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Isolation and characterization of a heavy metal resistant *Comamonas* sp. from industrial effluents

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Abstract

In this study, eight bacterial isolates showing a suitable resistance to heavy metals were obtained from electroplating effluents. The isolates were grown on nutrient agar including 1 mM of each heavy metal of cobalt (Co), chromium (Cr), mercury (Hg), zinc (Zn) and copper (Cu). The minimum inhibitory concentrations (MICs) of the isolates were determined using different concentrations of heavy metals (1-5 mM) by broth dilution method. One of the isolates showed higher MICs of 5, 5, 4, 3 and 3 mM when grown on Co, Cr, Hg, Zn and Cu, respectively and selected for further study. The bacterial identity was determined by various biochemical and physiological experiments. The optimum temperature and pH for the growth of the strain were 30˚C and 7, respectively. The isolate was resistant to ampicillin, cephalotin, amikacin and gentamicin. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belongs to the genus *Comamonas* with 99% similarity to *Comamonas testosteroni*; it was then designated as *Comamonas* sp. HM_AF12. Atomic absorption spectrometer analysis showed that 80% of chromium was removed from 5 mg/L of chromium solution after 1 h. The results suggest that *Comamonas* sp. HM_AF12 obtained from effluent owing to its high resistance to toxic heavy metals has great potential to be employed for bioremediation of chromium contaminated effluents.

Keywords: Heavy metals; *Comamonas*; multiple resistances; industrial effluent; bioremediation

1. Introduction

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress [1]. Industrial wastes containing toxic metals can arise from a wide variety of industrial processes. Today indiscriminate and uncontrolled discharge of metal-contaminated industrial effluents in the environment has become an issue of major concern. Heavy metals are difficult to be removed from the environment and usually accumulate in animal/plant tissues [2]. According to the World Health Organization (WHO), metals of most immediate concern include cadmium, chromium, cobalt, copper, lead, nickel, mercury and zinc [3]. These metals have exacting consequences on human such as brain damage, reproductive failure, nervous system failure and tumor formation [4]. Conventional chemical and physical remediation technologies exhibit several disadvantages such as high costs and generation of toxic slurries that are difficult to eliminate [5]. Bioremediation is a potential cost effective solution for the remediation of heavy metal –contaminated environment [6]. The bioremediation of heavy metals using microorganisms has received a great deal of attention, not only as a scientific novelty but also for its potential application in industry [1]. Microorganisms have acquired a variety of mechanisms for adaptation and response to heavy metals by several processes including exclusion, compartmentalization, complex formation and synthesis of binding proteins such as metallothioneins [7]. Microorganisms that are able to survive well in high concentration of heavy metals are of great interest as bioremediation agents because they can achieve different transformation and immobilization processes. Specifically, they conduct bioaccumulation based on the incorporation of metals inside the living biomass or biosorption, in which metal ions are adsorbed at the cellular surface by different mechanisms [8].

Many efforts have been devoted to the isolation of heavy metal resistant bacterial strains during the past years. *Pseudomonas aeruginosa* isolated from

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active sludge of industrial effluents were found to resist cadmium [9]. Zaki and Farag [10] isolated some copper resistant strains from tannery effluent. They found *Chryseobacter*ium sp., *Entrobacter* sp., *Stenotrophomonas* sp. were the most resistant strains. Cobalt resistance was shown in *C. testosteroni* isolated from contaminated soil [11]. Recently *Ralstonia pickettii* and *Sphingomonas* sp. were isolated from a copper mine tailing and shown to be resistant to high level of Zn, Cu, Pb and Ni [12].

Zolgharnein et al. [13] isolated several heavy metal resistant bacterial strains from sediment and water samples collected from the Persian Gulf and enclosed industrial areas. They found removal of lead and cadmium by some of the isolates was very efficient.

Despite many reports about microbial resistance to heavy metals, only a few attempts have been made to isolate and characterize heavy metal resistant bacteria in Iran [13]. The study of heavy metal resistant microorganisms isolated from polluted sites is of interest. It may provide new isolates and probably new genetic information on heavy metal resistance which could be exploited for bioremediation in future.

The objective of this study is the isolation and characterization of heavy metal resistant bacteria and selection of multiple metal resistant strains which might be useful in bioremediation.

