Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Bioresource Technology 101 (2010) 8599–8605

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Bioremediation of heavy metals by growing hyperaccumulaor endophytic bacterium *Bacillus* sp. L14

Hanjun Guo [a,b], Shenglian Luo [a,b,c,d,*], Liang Chen [a,b], Xiao Xiao [a,b], Qiang Xi [c], Wanzhi Wei [c], Guangming Zeng [a,b], Chengbin Liu [c], Yong Wan [a,b], Jueliang Chen [a,b], Yejuan He [c]

[a] College of Environmental Science and Engineering, Hunan University, Changsha 410082, People's Republic of China
[b] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, People's Republic of China
[c] State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha 410082, People's Republic of China
[d] School of Environment and Chemical Engineering, Nanchang Hangkong University, Nanchang 330063, People's Republic of China

A R T I C L E I N F O

*Article history:*
Received 13 March 2010
Received in revised form 8 June 2010
Accepted 19 June 2010
Available online 15 July 2010

*Keywords:*
Endophytic bacteria
Heavy metals
Bioremediation
Hormesis

A B S T R A C T

Heavy metal bioremediation by a multi-metal resistant endophytic bacteria L14 (EB L14) isolated from the cadmium hyperaccumulator *Solanum nigrum* L. was characterized for its potential application in metal treatment. 16S rDNA analysis revealed that this endophyte belonged to *Bacillus* sp. The hormesis of EB L14 were observed in presence of divalent heavy metals (Cu (II), Cd (II) and Pb (II)) at a relatively lower concentration (10 mg/L). Such hormesis was the side effect of abnormal activities increases of ATP-ase which was planned to provide energy to help EB L14 reduce the toxicity of heavy metals by exporting the cations. Within 24 h incubation, EB L14 could specifically uptake 75.78%, 80.48%, 21.25% of Cd (II), Pb (II) and Cu (II) under the initial concentration of 10 mg/L. However, nearly no chromium uptake was observed. The mechanism study indicated that its remediation efficiencies may be greatly promoted through inhibiting the activities of ATPase. The excellent adaptation abilities and promising remediation efficiencies strongly indicated the superiority of this endophyte in heavy metal bioremediation at low concentrations, which could be useful for developing efficient metal removal system.

© 2010 Elsevier Ltd. All rights reserved.

## 1. Introduction

Heavy metal pollution by industrial activities and technological development is posing significant threats to the environment and public health because of its toxicity, non-biodegradability and bio-accumulation (Bahadir et al., 2007; Pérez-Marín et al., 2008; Reddad et al., 2003). Trace of heavy metals such as cadmium (Cd (II)), lead (Pb (II)), copper (Cu (II)) and chromium (Cr (VI)), which are commonly presented in contaminated soils, can enhance Fe deficiency symptoms both in microbes and plants (Baysse et al., 2000; Yoshihara et al., 2006; Christian et al., 2008), thus affecting their growth negatively. Furthermore, they can combine with sulfhydryl groups of proteins, restraining the activity of enzymes. The permanent existences of cadmium (Cd (II)), lead (Pb (II)), copper (Cu (II)) and chromium (Cr (VI)) in polluted ecosystems threaten the health of entire human beings all the time (Nogaw and Kido, 1996).

A lot of physicochemical strategies, such as filtration, chemical precipitation, electrochemical treatment, oxidation/reduction, ion exchange, membrane technology, reverse osmosis, and evaporation recovery, have been developed for removing heavy metals from the polluted water (Xiao et al., 2010). However, most of them appear to be expensive, low efficient, labor-intensive operational or lack of selectivity in the treating process (Chen et al., 2008; Tang et al., 2008).

Bioremediation, which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention in recent times to clean up a polluted environment (Farhadian et al., 2008; Radhika et al., 2006; Malik, 2004; Gadd, 2000). Bioremediation, being *in situ* treatment, provides a safe and economic alternative to commonly used physicochemical strategies (Bai et al., 2008). However, it seems not feasible at present. On one hand, it is difficult to obtain such valuable microbes from numerous kinds of microorganisms for heavy metal bioremediation. As well as, the adaptation abilities and the remediation efficiencies of reported microorganisms are not enough for practical application. On the other hand, the mechanisms of bioremediation still need further understanding. As it known, the mechanisms may facilitate the remediation efficiencies of valuable microbes and finally enable them feasible for practical application.

Plant-associated bacteria play a key role in host adaptation to a changing environment (Sturz and Nowak, 2000). Plant-associated

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, People's Republic of China. Tel./fax: +86 731 88821967.
*E-mail address:* sllou@hnu.cn (S. Luo).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.06.085

Author's personal copy



bacteria isolated from rhizoplane and phylloplane surfaces are known as epiphytes (Andrews and Harris, 2000) whereas those isolated from the interior of tissues, which they inhabit without causing harm to the host, are called endophytes (Azevedo et al., 2000; Kuklinsky-Sobral et al., 2004), with some bacterial populations fluctuating between endophytic and epiphytic colonization (Hallmann et al., 1997). Rhizobacteria are the most studied plant-associated bacteria and are often fond to have beneficial effects on plant growth, e.g., via the provision of essential elements, inhibition of colonization by pathogenic microorganisms, or by helping the plant to overcome stress responses to environmental insults (Mastretta et al., 2009). Similar beneficial effects have also been described for endophytic bacteria. Certain endophytic bacteria have been shown to enhance plant growth, increase plant resistance to pathogens, drought and even herbivores, such that their commercial potential has received much study (Sturz and Nowak, 2000; Azevedo et al., 2000; Reiter et al., 2002). Our previously study also showed that endophytes of a hyperaccumulator may be potential resources of highly efficient biosorbent for heavy metal biosorption (Xiao et al., 2010). Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high-stress environment of the soil (Sturz and Nowak, 2000).

