioremediation of selenate by *Proteus hauseri* strain QW4

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ABSTRACT

Background and Objective: Selenium is essential for biological systems at low concentrations and toxic at higher levels. Heavy metals and metalloids such as selenium are major contaminants in 40% of hazardous waste sites. Thus, bioremediation has been considered as an effective means of cleaning up of selenium-contaminated sites.

Materials and Methods: In this study, 30 strains were isolated from wastewater samples collected from selenium-contaminated sites in Qom, Iran using the enrichment culture technique. One bacterial strain designated QW4, identified as *Proteus hauseri* by morphological, biochemical and 16S rRNA gene sequencing was studied for its ability to tolerate different concentrations of sodium selenate (100-800 mM). Also, the disk diffusion method was performed to determine resistance to some antibiotics

Results: Strain QW4 showed maximum minimum inhibitory concentration (MIC) to selenate (760 mM). The maximum selenate removal was exhibited at 35 ºC, while the removal activity reduced by 30.7% and 37% at 25 ºC and 40 ºC, respectively. The optimum pH and shaking incubator for removal activity was shown to be 7.0 and 150 rpm, with 60.2% and 60.3%, respectively. This bacterial strain was resistant to some antibiotics.

Conclusion: The concentration of toxic sodium selenate (1000 µg/ml) in the supernatant of the bacterial culture medium decreased by 100% after 2 days and the color of the medium changed to red due to the formation of less toxic elemental selenium. Also, our results imply that heavy metal pollution may contribute to increased antibiotic resistance through indirect selection.

Keyword: Bioremediation, MIC, *Proteus hauseri,* Sodium selenate.

INTRODUCTION

Selenium is the 34th element on the periodic table and has chemical properties resembling those of sulfur (1). It is an essential micronutrient for animals and microorganisms, , used in the synthesis of the seleno-amino acids selenocysteine and selenomethionine (2,3) and also plays an essential dietary role in humans due to its ability to form the enzyme glutathioine peroxidase which is involved in fat metabolism (4). However, exposure to higher concentrations of selenium is toxic (1). High intake of selenium in humans can lead to respiratory distress, liver and kidney necrosis and cell death. Chemical detoxification of polluted sites can be expensive and often results in secondary effects in the environment (5). Therefore, it is important to understand how environmental selenium is controlled.

Agricultural drainage, industrial effluents and sewage sludge are major sources of various toxic forms of selenium (6). On average most soils contain between 0.01- 2 mg Se/kg while seleniferous soils may have

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as much as 5 mg Se/kg. Natural waters generally have Se concentrations < 0.01 mg/L but agricultural drainage waters often have Se concentrations of 140 to 1400 mg/L (7).

Selenium occurs naturally in four oxidation states, i.e., selenate (+VI), selenite (+IV), elemental selenium (0), selenide (-II), and organic selenium (-II), and it is known that prokaryotes play a major role in its oxidation and reduction (1, 8).

Bioremediation is the application of biological systems to the clean-up of organic and inorganic pollution, with bacteria and fungi (9). Apparently, microbes that can reduce selenate and selenite are not restricted to any particular group/subgroup of prokaryotes and examples are found throughout the bacterial and archaeal domains (10). Bacterial reduction of selenate to elemental selenium is an important biogeochemical process in an aquatic environment. In this system, selenate can be used in microbial respiration or dissimilatory selenate reduction, as a terminal electron acceptor for growth and metabolism (11).

The optimization of biological remediation processes depends on an understanding of the biology involved and, if bacterial inoculation is needed, the identification and characterization of microorganisms that can best carry out the desired remediation. The aim of this research was an attempt to isolate and characterize microorganism capable of transforming toxic SeO42- via SeO32- to non-toxic elemental selenium and to investigate its ability in selenite removal from contaminated sites. Also, antimicrobial test was performed to determine resistance or sensitivity to some antibiotics.

MATERIALS AND METHODS

Chemicals. Sodium selenate anhydrous was obtained from Alfaesar (Germany) and sodium sulfide and thionine dye were bought from Merck (Darmstadt, Germany). The stock solutions were prepared in distilled water and maintained at 4 ºC following sterilization by microbiological filter (0.22 µm). Working solutions were stored at 4 ºC for up to 5 days.

