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Isolation of Zinc Resistant Bacteria from Karun River in Iran and Evaluation of their Growth Kinetics

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Zinc is an essential metal ion but at elevated concentrations it can be toxic and has inhibitory effects on cell activities. There are many industries along Karun river's bank (longest river in Iran) discharging their zinc containing effluents to this river. The objectives of the present study were isolation and identification of zinc resistant bacteria from water and sediments of Karun River and study their growth kinetics. Sampling were done from water and sediments of 4 stations in 3 seasons, summer, autumn and winter. Zinc content of samples was measured by atomic absorption spectrophotometer. Bacterial numbers were count in both medium containing and without zinc. Bacterial isolation was performed by primary enrichment and direct plating methods. Isolates were examined for their resistance and growth kinetics in presence of zinc. Bacterial number was obtained 4.278 in medium containing zinc that was higher than control medium without zinc. *Pseudomonas* sp., *Escherichia coli*, *Salmonella* sp., *Citrobacter* sp. were isolated as zinc resistant bacteria. Some of these bacteria were able to tolerate 1000 mg/l of zinc. The maximum zinc uptake was found to be about 68% by *Pseudomonas* sp. Results of this experiment showed that presence of zinc cause growth cease of many bacteria. Using primary enrichment method compared with direct plating caused better isolation of resistant bacteria. Isolated bacteria were able to remove zinc metal.

Key words: Zinc resistant bacteria, Bioelimination, Heavy metals, Karun River.

Unlike organic pollutants, heavy metals are not biodegradable and undergo a global eco-biological cycle. Heavy metal anthropogenic sources have resulted in the increased levels of these metals in many aquatic environments[1]. Heavy metals are also a serious environmental problem due to their stability, bioaccumulation and biomagnification in food chains[2]. Although some of heavy metals are essential like zinc that low level of this metal is necessary for optimal growth and metabolism of microorganisms[3,4], but at elevated concentrations, it can be toxic and effect on cell activities[5,6]. Zinc toxicity is caused by its interaction with sulfhydryl groups and other important enzymes [7].

The most important industrial source of anthropogenic zinc in environment comes from galvanization, electroplating, manufacture of batteries and other metallurgical industries [8]. The world health organization has recommended the maximum concentration of zinc in drinking water as 3.0 mg/l[9].

So because of heavy metals undesirable effects, it is necessary to employ an economical and efficacious option to speed up cleaning process in polluted sites. The ideal process is bioremediation include usage of biological systems to adsorb heavy metals from solution and return the environment altered by contaminants to its original condition[10,11].

Karun River is the longest and the most important river in in south western of Iran. Shipping

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on the lower course of the Karun has become increasingly important owing to oil drilling and refining in vicinity. The organic and inorganic pollution of Karun River has been attributed to the effluent discharges from industries sited along its bank, such as metallurgical, oil and petrochemical industries[12].

The present study is aimed at isolation and identification of zinc resistant bacteria from Karun River and investigation of their growth kinetics and zinc elimination ability.

MATERIALS AND METHODS

Sampling area

Four Sampling stations were selected based on slop of the beach, river path, industries establishment and their sewages output and accessibility for sampling[12]. Dez, Gargar, Ommoltamir and Samen Bridge stations were determined from Bandegir to Khorramshahr city. Triplicate sampling was done from water and sediments of selected stations in summer, autumn and winter. Samples were aseptically placed near ice and immediately transferred to laboratory[13].

Measurement of samples zinc content

The zinc content in all samples was obtained after adding acid nitric and chloridric acid and filtration through 42µm Watman filter and was analyzed using Atomic Absorption Spectrometer[14,15].

Bacterial counting

Viable plate count method was used for quantitative determination of bacterial populations. Serial dilution of samples from 10^{-1} to 10^{-9} were prepared by physiological serum. Then they were cultured by surface plate method on nutrient agar mediums with (260 mg/l) and without zinc and incubated at 30°C for 48 hours. At the end of the incubation period, bacterial colonies were counted in all plates [16].

