



**International Journal of Biological
&
Pharmaceutical Research**
Journal homepage: www.ijbpr.com

IJBPR

STUDY THE HEAVY METALS TOLERANCE, BIOSORPTION AND ANTIBIOTIC RESISTANCE OF *BACILLUS CEREUS* ISOLATED FROM DIESEL FUEL POLLUTED SOIL

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ABSTRACT

The heavy metals tolerance (chromium, cadmium, lead and zinc), biosorption of these ions and resistance of antibiotics were studied by using *Bacillus cereus* bacterium isolated from soil contaminated with diesel fuel. From 10 samples of soil polluted with diesel fuel, 7 isolates identified as rod of spore former (*Bacillus* spp.) and 3 of these isolates (DB.1, DB.2, and DB.5) were identified as species (*B. cereus*), the *Bacillus* isolate DB.5 (*B. cereus*) showed the highest MIC, where the range was 200 mg/L for Zn to 750 mg/L for the Cr, and this isolate selected for the other experiments. It was found that the isolate exhibited resistance to most of the tested antibiotics. Where the isolate was resist to amoxillin, cloxacillin, cephalixin, ceftriaxone, cefoxitin and trimethoprim, also it was sensitive to erythromycin, azithromycin and gentamycin and intermediate sensitive to vancomycin. The effect of chromium on the growth of *B. cereus* in Cr containing medium in contrast with control, indicated that *B. cereus* without chromium (Cr) revealed a lag phase of 2-3 hrs, after this the bacterium showed accelerated growth rate with maximum growth at 20 hrs. The bacterium with Cr stress (treated) however, showed lag phase of 4-8 hrs and maximum growth at 24hrs. but the number of bacteria was less than in control. It was found that *B. cereus* attained its maximum Cr biosorption of 73% after 48 hrs of incubation, while the removal efficiency of Cd, Pb and Zn ions from aqueous solution was 51, 45, and 16 % respectively after 48 hrs of incubation. Study showed that the metals tolerant *B. cereus* can be used for heavy metals bioremediation and this tolerance was correlated with antibiotic resistance.

Key Words: *Bacillus cereus*, Heavy metals, Antibiotics, Resistance, Biosorption.

INTRODUCTION

The discharge of heavy metal into the environment as a result of agricultural, industrial and military operations, and the effect of this pollution on ecosystems and human health have been of concern for some years (Essa *et al.*, 2002). The microorganisms surviving the petroleum stress tends to adapt by its ability to limit environmental yoke (resistance) and limit the harm caused by environmental stress (tolerance), which will enable them an ecological and evolutionary advantages to

counteract heavy metals, antibiotics and organic solvent stress in contaminated site (Edward *et al.*, 2012). An increasing problem for the treatment of different infections disease is bacterial resistance to antibiotic and other antimicrobial agents. It is thought that a correlation exists metals tolerance and antibiotic resistance in bacteria (Enne *et al.*, 2001). To survive under metal-stressed conditions, bacteria have evolved several types of adaptation mechanism to tolerate the uptake or heavy metals ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and the reduction of the heavy metals ions to a less toxic state (Montuella *et al.*, 1994). Microbial survival in polluted soil on intrinsic biochemical and structural properties, physiological and/or genetic

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adaptation including morphological changes of cells (Wuertz and Mergeay, 1997).

The purpose of this study was to isolation of *Bacillus cereus* bacteria from soil contaminated with diesel fuel, determine the maximum inhibitory metal concentration (MIC) for this local isolate, biosorption of heavy metals, also study the antibiotic resistance and correlation between the antibiotic resistance and heavy metal tolerance.

MATERIALS AND METHODS

Sampling

Diesel fuel polluted soil samples were collected in sterilized polystyrene bags of one liter capacity from different 10 areas, Baghdad, Iraq. Samples were transferred for microbial analysis.

