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Bioremediation of heavy metals by using bacterial mixtures

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ABSTRACT

Environmental pollution by heavy and toxic metals because of mining, metallurgic processes, and other chemical industries is a worldwide problem affecting both human health and the environment. The aim of this study was to investigate the synergistic effect of bacterial mixtures on the bioremediation of a mixture of Pb, Cd, and Cu from contaminated soils. Compared to the single culture method, the bacterial mixtures showed higher growth rate, urease activity, and resistance to heavy metals. Four bacterial strains were isolated and identified from bacterial mixtures—*Viridibacillus arenosi* B-21, *Sporosarcina soli* B-22, *Enterobacter cloacae* KJ-46, and *E. cloacae* KJ-47, which obtained from an abandoned mine site in Korea and showed effective microbially induced calcite precipitation (MICP). The following parameters were monitored during the course of the experiment: optical density, pH, urease activity, calcite production, tolreance to heavy metals, and impermeability test. Synergistic effects on the remediation of various heavy metals via modification of the bacterial mixtures were observed and, after 48 h, remediation of 98.3% for Pb, 85.4% for Cd, and 5.6% for Cu were recorded. Compared with single strain cultures, the bacterial mixtures demonstrated greater resistance and efficiency for the remediation of heavy metals. Thus, our results show that the use of bacterial mixtures is useful in the bioremediation of heavy metals from the contaminated environment.

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1. Introduction

The accumulation of heavy metals in water, sediments, and soils has led to serious environmental problems. In recent years, several processes have been developed with the aim of reducing or recovering heavy metals from contaminated environments (Akinci and Guven, 2011). Physical and chemical approaches are capable of removing a broad spectrum of contaminants, but the main disadvantages of these methods lie in the increased energy consumption and the need of additional chemicals (Ilhan et al., 2004). In recent years, the processes such as bioleaching, biosorption, and bioprecipitation are all based on the use of microorganisms that have the ability to solubilize, adsorb, or precipitate heavy metals (Ballester et al., 1992; Zouboulis et al., 1997).

To date, most of the research on microbially induced calcite precipitation (MICP) has been confined to ureolytic bacteria, with specific focus on the catalysis of urea hydrolysis (Ferris et al., 2003), efficiency of calcite production (Muynck et al., 2010; van Paassen et al., 2010), and modification of soil physical properties by model bacteria (Burbank et al., 2011; De Jong et al., 2010). The MICP has been shown to increase the shear strength of porous materials (De Jong et al., 2006; Harkes et al., 2010). MICP arises when the following reaction catalyzed by urease: $(NH_2)_2CO + 2H_2O \rightarrow 2NH^{4+} + CO_3^{2-}$ occurs in the presence of dissolved calcium ions, leading to the precipitation of calcium carbonate crystal: $Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3(s)$.

The crystals formed by this process create bridges between particles, thus improving the strength and stiffness of the material (Harkes et al., 2010). Urease-induced $CaCO_3$ can fill pore spaces within various soil matrices and cement soil grains together to form sandstone (Burbank et al., 2011; Deepak et al., 2009; De Jong et al., 2006). Precipitation of $CaCO_3$ induced by the urease-catalyzed hydrolysis of urea has been shown to change the engineering properties of geomaterials (Burbank et al., 2011; Whiffin et al., 2007).

Bacterial mixtures systems have long been used to study the interactions between cell populations and fundamental cell–cell interactions. Recently, these systems have been of particular interest to synthetic biologists for the study and engineering of complex multicellular synthetic systems. At the basic level, a co-culture is a cell culture set-up, in which two or more populations of cells are grown with some degree of contact between them (Goers et al., 2014). The ultimate aim of the bacterial mixtures system is to deliver societal benefits via its industrial, medical, and environmental applications (Chen, 2012; Kitney and Freemont, 2012).

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Therefore, many bacterial mixtures systems are developed for future industrial, medical, or environmental applications. Although the synergetic interactions between metals and ureolytic bacteria have attracted a fair share of attention, the effects of metal contaminated environments on bacterial mixtures growth are still unknown.

The utilization of microorganisms with proven remediation potential and survivability in the contaminated environment is crucial for a successful bioremediation. In view of this, the present paper aims to study the remediation capacity of heavy metals by pure and mixed bacterial cultures, for bioremediation process applications.

2. Materials and methods

2.1. Microorganisms and culture conditions

Four bacterial strains were isolated from an abandoned mine soil in our previous work (Kang et al., 2015). These strains include *Viridibacillus arenosi* B-21 (B-21), *Sporosarcina soli* B-22 (B-22), *Enterobacter cloacae* KJ-46 (KJ-46), and *E. cloacae* KJ-47 (KJ-47). Three sets of experiments were conducted under aerobic conditions using defined co-culture—A1, mixed bacterial cultures of B-21 and B-22; A2, mixed bacterial cultures of KJ-46 and KJ-47; A3, mixed bacterial cultures of B-21, B-22, KJ-46, and KJ-47. These strains were pre-selected based on their high levels of urease activity and calcite production. Also, these strains had the highest heavy metal tolerance. Their sequences have been deposited in GenBank with accession numbers KJ671467 (B-21), KJ485701 (B-22), KF598853 (KJ-46), and KF598854 (KJ-47). The bacterial mixtures were routinely grown at 30 °C in YA (yeast extract 20 g/L and ammonium sulfate 10 g/L at pH7) broth. The final pH of the medium was adjusted to 7.0.