Isolation and identification of zinc resistant bacteria

Microorganisms were isolated by enrichment method and direct plating on solid medium. In order to enrichment operations, 10 ml of water samples or 100 gr of sediments were added to 90 ml of Luria Bertani broth medium containing 260 mg/l zinc chloride and incubated at 30 °c for 48 hours. Then 0.1 ml of mediums were cultured on nutrient agar by surface culturing method. Pure culture of obtained colonies were prepared. The isolated microorganisms were subjected to staining and various biochemical procedures. Identification was done according to Bergey's manual of systematic bacteriology[14].

Analysis of bacterial resistance

After preparing bacterial suspension according to McFarland standards, they were cultured in LB broth medium containing 100, 400, 600, 800, 1000, 1200, 1400 mg/l zinc chloride and incubated at 30 °C for 48 hours[17].

Growth kinetics

Two resistant bacteria from every season were selected and their suspension were prepared according to McFarland standard. The bacterial strains were grown in 3 different LB broth medium: 1) containing zinc chloride, 2) without zinc chloride (control) and 3) mid exponential phase of bacterial growth. Optical density of each medium was read at 540 nm with a spectrophotometer immediately after bacterial inoculation. Medium were incubated at previously described condition and after 12 hours, 2 ml of each culture were taken for OD value determination hourly[17,18].

Preparing bacterial suspension for bioelimination studies

The bacterial suspension were inoculated in LB broth medium containing 500 mg/l zinc chloride at 30°C for 48 hours and cell harvested by centrifugation at 6000 rpm for 10 min. Supernatants were filtered by 42 µm Watman filter. Harvested cells were washed once in distilled water and kept at 105°C for a night to convert to dry mass. Dry mass was digested with 15 ml of concentrated nitric acid and kept at 100°C for an hour. Then its volume reached to 5 ml by distilled water[17,19,20].

Measurement of zinc bioelimination

Experiment was carried out to study the bioelimination of zinc using isolated bacterial cells. The bacterial cells were added to mediums containing zinc for different time interval. The residual zinc concentrations in solution obtained after filtration through 42 µm Watman filter and were analyzed for zinc in an atomic absorption spectrophotometer with flame method.

Statical analysis

The statistical analysis concerning the number of bacteria were performed using analysis of variance (ANOVA), and the Duncan test was conducted employing the SPSS software.

RESULTS

Analysis of zinc content in Karun River did not show dangerous levels of toxicity for human consumption but revealed variation at ppb level in different months. Maximum and minimum of zinc was determined in water and sediments samples of Samen Bridge and Gargar stations respectively.

Logarithmic average number of bacteria in medium containing zinc (4.278) was less than in control medium (5.333) and indicated significant difference at the 1% level. Maximum resistance bacterial number obtained 4.998 and 4.635 from Samen Bridge and Ommoltamir stations respectively and the least number was 3.440 from Gargar station. There was a statistically significant difference between Gargar and the other stations in number of resistant bacteria.

Gram negative bacteria (72%) was more than gram positive (28%) and showed significant difference at the 1% level. Maximum percentage of the identified bacteria belonged to *E. coli* that was isolated from all stations in all seasons and minimum percentage related to *Citrobacter* sp, *Enterococcus* sp, *Micrococcus* sp.

The isolates tolerated 200-1000 mg/l zinc. *E. coli* and *Pseudomonas* sp. indicated the most resistance against zinc (1000 mg/l). The most and the least resistance to zinc related to isolates of Samen Bridge (688.889) and Gargar station (416.667) respectively with a significant difference at the 5% level.

Bacterial growth kinetics.

Presence of zinc in medium resulted in log phase in bacterial growth. *Bacillus* sp. showed the longest log phase indicating minimum adaptation with the presence of zinc in its medium (Fig. 1).

