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Original Research

The resistance of locally isolated *Serratia marcescens* to heavy metals chlorides and optimization of some environmental factors

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Abstract
The aim of this study is the evaluation the resistance of S. marcescence obtained from soil and water to metals chlorides (Zn+2, Hg+2, Fe+2, Al+3, and Pb+2). Four isolates, identified as Serratia marcescence and S. marcescena (S4) were selected for this study according to their resistance to five heavy metals. The ability of S. marcescena (S4) to grow in different concentrations of metals chloride (200-1200 µg/ml) was tested; the highest concentration that S. marcescence (S4) tolerate was 1000 µg/ml for Zn+2, Hg+2, Fe+2, AL+3, pb+2 and 300 µg/ml for Hg+2 through 24 hrs incubation at 37 Co. The effects of temperature and pH on bacteria growth during 72 hrs were also studied. S. marcescence (S4) was affected by ZnCl2, PbCl2, FeCl2, and AlCl3 during 24 hrs, while mercury causes no bacterial growth. S. marcescence (S4) showed growth in temperature range of 30-50 Co in presence of 4 metals. The isolates showed the ability to grow in different pH values (4, 7 and 9) in presence of four metals in all pH values (1000 µg/ml) and un-ability to grow with 300 µg/ml Hg+2. The highest Zn+2 removal ratio was 75% then Pb+2 55% while Fe+2 has the lowest removal ratio (48%). The study was conducted in the central lab of College of sciences/University of Baghdad/Iraq in 2011-2012. It was conclude that the identified heavy metal resistant bacteria could be useful for bioremediation of heavy metals in the contaminated soil and water.

INTRODUCTION

Serratia marcescence produces prodigiosin pigment which shows a high sensitivity to toxicants [1]. Heavy metals pollution is one of the most serious environmental problems facing life on earth; it influences all living organisms in aquatic, terrestrial and air habitats [2,3]. Chemical and physical weathering of igneous and metamorphic rocks and soils often releases heavy metals into the sediment and into the air [4,5]. Heavy metals in surface water systems can from natural or anthropogenic sources. The combustion of fossil fuels pollutes the atmosphere with metal particulates that eventually settle to the land surface [6]. Heavy metals differ from other toxicants because they are not metabolically degradable and accumulate in living tissues resulting in death or serious health threats [7]. In high concentrations, heavy metal ions react to form toxic compounds in bacterial cells as a mechanism of bacterial tolerance to heavy metals. To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state [3]. Many bacterial species have gene that control resistance to specific toxic metals; this resistance is determined by extra chromosomal DNA molecules for toxic metal ions including Ag, Cd, Co, Hg, Ni, Pb, Te, Zn and other toxic metals [8]. Many soil sites in industrialized areas are contaminated with high concentrations of metals; these pollutants accumulated in the ecosystem and human body and cause serious toxicity [9]. One of the available strategies to remove

metals from the soil is to make use of an ecologically sound, safe, and cost effective rhizoremediation through the symbiotic relationship of plants and microbes in the rhizosphere. *Serratia* sp. K1RP-49 isolated from the rhizoplane of the barnyard grass, and *Echinochloa crusgalli* grown in oil contaminated soil can produce various organic acids that aid in accumulating metals [2]. The present study was designed to evaluate the tolerance of locally isolated *Serratia marcescence* to heavy metals chloride and the influence of some environmental factors in this respect.

MATERIALS AND METHODS

*Heavy metals stock solution*s

Stock solutions were prepared in a concentration of 10 mg/ml by dissolving 0.5gm of the chloride salts of Hg, Fe, Pb, Zn and Al in 50ml of distilled water.

Growth Media

Nutrient agar and broth were prepared and sterilized by autoclaving at 121 °C for 15 min. Different concentrations (100-1000 µg/ml) of the heavy metals chloride were added into the medium after cooling to 50°C.

Soil Samples Collection

Ten samples of the soil were collected from different areas in Baghdad governorate in sterile plastic bags, and preserved at 10^{o}C until they are used to isolate the bacteria.