2.2. Preparation of heavy metal stock solutions

For the preparation of 1 M mixtures of heavy metal stock solutions the necessary quantities of: $CdCl_2 \cdot 5H_2O$ (Kanto Chemical Co., Ltd., Tokyo, Japan), $PbCl_2$ (Junsei Chemical Co., Ltd., Tokyo, Japan), and $CuCl_2$ (Samchun Pure Chemical Co., Ltd., Korea) were dissolved in Milli-Q water. All solutions were filtered through a 0.22 μm filter (Pall Co., MI, USA). Working concentrations of heavy metal mixture were obtained by serial dilutions. The stock solutions were stored in the dark at 4 °C.

2.3. Measurement of urease activity and calcite production

Experimental conditions were the same as for the bacterial mixtures growth described above. To establish the bacterial mixtures, first, a bacterial culture was added to the experimental flasks and incubated at 30 °C for 24 h with continuous agitation at 200 rpm.

Urease activity was determined using the phenol-hypochlorite assay (Natarajan, 1995). The bacterial suspension (250 μL) was added to 250 μL of 0.1 M sodium phosphate buffer containing 500 μL of 3 M urea solution. The mixture was incubated at 37 °C with regular time intervals. Subsequently, 2 mL of phenol nitroprusside solution were added to an alkaline hypochlorite solution and, then, incubated at 50 °C for 40 min. After incubation, absorbance was measured at 626 nm with ammonium chloride (0–10 μM) as a standard. One unit of urease activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μM urea per min.

To produce calcite, the isolates were cultured in YA broth for 24 h and subcultured onto BPU broth (3 g/L beef extract, 5 g/L peptone, and 20 g/L urea at pH 7) at 30 °C for 72 h with continuous agitation at 200 rpm. The bacterial suspension (500 μL) was added to 500 μL of 350 mM calcium chloride dihydrate solution and the mixture was centrifuged at 16,179 × *g* for 5 min at 25 °C to collect the precipitate. Finally, the precipitate was dried at 50 °C for 48 h prior to being weighed.

2.4. Determination of the minimum inhibitory concentration

The tolerance experiments were carried out in 250 mL conical flasks, each containing 45 mL of YA broth. Approximately equal numbers of cells from the four isolated strains were used and the bacterial mixtures with an initial cell density of 1×10^8 cells/mL for each of the isolated strains. All cultures were incubated at 30 °C and agitated at 200 rpm. To study the influence of heavy metals on bacterial growth, bacterial mixtures were exposed to a mix of heavy metals with individual concentrations ranging from 0 to 10 mM.

2.5. Impermeability test

The single cultures and bacterial mixtures grown in YA broth overnight were harvested by centrifugation at 6000 × *g* for 5 min, washed twice, and resuspended in a 0.9% sodium chloride solution at a final OD_{600} of 1.0 (equivalent to 1×10^8 cells/mL). Sterile silica sand (200 g, 0.45–0.7 mm, Joomoonjin Sand Co. Ltd., Korea) was mixed with 10 mL urea (40 g/L) and calcium chloride dihydrate solution (25 g/L). The sand slurry (40 g) was packed into a 25 mL plastic column (Corning Co. Ltd., Corning, NY, USA). After being dried for 48 h at 50 °C, the columns were run once by gravity with 10 mL of cell suspension. The columns were stored for 48 h to allow calcite crystal growth. Then, 2 mL of crystal violet (CV) was pipetted onto the packed sand column. The degree of impermeability was determined by measuring the migration distance of CV.

2.6. Bioremediation of heavy metal

Five milliliters of the overnight pre-culture were inoculated into 45 mL of YA broth contained in 250 mL conical flasks. The YA broth contained 5 mM urea and 25 mM $CaCl_2$, which were filter sterilized using a 0.22 μm filter, and was supplemented with 2 mM of heavy metal mixture. The flasks were incubated in a shaking incubator (200 rpm) at 30 °C for 48 h. Control sets without any added bacterial cells were also included in all experiments. After incubation, the cultures were centrifuged at 8000 × *g* for 15 min. The heavy metal bioremediation by single cultures and bacterial mixtures (A3) were calculated as the percentage difference between the initial and final concentrations of heavy metals present in the supernatants. Concentrations of heavy metals were measured using a Perkin-Elmer OPTIMA-7300DV inductively coupled plasma optical spectrometer (ICP-OES, PerkinElmer, Inc., USA).

2.7. Column test

The silica sand samples were sieved, washed, air-dried, and sterilized before the experiments. The pH of the sand suspended in distilled water was 8.5. The heavy metal bioremediation studies were performed in 50 mm diameter and 500 mm length columns (OMG Chemical Co., Korea), containing 1.0 kg silica sand supplemented with 2 mM mixture of heavy metals. The heavy metals were thoroughly mixed into the sand while in solution form. The bacterial mixtures culture (A3, equivalent to 10^8 CFU/mL) was grown overnight in YA broth. The control treatment consisted of the same set-up as the experimental treatment but without the addition of bacterial cells. Bioremediation was determined by measuring the time required for 100 mL of 5 mM urea and 25 mM calcium chloride to pass through the column. The experiment was performed at room temperature (25 °C) and run for 3 d. After the experiment

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