2. Materials and methods

2.1. Sample collection and growth condition

The industrial effluent samples were collected under aseptic conditions in sterilized plastic containers from electroplating factories in Islamshahr, located in the south-west of Tehran, Iran and transported to the microbiology laboratory, Islamic Azad University, Islamshahr Branch, Iran for bacteriological analysis. Stock solutions of the metal salts (1 M) including $CoCl_2.6H_2O$, $ZnSO_4.7H_2O$, $CuCl_2.5H_2O$, $K_2Cr_2O_7$ and $HgCl_2$ were prepared in distilled water and sterilized by filter membrane (0.22 µm). The samples were directly streaked on nutrient agar plates supplemented with 1 mM concentration of each metal. Plates were incubated at 30˚C for 2 days. Eight colonies differing in morphological characteristics were selected and subcultured on nutrient agar containing 1 mM of each metal to obtain purified cultures.

2.2. Determination of MIC

The MICs of the metals (Cr, Co, Hg, Zn and Cu) for eight isolates were determined by dilution method [14]. Metals were added separately to nutrient broth (NB) at a concentration of 1-5 mM. The lowest concentration of metal that inhibited the bacterial growth was considered as MIC. The experiments were carried out in triplicate. The bacterial strain that showed the highest MICs was selected for further analysis.

2.3. Antimicrobial resistance test

The antibiotic resistance pattern of the selected isolate was assayed by disc diffusion method [15]. After incubation, the organism was classified as sensitive or resistant to antibiotic according to the diameter of inhibition zone given in standard antibiotic disc chart. Discs containing the following antibiotics were tested: ampicillin (Am 10 µg/ml), ceftriaxone (Cro 30 µg/ml), tetracycline (Te 30 µg/ml), cefalotin (Cf 30 µg/ml), gentamicin (Gm 10 µg/ml), co-trimoxazole (SXT 25 µg/ml), amikacin (AN 30 µg/ml) and nalidixic acid (NA 30 µg/ml).

2.4. Biochemical and physiological properties

Physiological and biochemical tests were performed as previously described [16]. The selected bacterial isolate was identified by morphological and biochemical methods in accordance with Bergey's Manual of Systematic Bacteriology [17].

Growth studies of the isolate were performed in 250 ml flasks containing 0.5 mM concentration of Co, Cr, Hg, Cu and Zn. Flasks were inoculated with 1% of the overnight culture at 30˚C and agitated at 150 rpm. Growth was monitored by measuring the absorbance at 600 nm using spectrophotometer (Beckman). The experiments were carried out in triplicate.

2.5. Molecular Identification

The isolated bacterium was identified based on the sequencing of 16S rRNA gene. Genomic DNA was extracted from the isolated bacterium using a DNA extraction kit (Roche, Germany). Bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using bacterial universal primers, F (5′ AGAGTTTGATCATGGCTC3′) and R (5′AAGGAGGTGATCCAGCC3′). The PCR mixture contained 10 pmol of each primer, 10 ng template DNA, 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM dNTP mix, 1.25 u Taq DNA polymerase (Fermentas, Lithuania), in a final volume of 50 µl. PCR amplification was performed under the following conditions: 5 min at 95˚C followed by 30 cycles of 30 s at 95˚C, 60 s at 45˚C and 60 s at 72˚C, followed by 5 min at 72˚C using Primus thermal cycler. The PCR product of 16S rRNA gene was extracted from a 1% agarose gel

(Fermantas, Lithuania) using Gel DNA Extraction kit (Fermantas, Lithuania) according to manufacturer's instruction. The purified PCR product was sequenced using an automated sequencer by Microgene Company (South Korea). BLAST analysis was performed through the National Center for Biotechnological information online services (htt://www.ncbi.nlm.nih.gov/BLAST).

2.6. Nucleotide sequence accession number

The 16S rRNA sequence determined in this study was deposited in the GenBank database under the following accession number JN573358.

2.7. Phylogenetic analysis

Multiple sequence alignments were performed using MEGA version 4 [18] after obtaining multiple alignments of data available from public databases by Clustal W [19]. Phylogenetic tree was inferred by using neighbor joining tree making algorithm [20]. An evolutionary distance matrix was generated for neighbor joining algorithm with the help of Jukes & Countor distance model [21] and bootstrap analysis (1000 resamplings).