From the Fig. 2,3 and 4 it is evidence that zinc addition during experiment (OD=0.5) led to

Table 1. The amount of Zinc elimination by resistant bacteria

Season	Bacteria Zn	Initial Zn content	Supernatant	Mass cell Zn concentration	Eliminated Zn	Elimination percentage
Summer	*Pseudomonas* sp.	500	131.34	27.43	341.23	68.25
Autmn	*Salmonella* sp.	500	152.53	77.287	270.19	54.04
Winter	*Pseudomonas* sp.	500	239.2	64.56	196.24	39.23
Control	-	500	454.74	-	45.26	9.053

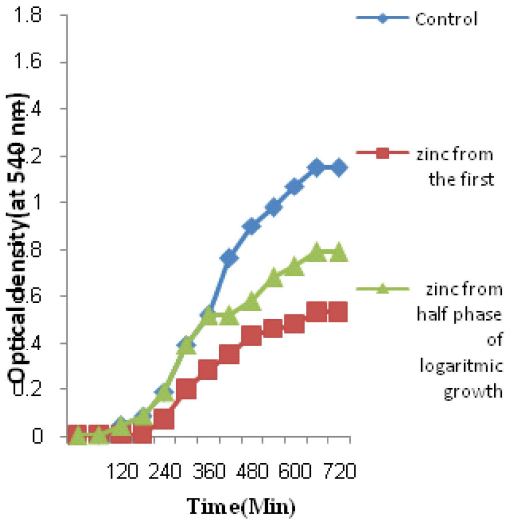

Fig. 1. Growth curves of *Bacillus* sp isolated in winter

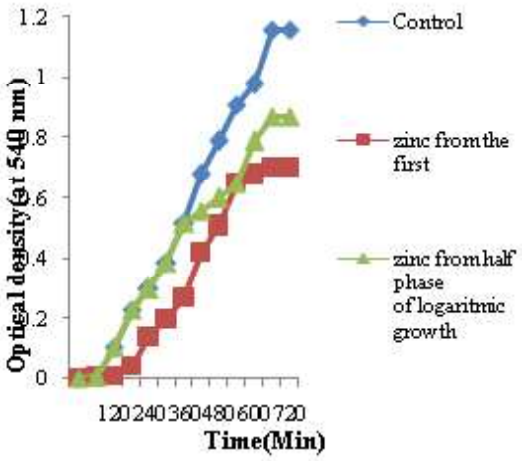

Fig. 2. Growth curves of *Pseudomonas* sp isolated in winter

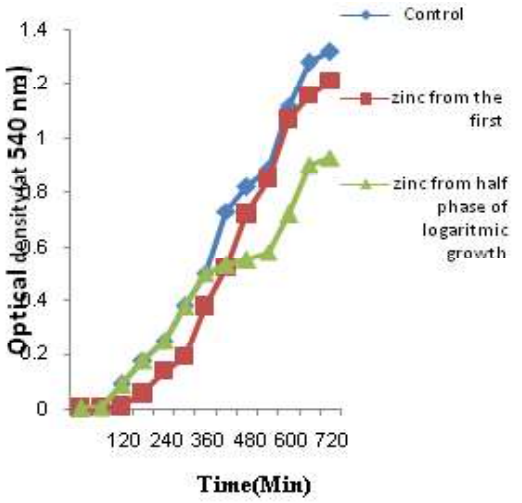

Fig. 3. Growth curves of *Salmonella* sp isolated in autumn

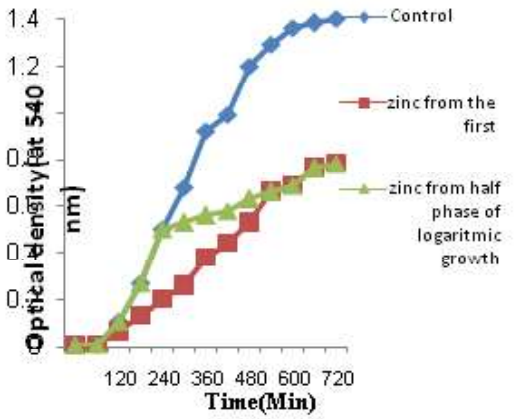

Fig. 4. Growth curves of *Citrobacter* sp isolated in autumn

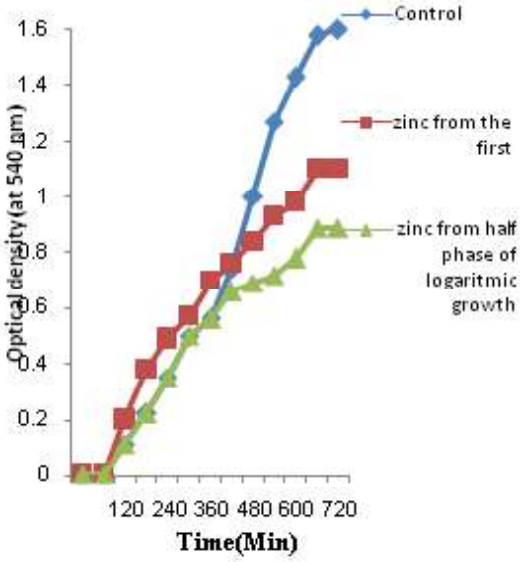

Fig. 5. Growth curves of *Escherichia coli* isolated in summer

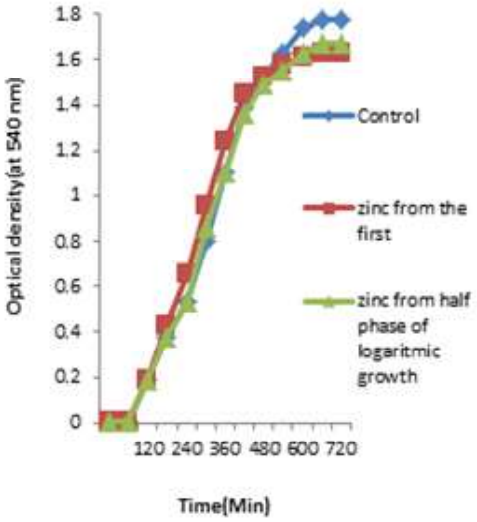

Fig. 6. Growth curves of *Escherichia coli* isolated in summer

growth decrease for *Salmonella* sp, *Citrobacter* sp and *Pseudomonas* sp

As it is seen in Fig. 5, *E.coli* showed the best growth in presence of zinc *Pseudomonas sp.* had maximum growth rate in presence of zinc. Zinc addition in the mid phase of logarithmic growth didn't decrease this bacteria growth, instead resulted in bacteria growth intensification (Fig. 6).

Maximum and minimum of zinc elimination was 68.25% by *Pseudomonas* sp. and 19.3% by *Bacillus* sp. respectively. Zinc was more eliminated in medium containing bacteria than control medium with a significant difference at the 5% level (Table 1).

DISCUSSION

As it was mentioned, zinc concentration did not indicate any alarming level of toxicity for human consumption in Karun River. Similar results have been reported earlier. Diagomanolin *et al*. (2004) concluded that average concentration of

zinc in Karun River is less than maximum determined drinking concentration by WHO and EPA[12].

According to obtained results, bacteria in metallic medium were more than control that it can because of metal inhibitory effect on bacterial growth. Sugarman *et al*. (1982) indicated the same results that metallic medium bacteria were so less than control[21].

In current investigation, isolated bacteria had more growth and resistant in presence of zinc and more ability to zinc removal. It can be concluded that primary enrichment led to expression of zinc resistant gene that enhance bacterial compatibility to zinc presence and so increase their growth[22]. For isolation of zinc resistant bacteria, Talat (2000) used primary enrichment method and Bhadra *et al*. (2007) used direct plating[23,18] like present study. Results of previous studies indicated that isolated bacteria by direct plating were not resistant to the concentrations higher than isolation required amount.

