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Microbially-induced Carbonate Precipitation for Immobilization of Toxic Metals

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Abstract

Rapid urbanization and industrialization resulting from growing populations contribute to environmental pollution by toxic metals and radionuclides which pose a threat to the environment and to human health. To combat this threat, it is important to develop remediation technologies based on natural processes that are sustainable. In recent years, a biomineralization process involving ureolytic microorganisms that leads to calcium carbonate precipitation has been found to be effective in immobilizing toxic metal pollutants. The advantage of using ureolytic organisms for bioremediating metal pollution in soil is their ability to immobilize toxic metals efficiently by precipitation or co-precipitation, independent of metal valence state and toxicity and the redox potential. This review summarizes current understanding of the ability of ureolytic microorganisms for carbonate biomineralization and applications of this process for toxic metal bioremediation. Microbial metal carbonate precipitation may also be relevant to detoxification of contaminated process streams and effluents as well as the production of novel carbonate biominerals and biorecovery of metals and radionuclides that form insoluble carbonates.

Keywords: Toxic Metals; Urease; Biomineralization; Bioprecipitation; Calcium carbonate; Calcite; Bacteria; Fungi

1. INTRODUCTION

With rapid urbanization and increasing populations, increasing industrial development is inevitable despite awareness of possible adverse effects on human health and the environment. Various industrial wastes such as those from mining and metal refining, fuel and energy production including atomic energy, iron and steel production, aerospace industries, and many others, contain toxic metals which are directly or indirectly discharged into the environment causing pollution (Bishop, 2002). Metals are regarded as the main soil contaminants in many countries (Guimaraes et al., 2010). Important pollutants include toxic metal(loid)s such as Cu, Cr, Cd, Hg, Sb, Pb, As, Co, Zn, and Sn, and radionuclides such as Sr, U, Th, Am, and Ra (Singh et al., 2011; Wuana et al., 2011).

The contamination of soil with toxic metals affects human health directly or indirectly in addition to causing great economic losses (Zinjarde et al., 2014). The behaviour of metals in soil always makes them challenging substances to decontaminate as they may form complexes with naturally-occurring substances, bind to soil components, and precipitate as insoluble mineral forms. All soils naturally contain trace levels of metals; however, when this level exceeds tolerable concentrations, it results in pollution. In soils, metals may dissolve in the soil solution, occupy exchange sites or be adsorbed on inorganic soil constituents, associate with insoluble soil organic matter or precipitate as pure or mixed solids (Shuman, 1991) as well as be accumulated by the biota (Gadd, 2010).

Conventional methods for the treatment of contaminant metals in soil include

physico-chemical methods that suffer from high costs associated with energy and chemical consumption in addition to possible emission of secondary pollutants (Krishna & Philip, 2005). Phytoremediation methods are also highly popular and have attempted used for in situ remediation of heavy metals. However, this also has limitations because of the dependence on plant growth conditions such as climate, geology, altitude and temperature (Achal et al., 2012a). Phytoremediation may also be a long-term method to clean the soils because of the low amounts of metals that can be accumulated by plants before toxic symptoms result.

There have been various reports of bacterial decontamination of metal-polluted soils. Fundamental processes that enable bioremediation include changes in pH and/or redox reactions, increases or decreases in solubility by means of complexation or precipitation, and adsorption or uptake of pollutants (Smith et al., 1994). Different oxidation states of many metal(loid)s are of differing mobility and toxicity meaning that variations in soil redox potential may affect microbial redox transformations and result in failure to stabilize a metal in contaminated soil (Achal et al., 2012a).

When a problem associated with a bioremediation method exists, it may be solved with an advanced or unexplored approach. Biotechnology applied to the remediation of metal pollution has been a topic of great interest for many years. Various enzymic systems have been used effectively for the remediation of different organic pollutants (Nessner Kavamura et al., 2010), including those from bacteria and fungi (Ruggaber and Talley, 2006). Most of the degradative enzymes involved in organic bioremediation are mono- or di-oxygenases, oxidoreductases, dehalogenases,

cytochrome P450 mono-oxygenases, enzymes involved in lignin degradation and phosphotriesterases (Pieper, Martins dos Santos, & Golyshin, 2004; Rao et al., 2010). However, there are many enzymes which are less studied. Microbial urease, a type of hydrolase, is one such enzyme, which has been demonstrated to have an effective role in the immobilization of various metals as insoluble carbonates. This article therefore reviews the properties and applications of urease for toxic metal immobilization and discusses future prospects for the use of ureolytic microorganisms in bioremediation and metal biorecovery.