In this study, endophytic bacteria L14, which was isolated from the leaf of cadmium hyperaccumulator *Solanum nigrum* L., has been chosen for the bioremediation of synthetic solutions of Cd (II), Pb (II), Cu (II) and Cr (VI). In addition, the mechanisms of bioremediation were also investigated and discussed to evaluate the possibility of heavy metal remediation enhancement for subsequent studies and eventually practical application.

## 2. Methods

### 2.1. Preparation of reagents and medium

All reagents used were of analytical grade and purchased from shanghai Pharmaceutical Co. Ltd., in China. The 1000 mg/L Cd (II), Pb (II), Cu (II), Cr (VI) and Zn (II) stock solution was prepared by dissolving the exact quantities of the $3CdSO_4{\cdot}8H_2O$, $PbSO_4$, $CuSO_4{\cdot}5H_2O$, $K_2Cr_2O_7$ and $ZnSO_4{\cdot}7H_2O$ in deionized–distilled water. The working concentration of Cd (II), Pb (II), Cu (II), Cr (VI) and Zn (II) solution was prepared from suitable serial dilution of the stock solution. The deionized–distilled water used in this experiment was obtained from a Milli-Q system (Millipore USA).

Putative endophytic bacterial strains which isolated from plants were maintained and activated in LB medium comprising of 10 g tryptone, 5 g yeast extract, 5 g NaCl per litre. The pH of the medium was adjusted to 7.2–7.4.

### 2.2. Isolation and conservation of endophytic bacteria

Putative endophytic bacterial strains were isolated from surface-sterilized *S. nigrum* L., a cadmium hyperaccumulator, collected at the sewage discharge canal bank of Zhuzhou Smeltery (27°52′N, 113°05′E). A total of six plants were collected. Picked plants were put into plastic pot and processed the following day. Root, stem and leaf of each plant were analyzed separately. Endophytic bacteria were isolated after removing epiphytes by surface disinfection using serial washing in 70% ethanol for 3 min, sodium hypochlorite solution (2% available chlorine anion) for 30 min and rinsed three times in sterilized distilled water (Barzanti et al., 2007). The disinfection process was checked by plating a 100 μL sample of the sterile distilled water used in the final rinse onto LB medium and incubating the plates at 28 °C for 2–14 days. After surface disinfection, the root, stem or leaf tissue was cut and triturated in 10 mL of sterile PBS contained in a 50 mL conical flask maintained at 28 °C and agitated at 150 rpm for 1 h, after which appropriate dilutions (100 μL) were plated onto LB medium and incubated at 28 °C for 2–14 days. After incubation, colonies were picked off the plates, inoculated on 10% LB agar slants, incubated at 28 °C for 2 days and stored at 4 °C. All the isolation processes were operated in the SW-CJ-1FD clean bench (Airtech, China) (ISO14644-1, class 5) of our disinfection chamber (ISO14644-1, class 6).

### 2.3. Identification of selected EB L14

The selected EB L14 strains were identified by determination of 16S rDNA gene sequences. Colony PCR was performed from live cells cultured on solid LB medium and the 16S rDNA were amplified by PCR using the following primers 27 f (5′-GAGTTTGATC ACTGGCTCAG-3′) and 1492r (5′-TACGGCTACCTTGTTACGACTT-3′) (Byers et al., 1998). Amplification was performed for 30 PCR cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min. The amplified DNA was purified with TaKaRa Agarose Gel DNA Purification Kit (TaKaRa, China) and sequencing was performed at TaKaRa Biotechnology Company, Limited (Dalian, China). The 16S rDNA sequence was compared against the GenBank database using the NCBI Blast program.

### 2.4. Heavy metals resistance of EB L14

The minimal inhibitory concentration (MIC) of heavy metals at which no colony growth occurred was determined in triplicate in LB liquid medium with a series concentration of Cu (II), Cd (II), Pb (II) and Cr (VI) (1–15 mM). All the fermentations were carried out in 250 mL Erlenmeyer flasks with 50 mL working volume. The cultures were incubated in a rotary incubator shaker (New Brunswick Scientific, Co., Inc., NJ, USA) for at least 1 week at 28 °C and 150 rpm, checking bacterial development by absorbance at 600 nm every 24 h, MIC was considered to be the lowest concentration of heavy metals at which completely inhibited bacterial growth in LB medium.

### 2.5. Analytical technique

The optical densities of the cultures were determined by using UV–vis spectrophotometer (Cary 300, Varian, USA).

The concentrations of heavy metal ions were determined by the flame atomic absorption spectrometry (FAAS) using Z2000 polarized zeeman atomic absorption spectrophotometer (Hitachi, Japan). The hollow cathode lamp was operated at 5 mA and the analytical wavelengths were set at 228.8, 283.3, 359.3 and 324.8 nm for detection of Cd (II), Pb (II), Cu (II) and Cr (VI), respectively.

### 2.6. Effects of heavy metals on growth curve

The endophytic bacterial L14 was inoculated into 100 mL LB medium in 250 mL conical flakes and incubated in a shaker at 150 rpm for 24 h at 30 °C. The cells were grown to late exponential phase, and then inoculated into 10 and 100 mg/L Cd (II), Pb (II), Cu (II) and Cr (VI) with 1‰ inoculation. Growth was determined from the optical density at 600 nm ($OD_{600}$). Optical densities were carried out for 24 h. All the experiments were performed in triplicate.

