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Heavy metal bioaccumulation and cation release by growing *Bacillus cereus* RC-1 under culture conditions

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ABSTRACT

In an effort to explore the detoxifying mechanisms of *B. cereus* RC-1 under heavy metal stress, the bioaccumulation by growing cells under varying range of pH, culture time and initial metal concentration were investigated from a perspective of cation release. The maximum removal efficiencies were 16.7%, 38.3%, 81.4% and 40.3% for Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}, respectively, with initial concentrations of 10 mg/L at pH 7.0. In presence of Cu^{2+} or Zn^{2+}, large quantities of cations were released into the medium in descending order of $Na^{+} > K^{+} > Ca^{2+} > Mg^{2+}$, while bioremoval of the two essential metals Cd^{2+} and Pb^{2+} was accompanied with cellular Na^{+} and Mg^{2+} uptake from the medium, respectively. The relative mean contributions of intracellular accumulation to the total removal were approximately 19.6% for Cu^{2+}, 12.8% for Zn^{2+}, 51.1% for Cd^{2+}, and only 4.6% for Pb^{2+}. Following exposure at high concentration, *B. cereus* RC-1 could keep intracellular Cd^{2+} concentrations constant, possibly by means of a Cd-efflux system whose activity coincided with uptake of Na^{+}, and reduce intracellular Pb^{2+} concentration due to the effect of Mg^{2+} on limiting Pb^{2+} access to the cells. Cellular morphology, surface functional groups and intracellular trace elements were further investigated by SEM-EDX, TEM-EDX, FTIR and ICP-MS analysis. The phenomena that removal of Cd^{2+} and Pb^{2+} coincided with uptake of Na^{+} and Mg^{2+}, respectively, inspires a novel research perspective towards the study of protective mechanism of bacterial cells against the toxicity of heavy metals.

1. Introduction

Water pollution owing to hazardous heavy metals is an important environmental problem throughout the world. Given the high toxicity and non-biodegradation of heavy metals, there is an increasing risk of heavy metal contamination in water, causing significant threats to human health through the bioaccumulation and biomagnification via the food chain (Bharagava et al., 2017; Goutam et al., 2018). Biological removal including biosorption and bioaccumulation, has been regarded as a cost-effective technique for the treatment of wastewaters containing heavy metals (Volesky, 2007; Saxena et al., 2016).

For a given microorganism, the efficiency of bioremoval by targeted microbial activity depends on factors such as culture age, cell form, pH, contact time, and initial metal concentrations in solution (Wang and Chen, 2006). Among these influential factors, microbial strain in the cell form of dead or living (resting state) has been extensively studied for the bioremoval of heavy metals (Du et al., 2012; Huang et al., 2013; Wu et al., 2016; Li et al., 2017), but the bioaccumulation by growing cells under culture conditions has rarely been reported in the literature. In this regard, few studies with actively growing cells have demonstrated those microbes can efficiently remove heavy metal ions from a growth medium, especially when the metal concentration was relatively low (Mishra and Malik, 2012; Li et al., 2014; Gola et al., 2016). Specifically speaking, Mishra and Malik (2012) reported that the removal efficiencies of growing *Aspergillus lentulus* between 34% for Ni^{2+} and 71–78% for Cu^{2+}, Cr^{3+} and Pb^{2+}. Similar findings were reported with growing *Zygosaccharomyces rouxii*, in which the removal efficiency achieved 94% when initial Cd^{2+}concentration was less than 0.04 mmol/L (Li et al., 2014), while the removal efficiencies by growing *Beauveria bassiana* were 61–75% for Cu^{2+}, Cr^{3+}, Cd^{2+}, Zn^{2+} and Ni^{2+} (Gola et al., 2016). These above studies demonstrate that the bioaccumulation by growing fungal cells provide a promising technology for treatment of wastewaters polluted with heavy metals. Furthermore, the direct use of actively growing cells simplifies controlling the system and

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reduces operation costs, by avoiding the need for a separate biomass production process (Chojnacka, 2010).

Despite these insights, in few of the existing literature, the release of cations during cell growth should be taken into account when apply growing cells for heavy metals removal under culture conditions. Bioaccumulation depends on active metabolic transport across the cell membrane, which can involve both essential and non-essential heavy metals (Ferguson and Deisenhofer, 2004). During this, actively growing cells release some light metal ions such as K^+, Ca^{2+}, Na^+ and Mg^{2+} to balance the uptake of heavy metal ions (Ye et al., 2013). This cation release during the bioaccumulation should be examined in detail, to fully understand the processes of heavy metal removal under culture conditions.

Our previous work has demonstrated that actively growing *Bacillus cereus* RC-1 is tolerant to highly toxic Cd^{2+} concentrations, due to the presence of an energy-dependent efflux system that protects the cells (Huang et al., 2014). The feature of cation release during protective operation of this efflux system is the main subject of this study. We investigated the effects of pH, culture time and initial metal concentrations on cation release in the removal of two essential metals Cu and Zn and two non-essential metals Cd and Pb. The roles of intracellular accumulation and extracellular adsorption were established under different heavy metal concentrations, and bioaccumulation mechanisms were investigated by SEM-EDX, TEM-EDX and FTIR analysis, to provide insights on the detoxifying mechanisms of growing cells in response to heavy metal stress.