2. UREASE

Urease (or urea amidohydrolase) was discovered around 150 years ago. The first ureolytic microorganism, *Micrococcus ureae*, was isolated from urine in 1864 by van Tieghem. However, Musculus obtained the first ureolytic enzyme in 1874 from putrid urine, and as proposed by Miquel in 1890, it was named urease (see Mobley & Hausinger, 1989; Mobley et al., 1995; Krajewska, 2009). Initially, the ureolytic enzyme was considered to be a potent virulence factor in pathogenic bacteria such as *Helicobacter pylori*, *Proteus mirabilis*, *Campylobacter pyloridis* and *Staphylococcus saprophyticus*. However, it was subsequently found that urease is produced by many taxonomically diverse bacterial species, including normal non-pathogenic microbiota from terrestrial and aquatic habitats (Graham et al., 1987; Jones & Mobley, 1988; Gatermann & Marre, 1989; Dunn et al., 1990). Mobley and Hausinger (1989) have highlighted the significance of urease as a virulence factor in animal pathogenesis, its

role in ruminant metabolism and in environmental transformations of urea-based compounds. Furthermore, Mobley, Island, and Hausinger (1995) reviewed numerous urease gene clusters for which the entire nucleotide sequence was known in addition to exploring mechanisms by which urease gene expression is regulated in different bacterial species.

Urease belongs to the hydrolase class and superfamily of amidohydrolases and phosphotriestreases with EC number 3.5.1.5. Urease hydrolyzes urea to yield ammonia and carbamate, which is unstable and spontaneously forms carbonic acid and ammonia upon further hydrolysis. Urease activity is widely found among prokaryotes, as well as in eukaryotes including fungi and plants (Blakeley & Zerner, 1984; Li, Csetenyi & Gadd, 2014). To date, the widest analytical application of urease has been for the quantification of urea in blood and urine (Francis, Lewis, & Lim, 2002). Recently, there has been a growing demand for urease in applications in other areas, such as food production (Krajewska, 2009).

Urease plays an essential role in the nitrogen metabolism of terrestrial and aquatic microorganisms. Ureolytic activity minimizes crop damage during urea fertilization of agricultural soil, and solves the problem of fixed nitrogen availability (Mobley & Hausinger, 1989). Such urease activity is attributable to a variety of soil microbes. Lloyd and Sheaffe (1973) reported that 17-30% of the cultivable bacterial population from soil produced urease. This urease activity in soil is known to be extracellular and is stabilized by association of the urease with certain soil components (Mulvaney & Bremner, 1981). Urease levels in different soils vary. The cellular content of urease

among microbes also varies, suggesting different regulatory mechanisms of urease production. Urease production in many microbes may be tightly regulated in conjunction with the nitrogen regulatory system, which is controlled by a complex cascade that ultimately triggers ribonucleic acid (RNA) polymerase synthesis, recognizing specific promoters of nitrogen-regulated gene products (Mobley & Hausinger, 1989). In some microbial species urease production is dependent on the presence of urea, which acts as an enzyme inducer, while other microbial species produce urease constitutively. It has also been demonstrated that urea can significantly increase soil respiration but may not influence soil urease activity (Margesin, Zimmerbauer, & Schinner, 2000). In brief, urease production has been demonstrated to be constitutive, or inducible and repressible (Mobley & Hausinger, 1989). Most earlier studies on environmental urease have been confined to its significance in soil chemistry and agricultural practice. More recent studies have shown that urease-producing microbes show considerable potential for mediating metal bioprecipitation through the formation of insoluble metal carbonates (Fujita et al., 2000, 2004, 2008; Achal, Pan, & Zhang, 2011; Li, Chen & Guo, 2013; Li et al., 2014, 2015).