2.8. Chromium removal

The isolated microorganism was cultured in NB at 30°C for 24 h and then harvested by centrifugation at 5000×g for 15 min. 0.5 g of harvested cells (wet weight) were resuspended in 100 ml of 5 mg/L chromium solution. The suspended solution was shaken at 100 rpm at 30°C and the samples were taken and centrifuged at 5000×g every 5 min for the first 20 min and an interval of 20 min for 2 h. The supernatant and biomass fractions from each specific time were analyzed for the remaining chromium by atomic absorption spectrometry (Philips, PU 9100X). For determination of chromium concentrations in the biomass, bacterial cells were heated to 105°C overnight. The dried cells were dissolved in 1 mL 95% nitric acid (Merck Co.), mixed well and incubated in a water bath at 100°C for 1 h. The mixture was later cooled to 25°C and diluted to 10 mL with sterile distilled water. All the above experiments were performed in triplicate.

2.9. Statistical analysis

Analysis of variance was used to compare treatment means. All the statistical analyses were carried out using SPSS 16.0.

3. Results

A total of eight bacterial strains were isolated from electroplating effluent. They were grown on a culture medium containing 1 mM of each heavy metal of choice. Then, the MICs of 5 metal ions were determined against the isolates. The MIC results indicated that all isolates had high resistance to one or more metal ions. However, one isolate was shown to be resistant against all metal ions and was selected for further analysis (Fig. 1). The bacterial growth was remarkably decreased after addition of 5 mM Cr and Co, 3 mM Cu and Zn, and 4 mM Hg. The highest MIC was observed for Cr and Co (5 mM). The order of toxicity of the metals to the selected strain was found to be Cu=Zn>Hg>Cr=Co. The bacterial strain also exhibited antibiotic resistance characteristic of ampicillin, cephalotin, amikacin and gentamicin (Table 1).

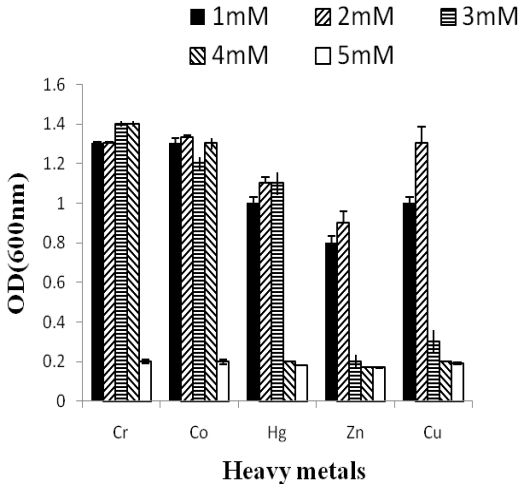

Fig. 1. Minimum inhibitory concentration (MIC) of heavy metals for *Comamonas* sp. HM_AF12 isolated from effluent. Values indicate the means ± standard deviation (SD) of three independent experiments.

Table 1. Microbiological characterization of *Comamonas* sp. HM_AF12.

Characteristic	Results
Gram staining	-
Morphology	Rod
Spore	-
Motility	+
Catalase	+
Oxidase	+
Indole	-
Pigment production	-
Acid from:	
Glucose	-
Sucrose	-
Xylose	-

Table 1. (Continued)

Hydrolysis of:	
Starch	-
Urea	-
Gelatin	-
Utilization of Citrate	+
Antibiotic sensitivity to:	
Tetracycline	+
Gentamicin	-
Nalidixic acid	+
Cephalotin	-
Ceftriaxone	+
Co-trimoxazole	+
Amikacin	-
Ampicillin	-

+, substrate is utilized, substrate is hydrolyzed, strain is antibiotic sensitive. - , substrate is not utilized, substrate is not hydrolyzed, or strain is antibiotic resistant.

The major physiological and biochemical properties of the isolate were summarized in Table 1. The strain had a wide pH tolerance of 5.0–10.0, and salt tolerance was up to 5% NaCl. The optimum temperature and pH for growth were obtained at 30˚C and 7, respectively. The bacterial growth was completely inhibited at 40˚C showing the isolate is a mesophilic bacterium.

The growth curve of the isolate in the presence of heavy metals (Cr, Co, Hg, Cu and Zn) and untreated media (without heavy metals) as a control sample were exhibited in Fig. 2. It was found that the growth of the isolate was decreased in the presence of heavy metals.