Metal salts and preparation of stock solution of metal ions

The following metal salts were used to prepare the stock solution of metal ions in aqueous form: Potassium dichromate (K₂Cr₂O₇), Lead nitrate (Pb (NO₃)₂) and Cadmium nitrate (Cd (NO₃)₂) and Zinc nitrate Zn (NO₃)₂. For each of the metal ions, a stock solution was prepared separately by dissolving 1gm of the metal salts in 1000 ml of distilled water. The solutions were prepared using a standard flask. The range of concentrations used was prepared by serial dilution of the stock solution with deionized water (Samarth *et al.*, 2012).

Isolation of heavy metals tolerant bacteria by enrichment

For isolation and enumeration of bacteria, 1ml samples were inoculated in 100 ml Nutrient broth (NB) medium supplemented with 100 mg/L concentration of heavy metals and grown in a shaker incubator at 30 °C with 160 rpm for 5 days. The culture was then plated on NB agar plates supplemented with similar heavy metal concentration using spread plate method and incubated at 30 °C for 24 to 48 hrs. Initially the bacterial isolates were identified on the basis of colony morphology, gram staining and biochemical tests. Total number of heavy metal tolerant bacteria was expressed as CFU/ml. A single strain which is gram positive rod with endospore was capable of growing at this condition and was selected for further experiments. The isolate was preserved in NB slants at 4 °C as well in glycerol stocks at -20 °C (Khatun *et al.*, 2012).

Determination of MIC

Minimum Inhibitory Concentration (MIC) of metals such as Lead (Pb), Zinc (Zn), Chromium (Cr) and Cadmium (Cd) was determined for different bacterial isolates by broth dilution method (Colomiris *et al.*, 1984). Eight concentrations (100-800 ppm) of each metal were mixed with Brain Heart Infusion Broth. Based on the evaluation, Minimum Inhibitory Concentration (MIC) was

determined at 37°C for 24hrs. The minimum concentration of heavy metals at which no turbidity was observed by spectrophotometer at 660nm was considered as the MIC of bacterial isolates against heavy metals.

Antibiotic sensitivity test

Bacillus cereus isolate was tested for its sensitivity to antibiotics by means of a disc diffusion method. The bacteria were investigated using antibiotics disc containing amoxillin (25µg), cloxacillin (10µg), cephalexin (30µg), cephterixan (30µg), cephexitin (30µg), gentamycin (10µg), trimethoprim (5µg), erythromycin (15µg), azithromycin (15µg), vancomycin (30µg) and gentamycin (10µg). The antibiotics disc were spaciouly placed on Mueller - Hinton agar plates previously seeded with 6 hrs broth cultures of *Bacillus cereus* isolate. The plates were incubated at 37° C for 18-24 hrs. The different zones of inhibition were measured to the nearest millimeter and interpreted as sensitive, moderate sensitive and resistant based on the interpretation table recommended by the disc manufacturer (Whong and Kwaga, 2008).

Growth curves of *Bacillus cereus*

The effect of Chromium on growth of bacteria was checked by counting the number of cells in the medium by using viable count method. The cells were grown in their respective media with 300µg/ml of Chromium. The aliquots (2ml) of bacteria incubated medium were taken out at regular intervals of one hour for 48 hrs. The growth was compared with that of control culture, which contained no added chromium ions. Growth curves were prepared by plotting a graph between time (hours for bacteria) of incubation along the X-axis and number of cells per ml along the Y-axis (Muneer *et al.*, 2013).

Experiments of biosorption

Experiments of heavy metals biosorption were done in Erlenmeyer flasks containing 150 ml of each samples and 15.0 ± 1.0 mg of cells. To ensure equilibrium, cells and waste were maintained in contact for 48 hrs, under constant agitation 120 rpm at 30°C and pH equal to 6. In all experiments, cells were obtained from only one cultivation and collected from the same flask at the same growth stage. After 48 hrs, cells were separated from the medium and residual metal concentrations were monitored by Atomic Absorption Spectrophotometer. Experiments were done in triplicate. The optimum pH and temperature maintained for the growth of microorganisms in the batch culture. The percentage of Cr (VI) removal, i.e., *R* (%) was calculated using the following equation (Kumar *et al.*, 2010)

$$R(\%) = \frac{(C_0 - C_e)}{C_0} \times 100$$

where C_0 and C_e represent initial and final Cr(VI) concentration.

