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Immobilization of heavy metal contaminated mine wastes using *Canavalia ensiformis* extract

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ABSTRACT

This study examined the ability of crude extracts of *Canavalia ensiformis* to catalyze the precipitation of calcium carbonate ($CaCO_3$) in columns packed with heavy metal contaminated mine waste collected from an abandoned mine site, and examined the effect of $CaCO_3$ precipitates on the leaching of heavy metals out of such waste. X-ray diffraction and scanning electron microscopy were employed to confirm $CaCO_3$ precipitation and to characterize the morphology of the resulting material. Urease in the *C. ensiformis* crude extracts catalyzed the hydrolysis of urea, leading to the formation of $CaCO_3$ precipitates that formed bridges between the particles in the mine waste. In column experiments, the amounts of the heavy metals As, Mn, Zn, Pb, Cr, and Cu in leachates from the mine waste were reduced by 31.7%, 65.7%, 52.3%, 53.8%, 55.2%, and 49.0%, respectively, when the waste was treated with *C. ensiformis* crude extract. This reduction can be attributed to immobilization of heavy metals within the mine waste as a result of $CaCO_3$ precipitation. Comparison of the microbial communities in mine waste columns that were untreated or incubated for 2 weeks with *C. ensiformis* crude extract and purified urease, using PCR-DGGE of 16S rDNA, showed that a greater diversity of microorganisms was present in the columns treated with *C. ensiformis* crude extract and purified urease. These findings suggest that crude extracts of *C. ensiformis* may be used to stabilize and immobilize heavy metals in contaminated mine waste to prevent further dispersion to the surrounding environment.

1. Introduction

Mine waste materials in and surrounding abandoned mines make these areas difficult to revegetate, as such materials are typically deficient in nutrients such as nitrogen and phosphorus, but rich in toxic heavy metals and metalloids, such as Pb, Zn, Cu, Cd, Mn, Ni, and As. Despite the negative impact of mine tailings worldwide (Mendez and Maier, 2008; Zhu et al., 1999), only a small number of studies have sought to address the issue of remediating mine sites through primary succession (Dobson et al., 1997) and ecological reconstruction (Shu et al., 2005). Heavy metal contamination of natural habitats as a result of mining may affect the health of organisms and the environment because of the toxicity of these substances and the difficulty of remediating affected sites (Houben et al., 2012; Pruvot et al., 2006). Bioremediation processes are considered superior to physicochemical methods, such as ion exchange, electrochemical treatment, precipitation, and heat evaporation, because of their lower cost and greater efficiency in treating systems in which metal concentrations are low (Bogdanova et al., 1992; Gadd and White, 1993). Bacteria, molds, yeasts, and seaweed have been used to remove metals from wastewater (Ahluwalia and Goyal, 2007; Davis et al., 2000).

One approach to the remediation of heavy metal contaminated mine waste is to bind the waste particles together, thereby minimizing leaching of the contaminants into the surrounding environment. Such immobilization can potentially be achieved through the precipitation of calcium carbonate ($CaCO_3$) by urease-producing organisms such as microbes and plants, a process that is exploited in several industrial applications (Bang et al., 2001; De Muynck et al., 2010; DeJong et al., 2006; Lee et al., 2006; Ramachandran et al., 2001; Rusznyák et al., 2012). Indeed, many soil microorganisms have been reported to contribute to $CaCO_3$ precipitation. However, this process, known as microbiologically induced calcite precipitation (MICP), is dependent on urease enzyme activity (De Muynck et al., 2010; DeJong et al., 2006; Le Metayer-Levrel et al., 1999; Nemati and Voordouw, 2003; Ramachandran et al., 2001), and to date few types of soil bacteria capable of constitutive or inducible expression of urease have been identified and characterized in regard to urease expression (Burbank et al., 2012; Mobley and Hausinger, 1989). Nevertheless, MICP has been shown to increase the shear strength of porous materials (DeJong

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et al., 2006; Harkes et al., 2010; Le Metayer-Levrel et al., 1999; Nemati and Voordouw, 2003; Whiffin et al., 2007).

MICP arises when the following reaction catalyzed by urease (urea amidohydrolase; EC 3.5.1.5):

$$(NH_2)_2CO + 2H_2O \rightarrow 2NH_4^+ + CO_3^{2-}$$

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., 2012; Deepak et al., 2009; DeJong 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; DeJong et al., 2006; Whiffin et al., 2007). Few studies, however, have assessed the application of MICP to heavy metal contaminated environments. One such study investigated microbially induced $CdCO_3$ precipitation in cadmium contaminated soils through urea hydrolysis (Li et al., 2010), while another isolated Pb-resistant ureolytic bacteria and utilized the urease activity of these strains for Pb removal through biomineralization (Kang et al., 2015).

The most extensively studied plant urease is that produced by the jack bean (*Canavalia ensiformis*), and the complete jack bean embryo-specific urease gene has been cloned in *Escherichia coli* (Riddles et al., 1991). A comparison of $CaCO_3$ formation via reactions catalyzed by plant (*C. ensiformis*) and bacterial (*Bacillus pasteurii*) ureases (Sondi and Salopek-Sondi, 2005), suggested that the urease from *C. ensiformis* crude extracts may be a viable alternative to bacterial ureases for the biomineralization of $CaCO_3$. Additionally, biomineralization of $CaCO_3$ with *C. ensiformis* crude extracts has the advantages that it is less sensitive to seasonal variations than bacterial cultivation, making it more effective for the bioremediation of heavy metal contaminated soil. These factors, combined with the potential utility of the products of $CaCO_3$ biomineralization, have prompted suggestions that this process may provide the basis for a new method of in situ formation of $CaCO_3$ for various soils including mine wastes (Dhami et al., 2013; Warren et al., 2001).