3. BIOMINERALIZATION

Biomineralization is the process by which organisms form minerals (Lowenstam & Weiner, 1989; Ben Omar, Arias, & González-Muñoz, 1997; Gadd, 2010)). The process of biomineralization can be categorized into biologically-induced

mineralization (BIM) and biologically-controlled mineralization (BCM) (Bazylinski, 2001; Northup & Lavoie, 2001; Gadd, 2010; Fouke, 2011; Benzerara et al., 2011; Phillips et al., 2013; Li, Csetenyi, & Gadd, 2014; Rhee, Hiller, & Gadd, 2015). BCM depends on the cellular activities of the biomineralizing organism (e.g. coccolithophores, diatoms and magnetic bacteria) which directly influence the nucleation, growth and morphology of the produced biominerals and control the final biomineral locations (Bazylinski, 2001; Mukkamala, Anson, & Powell, 2006; Gadd, 2010). In the context of BIM, the organism modifies its local microenvironment to create appropriate physico-chemical conditions for the precipitation of minerals (Gadd, 2010; Gadd et al., 2012; 2014; Li et al., 2014, 2015). Most microbial biomineralization processes therefore usually refer to biologically-induced mineralization (Burford, Hillier, & Gadd, 2006; Uroz et al., 2009; Gadd, 2010; Li, Csetenyi, & Gadd, 2014; Rhee, Hiller, & Gadd, 2015).

Calcium carbonate is a major biomineralization product (Berman *et al*., 1990; Lakshminarayanan, Kini, & Valiyaveettil, 2002; Perito & Mastromei, 2011) and calcite ($CaCO_3$) precipitation is a common microbially-mediated phenomenon in the biosphere (Ehrlich, 1998; Castanier, Levrel, & Perthuisot, 1999). Carbonates, especially calcite ($CaCO_3$) and dolomite ($CaMg(CO_3)_2$), are often found as limestones on the Earth's surface (Ehrlich & Newman, 2009). Moreover, 13% of the total land surface of the Earth is occupied by the near-surface calcretes and dolocretes in the soil environment and they are important carbon reservoirs in the Earth's lithosphere (Ehrlich & Newman, 2009; Goudie, 1996). A significant proportion of such carbonate

minerals at the Earth's surface is of biogenic origin, and many microorganisms, including bacteria and fungi, can deposit calcium carbonate extracellularly (Verrecchia, Dumont, & Rolko, 1990; Goudie 1996; Yamanaka, 1999; Verrecchia, 2000; Burford, Hillier, & Gadd, 2006; Navarathna et al., 2010; Barua *et al*., 2012; Li et al., 2014, 2015). Calcium carbonate precipitation by bacteria is generally regarded to be inducible and the type of mineral produced is largely dependent on environmental conditions (Rivadeneyra et al., 1994; Ben Omar, Arias, & González-Muñoz, 1997; Brennan, Lowenstein, & Horita, 2004). Bacteria involved in the nitrogen cycle are important organisms for calcium carbonate precipitation in various environments through the production of urease which mediates the precipitation of $CaCO_3$, a process known as microbially-induced calcium carbonate precipitation (MICP) (Achal, 2015).

3.1 Microbially-induced calcium carbonate precipitation

Microbially-induced calcium carbonate precipitation (MICP) by urease-producing bacteria involves a series of biochemical reactions. Apart from urease, the process requires calcium ions at a concentration that permits precipitation of carbonate, while nucleation sites with a strong affinity for cations enable the accumulation of calcium ions on cell walls.

In MICP, urease hydrolyses urea into ammonia and carbamate (Eq. 1), which on subsequent hydrolysis releases ammonia and carbonic acid (Eq. 2). These products equilibrate in water to form bicarbonate and ammonium and hydroxyl ions (Eqs. 3

and 4), resulting in an increase in pH that ultimately shifts the bicarbonate equilibrium to form carbonate ions (Eq. 5). Metabolic CO_2 from respiration further contributes to an increase in the level of dissolved inorganic carbon in the microenvironment to enhance the precipitation of calcium carbonate (Hammes & Verstraete, 2002). The conditions of high pH favour the formation of CO_3^{2-} from HCO_3^- (Knoll, 2003). The increased carbonate concentration therefore leads to $CaCO_3$ precipitation around cells, and in media, in the presence of calcium ions (Eqs. 6 and 7).

