

Removal of anthropogenic lead pollutions by a potent *Bacillus* species AS2 isolated from geogenic contaminated site

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Abstract A total of 422 bacterial isolates were obtained from the lead (Pb) ore in north-eastern Iran. The Pb tolerances of these strains were studied using microbroth serial dilution approach and 35 strains could grow up to 3250 ppm Pb concentration. Of these strains, 10 of them represented qualitatively high levels of Pb adsorption and were selected for quantitative studies. Strain AS2 which is phylogenetically related to genus *Bacillus* showed the highest level of Pb remediation. The effects of different factors, including pH, initial Pb concentration, temperature and inoculum size, were studied on the remediation process. Pb remediation capacity was reached at 74.5 mg/g (99.5 % of initial Pb) at pH 4.5, temperature 30 °C, inoculum size 1.0 % (v/v) and an initial Pb concentration of 500 ppm after 24 h. Pb desorption capacity of strain was 66 %. The novel isolate could remove 98 % of Pb from the contaminated industrial wastes after 24 h. Pb uptaking to the cell surface was proven using scanning electron microscopic micrograph and energy-dispersive X-ray spectroscopy analysis. Most Pb removal efficiency was observed in the active cell culture as compared to the inactive cell and extracellular polymeric substances. The novel strain represents a good candidate for removal of environmental anthropogenic Pb pollutions.

Keywords Anthropogenic · Bioremediation · Geogenic · Lead (Pb)

Introduction

Lead (Pb) is a non-essential heavy metal. It causes serious health hazards, that is, permanent brain damage, learning disabilities, hearing loss, anaemia, insomnia, headache, dizziness, irritability, weakness of muscles, renal damages and heart disease (Naseem and Tahir 2001). Industrial activities, such as production of batteries, pigments and metal smelting, as well as manufacturing of products, such as Pb arsenate insecticides or Pb water pipes, are the main anthropogenic sources of Pb (Jarosławiecka and Piotrowska-Seget 2014; Ismail et al. 2013). Accordingly, large volumes of Pb-containing wastes from these industries are introduced into environments. To protect ecosystems, wastewaters must be treated to reduce Pb to below 0.05–0.10 ppm before discharging into the environment (Sağ et al. 1995). Different physical and chemical methods have been applied to treat Pb-containing wastes. These methods include adsorption, flotation, ion exchange, membrane filtration and precipitation (Chen et al. 2009; Kabbashi et al. 2009; Landaburu-Aguirre et al. 2009; Zhang et al. 2009). The physico-chemical methods are generally expensive, so the need for hazardous reagents produces other toxic chemicals and requires long treatment time. The biological systems apply a process called bioremediation to protect environments from toxic effects of hazardous components. Since the elemental form of Pb is toxic, it cannot be degraded or transformed (biotransformation/biodegradation) into a less dangerous material. However, the living systems (like bacteria) can precipitate bioavailable soluble and toxic Pb to insoluble forms

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(biosorption) and reduce drastically its availability and toxicity. The major advantages of the biosorption approach are its high effectiveness in reducing the heavy metal ions and the low cost of the process (Macek and Mackova 2011). Various groups of microorganisms like *Bacillus cereus*, *Arthrobacter* species, *Corynebacterium* species (Roane and Kellogg 1996; Trajanovska et al. 1997; Zanardini et al. 1997), *Pseudomonas marginalis*, *Pseudomonas vesicularis*, *Enterobacter* species (Hasnain et al. 1993; Trajanovska et al. 1997), *Saccharomyces cerevisiae* and *Penicillium* species (Chen and Wang 2007; Sun and Shao 2007) were reported for their ability to remediate Pb contaminations using the adsorption method.

Heavy metals like Pb are found in the environments as the natural sources (geogenic) of parent rocks and metallic minerals (metalliferous ores) (Sparks 2005). The long-time contacts of microbial populations with this element in the contaminant regions result in the enrichments of resistant strains by the act of natural selection. Accordingly, these indigenous species are appropriate candidates for bioremediation of anthropogenic (Human made) pollutions. This study describes the isolation of Pb-tolerant and absorbing bacteria from Pb ore, and the influence of various biosorption-related variables, such as pH, inoculum size, temperature and initial Pb concentration, was studied on the selected strain. The ability of novel strain to remediate industrial Pb-containing wastes was also taken into account.