### 2.7. Bioremediation of cadmium, lead, copper and chromium by growing EB L14

The endophytic bacteria L14 were inoculated into 100 mL medium in 250 mL conical flasks containing 10 mg/L of Cd (II), Pb (II), Cu (II) and Cr (VI), and the flasks were incubated at 30 °C and agitated at 150 rpm. Samples were taken at predefined time intervals (0, 2, 4,

Author's personal copy



6, 8, 12 and 24 h). Then samples were divided into two parallel samples. One parallel samples were filtered through 0.22 μm filter units (Millipore, Ireland). The Cd (II), Pb (II), Cu (II) and Cr (VI) concentrations in the filtrate were analyzed with the methods mentioned above. The other parallel samples were determined from the optical density at 600 nm ($OD_{600}$). All experiments were done triplicate, yielding an experimental error of less than 5%. In all experiments, control sets, without any added bacterial cells were used to compare the metal uptake by the bacteria. Following metal uptake, cell viability was monitored by checking the colony forming units (CFU) counts on nutrient agar plates. CFU per mL of solution was determined before and after (24 h) the metal uptake. Plates were incubated at 30 °C overnight and CFU counts were determined.

### 2.8. ATPase activities in presence of cadmium and lead

ATPase was tested in the concentration of 10 mg/L cadmium (Cd (II)) and lead (Pb (II)) in 250 mL conical flakes. Samples were taken at predefined time intervals (0, 2, 4, 8 and 24 h). Then, the process of experiment was based on ATPases testing reagent provided by Nanjing Jiancheng Bioenginnering Institute.

### 2.9. Bioremediation of cadmium, lead, copper and chromium without supplement studies

The 50 mL cell suspensions after 24 h were harvested after centrifuging at 15,000*g* for 15 min. Then the cells were introduced into 10 mg/L Cd (II), Pb (II), Cu (II) and Cr (VI) aqueous solution in a 250 flask at pH 7.0 and equilibrated on orbital shaker for a specified time at 150 rpm and 30 °C. Samples were taken at predefined time intervals (0, 2, 4, 6, 8, 12 and 24 h). The residual Cd (II), Pb (II), Cu (II) and Cr (VI) concentration in the solution obtained after filtrating through 0.22 μm filter units (Millipore, Ireland). The Cd (II), Pb (II), Cu (II) and Cr (VI) concentrations in the filtrate were analyzed with the methods mentioned above. The amount of Cd (II), Pb (II), Cu (II) and Cr (VI) biosorbed was obtained by taking the difference between the initial and final concentration of Cd (II), Pb (II), Cu (II) and Cr (VI) (Radhika et al., 2006).

### 2.10. Subcellular fractionation

Subcellular fractionation was obtained by the method of Kumar and Upreti1 (2000). After 24 h of incubation, cells grown with and without cadmium were harvested by centrifugation (2576*g*, 25 min, 4 °C), washed twice with 0.03 mol/L Tris buffer containing $2.5 \times 10^{-3}$ mol/L EDTA, pH 8.0, and resuspended in the same buffer. To prepare spheroplasts, lysozyme was added to a final concentration of 200 mg/mL and cells were incubated for 30 min at 25 °C. All subsequent steps were carried out at 0–4 °C. Spheroplasts were collected by centrifugation at 2576*g* for 15 min and resuspended in 0.03 mol/L Tris buffer containing $3 \times 10^{-3}$ mol/L EDTA, pH 8.1. The supernatant obtained was the periplasmic fluid consisting of a peptidoglycan layer. The spheroplasts were then disrupted by two 15 s bursts with the Vibronic Ultrasonic processor and centrifuged at 2000*g* for 10 min to remove debris and unbroken cells. The resulting supernatant consisting of membrane and cytoplasmic fractions was centrifuged at 2576*g* for 150 min. The pellet consisting of both outer and inner membrane envelopes, now termed crude membrane.

## 3. Results and discussion

### 3.1. Isolation and identification of EB L14

Ninety-six endophytes were isolated. All of them were tested for heavy metal removal efficiency and EB L14 was selected due to its high removal efficiency of Cd (II), Pb (II) (data not shown). EB L14 was isolated from the leaf of cadmium hyperaccumulator *S. nigrum* L. The isolates were gram-positive bacteria; on the basis of morphological, physiological, biochemical characteristics (data not shown) and comparative analysis of the sequence with already available database showed that the EB L14 (610 bp) were close to the members of the genus *Bacillus* sp. and showed 100% homology with *Bacillus thuringiensis*. The sequence were deposited at GenBank (*Bacillus* sp. EB L14, accession no. GU570957).

### 3.2. Heavy metals resistance of EB L14

The bacterial strain EB L14 showed a high degree of resistance to heavy metals, especially to Cu (II), Cd (II) and Cr (VI). The minimal inhibitory concentration (MIC) of the strain in the liquid LB medium containing heavy metal ions was 10 mM (Cu (II)), 2 mM (Cd (II)), 12 mM (Cr (VI)), 4 mM (Pb (II)) and 5 mM (Zn (II)), respectively. The order of the toxicity of the metals to strain EB L14 was found to be Cd (II) > Pb (II) > Zn (II) > Cu (II) > Cr (VI). This result is different with our previous studies screening EB LRE07 from cadmium hyperaccumulator *S. nigrum* L., where Pb (II) is the most toxic metal for the strain (unpublished results). This phenomenon may be related to the different localization of the two endophytes: EB LRE07 was inhabited in the root interior of cadmium hyperaccumulator *S. nigrum* L, in which the cadmium concentration was relatively higher. While, EB L14 was isolated from the leaf of the host, in which the concentration of Cd (II) was relatively lower. The selective pressure of plants has its maximum effect on the bacterial populations (Siciliano et al., 2001). The high heavy metal resistance of EB L14 indicating its promising adaptation abilities for practical applications.

