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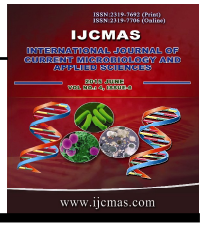

Original Research Article

Isolation and Characterization of Heavy Metal Resistant *Bacillus subtilis* spp. Collected from Water Sources of Taif Province of Saudi Arabia

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Heavy Metals, Water, *Bacillus subtilis*, Bioremediation and Molecular identification methods

A B S T R A C T

The aim of this study is to screen and characterize the resistance of the bacteria of heavy metals from different water resources in Taif. For this purpose, various metals such as Lead (Pb), Cadmium (Cd) and silver (Ag) with different concentrations (100- 1200 µg /ml) were used. Biochemical testes and RAPD PCR carried out. The findings showed that bacteria present have resistance over the heavy metals. Two resistant bacteria were identified as *Bacillus subtilis* spp based on 16Sr RNA analysis. RAPD PCR and the biochmical results indicated that all strains under this project could be from the same species. This bacteria can be used for bioremediation from water sources.

Introduction

Heavy metals are metalloids that are concerned with environment. A common definition is based on the weight of the metal which applies to all metals that weigh more than 5000 kg/m³; such as lead, zinc and copper. Heavy metals are naturally present in the ecosystem, with significant differences that emphasize on the importance of metals. However, the proportions are becoming more frequent recently due to industrial sources and industrial effluents and leaching metal ions from the soil into lakes and the pollution incident of waste derived fuels in particular. The pollution caused by the metals in the environment is dangerous for the ecosystem, for example, organic molecules present in the environment are destroyed as metals cannot be degraded like organic pollutants (Igwe and Abia, 2005). The toxicity emerged from the metals destroys the life of microorganism, animals and plants; whereas, the degree varies for different organisms. In addition, the metabolic activity and the structure of microbial communities are decreased by heavy metals (Giller *et al.*, 1998).

Several methods for the removal heavy metals from the environment can be used

such as Biotic methods, and Abiotic methods. The physiochemical processes are very expensive and generate secondary products (Celis *et al.,* 2000) whereas the biological processes are considered as cost effective and are environmentally friendly methods for the remediation of heavy metal contaminated soils (Congeevaram *et al.,* 2007). Microorganisms which have the ability to survive in highly concentrated heavy metals can be used as an agent of bioremediation through which immobilization and different transformation processes can be performed. The process of bioaccumulation is easily performed which is based on the incorporation of metals inside the biomass that absorbs the metal ions at the cellular surface through various mechanisms (Vijayaraghavan and Yeoung-Sang, 2008).

Bacteria such as *Bacillus sp.*, *Pseudomonas sp.* and *Klebsellia sp.* can be isolated through the usage of heavy metals whereas, the concentration of the heavy metals varies. Bacteria that grow on metals play an important in the cycling of biogeochemical of metal ions. According to there is a correlation between antibiotic resistance in bacteria and metal tolerance as both of them resist genes that are closely related on the plasmid of the bacteria or on the DNA chromosome of bacteria (Piddock, 2006; Haferburg and Kothe, 2010). Many researchers isolated and identified different species of heavy metal resistant bacteria from various water sources and soil were reported (Abo-Amer *et al.,* 2014; Aly *et al.,* 2015).

Materials and Methods

Sample collection

The water samples were collected from various water bodies' wells at Taif province in Saudi Arabia. The samples were taken in sterile plastic bottles and transported to laboratory in ice box for bacteriological analysis.

Biochemical tests

Several Biochemical tests were used for identification of isolated species**.** These tests were mainly Mannitol salt agar, starch hydrolysis and Gram Stains.

Heavy metal resistance

Nutrient agar medium containing various concentrations (0 and 100-1200 µg /ml) of the different heavy metal compounds (Ag, Pb and Cd) were prepared. A sterile wire loop was used to collect a loop full of the pure isolates and directly streaked on the surface of the heavy metals incorporated media. The plates were incubated at 37 ºC for 24 h. After the incubation period, the plates were observed for bacterial growth.

Determination of Minimum Inhibitory Concentration (MIC)

The bacteria isolates were determined by gradually increasing the concentration of heavy metals, each time on the nutrient agar plate until the bacterium failed to give colonies on the plate. Minimum inhibitory concentration (MIC) was noted when the isolates failed to grow on the plates after incubation.