Materials and methods

Site description, samples collection and analysis

Samples were collected from different sites of Tarik Darreh Pb Ore in north-eastern Iran (35°21'–35°28'N, 60°40'–60°49'E). The samples including soil, water and sediment were collected in sterile plastic containers and kept in the dark at an environmental temperature for a few hours before being analysed in the laboratory. Anions and cations were analysed using titration and atomic absorption methods (atomic absorption spectroscopy, AAS), respectively (Maiti 2004; Saad et al. 1998). Pb concentration was analysed using AAS-7000 (Shimadzu, Japan).

Culture media and growth conditions

Bacteria were isolated under aerobic conditions on Luria–Bertani (LB) agar (Merck). The pH of the medium was adjusted at pH 5.0 by 2 M HNO₃. All samples were serially diluted up to 10^{−6} and plated by spreading method. The plates were incubated at different temperatures of 20, 30 and 40 °C for 2 weeks. Pure isolates were obtained after

successive cultivation. The isolates were maintained on the slant LB medium at 4 °C and LB broth medium supplemented with 30 % (v/v) glycerol at −80 °C for short and long preservations, respectively.

Minimum inhibitory concentration (MIC) determination

Since Pb chemically precipitates in alkaline condition (Leung et al. 2001), the pH was adjusted to acidic condition (pH < 5.0) with 2 M HNO₃ in all Pb relative experiments. MIC determination was conducted in LB liquid medium by broth microdilution method. It was performed with 96-well, round-bottom microtiter plate. The final volume of wells was 200 µl. Each plate included positive control (bacteria without Pb), negative control (medium only) and serial twofold dilutions of Pb in LB medium. Bacterial inoculum was produced from mid-log culture in Pb-free LB medium. After the addition of a 100 µl inoculum containing 2×10^6 cells/ml to Pb-containing positive control wells, the plates were incubated at 30 °C. Bacterial growth was determined by measuring the turbidity at 600 nm. The lowest concentration of Pb which inhibited the bacterial growth was determined as the MIC of the isolate.

Quantitative and qualitative screening for Pb-absorbing bacteria

In order to evaluate the biosorptive potential of Pb-resistant bacteria, a rapid agar screening method was used, which was introduced by Pümpel et al. (1995). Briefly, bacterial strains were punctually inoculated in Pb-free LB agar and incubated for 48–72 h at 30 °C. The petri dishes were then overlaid by soft agar containing 500 ppm Pb and incubated for 24 h at 30 °C. Afterwards, Pb removal from solid media by microorganisms was visualized by exposing each plate to H₂S vapours, which was generated in situ by reacting sodium sulphide with hydrochloric acid in a stoichiometric ratio. Precipitation of lead sulphide (PbS) and halo formation around the colonies were considered for Pb biosorption capacity. For quantitative measurement of Pb adsorption, the liquid LB medium was supplemented with 100 ppm Pb concentration. Cultures were grown in 150 rpm at 30 °C for 24 h. After the incubation, supernatant was collected and Pb absorption was determined using AAS. Cultures containing 100 ppm Pb without inoculum were applied as negative control.

Factors affecting removal process

To understand the effects of various conditions on Pb removal, growth conditions or chemical components were varied one at a time. Organisms were cultured in 25 ml LB



medium containing Pb as PbNO₃ in 100-ml Erlenmeyer flasks incubated aerobically at 150 rpm in an orbital shaker. These variables consisted of various pH of the medium which was adjusted to pH 3.0, 3.5, 4.0, 4.5 and 5.0; initial Pb concentrations of 50, 100, 200, 300, 400, 500, 600 and 700 ppm; different incubation temperatures of 20, 25, 30, 35 and 40 °C; various inoculum sizes of 0.5, 1.0, 3.0, 5.0 and 7.0 % (v/v); and incubation time up to 36- and 2-h intervals. Residual Pb was measured using AAS.

