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RESEARCH ARTICLE

BIOREMEDIATION OF TOXIC HEAVY METALS BY WASTE WATER *ACTINOMYCETES*

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ABSTRACT

Efficient and safe wastewater treatment methods with low costs with minimum requirements are urgently needed instead of conventional and currently used methods. The aim of the present study was to isolate resistant microbes from wastewater treatment plant and selection of the most active isolate for bioremediation process. Three different waste water samples were collected from Bani Malek, heated and used for actinomycetes isolation on starch nitrate agar with Cu++ and Cd++. All plates were incubated at 45°C for 10 days and the obtained bacterial colonies were purified and preserved. Growth and resistance to different concentration of heavy metals including Cu++, Cd++, Ni++ and Pb++ of the five obtained bacterial isolates were determined in minimum broth medium. The isolate WW1 was the most resistant isolate, thus it was identified as *Streptomyces* sp WW1 using different methods including morphological, physiological and biochemical methods .The selected and identified *Streptomyces* sp was used for removal of heavy metals from treated waste water of Bani Malek Plant. The removal percentages (%) were 12%, 22%, 16%, 24%,12% and 11% for Cd++, Cr++, Cu++, Fe++, Ni++ and Zn++. Moreover, it was 79 and 32% for Mn++ and Pb++ respectively. In conclusion, actinomycetes genus *Streptomyces* can be used safely for heavy metal bioremediation processes.

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INTRODUCTION

Pollution of water and soil is a major problem in the world, as much of the population use soil as cultivable land and groundwater as a major source of drinking water. Contamination with heavy metals causes a potential health hazard and toxicoses in humans and animals (Volesky and Holan, 1995). Microorganisms have an excellent role in removal of heavy metals and toxic minerals and there are many various biological and biogeochemical cycles and mechanisms transform soluble and insoluble forms of xenobiotics to non toxic or less toxic forms. Gadd (2000) reported the importance of bioremedial treatment processes for solid and liquid wastes. Recovering of metals from waste streams using of microorganisms has raised growing attention (Watanabe, 1997). A wide range of life or dead biomass of bacteria, algae and fungi were used as biosorbents for heavy-metal remediation (Gadd 1993; Diels *et al*., 1993; Kotrba *et al*., 1999). Bacteria process different strategies to remove heavy metals from soil and water environments and bacterial resistance to copper, cadmium, arsenic, mercury and lead have been documented (Silver and Phung 1996; Nies and Brown 1998; Xu *et al*., 1998) and several bacterial genes responsible about heavy metal tolerance have been detected (Cai *et al*., 1998; Xiong and Jayaswal 1998; Hassan *et al*., 1999). The filamentous bacteria (Actinomycetes), belonging to Gram-positive bacteria, live mainly as a significant component of soil and sometimes in marine and aquatic sediments and have many roles in removal of different heavy metals and pollutions (Siñeriz *et al*., 2005). Copper is needed at low concentration (<0.1 µM) to normal functions of living cells but it is very toxic at high concentrations because its production of free radicals that caused a serious damage to the cell (Gutteridge and Wilkins 1983). Nevertheless, bacteria exposure to toxic levels of copper has led to the development and evolution of various genetics mechanisms that regulate the uptake and resistance to copper (Cooksey, 1993). Plasmid pRJ1004, that confers copper resistance to *E. coli*, is one example of these mechanisms (Tetaz and Luke, 1983) is a conjugate 78-megadalton plasmid found in an *E. coli* strain isolated from the effluent of a piggery where pigs were fed a supplemented diet with copper sulphate Trajanovska *et al*. (1997) reported the detection of copper and other heavy metals resistance genes in Gram-positives and Gram-negatives isolated from a lead contaminated area. There is still less information about the genes that codifies heavy metal resistance in actinomycetes. Concerning mercury resistance in *Streptomyces*, it is related to the presence of giant linear plasmids (Ravel *et al*., 2000 a, b).

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Cadmium is a very toxic metal and has been found in the environment at increased concentrations producing different pathologies in humans and animals. The accelerated growing of industrial activities producing this contamination has increased the cadmium liberation at a higher rate than the one of the natural geochemical processes (Abbas *et al*., 2014). The monitoring of the viability of cadmium resistant actinomycetes in culture medium and in soil samples is of considerable importance because of the potential capacity of these strains in the bioremediation of cadmium (Amoroso *et al*., 1998). The aim of the present study was isolation and characterization of an actinomycete isolate resistant to some toxic heavy metals.

