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Bioremediation of lead by ureolytic bacteria isolated from soil at abandoned metal mines in South Korea

Chang-Ho Kang [a], Soo Ji Oh [a], YuJin Shin [a], Sang-Hyun Han [a], In-Hyun Nam [b], Jae-Seong So [a,*]

[a] Department of Biological Engineering, Inha University, 100 Inharo Nam-gu, Incheon 402-751, Republic of Korea
[b] Geologic Environment Division, Korea Institute of Geoscience and Mineral Resources, Daejeon 305-350, Republic of Korea

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ABSTRACT

Lead (Pb) is one of the most widely present heavy and toxic metals in the environment. Application of Pb-resistant bacteria to biomineralize this toxic metal could be utilized in the Pb bioremediation process. In this study, 93 ureolytic bacteria were newly isolated from the soil of abandoned metal mines. Among the isolates, *Enterobacter cloacae* KJ-46 and KJ-47 were selected for subsequent studies. When the biomineral samples were analyzed using an inductively coupled plasma, high removal rates (ca. 60%) of Pb were observed, following 48 h of incubation. Scanning electron microscopy showed $PbCO_3$ precipitates with a diameter of approximately 5 μm. Energy dispersive X-ray spectroscopy confirmed the presence of Pb along with carbon (C) and oxygen (O) within the $PbCO_3$ crystals. The results demonstrated that application of the biomineralization process based on stimulation of urea hydrolysis could be possible and potentially useful for soil bioremediation applications.

1. Introduction

Contamination of natural habitats by heavy metals through industrial and agricultural activities has the potential to affect the health of organisms and the environment because of the toxicity of these substances and difficulty in their remediation (Bahadir et al., 2007; Perez-Marin et al., 2008). Bioremediation processes are very attractive in comparison with physicochemical methods, such as electrochemical treatment, ion exchange, precipitation, re-verse osmosis, evaporation, and sorption, for heavy metal removal, because they can be lower in cost and more efficient for low metal concentrations (Bogdanova et al., 1992; Gadd and White, 1993). There are a number of biomaterials that can be used to remove metal from wastewater, such as molds, yeasts, bacteria, and seaweeds (Davis et al., 2000; Waisberg et al., 2003).

Lead (Pb) requires special attention as it is a cumulative poison because of its high toxicity and long-term persistence in the environment. It has been well documented that prolonged exposure in humans to lead can cause anemia, reproductive impairment, renal failure, and neurodegenerative damage (Lam et al., 2007). High concentrations of Pb occur unnaturally in soils through mining and smelting activities, combustion of leaded gasoline, land application of sewage sludge, and disposal of batteries and other Pb-bearing products (Lin et al., 1998). However, little information is available on the application of microbial cells as inoculums to augment the remediation of Pb in contaminated soils.

Urease (urea amidohydrolase) is an enzyme that hydrolyzes urea into one mole of carbonate and two moles of ammonia per mole of urea, as shown in the following reaction:

$$2\ (NH_2)_2CO + 2H_2O \rightarrow CO_3^{2-} + 2NH_3$$

Urea is released in large quantities into the environment, both as a waste product of human and animal urine as well as through the degradation of uric acid and many other nitrogenous compounds (Visek, 1972). Urease activity is known to be widespread in soil and water organisms (Fujita et al., 2008).

The evidence of microbial involvement in carbonate precipitation has subsequently led to the exploration of this process in a variety of fields. A first series of applications are situated in the field of bioremediation. In addition to conventional bioremediation strategies which rely on the biodegradation of organic pollutants (Chaturvedi et al., 2006), the use of microbially induced carbonate precipitation (MICP) has been proposed for the removal of metal ions. However, most reports still focus on the microbe-heavy metal cation adsorption issues (Gadd and Fry, 1992; Ryan et al., 2005). According to these studies, the release of CO_3^{2-} and NH_4^+ ions (with increasing pH) in the presence of metal ions

* Corresponding author. Tel.: +82 32 860 8666; fax: +82 32 872 4046.
E-mail address: sjaeseon@inha.ac.kr (J.-S. So).

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induces carbonate precipitation. Although carbonate precipitation of heavy metals is an interesting bioremediation mechanism, very few reports are available on carbonate precipitation of Pb.

The objectives of this study were to isolate and characterize Pb-resistant ureolytic bacteria and to utilize the urease activity of these strains for Pb removal through biomineralization. To determine the efficiency of biomineralization, precipitates were characterized using scanning electron microscopy (SEM) and energy dispersive X-ray spectrometry (EDX).

2. Materials and methods

2.1. Sample collection and isolation

Urease-producing bacteria were isolated from soils at abandoned metal mine sites in Gangwondo, Korea (37°59′28.89″N; 127°57′17.51″E). To collect soil samples (ca. 600 g), the top 10 cm of soil was aseptically digged from a 10 cm^2 area into sterile stainless steel bottles and stored in ice. Soil samples were serially diluted and spread onto plates containing BPU agar (3 g L^{-1} beef extract, 5 g L^{-1} peptone, 20 g L^{-1} urea, 10 g L^{-1} micro agar containing 100 μg mL^{-1} cycloheximide; pH 7). The plates were incubated at 30 °C for 3 days. Isolated strains were inoculated on YA plates (yeast extract 20 g L^{-1}, ammonium sulfate 10 g L^{-1}; pH 7) along with $PbCl_2$ with increasing concentrations from10 mM to 50 mM and incubated at 30 °C for 24 h. Isolated strains were stored at −20 °C in YA broth containing 25% (v/v) glycerol. All the strains were cultured at 30 °C with aeration at 200 rpm.