Biological removal efficiency (BRE) and biological removal capacity (BRC) were calculated as shown in the following formula:

$$\text{BRE (\%)} = \frac{\text{Primary lead concentration (non inoculated)} - \text{Residual lead concentration (inoculated)}}{\text{Primary lead concentration (non inoculated)}} \times 100 \quad (1)$$

$$\text{BRC (mg/g)} = \frac{\text{Primary lead concentration (non inoculated)} - \text{Residual lead concentration (inoculated)}}{\text{Bacterial biomass (dry weight)}} \quad (2)$$

Each experiment was carried out in three independent batches in triplicates (nine replications). The standard error of the mean was calculated and was shown as error bars on the figures. One-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test were performed using Statistical Package for Social Science (SPSS) ver. 16 software (IBM Co).

Comparing active–inactive cells and extracellular polymeric substances (EPS) in removal process

Fresh culture media of selected strain were prepared and divided into three parts. Two parts were inactivated by autoclave and treatment by 1 mM sodium azide (Johnson et al. 2007), respectively. All the autoclaved (inactive), sodium azide treatment (inactive) and living cells (active) were harvested by centrifugation at 7500g for 10 min. To determine whether bacterial exopolysaccharides are involved in bioremediation, the EPS of the bacterial culture was extracted as described by Zhang et al. (2002). The ability of active and inactive cells as well as extracted exopolysaccharide to uptake Pb was measured as mentioned in “Quantitative and qualitative screening for Pb-absorbing bacteria” section.

Scanning electron microscopic (SEM) and energy-dispersive X-ray spectroscopy (EDS) analysis

Samples for SEM studies were harvested from LB liquid culture containing 500 ppm concentration of Pb at the optimum condition. Cells pellets were freeze-dried and

coated with gold using a sputter coater (EmscopeSc 500). SEM analysis was performed using a LEO.1450.VP/REM (Oberkochen-Zeiss, Germany). EDS elemental analysis was carried out on non-treated bacterial cells as well as bacterial cells interacted with Pb using an ESEM FEI Quanta 200, high-resolution electron microscope.

Measurement of Pb desorption capacity

Bacterial cells were harvested from adsorption experiments and washed with distilled water to remove unbound Pb(II). The biomass was transferred to desorbent solution containing HNO₃, Ca(NO₃)₂ and ethylenediaminetetraacetic acid (EDTA; 0.10 and 0.01 M, respectively), and the solution was shaken for 18 h. The biomass was collected and analysed to determine the concentration of Pb(II) after desorption (Deng et al. 2007). Desorption capacity was calculated as follows:

$$\text{Desorption capacity (\%)} = \frac{\text{Amount of desorbed lead}}{\text{Amount of adsorbed lead}} \times 100 \quad (3)$$

Removal of anthropogenic Pb contamination in batch fermentation

Remediation of Pb-contaminated wastes from local batteries and dye industries was carried out in a 1 L batch fermentation system with waste Pb added at 500 ppm concentration to the optimal removal of LB culture. Residual Pb was measured using AAS.

Molecular identification of the strain

The genomic DNA of the strain was extracted using DNA extraction kit (Thermo scientific, Lithuania) according to the manufacturer's recommended procedure, and the 16S rRNA gene was amplified using the bacterial universal primers 27F and 1492R (Lane et al. 1985). Amplification reactions contained 1.5 µl of each primer, 1 µl of 10 mM deoxynucleotide (dNTP), 5 µl polymerase chain reaction (PCR) buffer, 1.5 µl of 50 mM MgCl₂, 4 µl template DNA, 0.25 µl DNA polymerase and 35.25 µl dH₂O, in a final volume of 50 µl. The following conditions were used in the amplification of 16S rRNA gene: 94 °C for 2 min, followed by 30 cycles of 94 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s, with a final 7-min extension at 72 °C. PCR products were purified with the DNA purification kit (Bioneer, South Korea), according to the manufacturer's protocol. The sequencing was conducted on an ABI 3730XL DNA sequencer at MacroGen (Seoul, South Korea).



Results and discussion

Chemical characterization of sample and isolation of bacteria

Several water, soil and sediment samples from Tarik Darreh Ore were obtained and used in this study (pH of the samples was neutral to slightly alkaline (7.3–7.8). The Pb concentration in the soil samples was 400 mg kg^{-1} . Concentration of major cations and anions was as follows (mg/L): Ca^{2+} (80), Mg^{2+} (22.4), Na^{+} (47.5), K^{+} (5.2), HCO_3^{-} (317.6), SO_4^{2-} (53.4) and Cl^{-} (32.8). The LB agar-isolating medium yielded high numbers of colonies, and the range was 2.5×10^6 – 4.4×10^6 CFU/ml. A total of 422 pure isolates were obtained by successive cultivation. These strains were characterized as Gram-positive cocci (33 isolates), Gram-negative cocci (10 isolates), Gram-positive bacilli (285 isolates) and Gram-negative bacilli (94 isolates).