DNA extraction and RAPD PCR

DNA Extraction

Chromosomal DNA from two bacteria were extracted. The DNAse Tissue Kit (Qiagen) was used for this study with the manufacturer's instructions. A fresh single colony from nutrient agar plate was used to inoculate in 3ml of nutrient broth and incubated overnight at 37ºC. Then diluted

1/50 to fresh nutrient broth medium and was grown at 37ºC, with shaking, to an OD 600 approximately 1. An aliquot of 1.5 ml of culture was centrifuged at 8,000 rpm for 2 min. and cells were then processed as instructed in the manufacturer's manual, except that the final elution of DNA from the filter was in sterile 0.1x TE. DNA was stored at -20ºC.

RAPD Polymerase Chain Reaction (PCR)

Primer Design

Three random primes were chosen as universal (Table 1)

RAPD Polymerase Chain Reaction (PCR)

Amplification reaction was performed in 25 ml volume mixtures consisting of 1× PCR buffer (10 mm Tris-Hcl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 2.5 mM dNTP, 5.0 µm of each RAPD primers (Table I-1), 50 ng of template DNA and 2U Taq DNA polymerase.

A single primer was used in each PCR reaction. Amplifications of DNA fragments were carried out by using a thermal cycler with the following cycling profile: pre-denaturation at 95°C for 2 min, followed by 45 cycles of amplification (denaturation at 94°C for 1 min, extension at 72°C for 2 min) ending with a final extension at 72°C for 4 min.

Agarose gel electrophoresis

DNA fragments were separated by horizontal gel electrophoresis. Typically a 0.8 % (w/v) agarose gel was prepared containing 0.2 µg ml ethidium bromide and submerged in TAE electrophoresis buffer. DNA samples were mixed with 6X DNA sample buffer in a 5:1 ratio and loaded into gel wells. Electrophoresis was typically at 70 V for 36 min. Gels were visualized using UV.

Nucleotide sequencing and alignment

16S rDNA sequencing of isolated strain was carried out by MacroGen (Korea). Sequences obtained were compared to the non-redundant nucleotide database at the National Centre for Biotechnology Information by using their world wide website, and the BLAST (Basic Local Alignment Search Tool) algorithm.

Isolation of plasmids

Plasmids of bacterial isolates were investigated by QIAprep spin miniprep kit. The DNA samples were subjected to electrophoresis along with standard supercoiled DNA ladde**r.**

Results and Discussions

Isolation of Heavy Metal Resistant Bacteria:

In the present study, bacteria were identified and characterized as heavy metal resistant bacteria, isolated from water wells from Taif. The enrichment isolation technique was used to isolate metal resistant bacteria. Isolated colonies were picked up from plates according to their different form and purified by subculturing onto fresh nutrient-metal agar plates using the streak-plate technique.

Thirteen isolates were screened from initial level of heavy metal supplemented LB medium. Two bacteria were isolated and found resistant to the tested heavy metals in this study. These two isolated bacteria were chosen for more investigation. These bacteria showed high resistance to three heavy metals used in our study (Table 2, 3 and 4). Both strains found more resistant to

higher concentrations of Ag and Pb till 1000 μg/ml followed by Cd till 900 μg/ml.

Results of biochemical testes:

All the strains used under this study were Gram positive (G +) and negative under Starch hydrolysis and Mannitol salt Agar however, the results of Maltose hydrolysis Test was positive

Isolation of plasmids

No plasmid was found in both isolates bacteria.

RAPD PCR

The two unknown resistance bacteria used in this study were identified by RAPD PCR technique with three random primers (Table 1). However, only one primer (*OPE-21*) (Fig. 1) yielded particularly discriminatory patterns and revealed different bands ranging from 400 to 1200 bp. From these results we could concluded that both samples in lanes 1 and 2 were more closely which may confirm that they were originated from same species. The degree of the heavy metals resistance to the highest concentration in the media was evaluated based on the ability of the isolated bacteria to grow on the subsequent higher concentrations.

The two of the bacterial isolates grew well in the presence of various concentrations of the heavy metals ranging from 100- 1000 μg /ml. (Table 2, 3 and 4). Among the bacterial isolates OSTA M1 and OSTA M2 showed multiple-metal resistance to high concentration and Ag metals. In addition both isolates shown less resistance to Cd. Microorganism possess different mechanisms to deal with high concentrations of heavy metals (Campos Garcia, 1997) (Figure 1). Result of RAPD PCR with *OPE20* primer as; First lane from the left marker (100 pb), lane 1 (strain OSTA M1), lane 2 (strain OSTA M2). Many studies have revealed that certain microorganism can resist the toxicity of heavy metals even through the acquisition of specific resistance systems such as efflux and uptake mechanisms, extracellular precipitation (Bezverbnaya, 2005). *B. megaterium* resistant to Pb, Cd, Cu was isolated and Cr metals (Velusamy *et al.*, 2011). In addition different genra and species of heavy metal resistant bacteria (*B. aquimaris* and *B. cereus)* from river water was also reported (Qiuzhuo *et al.*, 2014).