2.2. Preparation of Pb stock solutions

Stock solutions were prepared as $PbCl_2$ (Junsei Chemical Co., Ltd., Tokyo, Japan). The 1 M Pb(II) stock solutions were prepared by dissolving the exact quantities of $PbCl_2$ in Milli-Q water and filtering through a 0.22 μm filter (Pall Co., MI, USA). Working concentrations of Pb(II) were obtained by serial dilution. The stock solutions were stored in the dark at 4 °C.

2.3. Biochemical tests and bacterial identification

After incubation, the colonies that grew were purified and identified with Gram staining (Claus, 1992) and conventional biochemical tests. Basic local alignment search tool (BLAST) analysis was performed to compare the sequences with available DNA sequences from the National Center for Biotechnology Information (NCBI) database. The sequences were aligned using the PHYDIT (http://plaza.snu.ac.kr/~jchun/phydit/) program, the alignment manually corrected and a phylogenetic tree was constructed by the neighbor-joining method using MEGA 5.0 software (Tamura et al., 2011).

2.4. Measurement of calcite production and urease activity

The isolates were cultured in YA broth for 24 h and sub-cultured to BPU broth at 30 °C for 72 h with continuous aeration at 200 rpm. The bacterial suspension (500 μL) was added to 500 μL of calcium chloride dihydrate solution (350 mM). The mixture was centrifuged at 16,179 × *g* for 5 min at 25 °C to collect the precipitate. The precipitate was dried for 24 h at 50 °C and weighed. Urease activity was determined using the phenol-hypochlorite assay (Natarajan, 1995). The bacterial suspension (250 μL) was added to 250 μL of sodium phosphate buffer (0.1 M) containing 500 μL of urea solution (3 M). The mixture was incubated at 37 °C for 3 days. Subsequently, 2 mL of phenol nitroprusside solution was added to alkaline hypochlorite solution and then incubated at 50 °C for 10 min. After incubation, absorbance was measured by UV/Visible Spectrophotometer (Ultrospec 2000; Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 626 nm. Ammonium chloride (0–10 μM) was used as a standard. One unit of urease activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μM urea per minute.

2.5. Ure*gene PCR amplification*

*Ure*C gene amplicons were obtained by polymerase chain reaction (PCR) for six of the bacterial isolates used in this study. For amplification of the 323 bp amplicons from the *ure*C gene, 2 universal primers, *ure*C-F (5′-AAG STS CAC GAG GAC TGG GG-3′) and *ure*C-R (5′-AGG TGG TGG CAS ACC ATS AGC AT-3′) were used (Gresham et al., 2007). PCR amplification was performed under the following conditions: initial denaturation for 5 min at 94 °C; 30 cycles of denaturation for 1 min at 94 °C, annealing for 1.5 min at 53 °C, extension for 2 min at 72 °C; and a final extension for 7 min at 72 °C. The PCR products were analyzed by 1% agarose gel electrophoresis.

2.6. Minimal inhibitory concentration test

This test was done in order to determine the concentration of Pb that prevents bacterial growth. Isolated strains cultured for 24 h were diluted to 1.0 of optical density at 600 nm (OD600) in YA broth. Different concentrations of Pb (100, 50, 25, 12.5, and 6.25 mM) and 100 μL (10% v/v) of the dilute culture were inoculated into each YA broth. After incubating for 24 h and aeration at 200 rpm in 30 °C, the lowest concentration of Pb that inhibited bacterial growth was considered the minimal inhibitory concentration (MIC) (Amoozegar et al., 2012).

2.7. Impermeability test

Cells grown in YA broth overnight were harvested (6000 × *g*, 5 min), washed twice, and resuspended in 0.9% sodium chloride solution at a final OD_{600} of 1.0. Sterile silica sand (200 g, 0.45–0.7 mm, Joomoonjin Sand Co., Ltd., Korea) was mixed with 53.3 mL urea $CaCl_2{}^{-1}$ solution (40 g L^{-1} urea and 25 g L^{-1} as calcium ion). Sand slurry (50 g) was packed into a 25 mL plastic column (Corning Co., Ltd., USA). Columns were fed once by gravity with 10 mL of cell suspension. 10 mL of flow-through was reloaded onto the column again. The columns were set aside for 8 h to allow calcite crystal growth. Subsequently, 2 mL crystal violet (CV) was pipetted onto a packed sand column. The degree of impermeability was determined by measuring the migration distance of CV. The maximum amount of cells that could produce optimum bioremediation by treating sand with different concentrations of bacteria were also identified.

2.8. SEM and EDX analyses

A modified SEM method was used to observe the lead carbonate crystals that were produced. Harvested calcium carbonate crystals were suspended in water and covered with a glass plate and then dried in an oven at 50 °C. After complete drying, the samples were sputter-coated with platinum. A field emission SEM (S-4300; Hitachi, Tokyo, Japan) and an EDX spectrometer (EX-350, Horiba Ltd., Japan) were used to study the morphological features and elemental composition of the crystals.

2.9. Removal of Pb

5 mL of 12 h cultures were inoculated into 45 mL of YA broth in 100 mL conical flasks containing 2 mM of Pb and 0.5 M urea. The flasks were incubated in a shaking incubator (200 rpm) at 30 °C for