MIC determination and selection of Pb-absorbing bacteria

A total of 36 strains could not tolerate the minimum amount of Pb and only grew in the absence of Pb. The MIC for other strains was as follows: 100 ppm (40 strains), 500 ppm (34 strains), 1000 ppm (48 strains), 1500 ppm (79 strains), 2000 ppm (97 strains) and 3000 ppm (52 strains). A total of 35 strains showed the highest Pb tolerance and could grow up to 3125 ppm initial Pb concentration. Among the last group, eight strains showed the highest qualitative Pb absorption (Fig. 1) and were selected for further quantitative analysis. One strain was designated as AS2 that uptakes remarkable amount of initial Pb

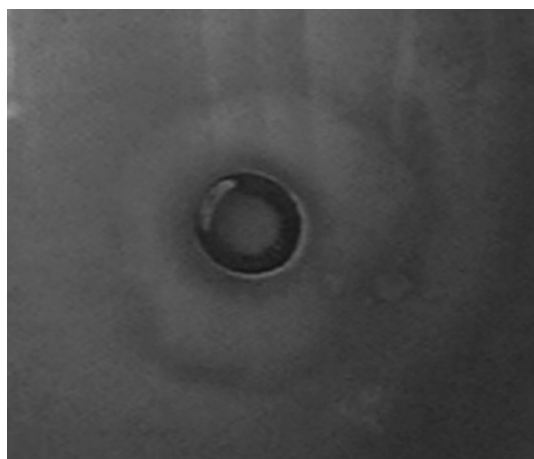


Fig. 1 Assay of Pb absorption activity on plate with solid isolation medium supplemented with Pb (500 ppm). Clearing zone of Pb absorption formed around the colony

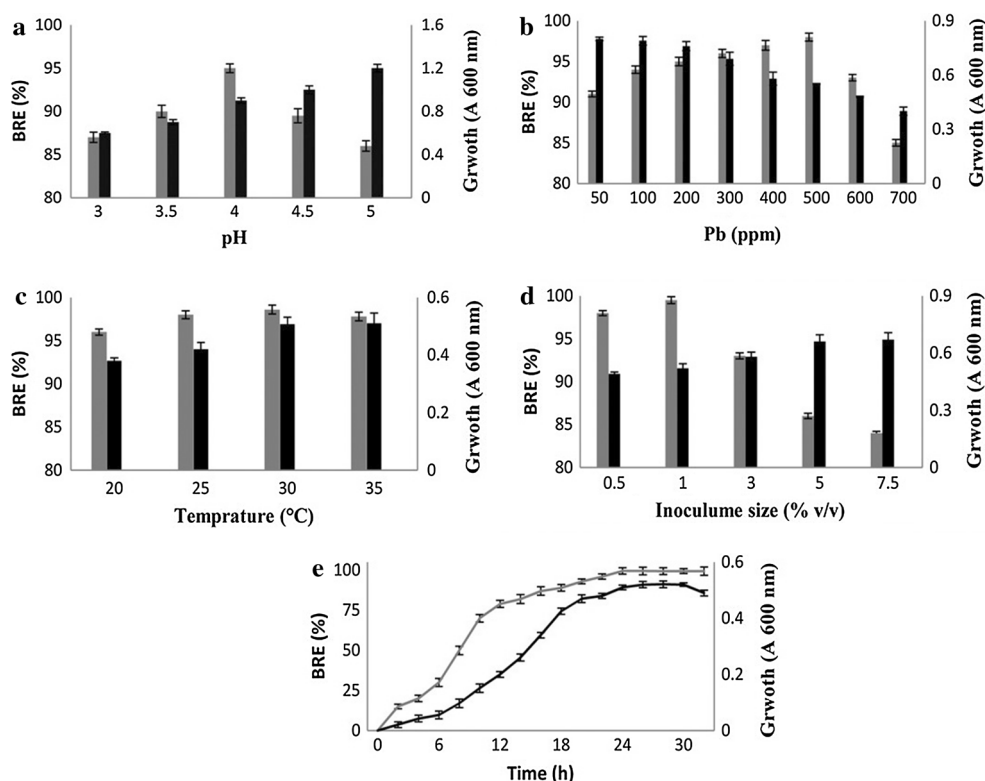
concentration (BRE 87 %; BRC 109 mg/g) and was selected for optimization studies. Geogenic contaminated sites were reported to be good sources for heavy metal tolerance microbes with potential applications in bioremediation. Arsenic-, cadmium-, copper- and zinc-resistant bacteria were isolated from gold, lead–zinc, copper and silver mine soils, respectively (Li et al. 2013; Piotrowska-Seget et al. 2005; Zhu et al. 2012). Heavy metal tolerance of the microbial community in these environments could be acquired by genetically tolerance elements (genes involving in metal resistance are mostly presented in plasmids) and shift in species composition (Hu et al. 2007). More than 90 % of bacterial strains obtained from Pb ore in this study could tolerate at least 100 ppm Pb. There are a wide range of Pb tolerances in scientific reports: *Pseudomonas aeruginosa* ASU 6a (Gabr et al. 2008) was able to survive under the maximum range of Pb concentration up to 500 ppm, *Enterococcus faecum* Pb12 tolerates 800 ppm Pb (Bhakta et al. 2012), and *Achromobacter* species TL-3 isolated from activated sludge samples tolerates 1500 ppm Pb in Luria broth medium (Batta et al. 2013). The novel strain AS2 which was isolated from Pb ore in this study showed the maximum MIC to be higher than previous report and could survive up to 3125 ppm Pb.