Sample collection and isolation of metalloid-tolerant bacterial strains. Selenium-contaminated water samples were collected from industrial area of Qom in Iran. Totally, 30 bacterial strains were isolated using the enrichment culture technique at 34 ºC in a shaking incubator (150 rpm) and pH 7.0 for 48 h in LB broth (Luria Bertani broth) supplemented with 10 mM sodium selenate under aerobic conditions. Red colonies, indicating reduction of selenate, were re-streaked on LB agar without selenate to confirm that the colour was not due to pigmentation. The pure cultures were isolated and maintained on selenate supplemented plates. Filter sterile 10 mM sodium salts of selenate were added to the LB medium after autoclaving (12, 13). Among the strains isolated, the strain named QW4 showed the highest tolerance toward this sodium selenate and was selected as a model strain for further experiments.

Determination of minimum inhibitory concentration (MIC). In order to determine MICs, the strains were grown in LB agar medium supplemented with sodium selenate at increasing concentrations (100-800 mM) and incubated at 34 ºC, for 72 h. Each plate was prepared in triplicates (13, 14).

Characterizations of the bacterial isolate. Morphological characterization such as colony and cell morphology, Gram-reaction, motility and other identification tests were performed as described by Ghosh *et al*., (2006) (15). Physiological and biochemical tests were carried out according to the standard protocols described by Smibert and Krieg (1994) and Ventosa (2004) (16, 17).

To determine the optimum temperature and pH for the growth of the strain, the cultures were incubated at a temperature range of 15-50 ºC with intervals of 5 ºC and pH values of 5-10.5. pH values below and above 6 were adjusted by sodium acetate and Tris-HCl buffer, respectively. Also, growth of the strain was evaluated at different % NaCl values (0- 30 % NaCl) (13, 18).

Antimicrobial susceptibility test was done by the disc-diffusion method on Mueller–Hinton agar plates. Isolated strain was screened for susceptibility to a panel of 15 antibiotics discs (Padtan Teb, Iran) viz: penicillin (P_{10}), ampicillin (AM_{10}), cephalothin (CF_{30}), cefazolin (CZ_{30}), streptomycin (S_{10}), gentamicin (GM_{10}), chloramphenical (C_{30}), tetracycline (T_{10}), erythromycin (E_{15}), trimethoprim sulfamethoxazol (SXT), nalidixic acid (NA_{30}), ciprofloxacin (CP_5), vancomycin (V_{30}) and polymyxin B (PB_{300}) (18).

Selenate removal experiments. Cells were cultured in 100 ml Erlenmeyer flasks containing 25 ml of LB broth supplemented with 1000 µg/ml sodium selenate. The basal medium (LB broth + sodium selenate) was inoculated with 1% of 1.5×10^8 cfu/ml of the bacterial suspensions and incubated aerobically at 35 ºC and pH value of 7 on a shaking incubator (150 rpm) for 2 days. The cells were centrifuged at 10000 rpm for 10 minutes and the supernatants were used to determine the residual sodium selenate through slightly modified kinetic spectrophotometric method based on the catalytic role of selenite in reducing thionine dye by sulfide ions (13, 19). For the measurement of Se^{6+} sorption, selenate was first reduced to selenite by mixing with concentrated HCl (2:1, by vol.) in a screw–capped tube in a 90 ºC water bath for 1 h (20) and assay was followed as above. All selenium measuring experiments were performed with acid-washed glassware and deionized water.

Factors affecting selenate removal. Capacity of selenate removal by the strain was evaluated at different pH values (5-10.5) and temperatures (15-40 ºC) and on a shaking incubator (50-200 rpm) in basal medium supplemented with 1000 µg/ml sodium selenate. To evaluate the effect of initial selenium concentration, selenate removal was monitored in basal medium supplemented with varying concentration of sodium selenate (200-2000 µg/ml). All experiments were done in triplicate.

Phylogenetic analysis. Genomic DNA of the isolate was extracted with a genomic DNA extraction kit (Cinnagene) by following the manufacturer's recommended procedure. The 16S rRNA gene was amplified using the universal primers 8F (5'-AGAGTTTGATYMTGGCTCAG- 3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3'). The amplification was done by initial denaturation at 95 ºC for 5 minutes; subsequent denaturation at 95 ºC for 1 minute; annealing at 66.6 ºC for 1 minute; extension at 72 ºC for 1 minute and final extension at 72 ºC for 10 minutes. The PCR product was directly double-strand sequenced by Seqlab Laboratory (Germany). The analysis of DNA sequences and homology searches were completed using the BLAST algorithm for the comparison of the nucleotide query sequence against a nucleotide sequence database. Multiple sequence alignments were done using CLC Sequence Viewer version 6.5.1. Phylogenetic tree was inferred using the neighbor-joining method as implemented in the software.