MATERIALS AND METHODS

Waste water collection

Some actinomycete strains from Bani Malek Wastewater Treatment Plant, Jeddah, Saudi Arabia were isolated to study their activities for biological treatment and removal of heavy metals from the wastewater. For this purpose, 3 wastewater samples representing the main stages of treatment in the plant, namely: the influent, the secondary sedimentation tank and the effluent were collected. The collected sample was transferred to a sterile plastic container and taken immediately to the laboratory and maintained at 4°C for further studies. The effluent was examined, characteristics and the concentrations of some heavy metals (Cu, Fe, Mn, Pb and Zn ions) were determined (APHA, 1992).

Isolation of resistance isolates

Wastewater was heat treated to 80°C for 15 min and used to isolate thermostable actinomycetes using minimal medium (g/l): 0.5 L-asparagine, 0.5 K_2HPO_4, 0.2 $MgSO_4$, 0.01 $FeSO_4$ and 10 D-glucose. Agar (15-20 g/l) was used to solidify the medium and as previously reported for actinomycetes isolation, 10.0 µg/ml of nalidixic acid and cycloheximide were added to inhibit growth of Gram-negative bacteria and fungi (Amoroso *et al*. 1998). To the prepared sterile medium, 100 ppm of sterile $CuSO_4$ and $CdSO_4$ solutions were added to isolate resistant bacteria. About 0.1 ml of each diluted wastewater sample was spread on the agar surface and the plates were incubated at 45°C for 10 days and the purified isolates were maintained on agar slants of the same medium at 4°C until used. The colonies were microscopically analyzed and transferred to starch nitrate agar (Shirling and Gotlieb, 1966). Minimal medium supplemented with sterile filtered stock solutions of the heavy metals tested has to be used in resistance determination experiment. The tested heavy metals were $Cd(NO_3)_2$, $CuSO_4$, $NiCl_2$, or $Pb(NO_3)_2$.

Heavy metal quantification

The concentrations of each heavy metal ion (Cu, Fe, Mn, Pb, Cd, Cr, Ni and Zn) were measured by atomic absorption spectrophotometer, Perkin Elmer 2380 according to Gajan and Larry (1972) and El-Sawi *et al*. (1994).

Screening for HM resistance

Five thermo tolerant actinomycete isolates were screened in 250 Erlenmeyer flasks containing 50 ml of minimal broth medium with different conc. of HM. About 2 ml ($4x10^6$ cfu/ml) of the preculture each bacterium previously grown in starch nitrate broth medium was used to inoculate each flask. All flasks were incubated at 45°C for 5 days and growth was recorded at 550 nm.

Removal of HM by the selected isolate

The bacterial isolate with maximum growth in presence of HM was cultivated in 50 ml of filter sterile WW in 250 ml Erlenmeyer flasks. Each flask was inoculated with a 2 ml (4×10^6 CFU/ml) of the bacterial suspension, previously grown in starch nitrate medium for 2 days at 45°C and 120 rpm. The inoculated flasks were incubated at 45°C and120 rpm for 10 days. Finally, bacterial cells were collected by centrifugation at 10,000 rpm for 15 min and the cell-free supernatant was used for heavy metals analysis as described before. The efficiency of the actinomycete strain in treatment of the wastewater sample and removal of each HM was determined. The treatment experiment was run in triplicates.

Identification of the most resistant isolate to Cu and Cd

The isolate with the highest resistant to HM was identified on the basis of colony morphology, microscopic examination using light and electron microscopy, sensitivity to different antibiotics and biochemical and physiological tests (Aly *et al*., 2011a, b, 2012). Whole-cell sugar composition and analysis of diaminopimelic acid isomer were as described by Hasegawa *et al*. (1983). The present phospholipids and the methyl esters of the fatty acids were determined respectively, using by two-dimensional thin-layer chromatography (Hoischen *et al*., 1997) and gas chromatography (Butte, 1983).

Statistical analysis

Mean of three replicates ±standard deviation were recorded and difference between mean values was determined using Student's t-test. Differences were considered significant when probability was less than 0.05.