Factors affecting removal process

As shown in Fig. 2, a biological removal efficiency of 100 ppm initial Pb was 87, 90, 95, 90 and 86 % at pH 3.0, 3.5, 4.0, 4.5 and 5.0, respectively. Bacterial growth was increased as the pH of the medium increased. Pb removal capacities for the selected strain were (mg/g): 13.1, 13.6, 14.4, 13.5 and 12.8 at pH 3.0, 3.5, 4.0, 4.5 and 5.0, respectively. Pb adsorption was best at pH 4.0 ($p < 0.05$) for strain AS2, and this pH was applied in the following experiments. Concentration of hydrogen ion (pH) has significant influence on Pb removal capacity. Under pH condition of above 6, the accumulation of hydroxyl group (OH^{-}) results in the chemical precipitation of Pb as $\text{Pb}(\text{OH})_2$ and misunderstanding the bioremoval capacity. In contrast, high acidic condition ($\text{pH} < 2$) decreased the removal capacity as a result of competition for negatively charged binding sites between heavy metal cation and protons (H^{+}). However, the optimal pH condition is also depending on the metal-accepting groups (if any) on the bacterial surface. For example, phosphoryl groups are mainly in unprotonated form already at pH 2 ($\text{pK}_a \approx 1.5$), so bacteria with this uptaking groups can remove Pb at lower pH (Halttunen et al. 2007). Strain AS2 removed maximum Pb at pH 4.5 and postulated that it applied binding groups to higher pK_a than phosphoryl groups. Strain AS2 adsorbed 91, 92, 95, 96, 97, 98, 93 and 85 % of Pb at the initial concentrations of 50, 100, 200, 300, 400,



Fig. 2 Effects of pH, Pb concentration, temperature, inoculum size and incubation time on Pb removal efficiency (grey columns/line) and growth (black columns/line) of strain *Bacillus* species AS2