RESULTS AND DISCUSSION

From different 10 area in Baghdad, 10 samples of soil contaminated with diesel fuel were obtained 7 isolates identified as rod of spore former (*Bacillus* spp.) by morphological and microscopic characteristics, and 3 of these isolates (DB.1, DB.2, and DB.5) were identified as species (*B. cereus*) according to their biochemical characteristics (Whong and Kwaga, 2008). The previous results indicated that *B. cereus* strains isolated from refinery field have great potential for in situ remediation of diesel contaminated soil in oil refinery site (Kebria *et al.*, 2009). *B. subtilis* and *B. cereus* were found to be hydrocarbon degraders in polluted soil with diesel oil (Nwaogu *et al.*, 2008).

Table1. Showed the result of minimum inhibitory concentration of heavy metal (Cr, Cd, Pb, and Zn) against selected *Bacillus* spp. isolates. The range of MIC of the isolate was ranged from 100 mg/L to 750 mg/L. The *Bacillus* isolate DB.3 exhibited the lowest MIC, which ranged from 100-150 mg/L for the used heavy metals, while the *Bacillus* isolate DB.5 (*B. cereus*) showed the highest MIC, where the range was 200 mg/L for Zn to 750 mg/L for the Cr, and this isolate selected for the other experiments. Many studies indicated the ability of *B.*

cereus to resist and tolerance some heavy metals, Da costa and Duta (2001) referred that different *Bacillus* strains have the ability to uptake Cadmium, Zinc, Copper, and lead ions by bioaccumulation and the best results were obtained for *B. subtilis* and *B. cereus*. Our results agreement with what have been reported by Khatun *et al.* (2012) who mentioned that *B. cereus* was most tolerable to Cd and Cr compared to other metals (Co, Ni, Pb, and Hg).

The antibiotic susceptibility of *B. cereus* (DB.5) isolated from soil sample contaminated with diesel fuel was summarized in table 2. It was found that the isolate exhibited resist to most of the tested antibiotics. Where the isolates were resist to amoxillin, cloxacillin, cephalixin, ceftriaxone, cefoxitin and trimethoprim, also it was sensitive to erythromycin, azithromycin and gentamycin and intermediate sensitive to vancomycin. Many investigators had been reported the association between heavy metals and antibiotic resistance (Colomiris *et al.*, 1984, Verma *et al.*, 2001, Kamala- Kannan and Lee, 2008). The bacteria which subjected to organic solvent can exhibit high antibiotic and heavy metals resistance due to the siphon out antimicrobials by efflux pump (Nielson *et al.*, 2005). The majority of isolated strains were able to degrade different aromatic and aliphatic hydrocarbons also exhibited multiple resistance against antimicrobial agents (Mathe *et al.*, 2012).

Table 1. Minimum inhibitory concentration of some heavy metals against selected *Bacillus* isolates.

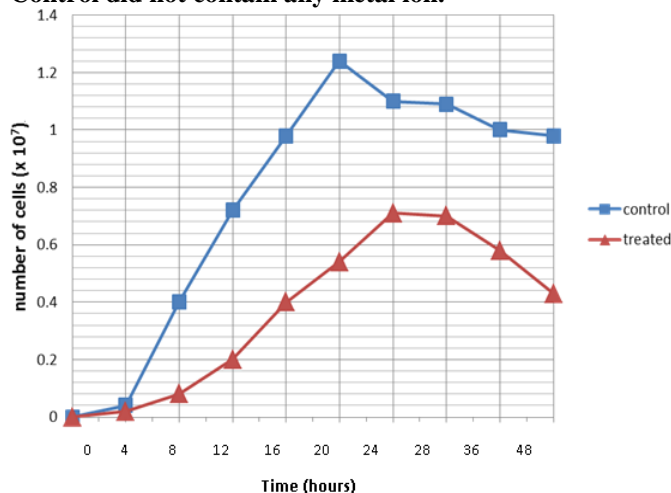
<i>Bacillus</i> isolates	Minimum Inhibitory Concentration (mg/L)			
	Cr	Cd	Pb	Zn
DB.1	300	200	150	350
DB.2	200	450	300	250
DB.3	150	100	150	150
DB.4	300	100	250	150
DB.5	750	600	400	200
DB.6	300	100	200	150
DB.7	350	250	300	300

Table 2. Antibiotic susceptibility of *B. cereus* (BD.5) isolated from soil sample contaminated with diesel fuel.