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (2)$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (3)$$

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \quad (4)$$

$$HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \leftrightarrow CO_3^{2-} + 2NH_4^+ + 2H_2O \quad (5)$$

$$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+} \quad (6)$$

$$Cell\text{-}Ca^{2+} + CO_3^{2-} \rightarrow Cell\text{-}CaCO_3 \quad (7)$$

MICP has been shown to have potential as a remediation strategy for toxic metals since toxic metals can also be precipitated as insoluble carbonates (Fujita *et al*., 2000, 2004, 2008; Achal, Pan, & Zhang, 2011; Li, Chen, & Guo, 2013). Furthermore, carbonates can be highly effective in further absorbing toxic metals (Plassard, Winiarski, & Petit-Ramel, 2000; Sipos et al., 2005).

Urease-based MICP has been applied to enhance the durability of building

structures by improving strength, reducing water permeation and corrosion (De Muynck, Belie, & Verstraete, 2010; Achal, Mukherjee, & Reddy, 2011; Achal et al., 2012b; Phillips et al., 2013) and for cementation of cracks and fissures (Ramachandran, Ramakrishnan, & Bang; 2001; Van Tittelboom et al., 2010). It has also been used as a "bio-grout" for ground permeability control and reinforcement (Whiffin, Van Paassen, & Harkes, 2007; DeJong et al., 2010; Akiyama & Kawasaki, 2012), and the restoration of historical monuments (Tiano, Biagiotti, & Mastromei, 1999). Urease-producing organisms have also been proposed for novel applications in the bioremediation of toxic metals and radionuclides through the formation of insoluble metal-containing carbonates (Table 1). A diagram showing how urease mediates metal carbonate bioprecipitation is shown in Figure 1. Toxic metals may also precipitate on the Ca mineral surface as discrete compounds or form mixed solid-solutions, e.g. $Cd_nCa_{n-1}CO_3$ (Papadopoulos & Rowell, 1988). The following sections discuss bioprecipitation of those metals where MICP has been successfully applied using urease-producing bacteria and fungi.

4. BIOPRECIPITATION OF METAL(LOID)S BY BACTERIAL-INDUCED CARBONATE PRECIPITATION

4.1 Arsenic

Arsenic, a crystalline metalloid, is highly toxic to all forms of life. The permissible limit of arsenic in soil is 24 mg kg^{-1} (USEPA 2009). The major sources of arsenic in soil are natural weathering from bedrock, atmospheric deposition, agricultural

materials and the coal industry. Arsenic is highly dangerous to human health as it can cause skin cancer, melanosis, and keratosis, as well as other physiological disorders (Singh et al., 2015). Removal of arsenic from contaminated soil is therefore very important and a great challenge using bioremediation methods. Arsenic exists in four oxidation states (O, -III, III and V) with arsenate [As(V)] and arsenite [As(III)] as predominant forms in contaminated environments. Due to the toxicity of arsenic, microorganisms possess mechanisms to resist its hazardous effects, mainly by active efflux, extracellular precipitation, chelation or intracellular sequestration (Kruger et al., 2013), Bioremediation may employ redox transformations of As via As(V) reduction and As(III) oxidation which can be carried out by a wide variety of As(V)-reducing and As(III)-oxidizing bacteria including *Chrysiogenes arsenatis*, *Sulfurospirillum barnesii*, *Bacillus arsenicoselenatis*, *Desulfitobacterium hafniense* and *Thiomonas arsenivorans* (see Yamamura & Amachi, 2014). While removing As from contaminated soil using *Bacillus selenatarsenatis* SF-1, Yamamura *et al*. (2008) successfully reported mobilization of As into the aqueous phase from contaminated soil through reduction of solid-phase As(V) and Fe(III); however, a maximum of 56% removal occured from soil containing 250 mg kg^{-1} As. Biovolatilization has also been used for As remediation and this resulted in about 2.2%–4.5% of arsenic removal from soil after a 30-day incubation using *Sphingomonas desiccabilis* and *Bacillus idriensis* (Liu *et al*., 2011). There was a significant rate of biovolatilization of As(V) and As(III) from culture medium by *Staphylococcus* sp. (Srivastava et al., 2012).