500, 600 and 700 ppm, respectively. However, increase in Pb concentration results in reduction of bacterial growth. The corresponding performances of removal capacity were (mg/g): 7.0, 14.2, 28.8, 43.6, 58.8, 73.5, 84.1 and 80.0 at a mentioned concentration of Pb, respectively (Fig. 2b). For removal efficiency, the highest bioremediation rate was observed at 500 ppm Pb concentration and was applied for further experiments. It could be explained that at low metal concentration, the biosorption of the biosorbents is not fully utilized. In contrast, the existence of infinite heavy metal concentration reduces the efficiency due to toxicity towards cells. Figure 2c represents the BRE amounts for strain AS2 as 96, 98.2, 98.6 and 98 % at temperature 20, 25, 30 and 35 °C, respectively, while the BRC were (mg/g): 73.0, 73.5, 73.9 and 73.9 at these temperatures, respectively. Pb removal was not different between 25 and 35 °C ($p < 0.05$). However, environmental temperature (about 30 °C) is more desirable for in situ remediation process (Vijayaraghavan and Yun 2008) and was selected for further studies. Strain AS2 removed 98, 99.5, 93, 86 and 84 % of the initial Pb at inoculum sizes (% v/v) 0.5, 1.0, 3.0, 5.0 and 7.0, respectively. The removal capacities for these variables were (mg/g) 73.8, 74.5, 70.2, 64.5 and 63.3, respectively. The highest bioremediation rate was observed at the 1.0 % (v/v) inoculum size (Fig. 2d). As shown in Fig. 2e, by increasing the incubation time up to 24 h, the amount of removal efficiency and capacity was

increased, but incubation time more than 24 h did not affect the rate of remediation. Most remediation was observed in the mid-log cells.

Role of active, inactive and EPS on Pb remediation/ SEM observation and EDS analysis

Active cells showed the highest biological removal efficiency (99.5 %). The BRE amounts for autoclaved inactive cells, sodium azide inactive cells and EPS were 67, 63 and 87 %, respectively. A SEM image (Fig. 3) depicts Pb-treated strain AS2 cells, indicating that the bacteria retain their shape and are visually unaffected after the Pb

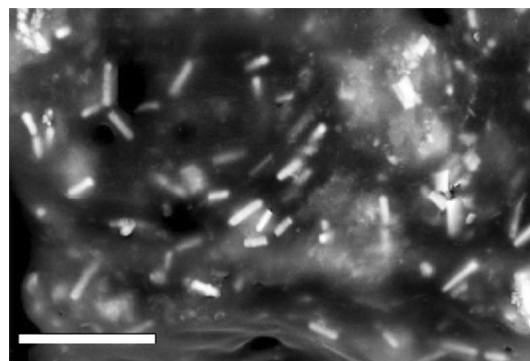


Fig. 3 Scanning electron microscopy of Pb-treated *Bacillus* species AS2. Bar 10 μm