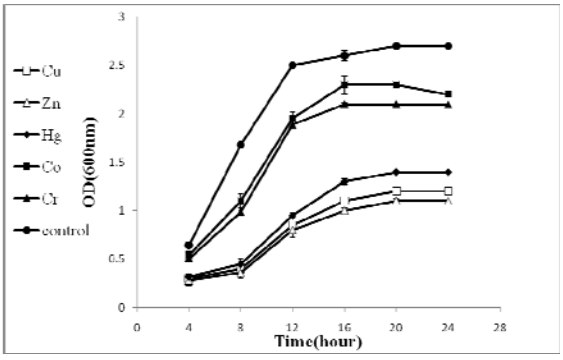

Fig. 2. Growth studies of *Comamonas* sp. HM_AF12 in the presence of heavy metals. Values indicate the means ± SD of three independent experiments.

The PCR product of 16S rRNA gene revealed ~1400 bp DNA band on agarose gel electrophoresis (Fig. 3). The 16S rRNA analysis revealed that the isolate belonged to the genus *Comamonas* in the β-subclass of the *proteobacteria,* subsequently designated as *Comamonas* sp. HM_AF12. It had 99% similarity with *Comamonas testostroni.* A phylogenetic tree was depicted showing the relationship of this isolate with the genus *Comamonas* (Fig. 4). According to the phylogenetic analysis, *Comamonas* sp. HM_AF12 and *Comamonas testostroni* are in the same branch supported by a high bootstrap value of 100% (Fig. 4).

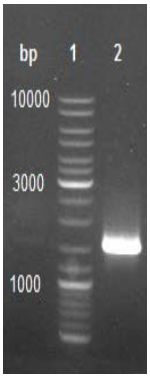

Fig. 3. Ethidium bromide-stained 1% agarose gel electrophoresis of 16S rRNA gene. Lane 1, 10 Kb DNA size marker; Lane 2, PCR product of 16S rRNA gene.

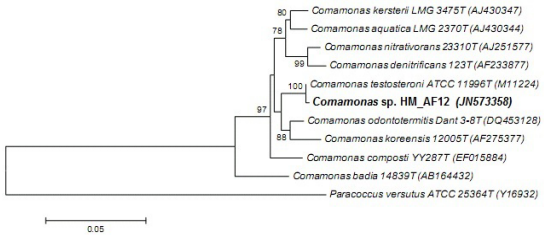

Fig. 4. Phylogenetic dendrogram derived from 16S rRNA sequence analysis (neighbor joining) showing the position of strain HM_AF12 within the genus *comamonas* in the family *Comamonadaceae*. Numbers at nodes are percentage bootstrap value based on 1000 resampled, datasets. Only values above 50% are given. *Paracoccus versutus* served as out group [34]. Accession numbers are given in parentheses. Bar, 0.05 sequence dissimilarity.

The removal of chromium by *Comamonas* sp. HM_AF12 in 5 mg/L of chromium solution is shown in Fig**.** 5. Chromium was removed up to 80% (approximately 100 mg/g dry weight) during a period of 60 min while the initial metal concentration was 5 mg/L. As shown in Fig. 5, chromium concentration rapidly decreased during the first 20 min in the supernatant.

4. Discussion

Bioremediation of metal pollutants from industrial waste water using metal resistant bacteria is a very important aspect of environmental biotechnology. The industrial effluents harbor a large number of bacteria resistant to high concentrations of heavy metals. This high level of resistance may represent

bacterial adaptation to the metals present in industrial effluents. For the purpose of defining metal resistance, those bacteria which are not inhibited by 1 mM of heavy metal ions were regarded as resistant [14]. The results of this study showed that *Comamonas* sp. HM-AF12 was particularly resistant to multiple metals. Similar results have been reported earlier [11, 22]. Since heavy metals are all similar in their toxic mechanisms, multiple tolerances are common phenomenon among heavy metal resistant bacteria [22].