RESULTS

Strain characterizations. Among the 30 strains of bacteria isolated from industrial wastewaters in Iran, one strain was selected for further study. In fact, strain QW4 showed maximum MIC to selenate (760 mM). The resistance of strain QW4 was associated with reduction of selenate to selenium and the formation of the red elemental selenium precipitate in the medium. Strain QW4 was shown to be a Gram-negative, motile, strictly aerobic rod, catalase-positive and non-oxidase. The strain QW4 could grow in a range of temperatures (15-50 ºC), pH conditions (5-10.5) and % NaCl range (0-30%). However, optimum growth was seen at 35 °C, pH 7.0 and 3% NaCl. Table 1, shows some characteristics of strain QW4. According to morphological, physiological and biochemical characterizations of the strain and in comparison to other studies, the strain was identified as *Proteus* strain QW4.

Also, antibiotic resistance patterns of strain QW4 as selected strain is shown in Table 2.

To confirm the identity of the isolate, PCR amplification, sequencing of the 16S rRNA gene were completed. The phylogenetic tree (Fig. 1) constructed by the neighbor-joining method indicated that the isolate QW4 was part of the cluster within the *Proteus* genus. Among the described species, the closest relative of isolate QW4 was *Proteus hauseri* (FR733709-1).

Selenate reduction experiments. Strain QW4, which showed the maximum resistance to sodium selenate, was selected for removal of selenate from contaminated environments. The effects of various environmental parameters in removing sodium selenate from culture medium by the strain were evaluated using sodium sulfide-thionine as an indicator. The strain also showed the reduction ability of selenate after 2 days in comparison with the control (a medium without the strain).

The decrease in the sodium selenate concentrations during growth is shown in Fig. 2. Typically, the maximum selenate removal in LB broth medium with a concentration of 1000 µg/ml sodium selenate was determined to be 100% after 2 days.

Table 1. Morphological, physiological and biochemical characteristics of strain QW4 as selected strain.

Strain Characteristic	Strain QW4	Strain Characteristic	Strain QW4
Cell type	Rod	**Catalase**	+
Gram Staining	-	**Oxidase**	-
Form	Irregular	**Motility**	+
Margin	Undulate	**Endol**	+
Elevation	Convex	**H_2S production**	+
Texture	Butyrus	**Voges-Proskauer test**	-
Opacity	Opaque	**Methyl red test**	+
Pigmentation	Cream	**Citrate Simmon**	-
TSI	K/A	**Diameter > 5 mm**	-
Growth limit in %NaCl	0-30	**Hydrolysis**	
Growth optimum in NaCl	3	Gelatin	+
Temperature limit of growth	15-50	Starch	-
Growth optimum of Temperature	35	Casein	-
Growth limit of pH	5-10.5	**Enzyme activity**	
Growth optimum of pH	7	DNase	-
Acid production from		Urease	+
Mannitol	-	Phenylalanine deaminase	+
D-glucose	+	Lysine decarboxylase	+
Lactose	-	Nitrate reduction	+
Salicin	-		

Sucrose	+	**Using of carbon sources**	
Xylose	+	Mannitol	-
Glucose (anaerobe)	-	D-glucose	+
Mannitol (anaerobe)	-	Lactose	+
Using of nitrogen sources			
L-Methionine	+	L-Tryptophan	-
L- Arginine	-	L- Lysine	-

Table 2. Antibiotic resistance patterns of strain QW4 as selected strain.

Group	**Antibiotics**	**Code**	**Resistance patterns of strain QW4**
Penicillins	Penicillin	P_{10}	R
	Ampicillin	AM_{10}	S
Cephalosporins	Cephalothin	CF_{30}	R
	Cefazolin	CZ_{30}	R
Aminoglycosides	Streptomycin	S_{10}	I
	Gentamicin	GM_{10}	S
Phenicols	Chloramphenical	C_{30}	I
Tetracyclines	Tetracycline	T_{10}	R
Macrolides	Erythromycin	E_{15}	R
Sulfonamides	Trimethoprim sulfamethoxazol	SXT	S
Quinolones	NAlidixic acid	NA_{30}	S
	Ciprofloxacin	CP_{5}	S
Glycopeptides	Vancomycin	V_{30}	R
Others	polymyxin B	PB_{300}	R