In current research, different gram negative and positive bacteria were identified as zinc resistant bacteria. Abundance percentage of gram negative bacteria was more than those of gram positive bacteria. Under unfavorable conditions, gram negative bacteria are able to pass each other resistance gene to zinc through conjunction process. Besides the first step the interaction of bacteria and zinc is passage of this metal through cell walls. Since gram negative bacteria have external membranes which impede the poisonous materials passage into cytoplasm, they are less affected to zinc than gram positive bacteria. Therefore they are more likely to be isolated from contaminated sites. Isolation of many gram negative and positive zinc resistant bacteria was reported in previous literature. Neweke *et al* (2007) were identified *Bacillus* sp. and *Salmonella* sp. as zinc resistant bacteria [24]. Bhadra *et al*. (2007) also indicated that some of the Torsa River isolates, having inducible zinc resistance, are members of the genus Pseudomonas and Bacillus[18]. In present study, *E.coli* was also isolated in addition to mentioned bacteria.

Bhadra *et al*. (2007) indicated that exposure of the cells to ppb levels of Zn^{2+}, comparable to the indigenous zinc ion concentration of Torsa River, could induce zinc resistance which is consistent with results of this experiment. Bacteria were resistant to 200-1000 mg/l of zinc[18]. Nweke *et al* (2007) isolated zinc resistant bacteria that they were exposed to Zn^{2+} concentration of 0.2 to 2.0 µm in a nutrient broth-glucose[24]. Talat (2000) reported that *pseudomonas aeroginosa* CMG can tolerate in a medium containing 20-25 µm zinc[23].

Resistance of the isolates of this research can be result of isolation or culturing methods. In most of previous research, isolation with just direct plating contribute to less bacterial resistance, but in this study they were isolated by primary enrichment and direct plating methods together. Primary enrichment causes bacterial adaptation to zinc stress leading tolerance increase to higher concentrations. Although observed resistances in different studies cannot be compared due to various reasons such as differences in used medium or methods. There are some past investigations that were used primary enrichment method for zinc resistant bacteria isolation. For example Al. Momani *et al*. (2006) isolated zinc resistant bacteria by this method and they can growth in presence of 1250 mg/l of zinc metal[19]

Growth curves of most isolates of present study follow bacterial growth curve. A sudden stress due to adding zinc to medium in mid phase of logarithmic growth did not prevent bacterial growth and just led to a short interval in growth of Bacillus sp. causing a shock due to sudden zinc presence in medium.

After inoculation of bacteria to metallic medium, they needed more time to adapt themselves to new conditions. Therefore during adaptation period, the length of lag phase naturally increased.

The maximum percentage of zinc eliminated is determined by *Pseudomonas* sp. that isolated from Samen station with highest level of zinc and its growth curve indicate a rapid growth in presence of zinc. It seems that the constant presence of zinc in the environment and using enrichment method have adapted this isolate to stressful conditions.

Zinc resistant bacteria can eliminate this metal from metallic medium. Zinc elimination was obtained from 19-68% in this experiment that this is a considerable results compared with other studies.

Al-Momani *et al*. (2006) isolated zinc

resistant bacteria from water, mud and soil samples collected from the Dead Sea Shore at Suwaymah with ability to eliminate 19.2% -82.84% of zinc after 3 weeks of incubation[19]. In other research Shakoori *et al*. (2003) reported the ability of zinc removal by 6 bacterial strain from 10%-28%[20].

CONCLUSION

The results of current study revealed that bacterial elimination is a major pathway to removal of heavy metal contamination in Karun River. Their high ability to remove zinc suggests them for field experiments and use in critical zinc pollution.

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