Rasulv *et al.* (2015) isolated and identified the resistant *Azotobacter chroococcum* XU1 bacteria which can remove silver from water by biosorption process. Many studies reported that *Bacillus subtilis* can survive in different environments. For example, Nanganuru *et al.* (2012) reported that the role of *B. subtilis* in the absorption, accumulation degradation and detoxification of plumb from environment. *B. subtilis* was the most tolerant to lead. *Bacillus subtilis* is able to accumulate lead ions in its cell wall (Beveridge *et al.*, 1989). Some strains of *Bacillus subtilis* were tolerant to Cu 2+ (Hookoom and Puchooa, 2013) and Ag (Clement and Jarrett, 1994). Tharannum *et al.* (2012) reported that *Bacillus subtilis* has the capacity to tolerate and grow at different degradation and detoxification of plumb from environment. The B. *subtilis* strain KPA (Khusro *et al.*, 2014) was able to tolerate lead. The *B. subtilis* is able to accumulate lead ions in its cell wall. Lead resistant bacteria isolated and it had the ability to precipitate of lead with in the cell (Levinson and Mahler, 1998). In addition, bacteria can resistant to Ag and Pb may be based on RND-driven transenvelope efflux in gram negative bacteria, efflux by P-type ATPases in gram-positive organisms, and additional complexation by intracellular compounds (Nies, 1999).

Table.1 RAPD primers and their sequences used in this study

Primer	Primer Sequence 5´-3´
OPE-20	ACGGTGACC
OPF-06	GGGAATTCGG
OPS02	CCTCTCTG

Table.2 In vitro growth of all two isolates in different concentrations of Pb metal

Pb metal concentration (µg/ml)	Strain OSTA M2	Strain OSTAM1
100	+	+
200	+	+
300	+	+
400	+	+
500	+	+
600	+	+
700	+	+
800	+	+
900	**+**	**+**
1000	**+**	**+**

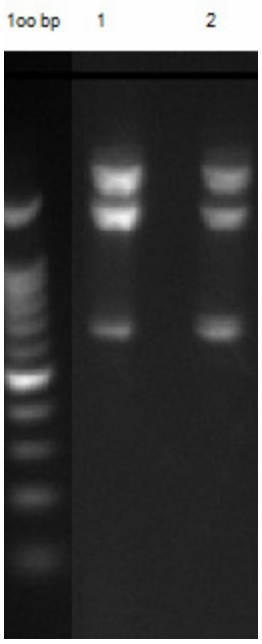
Table.3 In vitro growth of all two isolates in different concentrations of Cd metal

Cd metal concentration (µg/ml)	Strain OSTA M2	Strain OSTA M1
100	+	+
200	+	+
300	+	+
400	+	+
500	+	+
600	+	+
700	+	+
800	+	+
900	**+**	**+**
1000	**-**	**-**

Table.4 In vitro growth of all two isolates in different concentrations of Ag metal

Ag metal concentration (µg/ml)	Strain OSTAM2	Strain OSTAM1
100	+	+
200	+	+
300	+	+
400	+	+
500	+	+
600	+	+
700	+	+
800	+	+
900	+	+
1000	+	+

Figure.1 Result of RAPD PCR with OPE20 primer as; First lane from the left marker (100 pb), lane 1 (strain OSTA M1), lane 2 (strain OSTA M2)



The two strains (OSTA M1and OSTA M2) from different sources of water wells were isolated and analyzed using various methods and showed resistant to three different heavy metals (Ag, Cd and Pb) with various concentrations from 100 to 1000 µg/ml. Preliminary characterization using microscopic investigation has indicated that the selected stains were Gram positive bacteria and rod shaped in their structure.

Biochemical test were carried using starch hydrolysis; Mannitol. 16S rDNA and RAPD PCR results indicated a strong relationship between these bacteria strains and *Bacilli*. The strain was identified as *B. subtilis* sp. The multiple heavy metal resistant bacteria of our study can be applied for bioremediation processes.

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