Isolation and Identification of Serratia Species

One gram of each soil sample was added to 9 ml of sterilized water in test tubes; 0.5 ml of each sample suspension was spread on MacConkey agar plate and incubated at 28 C^{o} for 18 hrs. The colonies with red pigment were selected for further identification. The growing colony was streaked on nutrient agar without heavy metal chloride and this step was repeated until pure culture was obtained [9]. The shape and the colors of colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed by using Api 20E for the oxidase, catalase, urease, gelatin hydrolysis, DNase, esterase test, indol production and sugar fermentation [10].

Isolation and Identification of Heavy Metal Resistant Serratia

For the selective isolation of heavy metals resistant *Serratia*, heavy metals were incorporated in the media. Basal media nutrient agar (NA) loaded with heavy metals chloride ($AlCl_3$, $FeCl_2$, $ZnCl_2$, $PbCl_2$, and $HgCl_2$) was prepared separately. The concentration of each metal was maintained at 100µg/ml. Soil samples were diluted from 10^{-1}-10^{-3} then streaked on these media and incubated at 37°C for 24-48 hours [11]. Pure culture of bacterial isolates was stabbed into nutrient agar, then incubated at 37°C and stored in dark place at room temperature. This method may preserve bacteria for approximately one year [12].

Determination of Maximum Tolerable Concentration (MTC) Isolates

MTC of the heavy metal resistant *S. marcescens* (S4) was determined by gradually increasing the concentration of the metal 100µg/ml each time on NA plate. The starting concentration used was 100µg/ml. The culture that grows on the last concentration was transferred to the higher concentration by streaking on the plate. The maximum concentration of metal in the medium which support the growth was taken as MTC [13].

Effect of Heavy Metals on Bacterial Growth

Heavy metal resistant isolate (0.75%) was inoculated into 50ml of nutrient broth incorporated with MTC of heavy metals chloride like: Al, Fe, Zn, Pb and Hg that prepared separately; incubated at 37ºC for 3 days. Medium without metal but with bacterial inoculums (bacterial growth control) and medium with metal but without bacteria (a biotic control) served as controls. Bacterial count was recorded every 24 hrs using dilution to extinction method; also bacterial growth was measured in terms of optical density at 600 nm using spectrophotometer for 3 days at 24 hrs [14].

Effect of Temperature on Bacterial Resistance to Heavy Metals

The heavy metal resistant isolate was inoculated into 50ml of nutrient broth incorporated with different heavy metals chloride as mentioned above and incubated at different temperatures (28, 37 and 50 ºC) for 24 hours. Bacterial count was recorded by dilution to extinction method.

Effect of Different pH on Bacterial Resistance to Heavy Metals

The heavy metal resistant isolate was inoculated into 50ml of nutrient broth that prepared at different pH values (4, 7 and 9); and incorporated with different heavy metals chloride as mentioned above and incubated at 37 ºC for 24 hrs. Bacterial count recorded by dilution to extinction method; the pH was measured after 24 hrs incubation.

Adsorption of Heavy Metals Ions by Serratia marcescens

S. marcescens (S4) was grown in nutrient broth medium for 24hrs. Cells were separated by centrifugation at 6000rpm for 10min and washed three times with normal saline. 100ml from each heavy metal

solution at concentration of 150µg/ml was taken in 250ml flasks. Harvested cells were transferred to the metal solutions and incubated for 2 hrs at 37 ºC. Solutions were centrifuged at 6000rpm for 10min to separate the bacterial cells. The concentrations of three heavy metals Fe, Zn and Pb were measured by atomic adsorption spectrometer [15]. Adsorption of ions with bacterial cells was calculated as a ratio of ions removal %.

$R\ (\%) = (C_0 - C_1) / C_0 \times 100$

Where R = Removal Ratio (%); C_0= concentration of heavy metals ions in the original solution (µg/ml) and C_1 = concentration of heavy metals ions in the treated solution (µg/ml) [16]. The study was conducted in the central lab of College of sciences/University of Baghdad/Iraq in 2011-2012.

RESULTS

Isolation and identification of heavy metals resistant Serratia marcescens

Four isolates belonged to genus *Serratia* showed resistance to the heavy metals chloride Al^{+3}, Fe^{+2}, Zn^{+2}, Pb^{+2}, and Hg^{+2} at concentration of 100µg/ml. These isolates were identified as *Serratia marcescens* depending on morphological and biochemical characteristics. *Serratia* isolates showed difference in resistance to heavy metals chloride depending on the type of metals (Table 1).