R: Resistance, I: Intermediate, S: Susceptible

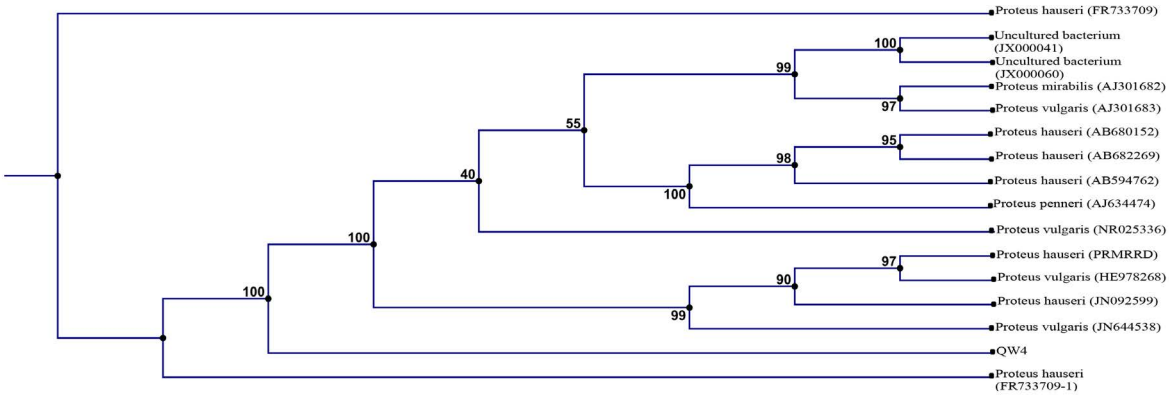

Fig. 1. Neighbor-joining tree showing the phylogenetic position of *Proteus hauseri* strain QW4 among members of rod Gram-negative bacteria.

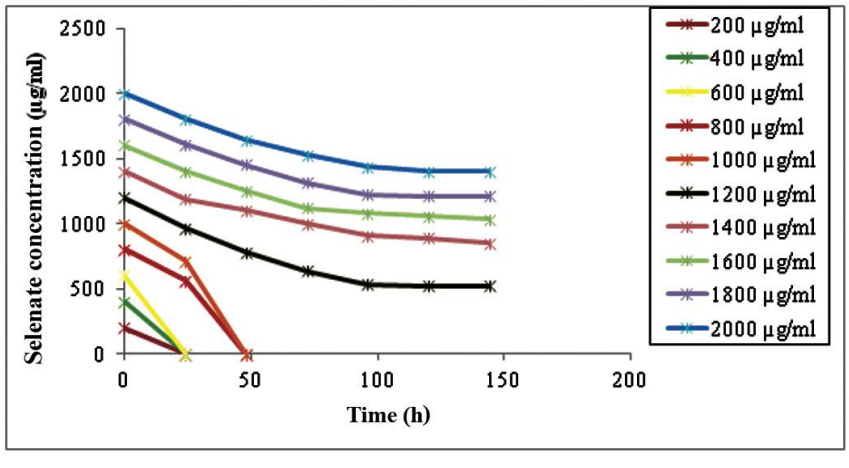

Fig. 2. Different concentrations effect of selenate on their removal by strain QW4 in LB broth medium (Temperature = 35 ºC, pH =7.2 ± 0.2, rpm =150). Reduction was monitored after 24, 48, 72, 96, 120, 144 h.

As shown in Figs. 3, 4 and 5, pH, temperature and rpm had significant effects on sodium selenate removal and the maximum removal occurred at pH value of 7.0, 35 ºC and 150 rpm. At higher and lower pH, temperature and rpm, the amount of sodium selenate removal was less. The reduced removal capacities at pH value of 7, temperature of 35 ºC and shaking incubator of 150 rpm were 60.2%, 42.1% and 60.3%, respectively.

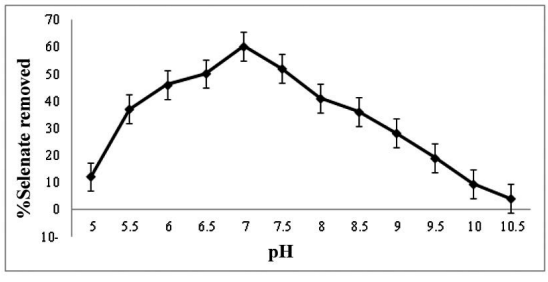

Fig. 3. Effect of pH values on selenate removal by strain QW4 in LB broth medium containing 1000 µg/ml selenate after 24 h (Temperature = 35 ºC, rpm =150).