### 3.3. Effects of cadmium, lead, copper and chromium on the growth

The growth of response curves of EB L14 at two concentrations (10 and 100 mg/L) of different heavy metals ions in Supplementary data showed that the lag phase as well as the optical density had been attained by the endophyte depended greatly on the concentration and toxicity of the heavy metals. At the concentration of 100 mg/L of cadmium ions (the most toxic metal among the five heavy metals), the lag phase was extended and the maximal cell density was reduced only below 23.86% of the control.

An unexpected phenomenon was observed at Cd (II), Pb (II), Cu (II) and Cr (VI) concentrations of 10 and 100 mg/L (Fig. 1). The relative growth indicates the growth efficiency in the presence of a metal compared to the growth in metal free medium. At the low concentration of 10 mg/L, as shown in Fig. 1, the relative growth of EB L14 in Pb (II), Cd (II), and Cu (II) medium were rapidly increased from 1 to 2.2, 2.6 and 3.2 at first 4 h, respectively, indicating the growth of EB L14 were advanced at this concentration of heavy metal. At the interval of 4–8 h, the relative growth curve in Pb (II), Cd (II), and Cu (II) were decreased sharply, and finally reached a plateau at about 1. The situation was basically conversed at the high concentration of 100 mg/L. The relative growth curves in Cd (II) and Cu (II) were below 1 during the 24 h of incubation indicating the toxicity of heavy metals restrain the growth of EB L14. However, the relative growth curve in Pb (II) was nearly the same as it in 10 mg/L Pb (II) medium. It was also noticed that the relative growth curves in these two concentrations of Cr (VI) medium were both below 1 during the 24 h of incubation.

The hormesis of EB L14 was induced by heavy metals. The ability to grow even at high metal concentrations is found in many microorganisms and maybe the result of intrinsic or induced mechanisms, as well as of other environmental factors (pH, speciation, redox etc.), which can reduce toxicity (Xiao et al., 2010; Zouboulis et al., 2004; Leedjärv et al., 2008). Life in the plant host,

Author's personal copy



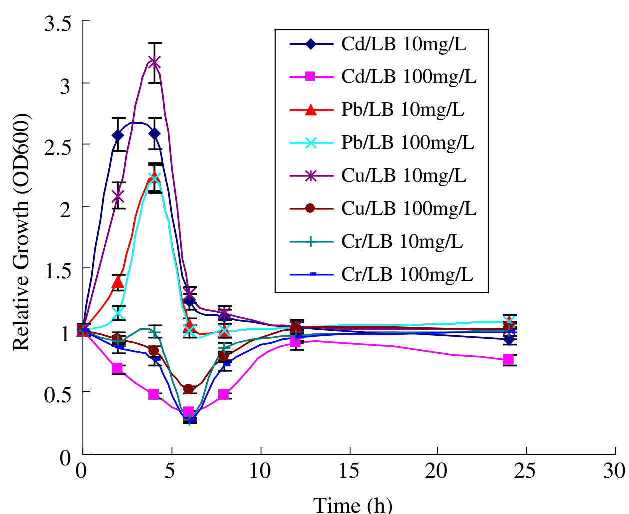


**Fig. 1.** Effect of heavy metals (cadmium, lead, copper and chromium) on the growth of EB L14. The relative growth indicates the growth efficiency in the presence of a metal compared to the growth in medium without the metal.

especially in the heavy metal hyperaccumulator, challenges the endophytes in many ways, which is reflected in the fact of a greater demand for energy in order to cope with the toxicity of pollutants. Subsequently, the continuous usage of energy (ATP) forces the cells into some altered growth rhythm, which, in the long term, results in enhanced growth of EB L14. The relative growth curves of EB L14 in the four heavy metals medium revealed that only the divalent heavy metal cations could force (induce) the ATPase to generate more ATP for the endophyte to cope with the toxicity of these divalent heavy metals. Among the three divalent heavy metals, the best induce effect was found for Cu (II) (the relative growth could increase up to 3.2) (Fig. 1). However, Pb (II) could induce the hormesis in both concentrations.

Such hormesis phenomenon was also observed in *Pseudomonas putida* KT2440 serials strains with functional CadA2 genes (Leedjärv et al., 2008). However, the hormesis of EB L14 was carried out at a relative higher concentration of heavy metals than KT2440 strains. This may due to the unique inhibition niches of EB L14.

Low remediation efficiencies and poor adaptation abilities to the toxic niches are the most two major factors which limited the application of heavy metal bioremediations by using living microorganisms. In this case, hormesis is no doubt a great characteristic which may facilitate their carrier to overcome these limits. The hormesis carriers such as EB L14 seems prefer to inhabit the toxic niches and actually could drive even more biomass under such tough conditions. Coincidentally, the remediation efficiencies of the microorganisms always depended on their biomass. The more biomass it could drive in the less time, the more remediation efficiencies it will be. As the results, it is not surprised that the microorganisms which not only possess heavy metal bioremediation ability but also show hormesis to the treatment substrate will be more feasible for practical applications. As the results, given the promising remediation abilities, the hormesis carriers such as EB L14 will have more advantages in practical applications.