Arsenic in soils is most commonly associated with its primary minerals derived

from bedrock, secondary minerals (primarily Fe oxy/hydroxides; sulfides) formed in the course of mineral weathering, and As adsorbed to mineral surfaces. Association of As with calcium minerals is well known (Chang & Jackson, 1957). The precipitation of Ca arsenates was shown in highly acidic waste pile leachates after association with carbonate subsurface layers (Juillot et al., 1999). Furthermore, significant adsorption of As on carbonate mineral phases has been reported (Goldberg & Glaubig, 1988; Roman-Ross et al., 2006). It was demonstrated that arsenate may substitute for CO_3^{2-} in calcite from travertine, suggesting the possibility of As immobilization through carbonate precipitation (Di Benedetto et al., 2006).

In order to improve the efficiency of As removal, Achal *et al*. (2012a) used *Sporosarcina ginsengisoli* CR5 for remediation of As(III) in contaminated soil. This ureolytic bacterium significantly reduced the As concentration in the exchangeable fraction of soil to 0.88 mg kg^{-1} in a soil supplemented with 500 mg kg^{-1} As(III). It was proposed that calcite production by the bacterium facilitated precipitation of a strong arsenic-calcite complex leading to reduced As mobility. Such an immobilization process may enable metal(loid)s to be transformed in situ into insoluble and chemically inert forms and are applicable to removing metals from aqueous solution (Gadd, 2004; 2010). Analysis of the mineralogical products in MICP-treated As contaminated soil samples showed that various minerals such as gwihabaite, calcite, vaterite and aragonite were formed along with As(III)–calcite co-precipitation products (see Figure 2). Such co-precipitation is unaffected by the oxidation state of arsenic which confirms the efficiency of calcite as an effective

scavenger of a variety of metals (Rouff et al., 2004; Alexandratos, Elzinga, & Reeder, 2007). Urease-producing bacteria have therefore been shown to be effective for immobilization of high amounts of arsenic and are therefore potential candidates for application in arsenic contaminated sites.

4.2 Cadmium

Cadmium (Cd) is a non-essential heavy metal, naturally present in soils and enriched by anthropogenic and agricultural activities. It occurs typically in the range of 0.1 and 1.0 mg kg^{-1}. Cd contaminated soils pose a threat to human health through consumption of cereals or other crops grown in such soil (Smolders & Mertens, 2013). Cd can form complexes with various anions, such as Cl^-, SO_4^{2-}, CO_3^{2-} and PO_4^{3-} (Makino et al., 2006) and this property makes it a suitable candidate for immobilization by MICP. Though various methods of Cd bioremediation from soil have been suggested, immobilization of Cd is generally recognized as the most practical technology as it does not affect agricultural activity (Wang et al., 2014). Cadmium sorption has been studied in calcareous soil which implicated the efficiency of calcium carbonate in Cd removal (O'Connor, O'Connor, & Cline, 1984). Waste oyster shells containing high amounts of $CaCO_3$ were used to stabilize Cd contaminated soils (Ok, Lim, & Moon, 2011). Addition of calcium chloride was reported as the most appropriate soil-washing treatment for Cd contaminated soil and this resulted in 55% Cd removal from the exchangeable soil fraction (Makino et al., 2006). However, the major drawback of such an approach was that this was not

sufficient to remove the high remaining amount of Cd. There is scope therefore to enhance Cd bioremediation using urease-producing organisms that would lead to further Cd immobilization.

Terrabacter tumescens, a urease-producing bacterium, was reported to effectively remove more than 90% Cd within 72 h when 2 g L^{-1} $CdCl_2$ was present in laboratory media (Li, Cheng, & Guo, 2013). The Cd in solution was assumed to precipitate as cadmium carbonate ($CdCO_3$). Li et al. (2013) also found effective immobilization of other metals such as Ni, Cu, Pb, Co and Zn using *T. temescens*, which were precipitated as $NiCO_3$, $CuCO_3$, $PbCO_3$, $CoCO_3$ and $ZnCO_3$. These biominerals exhibited different morphologies and were rhombohedral, needle-like or spherical in shape, and of size 10-50 μm (see Figure 3).