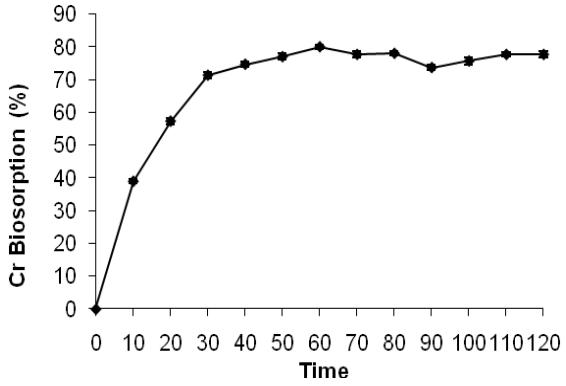

Fig. 5. Removal of chromium by *Comamonas* sp. HM_AF12 isolated from electroplating effluents in solution containing 5 mg/L chromium. Values indicate the means ± SD of three independent experiments.

The results of this study indicated that *Comamonas* sp. HM_AF12 was closely related to members of the genus *Comamonas* on the basis of 16S rRNA sequence analysis. The major physiological properties of this strain were in agreement with those described for the genus *Comamonas* [17]. The members of the genus *Comamonas* are gram-negative, strict aerobes and frequently occur in diverse habitats, including activated sludge, marshes, marine habitats and plant and animal tissues [23, 24]. They have many genes for transportation and signal transduction, which allows the cells to respond and adapt to changing environments. Their diversified niches make them environmentally important and also suggest that they represent a group of bacteria that can adapt very well, both ecologically and physiologically, to environment [25]. The previous reports showed that some bacteria related to genus *Comamonas* have displayed resistance to heavy metals. It was reported that *Comamonas* sp. BS501 had a high level of resistance (5 mM) to cobalt/nickel [26]. Cai et al. [27] isolated some highly arsenite-resistant *Comamonas* (MIC>20 mM) from the highly arsenic-contaminated soil. Staniland et al. [11] showed that *C. testosteroni* TDKW exhibited resistance to high concentrations of iron and manganese, and concentrations above 4 mM of Co completely inhibited growth of *C. testosteroni*. *Comamonas* sp. HM_AF12 was more resistant to Co than C. *testosteroni* TDKW and had also high resistance to Cr, Hg, Zn and Cu (Fig. 1). The results indicated that *Comamonas* sp. HM_AF12 was able to grow well in the presence of 0.5 mM of heavy metals. Although the growth pattern of the isolate was not significantly different from that of control, the growth of the isolate was decreased in the presence of heavy metals. These observations were in agreement with previous reports [28, 29]. The ability of microbial strains to grow in the presence of heavy metals would be helpful in the waste water treatment where microorganisms are directly involved in the decomposition of the organic matter in biological processes because of the inhibitory effect of heavy metals in a common phenomenon that occurs in the biological treatment of wastewater and sewage [30].

A high degree of metal resistance associated with multiple antibiotic resistances was detected in the selected isolate (Table 1). In most of the studies metal resistance is linked with antibiotic resistance [31, 32]. Similarly Filali et al. [30] studied waste water bacterial isolates which were resistant to heavy metals and antibiotics. The exposure to heavy metals results in the selection of bacterial strain also able to resist antibiotics. This happens because genes encoding heavy metals are located together with antibiotic resistance genes. Under conditions of metal stress, metal and antibiotic resistance in microorganisms possibly help them to adapt faster by the spread of resistant factors than by mutation and natural selection [33].

Ma et al. [25] reported that the *C. testosteroni* has heavy metal efflux pumps and efflux transporters for various drugs. The genes encoding these pumps and transporters possibly confer additional resistance to heavy metals and drugs to *C. testosteroni*.

It was found that *Comamonas* sp. HM_AF12 was capable of taking up chromium. Maximum chromium removal was found to be as high as 80% when this strain is exposed to 5 mg/L chromium. The elevated rate of resistance to chromium and chromium removal capacity in this strain reflects an adaptive response to the presence of chromium in electroplating effluents.

The bioremediation of toxic metals from industrial effluents is of great importance, not only because of decontamination effect but also because this process protects the activated sludge of sewage treatment plants from action of toxic compounds. The use of microorganisms specifically adapted to high concentrations of heavy metals will increase the ability to remediate heavy metal contaminated effluents. The results of the present study suggest

that *Comamonas* sp. HM_AF12 obtained from effluent owing to its high resistance to toxic heavy metals will be a good candidate for bioremediation of highly polluted effluents in the future.

Acknowledgments

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