Table 1. Biochemical tests of S. marcescens (S4)

Test	Result
Gram stain	-
Cell shape	Rod
Catalase	+
Oxides	+
DNase	–
Urease	–
Gelatinase	+
Esterase	+
Lactose fermentation	–
Glucose fermentation	+
Sucrose fermentation	+
Indol production	–

(+) positive result; (–) negative result

Determination of MTC (Maximum Tolerable Concentration)

S. marcescens (S4) isolates that have the highest resistance to all heavy metals used in this study at a concentration of 100µg/ml were grown in heavy metals-incorporated media at different concentrations from 100-1200µg/ml to determine MTC. Results showed that MTC for all heavy metals was 1000µg/ml except for Hg which was 300µg/ml (Table 2).

Table 2. Growth of Serratia macescens on nutrient agar containing 100µg/ml of metals chlorides incubation at 37oC for 24-48 hrs.

Isolate code	Heavy metals chloride 100 µg/ml				
	$ZnCl_2$	$FeCl_2$	$AlCl_2$	$PbCl_2$	$HgCl_2$
S1	+	+	+	–	+
S2	+	–	+	–	–
S3	+	+	+	+	–
S4	+	+	+	+	+

Effect of heavy metals on bacterial growth

For determining the effect of heavy metals on bacterial growth, *S. marcescens* (S4) was grown in nutrient broth incorporated with MTC of heavy metals that prepared separately for 3 days. This isolates exhibited different growth patterns in the presence of different heavy metals. It was observed that growth of *S. marcescens* (S4) was affected by presence of $ZnCl_2$ and $FeCl_2$ during incubation period, while Hg causes no bacterial growth (Table 3 and Figure 1).

Table 3. Maximum tolerable concentrations of S. macescens (S4) to different heavy metals chlorides incubation at 37oC for 24-48 hrs

Heavy metals chloride	MTC (µg/ml)
$ZnCl_2$	1000
$FeCl_2$	1000
$AlCl_2$	1000
$PbCl_2$	1000
$HgCl_2$	300

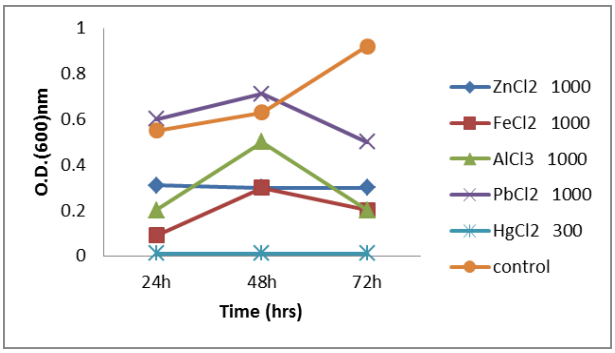

Figure 1. Growth of S. macescens (S4) in nutrient broth with different heavy metals chlorides incubation at 37oC for different time.

Effect of temperature on bacterial resistance to heavy metals

The removal of heavy metals ions from aqueous solutions by bacteria was not only affected by surface properties of the organism, but also by biosorption time and temperature. In studying the effect of temperature on the ability of *S. marcescens* (S4) to grow in the presence of certain concentrations of heavy metals, the results showed that temperature changes does not affect the absorption or adsorption of metal ions, and then on growth or inhibition of bacterial isolate. S4 isolate had clear growth in presence of $ZnCl_2$, $FeCl_2$, $AlCl_2$, and $PbCl_2$ at temperatures range from 28-50ºC, while there are no growth in the presence of $HgCl_2$ at all temperatures (Table 4).

Table 4. Effect of different heavy metals chlorides on the growth of S. macescens (S4) incubation at 37°C for 24, 48 and 72 hrs.