Lysinibacillus sphaericus CH-5 has been demonstrated to precipitate Cd based on ureolytic activity (Kang et al., 2014). This bacterium was isolated from an abandoned mine site and showed high urease activity (2.41 μmol min^{-1}) and produced 10 mg mL^{-1} calcite in broth containing beef extract, peptone and urea. Urease production was also evident in a consolidated sand column using *L. sphaericus* that resulted in improved mechanical properties. Urease production (1.72 μmol min^{-1}) after 48 h in the presence of 2 g L^{-1} Cd resulted in 99.95% Cd removal (Kang *et al.*, 2014). The precipitated Cd appeared mostly as spherical forms with a diameter of 10-40 μm, while XRD revealed calcite peaks along with otavite showing clear precipitation of Cd as carbonate.

Recently, Kumari *et al.* (2014) reported MICP for Cd immobilization from soil at

low temperature. *Exiguobacterium undae* YR10 was added to soil artificially contaminated with 100 mg $CdSO_4$ kg^{-1} soil, in the form of a bacterial culture grown in nutrient broth containing urea and calcium chloride. The experiments were terminated after 2 weeks and thereafter the soluble-exchangeable soil fraction contained 0.87 mg Cd per kg^{-1} soil at 25°C, and 1.2 mg Cd kg^{-1} soil at 10°C in the same fraction. The carbonate fraction of the soil had a significantly higher Cd concentration, suggesting that most of the Cd was either converted to $CdCO_3$ or co-precipitated with calcite. Although $CdCO_3$ is sparely soluble in the soil solution, it may combine with $CaCO_3$ and remain immobilized (Kumari et al., 2014). In more recent research, calcium and cadmium carbonate biomineralization by the ureolytic fungus *Neurospora crassa* has been reported (Li, Csetenyi, & Gadd, 2014). The Cd precipitates were identified as pure otavite ($CdCO_3$). This suggested an important role for ureolytic microbes in providing a means of metal biorecovery as well as bioremediation (Li, Csetenyi, & Gadd, 2014).

4.3 Chromium

Chromium (Cr) is often considered to be a "local source" contaminant and presumed not to constitute a widespread environmental problem (Samborska, Stepniewska, & Stepniewski, 2004). However, its toxic effects cannot be ignored. It contaminates soils from metallurgy operations, electroplating, production of paints and pigments, tanning, wood preservation, chromium chemical production, and pulp and paper production. Cr exists primarily in two different oxidation states as Cr(III) and Cr(VI), of which

Cr(III) is non-toxic and exhibits limited environmental disruption, while Cr(VI) is highly mobile, soluble and toxic with strong oxidazing properties (Zhang & Li, 2011). The disposal of Cr-containing wastes over large areas has led to extensive contamination of soil in many parts of the world. The sites around such dumping zones are highly prone to further contamination due to leaching and seepage of Cr(VI) into the groundwater (Zayed & Terry, 2003). In view of the seriousness of Cr(VI) pollution, efforts have been made based on a bioconsolidation approach involving urease-producing bacteria for the treatment of Cr-contaminated soils and slags.

Co-precipitation of Cr(VI), in which chromate incorporates into mineral structures, has been considered as an alternative means of limiting the mobility of chromate, although few studies address the interaction of Cr with calcium carbonate minerals (Tang et al., 2007). In one study, urease-producing bacteria were used to produce calcite and consequently entrap chromate. A calcifying ureolytic *Bacillus* sp. CS8 was used to consolidate Cr slag in the form of bricks of size 18 × 9.5 × 3.5 cm (Achal et al., 2013). The bioconsolidation resulted in a significant decrease in Cr(VI) in the exchangeable fraction that was 95% lower than the control. At the same time, the increased carbonate-bound Cr(VI) suggested preferential incorporation into the calcite during crystal growth (Tang *et al*., 2007). MICP was also tested to confirm its efficency in preventing metal leaching in soil column experiments. *Bacillus* sp. CS8 reduced the flow rate from a Cr slag column by reducing permeability due to a calcium carbonate layer being precipitated by the bacteria (Achal et al., 2013).