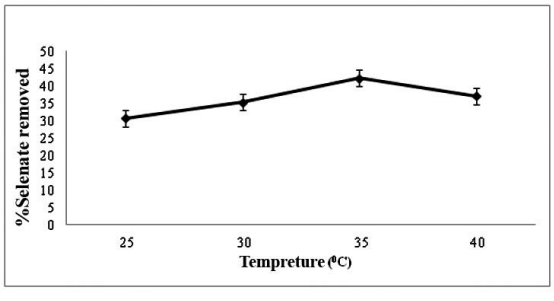

Fig. 4. Temperature effect on selenate removal by strain QW4 in LB broth medium containing 1000 µg/ml selenate after 24 h (pH =7, rpm =150).

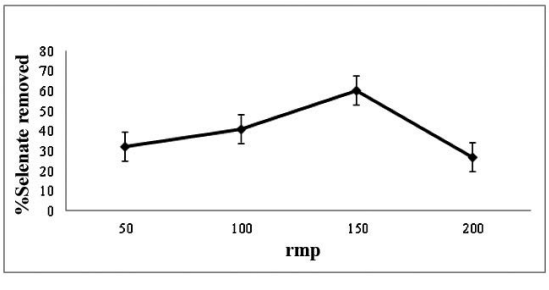

Fig. 5. Shaking incubator effect on selenate removal by strain QW4 in LB broth medium containing 1000 µg/ml selenate after 24 h (T= 35 ºC, pH =7).

DISCUSSION

Human activities have been contaminating the environment with toxic heavy metals and metalloids over the past 200 years and, consequently, have resulted in severe disturbance of ecological balance in most ecosystems (6). Microorganisms possess a high capacity for detoxifying and metabolizing Se (VI). Biodiversity of microorganisms harboring this capability attracts attention of researchers to look for novel microorganisms, elucidate new pathways or specific enzymes with superior oxyanion-metabolizing activity or different substrate specificity (11). Thus, introduction of a newly isolated bacterial strain and characterization of its oxyanion-processing capabilities were considered the goal of this research.

We isolated 30 strains from various industrial wastewaters in Iran and evaluated their resistance patterns to sodium selenate. Strain QW4 showed the maximum MIC (equal to 760 mM), which was much higher than that previously reported for Bacillus sp. SF-1 (21), *Enterobacter cloacae* SLD1a-1 (10), *Halomonas* sp. strain MAM (18), *Bacillus* sp. STG-83 (11), *Delftia tsuruhatensis* and *Pseudomonas sp*. (6) and *Pseudomonas sp*. CA5 (22). In fact, strain QW4 showed maximum oxyanion reduction and resistance ability for the first time in this research. Based on a partial 16S rRNA sequence, it was determined that strain QW4 was phylogentically related to the *Proteus genus.*

The isolated bacteria were pink to red when supplemented with selenate which was due to the accumulation of elemental selenium. This was an indication that selenate was reduced. Hunter *et al*., (2009) also observed red colonies indicating the formation of elemental red selenium as a reduction product of selenate in *Pseudomonas sp*. CA5 (22).

The genetically modified bacteria carry a plasmid containing genes conferring resistance to the antibiotics as well as resistance to toxic oxyanions of selenium (23). Strain QW4 showed resistance to penicillin, ampicillin, cephalothin, cefazolin, tetracycline, erythromycin, vancomycin and polymyxin B as well as resistance to selenate. Our results imply that heavy metal pollution may contribute to increased antibiotic resistance through indirect selection.

The biological methods and the effects of different environmental parameters were used to demonstrate the removal of sodium selenate by strain QW4. In 2003, Watts *et al.* reported that *Enterobacter cloacae* SLD1a-1 is able to remove 94.5% of 127 mMselenate at 37 ºC, pH 8 and 180 rpm (10). In 2006, 9 Bacillus strains were also isolated from sediments that 96.3% of 2137 µg/l selenate removed during 9 days at 35 ºC and pH 7 by Siddique et al. (24). The results obtained showed that under the following conditions a maximum removal of sodium selenate in the supernatant from 1000 µg/ml to 0 occurred after 2 days: pH 7.0,

temperature 35 ºC and 150 rpm. Generally, there was a good correlation between the optimal pH, temperature and rpm for growth and removal of selenate by strain QW4.

CONCLUSION

Conventional chemicals methods for removing toxic oxyanions are expensive and require high energy or large quantities of chemical reagents, while microbial reduction of these toxic oxyanions is cost effective and supports green technology. Transformation of selenate to elemental selenium could offer an important mechanism for the removal of toxic selenate from polluted environments. Strain QW4 was resistant to high concentrations of selenate and also, it reduced selenate to red elemental selenium under aerobic conditions. Therefore, this strain could be a good candidate for bioremediation of highly polluted effluents from industrial operations.

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