N. broth with Heavy metal chloride conc. (µg/ml)	Time (hours)		
	24h	48h	72h
	Bacterial No. (cell/ml)	Bacterial No. (cell/ml)	Bacterial No. (cell/ml)
$ZnCl_2$ 1000	10^5	10^5	10^5
$FeCl_2$ 1000	10^8	10^8	10^8
$AlCl_2$ 1000	10^{10}	10^{10}	10^{10}
$PbCl_2$ 1000	10^{10}	10^{10}	10^{10}
$HgCl_2$ 300	0	0	0
Control	10^{10}	10^{10}	10^{10}

Effect of different pH on bacterial resistance to heavy metals

At different pH values (4, 7 and 9), the growth of *S. marcescens* (S4) was not affected in presence of heavy metals such as $FeCl_2$, $AlCl_2$ and $PbCl_2$ compared to the control without metal amendment (Table 5 and Fig 2). Optimal conditions were reported at pH 9 and at temperature 37 C°. Strain of *Serratia* is inhibited at a pH<4.5 or at >45 C°.

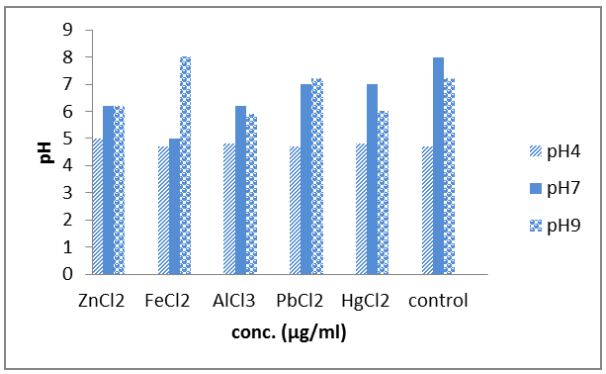

Figure 2. Variation of pH medium values on growth of S. macescens (S4) with ZnCl2 1000, FeCl2 1000 AlCl3 1000 PbCl2 1000 HgCl2 300 conc. (µg/ml).

Table 5. Effect of different temperatures on the growth (cell/ml) of S. macescens (S4) in presence of different metals chlorides.

Heavy metal chloride conc. (µg/ml)	Bacterial No. (cell/ml) Temperatures		
	28 ºC	37 ºC	50 ºC
$ZnCl_2$ 1000	10^4	10^4	10^4
$FeCl_2$ 1000	10^4	10^4	10^4
$AlCl_2$ 1000	10^5	10^5	10^5
$PbCl_2$ 1000	10^5	10^5	10^5
$HgCl_2$ 300	0	0	0
Control	10^5	10^5	10^5

Adsorption of Heavy Metals Ions by Serratia marcescens

Microorganisms may play significant role in the biogeochemical cycling of toxic heavy metals, also in cleaning upper remediating metal contaminated environments. *S. marcescens* (S4) showed the highest Zn^{+2} removal ratio (75%), while has the lowest removal ratio (48%) for Fe^{+2} (Figure 3).

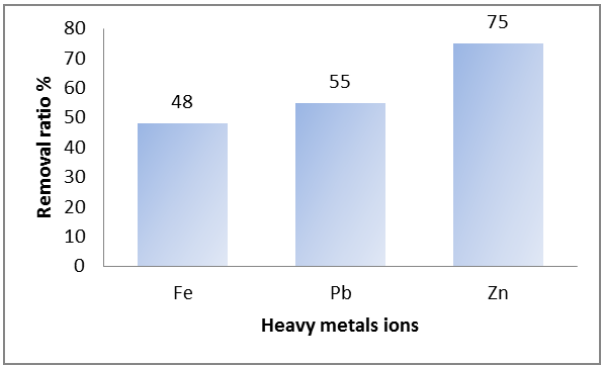

Figure 3. Adsorption of heavy metal chlorides by S. macescens (S4)

DISCUSSION

The toxicity is ranged from the highest toxic element (Hg^{+2}) to the lowest toxic element (Pb^{+2} and Al^{+3}). Low concentrations of certain heavy metals such as Zinc are essential for many cellular processes of bacteria. However, higher concentrations of these metals often are cytotoxic. Other heavy metals including Pb^{+2}, Cd^{+2}, Hg^{+2}, Ag^{+2} and Cr^{+2} have no known beneficial effects to bacteria cells, and others are toxic even at low concentrations [17]. The microbial resistance to heavy metals is attributed to a variety of detoxifying mechanisms developed by resistant microorganisms, such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal

Table 6. Effect of different pH values on the growth (cell/ml) of S. macescens (S4) in presence of different metals chlorides.