### 3.4. ATPase activities in presence of cadmium and lead

To investigate whether the hormesis of EB L14 was directly forced by continuous usage of energy (ATP), the ATPase activities of EB L14 were determined during the growth process with and without the presence of heavy metals. The two most toxic heavy metals (Cd (II) and Pb (II)) for EB L14 were chosen for this experiment. According to the relative growth results, the concentration of 10 mg/L was applied. The relative ATPase which indicated the ATPase activities of EB L14 in the presence of a metal compared to the growth in metal free medium activities were presented in Fig. 2. The results of relative growth under the same conditions were also included in Fig. 2 to clarify the relationship between ATPase activity and growth. It was noticed that the terms of relative ATPase activities and relative growth were highly similar. This suggested that the hormesis of EB L14 was directly forced by continuous usage of energy (ATP).

However, the intensities of relative ATPase activities were different in the presence of Cd (II) and Pb (II). The ATPase activities increased almost three times that of the control in presence of 10 mg/L Cd (II) after 4 h of incubation. While the ATPase activities only increased 1.83 times in presence of 10 mg/L Pb (II) under the same conditions. It seems like that the more toxic heavy metal for EB L14, the more energy (ATPase activities) would be driven by the inducement of the heavy metal. As we all know that microorganisms may have a greater demand for energy in order to cope with the toxicity of the pollutants (Leedjärv et al., 2008). The possible explanations for this phenomenon may be that the endophyte needs more energy to cope with the more toxic heavy metal.

### 3.5. Bioremediation of cadmium, lead, copper and chromium by growing EB L14

According to Fig. 3, the EB L14 seems preferred to removing divalent heavy metals, especially Cd (II) and Pb (II). The Pb (II), Cd (II), Cu (II) and Cr (VI) concentration reduces to 2.0, 2.5, 8.15 and 9.3 mg/L in about 24 h, amounting to about 80.48%, 75.78%, 21.25% and 2.92%. Furthermore, another unexpected phenomenon was observed at 10 mg/L concentration of Cd (II) and Pb (II) during the log phase of EB L14. Generally, the bioremediation rate of living microorganisms should be strongly dependent on the population of the cells. In this case, the decreasing rates of the heavy metal concentrations should be rapid at the log phase of the living microorganism, such as the heavy metal removal behaviors of *Rhodotorula* sp. Y11 (Li et al., 2008), *Ing5* (Sprocati et al., 2006), *Rhodobacter sphaeroides* (Bai et al., 2008) and *Arthrobacter strain* D9 (Malik, 2004). However, our observation indicated that the concentration of the heavy metal was hold in some kind of balance at the log

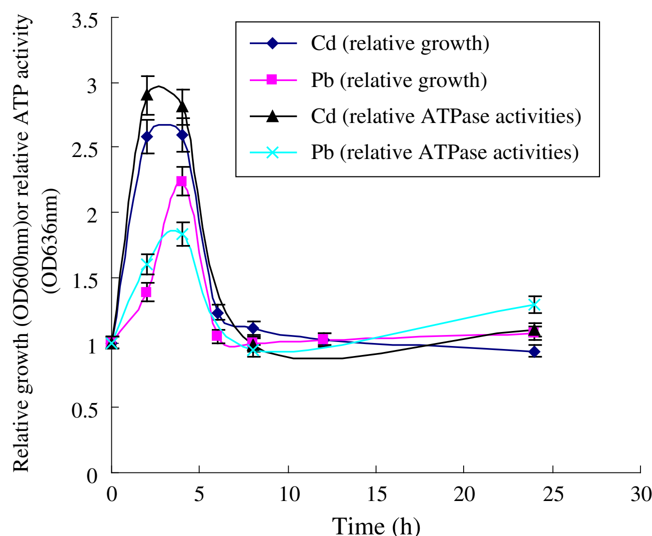


**Fig. 2.** The relative growth and relative ATPase activities of EB L14 in 24 h incubation.

Author's personal copy



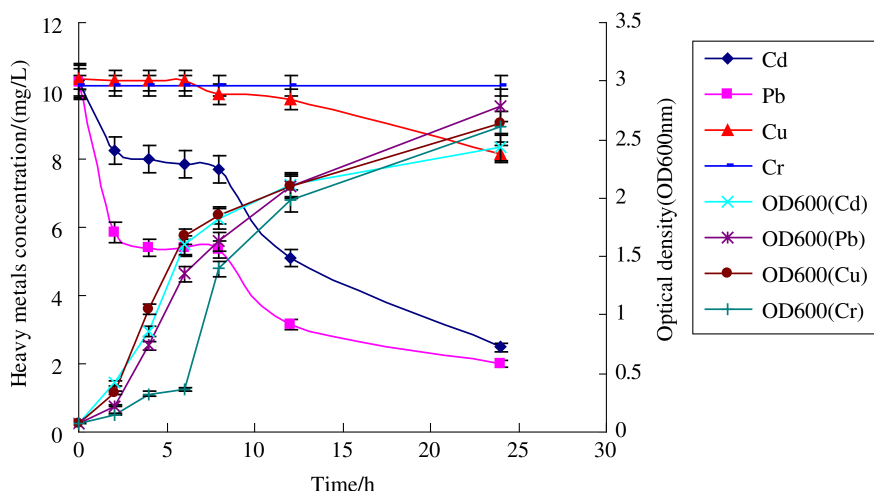


**Fig. 3.** The growth of heavy metal (cadmium, lead, copper and chromium) ions and heavy metals removal by EB L14.

phase. To the best of our knowledge, this phenomenon has never been found in any reported heavy metal bioremediation process of living microorganisms.