2. Materials and methods

2.1. Bacterial strain, growth conditions and metal solutions preparation

Bacillus cereus strain RC-1, a short rod-shaped, facultative anaerobic and gram-positive bacterium, was isolated from heavy metal-contaminated soil, Guangzhou, China (Huang et al., 2014). The bacterial strain was grown at 28 ± 2 °C in nutrient broth (NB) medium containing 10 g/L peptone, 3 g/L beef extract and 5 g/L sodium chloride with pH of 7.4 ± 0.2.

Heavy metal stock solutions of 1000 mg/L were prepared by dissolving their respective salts i.e. $CuCl_2{\cdot}2H_2O$, $ZnCl_2$, $CdCl_2{\cdot}2.5H_2O$ and $Pb(NO_3)_2$ in double distilled water (ddH_2O). These stock solutions were diluted to the desired concentration prior to use in the experiments.

2.2. Growth curve and heavy metal removal

Growth curves in presence of heavy metals were determined using a starter culture of 2 mL exponentially growing bacteria (OD_{600} = 1.2–1.5) suspended into 100 mL NB containing Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} in 250 mL conical flasks, which were incubated for 40 h at 28 ± 2 °C in an orbital shaker (150 rpm). Bacterial growth was monitored as absorbance at 600 nm by spectrophotometer (Shimadzu UV-2500, Japan), and after centrifugation at 6000 rpm for 10 min, heavy metal concentrations in the supernatant were measured by atomic absorption spectrometry (ZEEnit 700 P, Germany) at various time intervals within the 40 h of growth.

2.3. Cation release and heavy metal removal tests

Using the culture conditions described in Section 2.2, the pH was studied in the range of 3.0–7.0. The release of cations (K^+, Ca^{2+}, Mg^{2+} and Na^+) into the culture medium was measured by ion chromatography (ICS-900, Dionex, Sunnyvale, USA), and the residual heavy metals were determined by inductively coupled plasma mass spectrometry (ICP-MS iCAPQ, Thermo Fisher Scientific, USA). The effect of pH and culture time were studied under different metal concentrations of 20 mg/L Cu^{2+}, 50 mg/L Zn^{2+}, 100 mg/L Cd^{2+} and 50 mg/L Pb^{2+}, respectively. The effect of initial metal concentrations was also examined with initial concentration of 5–40 mg/L Cu^{2+}, 5–100 mg/L Zn^{2+}, 5–100 mg/L Cd^{2+}, 10–120 mg/L Pb^{2+}. A control experiment without the addition of bacteria was carried out to exclude spontaneous precipitation or loss of heavy metals.

The bioaccumulation capacity and removal efficiency were calculated using the following equations:

$$\text{Bioaccumulation capacity}(mg/g) = \frac{C_0 - Ce}{X} \quad (1)$$

$$\text{Removal efficiency}(\%) = \frac{C_0 - Ce}{C_0} \times 100\% \quad (2)$$

where C_0 and C_e are the initial and equilibrium metal concentration (mg/L), respectively, and X is the biomass concentration (g dry cells/L). The other conditions (temperature, agitation rate and adsorbent dosage) were identical to those used in the growth curve experiments. All experiments were carried out in triplicate and average values with standard deviation are shown.

2.4. Study of bioaccumulation mechanism

2.4.1. Extracellular adsorption and intracellular accumulation

Growing cells were inoculated into 100 mL NB containing different initial metal concentrations. After incubation of 24 h in previous section, cells were harvested by centrifugation and washed twice with sterile ddH_2O, and then suspended in 10 mM sterile EDTA for 10 min to remove the metal ions bound to the cell surface. Following centrifugation (6000 rpm, 10 min), the supernatant and cell pellets were used for the measurement of extracellular adsorption and intracellular accumulation of heavy metals, respectively, as previously described by Huang et al. (2014).

2.4.2. Intracellular trace elements measurement

The bacteria were inoculated into NB medium containing heavy metal ions and cultured for 24 h, after which the supernatant was collected by centrifugation at 6000 rpm for 10 min. The contents of trace elements Fe, Mn, Mo, Co, Cu, Zn, Ni and Se in the supernatant and original liquid medium were determined by ICP-MS, in order to calculate their concentration inside the cells after bioaccumulation.

2.4.3. SEM-EDX, TEM and FTIR analysis

Cells grown in absence and presence of metal at initial concentrations of 20 mg/L Cu^{2+}, 50 mg/L Zn^{2+}, 100 mg/L Cd^{2+} and 50 mg/L Pb^{2+} were prepared, and fixed with 2.5% glutaraldehyde at 4 °C overnight, washed three times with 1% osmium in phosphate buffer, and finally dehydrated using a gradient series of increasing ethanol concentration prior to SEM-EDX analysis (HITACHI S-3700N, Japan). Samples prepared for TEM were fixed in 1% osmium tetraoxide for 1.5 h, dehydrated and embedded in resin as previously described by Tyagi and Malik (2010) (JEOL JEM-1400, Japan). FTIR spectra were recorded in KBr pellets at room temperature using a FTIR spectrometer (PerkinElmer 2000, USA) in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} as described before (Bharagava and Mishra, 2018).

3. Results and discussion

3.1. Bacterial growth and heavy metal removal

Growth curves of *B. cereus* RC-1 were determined in absence and presence of four different heavy metals at five different concentrations, and the removal efficiency of the metals was recorded (Fig. 1). Bacteria entered exponential growth following a lag phase that was only slightly longer in presence of the metals. Stationary phase levels were similar to those in absence of metal for lower concentrations of Cu^{2+}(10 mg/L), Zn^{2+}(20 mg/L), Cd^{2+}(20 mg/L) and Pb^{2+}(10 mg/L), while higher metal concentrations resulted in lower final cell densities. Higher metal