The objective of this study was to exploit $CaCO_3$ biomineralization catalyzed by urease from *C. ensiformis* crude extracts to immobilize and stabilize heavy metals and metalloids in contaminated mine wastes, with the aim of developing a method for preventing pollution of surrounding environments. To achieve this, the crude extract of $CaCO_3$ precipitating *C. ensiformis* to a mine site was used in columns packed with abandoned mine waste. The performance of this system was compared with that of purified urease, including the degrees to which heavy metal concentrations in leachates were reduced and the change in shear strength. In addition, microbial communities were analyzed.

2. Materials and methods

2.1. Chemicals

ICP standard solutions (SPEX plasma standard, 1000 mg L^{-1} of H_3AsO_4, Mn, Cr, Cu, $Pb(NO_3)_2$, and Zn) in 2% nitric acid were purchased from SPEX (Metuchen, NJ, USA) and employed as standards for inductively coupled plasma (ICP) atomic emission spectroscopy (AES). Purified jack bean (*C. ensiformis*) urease (urea amidohydrolase; EC 3.5.1.5), urea, and calcium chloride were obtained from Sigma (St. Louis, MO). Phosphate buffer (3.5 g Na_2HPO_4 $2H_2O$ and 1 g KH_2PO_4 per liter of distilled water (pH 7.5)) was prepared as described (Nam et al., 2006; Nam et al., 2008). All chemicals were of the highest commercially available purity.

2.2. C. ensiformis crude extract preparation and column experiments

Mine waste samples were collected from an abandoned pyrophyllite mine enriched with various sulfide minerals in Busan, Korea and packed in columns. Column experiments were conducted under three conditions: no treatment (negative control), plant (*C. ensiformis*) crude extract treatment, and purified urease treatment (positive control). A nylon mesh and Advantec 5B filter paper were inserted in the bottom of glass columns (20 cm in length and 2.5 cm in internal diameter). Mine waste equivalent to 100 g on a dry basis was added in the columns.

Fifty grams of jack beans (*C. ensiformis*) in 20 mM phosphate buffer (pH 7.5) was disrupted using a homogenizer and passed through Advantec 5B filter paper. The samples were centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatants were collected and used without further purification. Purified urease (100 mg) was dissolved in 50 mM Tris-acetate buffer (pH 7.5).

For calcium carbonate ($CaCO_3$) precipitation experiments with *C. ensiformis* crude extracts and purified urease, 10 mL of a 20 mM phosphate buffer (pH 7.5) was added to mine waste packed columns containing 100 mg of urea and 200 mg of calcium chloride. To compare the $CaCO_3$ precipitation ability of *C. ensiformis* crude extract and purified urease, substrate mixtures (2 mL of each compound) were added to phosphate buffer suspensions. Every 12 or 24 h until 72 h, a set of triplicate columns and their corresponding controls were removed and analyzed by X-ray diffraction (XRD), scanning electron microscopy (SEM), and enzyme activity assays as described below. The leachate samples were analyzed for concentrations of metals by ICP-AES.

2.3. Measurement of urease activity

The protein content in each suspension was estimated by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976). Tubes containing 0.8 mL 50 mM Tris-acetate buffer (pH 7.5) and 1.0 mL 250 mM urea in the same buffer were equilibrated to 28 °C. The reactions were started by adding 0.2 mL of appropriately diluted enzyme or *C. ensiformis* crude extract and stopped after 10 min by adding 1.0 mL 10% trichloroacetic acid. The contents of each tube were transferred to a measuring flask (50 mL), 1.0 mL of Nessler's reagent was added, and sufficient distilled water was added to bring the volume in each tube to 50 mL (Das et al., 1998, 2002). The amount of ammonia liberated was determined based on the absorbance at 405 nm (Kumar and Kayastha, 2010; Prakash and Upadhyay, 2003), measured using a UV spectrophotometer (Biochrom, Cambridge, UK). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 mol of ammonia in 1 min under these test conditions.

2.4. Fall cone test

To investigate the effect of precipitated $CaCO_3$ on mine waste mechanical properties, a series of fall cone tests was performed. This test, which measures soil penetration of a cone of a specified mass (GEONOR, 2010; Tanaka et al., 2012), is a widely used simple method to determine the consistency and shear strength of soils (Hansbo, 1957). In the present study, the Swedish standard fall cone apparatus was used with different cone sizes. One cone had an apex angle of 30° and a mass of 100 g. The other two had an apex angle of 60° and a mass of 10 and 60 g respectively. The undrained shear strength (s_u, in kPa) of mine wastes can be determined by the relationship $s_u = 9.8\ KM/P^2$, where *K* is an empirical constant (0.8 for an apex angle of 30° and 0.27 for an apex angle of 60°), M is the mass of the cone (g), and P is the penetration depth (mm). Contaminated mine waste, urea, calcium chloride and distilled water were mixed, and *C. ensiformis*