It is known that metal resistance systems in bacteria are abundant and widespread, and the frequencies range from a few percent of the isolates in clean environments to nearly all isolates in a heavily polluted environment (Barkay and Schaefer, 2001; Zouboulis et al., 2004). Therefore, it was not surprised that EB L14 which was originally inhabited in the inner tissue of cadmium hyperaccumulator possessed these metal resistance systems. The metal resistance systems are known as flowing: (i) export mechanisms which extruded the metal ions from the cell by activated energy consuming transporter, such as ATPase, CBA transporters and cation diffusion facilitator (CDF) family transporters (Franke et al., 2003; Perron et al., 2004) and (ii) import mechanisms which involves the contribution of diffusion, sorption, chelation, complexation or micro-precipitation mechanisms, basically depending on the intrinsic surface properties of the cells.

According to these two kinds of metal resistance systems, the whole Cd (II) and Pb (II) removal processes of EB L14 could be divided to three stages. The first stage was lasted for about 2 h. At this stage, the import mechanisms were in charge resulting in the continuous metals concentrations decreases which were relative to the population of EB L14 (Fig. 3). The subsequent stage lasted from about 2 to 8 h after inoculation. Coincidentally, this period was not only the log phase of EB L14 but also the period when the ATPase activities of EB L14 were turned up by the inducements of Cd (II) and Pb (II) ions (Fig. 2). Clearly evidences showed that the ATPase of microorganisms was able to protect the cell from the toxic effect of heavy metals by exporting the cation (Leedjärv et al., 2008). These abnormal increases of ATPase activities indicated that the export mechanisms of EB L14 were turned up in presence of divalent heavy metals to protect the endophyte from the toxic effect. Therefore, the concentrations plateau of Cd (II) and Pb (II) (Fig. 3) may be the balanceable results of export and import metal resistance mechanisms of EB L14. The toxic effects of these two heavy metals finally brought down the ATPase activities after exposition to the last stage. As the results, these balances were broken. The import mechanisms were once again in dominant resulting in the decreases of metals concentrations (Fig. 3).

It was noticed that the export mechanisms had never been found so powerful that could balance with the import mechanisms during the uptake process in any other living microorganisms with the detoxification capacity of heavy metals. This unique property was first clearly shown in this endophyte of hyperaccumulator. Consequently, it would not be surprised that the divalent heavy metals (at least Cd (II) and Pb (II)) bioremediation efficiencies of EB L14 could be greatly promoted by inhibiting the export mechanisms of the endophyte.

### 3.6. Bioremediation of cadmium, lead, copper and chromium without supplement studies

In order to prove the above results that the concentration balances were functioned by energy consuming ATPase. The experiments, which were carried out in the absence with growth media, were examined. In these tests, 10 mg/L of Cd (II), Pb (II), Cu (II) and Cr (VI) were interacted with the bacterial cells ($10^8$ cells/mL). The results are shown in Fig. 4. The Cd (II), Pb (II), Cu (II) and Cr (VI) concentration slightly reduces to 4.18, 6.7, 9.5 and 9.7 mg/L in about 24 h, amounting to about 48.94%, 33.33%, 8.03% and 4.9% Cd (II), Pb (II), Cu (II) and Cr (VI) removal by biosorption, respectively. For ease the presence of Cd (II), Pb (II), Cu (II) and Cr (VI) are depicted in the same figure, wherein it is evident that complete bioremoval is effected in 24 h. Thus biosorption or abiotic precipitate also plays an important role in the overall bioremoval process. From Fig. 4, it was also clear to see that the concentration balance was not appearance, which indicated the export mechanism of EB L14 was energy consuming process.

### 3.7. Distribution and uptake of cadmium and lead in EB L14

The results of distribution and uptake of Cd (II), Pb (II) in EB L14 were presented in Table 1. Subcellular fractionation studies reveal that almost 80.8% and 76.5% of the Cd (II) and Pb (II) taken up by the cells, is found on the membrane fraction, whereas the presence of Cd (II) and Pb (II) in the cytoplasmic are only 5.5% and 7.4%, and on the cell wall are 13.7% and 16.1% of the total uptake, respectively. The distribution and uptake of Cd (II) and Pb (II) ions in EB L14 indicated that the removal and transformation of Cd (II) and Pb (II) ions are mostly carried out on the membrane fraction. It also indicated that the bio-accumulation played a significant role in the process of heavy metals removal.