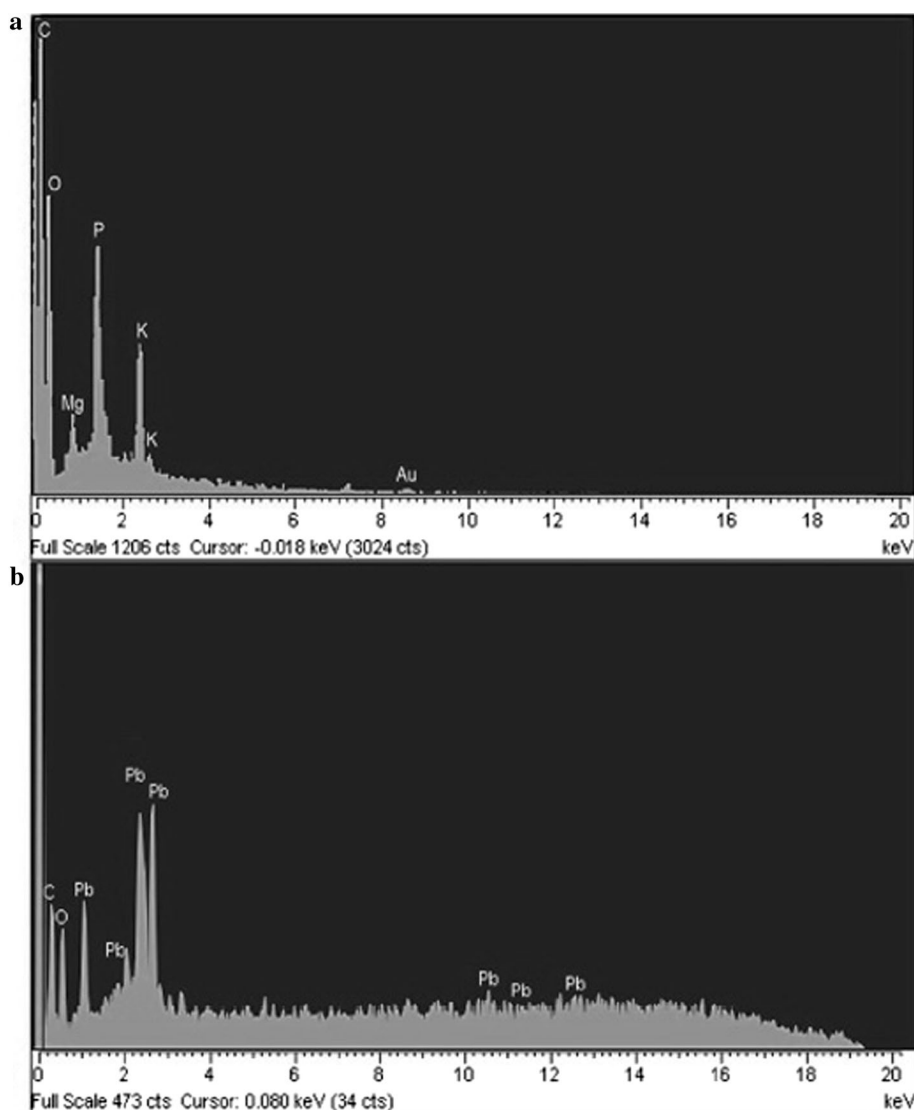
treatment. The image demonstrated exopolysaccharide around the cells adhesion zone to trap the Pb. EDS elemental analysis was carried out on bacterial cells before and after interaction with Pb. Figure 4a depicts the spectrum of pure bacterial cells, and Fig. 4b shows the presence of Pb. Metal ion can be observed in Pb-treated bacteria. Several mechanisms have been proposed to be involved in Pb biosorption: binding to phosphate, hydroxyl carbonyl and amino groups of peptidoglycan (Cabuk et al. 2006); teichoic acid and lipopolysaccharides (Beveridge and Fyfe 1985) of cell envelope; binding of toxic cation to extracellular polymers (like polysaccharides) (Pérez et al. 2008); precipitating Pb(II) inside/outside the cell in the form of an unusual phosphate compound $Pb_9(PO_4)_6$ (Naik and Dubey 2011); binding of Pb(II) by specific proteins like metallothioneins; and finally extracellular enzymes, such as superoxide dismutase, can also be engaged in Pb(II)

biosorption (So et al. 2001). The dependence of pH as well as EDS analysis in our work indicates that ion exchange is probably at least partly responsible for the observed metal binding. However, involvement of EPS was also confirmed by SEM micrograph and the fact that 67 % of the initial Pb could be absorbed in the cell-free polysaccharide extract. It was also hypothesized that Pb removal by strain AS2 is also an enzymatic dependent process, because the remediation rate decreased drastically in the inactive cells.

Remediation of Pb from industrial waste

Remediation of contaminated waste obtained from batteries and dye industries was analysed in a 1 L batch fermentation system. The concentration of Pb in these samples was as high as 1100 ppm. Twofold dilutions of these wastes were added to remediation culture at optimum condition. The bacterial

Fig. 4 Energy-dispersive X-ray analysis of non-treated (a) and treated (b) *Bacillus* species AS2



culture was able to reduce 500 ppm initial Pb concentration to less than 10 ppm (98.4 % removal efficiency) in 24 h. High anthropogenic removal efficiency by the culture of this strain as well as more than 66 % adsorption capacity appears to give desirable results for removal of Pb contamination from the environments.

Identification of Pb-adsorbing bacteria

The 16S rRNA gene sequences of strain AS2 were obtained (GenBank/EMBL/DDBJ accession number KT943756). Gene sequence analysis revealed strain AS2 to be a member of the genus *Bacillus*. The closest relative of this strain was *Bacillus sonorensis* NBCR101234^T, with a 16S rRNA gene sequence similarity of 99.5 %.

Conclusion

A novel indigenous bacterium from geogenic contaminated site with very high resistance to Pb (3125 ppm) and high biosorption activity (99.5 % of initial Pb) was isolated. This strain probably uptakes Pb via different pathways. Pb concentration, pH and inoculum size are the most effective variables on the remediation process. Anthropogenic Pb removal by the culture of this strain resulted in more than 98.4 % remediation of the initial Pb. Application of this strain with more than 66 % adsorption capacity appears to give desirable results for removal of Pb contamination from the environments.

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