Heavy metal chloride conc. (µg/ml)	pH4		pH7		pH9	
	Bacterial No. (cell/ml)	pH after incubation	Bacterial No. (cell/ml)	pH after incubation	Bacterial No. (cell/ml)	pH after incubation
$ZnCl_2$ 1000	10^2	5	10^5	6.2	10^5	6.2
$FeCl_2$ 1000	10^5	4.7	10^5	5	10^5	8
$AlCl_3$ 1000	10^5	4.8	10^5	6.2	10^5	5.9
$PbCl_2$ 1000	10^5	4.7	10^5	7	10^5	7.2
$HgCl_2$ 300	0	4.8	0	7	0	6
Control	10^5	4.7	10^5	8	10^5	7.2

efflux, etc. These mechanisms are sometime encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another [18]. Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological and/or genetic as well as environmental modification of metal speciation [19]. Microbes demonstrate various types of resistance mechanisms in response to heavy metals; these mechanisms may be encoded by chromosomal genes, but the most usual loci conferring resistance are located on plasmid [20]. Growth rate of *S. marcescens* (S4) in the presence of heavy metals (Zn^{+2}, Fe^{+2}, Al^{+3}, Pb^{+2}) were consistently slower than that of the control. Similar results have been reported previously [20]. Bacteria exposed to high levels of heavy metals in their environment have adapted to these stressful conditions by developing various resistance mechanisms. These mechanisms could be utilized for detoxification and removal of heavy metals from polluted environment. Mercury is one of the most toxic elements and the affinity of the mercury for thiol groups is stronger than the affinity of cadmium for sulfide [3]; it binds to sulfhydryl groups of enzymes, thereby inactivating vital cellular functions [21].

The dynamic adsorption processes of metals are passive and energy-independent process [22], and the high temperature was influential in the growth rate of bacterial isolates, but did not affect or has a minor effect in the components of the cell. Every type of bacteria has an optimum- minimum and maximum growth temperature. Temperatures below that which is optimum for growth depress the rate of metabolism of bacterial cells. Above the optimal temperature, the growth rate decreases and thermal death may occur [23]. The change in pH affects metal toxicity because many metal ions form complexes with various medium or buffer components and may be precipitated by phosphates, especially at pH near neutrality or higher [23]. *Serratia marcescense* is known for production of a red pigment, which is dependent on many factors including pH. It has been reported that the choice of pH also affects metal ions binding and production of red pigment which generally decreased as the pH falls, probably because of competition for binding sites by hydrogen ions on the sorption site. Thus, at lower pH and high concentrations of protons, protonation of the negatively charged binding sites of functional groups, thereby contributing to metal binding at low pH. Some of the functional groups will be positively charged and may not interact with metal ions. However, at higher alkaline pH values (8 and above), there are induction in the solubility of metals contributed of lower uptake rate [24]. The results indicated that the bactericidal activity is manifested with highest Zn^{+2} bioremoval ratio, which showed the lower tolerance and vice versa. The same result has been reported in literatures; as well as established an inverse relationship between tolerance and metal uptake; the microorganism accumulates more metal if it is less tolerant and accumulates less metal if it is more tolerant [25]. When using intact bacterial cells, one must consider that other processes besides surface adsorption could occur. These alternative processes include active uptake of the heavy metals into the cytoplasm through non-specific transport system and precipitation of metals at the cell surface [26]. However, some of heavy metals are necessary for *Serratia* life mainly copper, iron and zinc. These metal ions are essential trace elements that are required for the activity of many enzymes. Mercury exerts its toxicity by binding to sulfhydryl groups in the body and many derivatives are potent inhibitors of some enzymes. Also, Hg^{+2} block the transport of potassium and sugar into the cell. Meanwhile, iron affect prodigiosin pigment production; increasing or decreasing its concentration (0.1-2 mg/ml) inhibits the production pigment in *Serratia marcescens* [27]. Based on the reported data, we can conclude that the isolate strain of bacteria of the genus *S. macescens* (S4) is suitable organism for accumulating large amounts of Zn^{+2}, Pb^{+2} and Fe^{+2} from contaminated soil and water.

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