This result was different with other reports (Bai et al., 2008; Kumar and Upreti1, 2000) in which the cell wall were always responsible for almost all the uptakes. As indicated above, these kinds of uptakes were basically depending on the intrinsic surface properties of the cells wall which involves the contribution of diffusion,

Author's personal copy



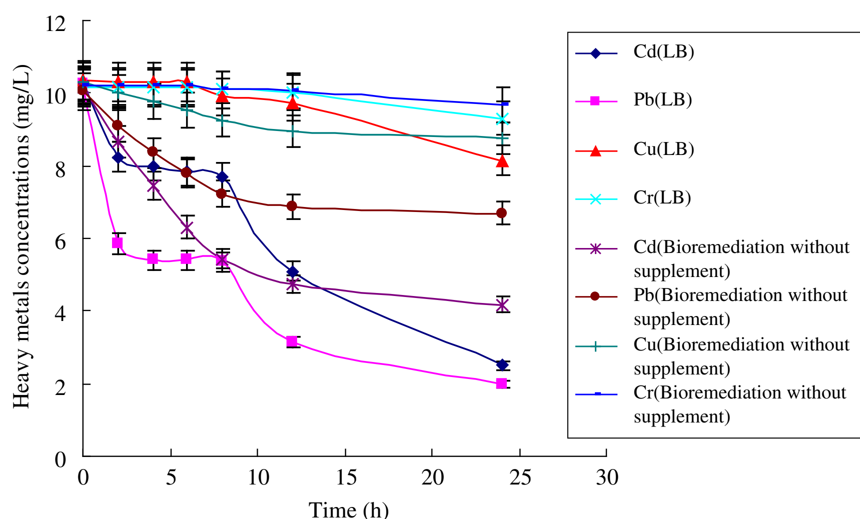


**Fig. 4.** Bioremediation of heavy metals (cadmium, lead, copper and chromium) by EB L14 with or without supplement.

**Table 1**
Distribution and uptake of cadmium and lead in EB L14 after 24 h of incubation.

| Fraction | Cadmium conc. (μg) | Lead conc. (μg) | Percentage ($Cd^{2+}$) (%) | Percentage ($Pb^{2+}$) (%) |
|---|---|---|---|---|
| *Culture broth* | | | | |
| Whole cells | 780 ± 24 | 825 ± 25 | 75.7 ± 2.3 | 80 ± 2.4 |
| Culture media | 250 ± 7 | 200 ± 8 | 24.3 ± 0.7 | 19.5 ± 0.8 |
| *Subcellular fraction* | | | | |
| Cell wall | 107 ± 4 | 133 ± 4 | 13.7 ± 0.5 | 16.1 ± 0.5 |
| Cytoplasmic | 43 ± 1.3 | 61 ± 2 | 5.5 ± 0.2 | 7.4 ± 0.2 |
| Membrane fraction | 630 ± 25 | 631 ± 30 | 80.8 ± 3.2 | 76.5 ± 3.6 |

The values are the means ± standard deviation ($n = 3$).

sorption, chelation, complexation or micro-precipitation mechanisms. Therefore, the bindings between the cell wall and heavy metals were physicochemical and could be easily broken by other competitors (e.g. cations, chelator etc.) resulting secondary pollution when used in bioremediation. Since the majority uptakes of heavy metals by EB L14 were not happened in the cell wall, it seems more feasible than other reported strains.

## 4. Conclusion

Our investigations revealed the practical bioremediation potential of EB L14 as followings: (i) the multi-metal resistance and hormesis of EB L14 exhibited excellent adaptation abilities for practical *in situ* bioremediation of heavy metals. (ii) At 10 mg/L of heavy metals, EB L14 could specifically uptake 75.78%, 80.48% and 21.25% of Cd (II), Pb (II) and Cu (II) within 24 h incubation. These efficiencies may be greatly promoted by inhibiting the activities of ATPase. (iii) The majority removal and transformation of Cd (II) and Pb (II) ions by EB L14 were carried out *in vivo* indicated that it would not cause seriously secondary pollution in bioremediation.


## Acknowledgements

This work was financially supported by a grant from National Science Fund for Distinguished Young Scholars (No. 50725825), the Key Program of National Natural Science Foundation of China (No. 50830301), Xiangjiang Water Environmental Pollution Control Project subjected to the National Key Science and Technology Project for Water Environmental Pollution Control (2008ZX07212-001) and the Key Special Science and Technology Project for Energy Saving and Emission Reduction of Hunan Province (No. 2008SK1002).


## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.06.085.

## References


Andrews, J.H., Harris, R.F., 2000. The ecology and biogeography of microorganisms on plant surfaces. Annurev. Phytopathol. 38, 145–180.
Azevedo, J.L., Macheroni Jr., M., Pereira, J.Q., Araújo, W.L., 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electron. J. Biotechnol. 3, 1–36.
Bahadir, T., Bakan, G., Altas, L., Buykgungar, H., 2007. The investigation of lead removal by biosorption. An application at storage battery industry wastewaters. Enzyme Microbiol. Technol. 41, 98–102.
Bai, H.J., Zhang, Z.M., Yang, G.E., Li, B.Z., 2008. Bioremediation of cadmium by growing *Rhodobacter sphaeroides*: kinetic characteristic and mechanism studies. Bioresour. Technol. 99, 7716–7722.
Barkay, T., Schaefer, J., 2001. Metal and radionuclide bioremediation: issues, considerations and potentials. Curr. Opin. Microbiol. 4, 318–323.
Barzanti, R., Ozino, F., Bazzicalupo, M., Gabbrielli, R., Galardi, F., Gonnelli, C., Mengoni, A., 2007. Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant *Alyssum bertolonii*. Microb. Ecol. 53, 306–316.
Baysse, C., DeVos, D., Naudet, Y., Vander monde, A., Ochsner, U., Meyer, J.M., Budzikiewicz, H., Schäfer, M., Fuchs, R., Cornelis, P., 2000. Vanadium interferes with siderophore-mediated iron uptake in *Pseudomonas aeruginosa*. Microbiology 146, 2425–2434.
Byers, H.K., Stackebrandt, E., Hayward, C., Blackall, L.L., 1998. Molecular investigation of a microbial mat associated with the great artesian basin. FEMS Microbiol. Ecol. 25, 391–403.
Chen, G.Q., Zeng, G.M., Tang, L., Du, C.Y., Jiang, X.Y., Huang, G.H., Liu, H.L., Shen, G.L., 2008. Cadmium removal from simulated wastewater to biomass byproduct of *Lentinus edodes*. Bioresour. Technol. 99, 7034–7040.
Christian, O.D., Aleš, S., Paulina, D., Andre, S., Wilhelm, B., Erika, K., 2008. Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp. Chemosphere 74, 19–25.
Farhadian, M., Vachelard, C., Duchez, D., Larroche, C., 2008. In situ bioremediation of monoaromatic pollutants in groundwater: a review. Bioresour. Technol. 99, 5296–5308.
Franke, S., Grass, G., Rensing, C., Nies, D.H., 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J. Bacteriol. 185, 3804–3812.
Gadd, G.M., 2000. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. Curr. Opin. Biotechnol. 11, 271–279.
Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F., Kloepper, J.W., 1997. Bacteria endophytes in agricultural crops. Can. J. Microbiol. 43, 895–914.
Kuklinsky-Sobral, J., Araújo, W.L., Mendes, R., Geraldi, I.O., Pizzirani-Kleiner, A.A., Azevedo, J.L., 2004. Isolation and characterization of soybean-associated