RESULTS

The treated waste water effluent sample was characterized and HM concentrations were recorded in Table 1. The detected HM in treated waste water effluent sample were Cu^{++}, Cd^{++}, Cr^{++}, Fe^{++}, Ni^{++}, Pb^{+++}, Mn^{++} and Zn^{++}. Five bacterial isolates were isolated in heavy metal containing medium, purified and preserved on starch nitrate agar slants. The 5 isolates were grown in minimum medium containing different concentrations (conc.) of Cd^{++}, Cu^{++}, Ni^{++} and Pb^{++}. In minimum medium, maximum growth was recorded in absence of HM and the bacterial growth was decreased with increasing concentration of HM (Table 2). No growth was recorded for isolates WW2 and WW5 at 120 ppm of Cd. The isolates WW3, WW4 and WW5 showed no growth 400 ppm of Cu, while isolates WW4 and WW5 had no growth at 200 ppm of Ni. Also, no growth was found for the isolates WW2, WW3, WW4 and WW5 at 400 ppm of Pb. The isolate WW1 was the most resistant one to the different HM. It was selected, characterized and identified using morphological, physiological and chemical characters (Tables 3, 4, 5, 6 and 7). It grows well on different agar

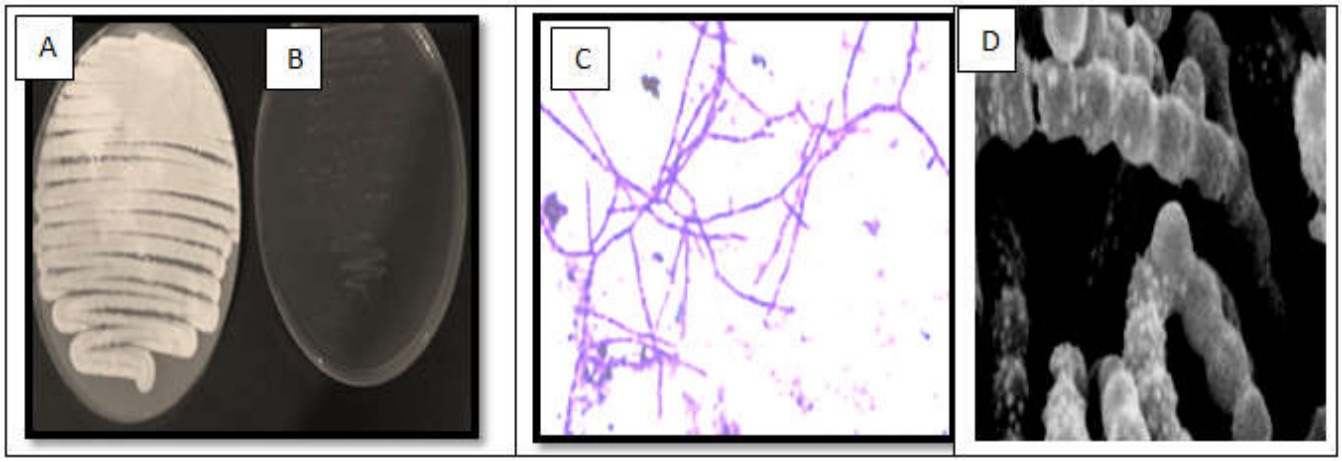

Fig. 1. The isolate WW1 on starch nitrate agar (A), on Minimal agar medium (B), under light microscope (C) and under electron microscope x 15000 (D)

Table 1. Characteristics of Bani-Malik wastewater effluent used for the isolation of heavy metal-resistant bacteria

Parameter	Value	Heavy metals (mg/l)	Value
Temperature	43±1.07	Cu^{++}	2.88± 0.07
pH	7.45±0.64	Cd^{++}	1.99 ± 0.43
Electrical conductivity (dS/m)	2.40±0.07	Cr^{++}	1.37 ±0.61
Total dissolved solids (mg/l)	151.2 ±4.8	Fe^{++}	1.50±0.22
Organic carbon (%)	3.5 ±0.59	Ni^{++}	0.032± 0.01
Organic matter (%)	6.13 ±0.07	Pb^{+++}	0.014± 0.07
COD mg/l	17.10±0.74	Zn^{++}	0.076±1.07

Table 2. Growth (absorbance at 550 nm, A_{550nm}) of the obtained actinomycetes in minimum broth medium with different concentrations of heavy metals after 5 days of incubation at 45°C and120rpm

Isolate/ Color	WW1/ Pink	WW 2/ Gray	WW3/ Yellow	WW4/ Gray	WW5/ White
Heavy metal conc. (ppm)					
Control (0.0)	1.92	2.40	1.22	0.99	1.33
Cd					
50	2.122	1.440	1.664	1.70	0.99
100	0.252	0.252	1.120	0.90	0.71
120	0.122	ND	0.122	0.24	ND
Cr					
50	1.003	2.122	1.006	1.068	0.570
100	0.180	0.252	0.212	0.412	0.110
120	0.119	0.122	ND	ND	ND
Cu					
100	1.123	1.052	1.616	1.00	0.730
200	0.552	0.442	0.442	0.612	0.390
400	0.222	0.212	ND	ND	ND
Fe					
50	2.002	2.125	1.466	1.13	1.170
100	0.952	0.252	1.112	0.682	0.490
200	0.422	0.127	0.212	0.192	ND
Ni					
50	1.199	1.122	0.966	1.66	0.970
100	0.352	0.252	0.612	1.12	0.790
200	0.022	0.022	0.112	ND	ND
Pb					
100	1.422	1.122	0.966	1.66	0.670
200	0.292	0.252	0.412	1.12	0.190
400	0.182	ND	ND	ND	ND