Antibiotic (conc. µg/ disc)	<i>B. cereus</i> State of susceptibility
Amoxilin (25)	R
Cloxacillin (10)	R
Cephalexin (30)	R
Ceftriaxone (30)	R
Cefoxitin (30)	R
Trimethoprim (5)	R
Erythromycin(15)	S
Azithromycin(15)	S
Vancomycin(30)	I
Gentamycin (10)	S

R = Resistance, S= sensitive, I=Intermediate

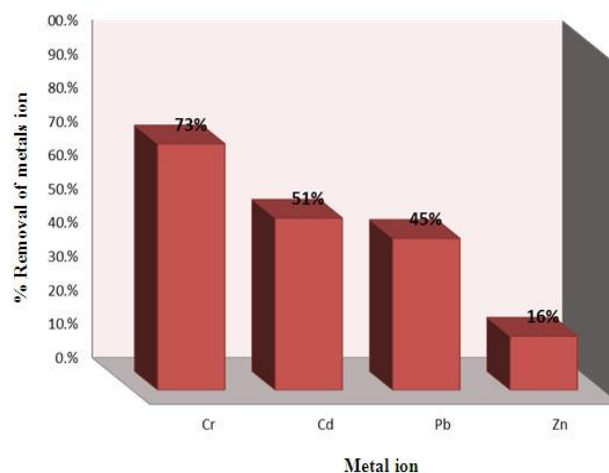
Fig 1. Growth curve of *B. cereus* in Cr containing media. Control did not contain any metal ion.



The effect of chromium on the growth of *B. cereus* (growth curve), in Cr containing medium in contrast with control (figure 1). It's clearly showed the characteristic phases during the growth of culture. The results indicated that *B. cereus* without metal (control) treatment showed lag phase of 2-3 hrs, after this the bacterium showed accelerated growth rate with maximum growth at 20 hrs. The bacterium with Cr stress (treated) however, showed lag phase of 4-8 hrs and maximum growth at 24hrs but the number of bacteria was less than in control. Due to selective pressure from the metal in the growth environmental, microorganisms have evolved various mechanisms to resist the heavy metals stress. Several metals resistance mechanism have been identified exclusion by permeability barrier, intra and extra cellular sequestration, active transport, efflux pumped and enzymatic detoxification (Rathnayake *et al.*, 2010).

Figure 2 showed the result of biosorption of metal ions (Cr, Cd, Pb and Zn) by *B. cereus* isolate (DB.5). It was found that *B. cereus* attained its maximum Cr biosorption of 73% after 48 hr. of incubation, while %

Fig 2. Biosorption of metal ions (Cr, Cd, Pb and Zn) by *B. cereus* isolate.



removal efficiency of Cd, Pb and Zn ions from aqueous solution was 51, 45, and 16 % after 48 hr. of incubation at 120 rpm, pH equal to 6 and temperature 30 °C. The enhancement in the metal sorption could be due to an increase in electrostatic interaction involving sites of progressively lower affinity for metal ions (Puranik and Pakniker, 1999). Our results showed high metal removal efficiency of living biomass of *B. cereus* (DB.5), thereby suggesting its possible application multiple removal in waste water treatment. Interaction between biomass, a live or not, and metals can take place intracellularly, extracellularly, or on the cell surface (Gadd and White, 1993). From the results can be concluded that the local isolate *B. cereus* exhibited multi resistant to heavy metals and antibiotics especially to Cr ion, Penicillin and Cephalosporins antibiotics. Also the isolate could grow in medium treated with Cr ion and could exploited in the bioremediation of Cr by the high biosorption capacity of this bacterium. In light of our observations, living biomass of *B. cereus* could be used efficiently and effectively in removal of heavy metals from the polluted environment.

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