Author's personal copy



bacteria and their potential for plant growth promotion. Environ. Microbiol. 6, 1244–1251.

Kumar, M., Upreti1, R.K., 2000. Impact of lead stress and adaptation in *Escherichia coli*. Environ. Res. B 47, 246–252.

Leedjärv, A., Ivask, A., Virta, M., 2008. The interplay of different transporters in the mediation of divalent heavy metal resistance in *Pseudomonas putida* KT2440. J. Bacteriol. 190, 2680–2689.

Li, Z.J., Yuan, H.L., Hu, X.D., 2008. Cadmium-resistance in growing *Rhodotorula* sp. Y11. Bioresour. Technol. 99, 1339–1344.

Malik, A., 2004. Metal bioremediation through growing cells. Environ. Int. 30, 261–278.

Mastretta, C., Taghavi, S., van der Lelie, D., Mengoni, A., Galardi, F., Gonnelli, C., Barac, T., Boulet, J., Weyens, N., Vangronsveld, J., 2009. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. Int. J. Phytoremediat. 11, 251–267.

Nogaw, K., Kido, T., 1996. Ital–Ital disease and health effects of cadmium. In: Chang, L.W. (Ed.), Toxicology of Metals. CRC Press, Boca Raton, FL, pp. 353–369.

Pérez-Marín, A.B., Ballester, A., González, F., Blázquez, M.L., Muñoz, J.A., Sáez, J., Meseguer Zapata, V., 2008. Study of cadmium, zinc and lead biosorption by orange wastes using the subsequent addition method. Bioresour. Technol. 99, 8101–8106.

Perron, K., Caille, O., Rossier, C., Van Delden, C., Dumas, J.L., Kohler, T., 2004. CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. J. Biol. Chem. 279, 8761–8768.

Radhika, V., Subramanian, S., Natarajan, K.A., 2006. Bioremediation of zinc using *Desulfotomaculum nigrificans*: bioprecipitation and characterization studies. Water Res. 40, 3628–3636.

Reddad, Z., Gérente, C., Andrès, Y., Thibault, J.F., Le Cloirec, P., 2003. Cadmium and lead adsorption by a natural polysaccharide in MF membrane reactor: experimental analysis and modelling. Water Res. 37, 3983–3991.

Reiter, B., Pfeifer, U., Schwab, H., Sessitsch, A., 2002. Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. *Atroseptica*. Appl. Environ. Microbiol. 68, 2261–2268.

Siciliano, S.D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., Ouellette, D., Roy, R., Whyte, L.G., Banks, M.K., Schwab, P., Lee, K., Greer, C.W., 2001. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl. Environ. Microbiol. 6, 2469–2475.

Sprocati, A.R., Alisi, C., Segre, L., Tasso, F., Galletti, M., Cremisini, C., 2006. Investigating heavy metal resistance, bioaccumulation and metabolic profile of a metallophile microbial consortium native to an abandoned mine. Sci. Total Environ. 366, 649–658.

Sturz, A.V., Nowak, J., 2000. Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. Appl. Soil Ecol. 15, 183–190.

Tang, L., Zeng, G.M., Shen, G.L., Li, Y.P., Zhang, Y., Huang, D.L., 2008. Rapid detection of picloram in agricultural field samples using a disposable immunomembrane-based electrochemical sensor. Environ. Sci. Technol. 42, 1207–1212.

Xiao, X., Luo, S.L., Zeng, G.M., Wei, W.Z., Wan, Y., Chen, L., Guo, H.J., Cao, Z., Yang, L.X., Chen, J.L., Xi, Q., 2010. Biosorption of cadmium by endophytic fungus (EF) *Microsphaeropsis* sp. LSE10 isolated from cadmium hyperaccumulator *Solanum nigrum* L. Bioresour. Technol. 101, 1668–1674.

Yoshihara, T., Hodoshima, H., Miyano, Y., Shoji, K., Shimada, H., Goto, F., 2006. Cadmium inducible Fe deficiency responses observed from macro and molecular views in tobacco plants. Plant Cell Rep. 25, 365–373.

Zouboulis, A.I., Loukidou, M.X., Matis, K.A., 2004. Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils. Process Biochem. 39, 909–916.