ND: not detected

Table 3. Cultural characteristics of the actinomycete isolate WW1, grown on different agar medium at 45°C

Media used	Growth	Color of aerial mycelium	Color of substrate mycelium	Presence of soluble pigment
Starch-nitrate agar	Heavy	Pink	Dark brown	-
In-organic salts-starch iron agar	Moderate	White	Dark brown	-
Tyrosine agar	Poor	Yellowish white	Yellow	-
Yeast extract-malt extract agar	Poor	Brown	Dark brown	-
Oatmeal agar medium (ISP-3)	Heavy	Pale yellow	Pale brown	-
Glycerol-asparagine agar	Moderate	Pale yellow	Yellow	-

- : Soluble pigment absent

Table 4. Morphological character of the selected isolate WW1

Tested character	Result	Tested character	Result
Gram stain	Gram positive	Spore surface	Spiny
Source of isolation	wastewater	Number of spore/ chain	9-29
Motility of spore	Absent	Aerial hyphae	Well developed
Shape of spore	Cylindrical (4-5, 5-6 µm)	Substrate mycelium	Well developed
Spore chain	Straight	Zoospore, Sporangium, Sclerichia, Fragmented mycelium	Absent

Table 5. Physiological characteristics of the isolate WW1

Character	Reaction	Character	Reaction
Melanin pigment on Tyrosine agar	-ve	Tolerance to 10% NaCl	+
Enzyme activities:		pH range	6-9
Proteolysis	+ve	Growth temperature:	10 - 55ºC
Lipolysis	+ve	Resistance to antibiotic	
Hydrolysis activities:		Penicillin	+
Chitin	+ve	Kanamycin	+
Gelatin	+ve	Rifampin	-
H_2S Production	-ve	Streptomtcin	+

Table 6. Utilization of different carbon and nitrogen sources using the selected isolate WW1

Utilization of different carbon source	Result	Utilization of different nitrogen source	Result
Positive control (glucose)	++	Na NO_3	++
Negative control	-	NH_4NO_3	++
D-mannitol	+ +	$NaNO_2$	-
Glycerol	++	NH_4Cl	++
Raffinose	-	Phenyl alanine	++
D- galactose	-	Valine	++
D-xylose	+ +	Peptone	++

-: No utilization. ++ Utilization

Table 7. Sugars, amino acids, phospholipids, and fatty acids of the cell wall or cell hydrolysate of the selected isolate MM10

Type of the reaction	Results	Type of the reaction	Results
Cell hydrolysate		Phospholipids	+
Diagnostic sugar	-	-Phosphatidylethanolamine	+
Cell wall amino acids		Saturated branched and unbranched fatty acids	+
-Diaminopimelic acid	+ (L-Form)		

Table 8. Percentage of removal of different heavy metals from industrial waste water using the selected isolate WW1

(mg/l)	Cd^{++}	Cr^{++}	Cu^{++}	Fe^{++}	Mn^{++}	Ni^{++}	Pb^{+++}	Zn^{++}
Before treatment (Raw waste water)	0.12±0.04	5.75±0.82	1.33±0.02	1.58±0.42	0.44±0.04	0.19±0.07	0.6±0.02	0.26±0.09
After treatment with the isolate WW1	0.009±0.02*	0.014±0.01*	0.05±0.01*	0.30±0.06*	0.096±0.04*	0.03±0.01*	0.41±0.05*	0.196±0.06*
Removal (%)	92.5	99.5	97	80	79	83	32	25

*: Significant results compared to control (Raw waste water)

medium without soluble pigment production. Aerial hyphae with long chain of spiny surface conidia and substrate mycelia were observed. It has the ability to hydrolyze different substrates and utilize different carbon and nitrogen sources. The activity of the selected isolate in HM removal was determined. Maximum removal percentage (99.5 %) was for Cr, 97 and 92.5 % were for Cd and Cu, respectively. Moderate removal percentages 80, 79 and 83 % were recorded by the selected isolate WW1 for Fe, Mn and Ni, respectively. The lowest removal activities 32 and 25% were recorded for Pb^{++} and Zn^{++} (Table 8).

DISCUSSION

Heavy metals are known as harmful pollutants in Wastewater with a negative effect on Biological Wastewater Treatment plant including microorganisms. Some heavy metals including lead and chromium even at low levels are toxic for microorganisms. As a rule, heavy metals have many negative effects on the growth of water microorganisms as they greatly depress their numbers. On one hand, the number of microorganisms depends on the total content and concentrations of particular forms of heavy metals (Sterritt and Lester, 1980). On the other hand, it is conditioned by several other factors, quantity and quality of organic matter, especially carbohydrate rich organic matter, pH, total exchange capacity, nutrient availability, moisture, temperature and oxygen availability. Heavy metals shift the structure of microbial populations, impoverish their diversity and affect species composition, reproduction and activity of indigenous microorganisms. Contamination of wastewater with high rates of heavy metals caused a significant decrease in the numbers of

bacteria in biological system (Majid, 2010). It is obvious that heavy metals are very danger contaminant in waste water and disorder of biological waste water treatment is as a result of this pollution (Akpor and Muchie, 2010).

Waste water contained different taxa of bacteria among them spore forming actinobacteria groups. Actinobacteria including streptomycetes are prominent soil bacteria with strong secondary metabolism enable them to cope with stress factors including toxic levels of heavy metals (So *et al*., 2000). Five resistant actinomycetes were isolated from wastewater at high temperature on starch nitrate agar with antibiotic and HM. Aly *et al.* (2011b) used the same medium with antibiotic to isolate resistant actinomycetes. Addition of Copper and Cadium to the isolation medium improves HM resistant isolate isolation. Copper and Cadmium are very toxic metals in the environment and at high concentrations produce different pathologies in humans and animals (Friberg *et al*., 1979).There is a lot of information available on copper resistance genetic mechanisms in Gram-negatives bacteria (Williams *et al*., 1993; Munson *et al*., 2000) but little has been done in Gram-positives especially actinomycetes. Mercury resistance in Streptomyces is related to the presence plasmids (Ravel *et al*., 2000a, b) while heavy metals resistance genes in Gram-positives isolated from a lead contaminated area was reported (Trajanovska *et al*., 1997). Some heavy metals are essential micronutrients and are incorporated into enzymes and cofactors (Fe^{+2}, Zn^{+2}, Mn^{+2}, Co^{+2}, Cu^{+}, Ni^{+2} and Mo^{+2}) but become toxic in high concentrations due to binding to enzymes and DNA or production of oxygen radicals (Lopez-Maury *et al*., 2002). Thus, bacterial cell must maintain a homeostasis that keeps optimum and non toxic level of heavy metals. Resistance factors of bacterial cells may maintain heavy metals intracellular at low levels or intracellular fractionation in non-harmful complexes (Eitinger and Mandrand-Berthelot, 2000). Thus, adaptation of a microbial populations lead to higher heavy metal resistance compared to populations of non-contaminated sites. Resistance to heavy metal may due to detoxification of oxygen radicals by superoxide dismutase overproducing strains (Geslin *et al*., 2001) and members of Streptomycetes have two superoxide dismutases, one iron- and one nickel-containing enzyme (Kim *et al*., 1998a,b).

Resistance mechanisms may include adsorption of generally positively charged heavy metal cations to bacterial cells walls. *Bacillus sphaericus* cell walls are commercially used for treatment of water (Raff *et al*., 2003). Heavy metal resistance in Gram-negative bacteria may due to efflux transporters (Mergeay *et al*., 2003; Nies, 2003, Merten *et al*., 2004). The most active isolate was identified according to morphological, physiological and chemical characters as described by Aly *et al*. (2011b, 2012, 2015). Industrial activities producing HM contamination has increased and the cadmium liberation at a higher rate than the one of the natural geochemical processes (Nriagu and Pacyma, 1988). The monitoring of the viability of cadmium resistant actinomycetes in culture medium and in soil samples is of considerable importance because of the potential capacity of these strains in the bioremediation of cadmium (Amoroso *et al*., 1998). Actinomycete strains isolated from heavy metals contaminated soils was resistance to copper at different concentrations and copper resistance may be widespread amongst actinomycetes growing in contaminated environments where bacteria which was expose to toxic levels (Siñeriz *et al*., 2005). Bacteria can evolve biochemical mechanisms to regulate metal uptake and resistant toxicity (Yang *et al*., 1993). In conclusion, HM resistant Streptomyces from waste water can be used for removal of different HM from wastewater.

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