In another study, soils artificially contaminated with 100 mg kg^{-1} Cr(VI) were

treated with ureolytic *B. cereus* YR5 which resulted in a significant decrease (92%) of Cr(VI) in the exchangeable fraction of the polluted soil and increased the carbonate-bound Cr(VI) fraction (Kumari et al., 2014). One report suggested the presence of urea enhanced Cr(VI) removal efficiency during electrochemical remediation of Cr(VI) in chromium slag. The Cr(VI) in the calcium carbonate structure showed resistance to gaseous reductants or solution-phase extractants (Thornton & Amonette, 1999; Hua et al., 2007) implying long-term stability of Cr(VI) incorporation in the calcium carbonate and prevention of Cr(VI) release.

4.4 Copper

Copper is a common soil contaminant (Santorufo, Van Gestel, & Maisto, 2012). Anthropogenic activities (such as application of sewage sludge, mine slags, industrial wastewaters, fungicides, and fertilizers) can lead to the elevation of copper to toxic levels in agricultural soils (Wang, Hua, & Ma, 2012; Hu et al., 2014; Anjum et al., 2015). Soluble and exchangeable metals such as copper are often considered as being the most potentially toxic in soil (Yang et al., 2006; Hu et al., 2014) and copper remediation from this soil fraction is therefore highly desired.

The versatility of *Kocuria flava* CR1 with a high tolerance to copper and urease producing ability has been documented for effective treatment of copper in contaminated soil (Achal, Pan, & Zhang, 2011). This bacterium produced a very high amount of urease (472 U ml^{-1}) in nutrient broth-urea media, establishing MICP for copper immobilization. Copper removal was 95% from a solution containing 500 mg

L^{-1} $CuSO_4.5H_2O$. The resulting precipitates were evaluated by FTIR and identified as calcium carbonate and aragonite (Vagenas, Gatsouli, & Kontoyannis, 2003). MICP using ureolytic bacteria was also effective in copper contaminated soil and 98% copper was immobilized from soil containing 340 mg kg^{-1} copper (Achal, Pan, & Zhang, 2011). Only 3.5 mg Cu kg^{-1} soil remained in the exchangeable fraction after treatment compared to 67 mg Cu kg^{-1} in untreated soil. Copper was also immobilized as $CuCO_3$ by the ureolytic bacterium *Terrabacter tumescens* (Li, Cheng, & Guo, 2013).

4.5 Lead

Lead (Pb) is a toxic metal that may pollute soil or water due to emission from automobiles, waste irrigation, pesticide application, mining and smelting, and ultimately may pose a health risk (Gworek, 1992; Li et al., 2009). Lead is also the most distinctive heavy metal contaminant of urban soils. Once it accumulates inside humans, it can cause neurodegenarative damage, DNA damage, apoptosis, cancer and various disabilities in children (Gworek, 1992; Li et al., 2009).

Urease based MICP has been shown to be highly effective in lead immobilization. A urease-producing *Kocuria flava* CR1 that grew well in nutrient media supplemented with 50 mM Pb was able to remove 80% Pb from the soluble-exchangeable fraction of contaminated soil (Achal *et al*., 2012c). The bioremediation efficiency of MICP was confirmed in terms of the distribution coefficient (γi) of each Pb fraction, indicating a significant increase in γi of carbonate-bound Pb, while at the same time

the γi of soluble-exchangeable Pb was reduced greatly. It was concluded that Pb immobilization by such a mechanism could be of considerable relevance because of its stability in a variety of geologic environments (Achal et al., 2012c). Another efficient urease producer, *Sporosarcina koreensis* (UR47) was reported to remove 99% lead from a solution containing 2 g L^{-1} $PbCl_2$ through MICP (Li, Cheng, & Guo, 2013).

A lead resistant *Bacillus* sp. KK1 isolated from Pb contaminated mine tailings effectively biomineralized mobile Pb (Govarthanan *et al.*, 2013). The lead mineral products were lead sulfide (PbS) and lead silicon oxide ($PbSiO_3$) (see Figure 4a). *Bacillus* sp. KK1 was used to treat lead contaminated mine tailings containing Pb 1050 mg kg^{-1} and this resulted in a 26% decrease in the exchangeable Pb fraction in the bioaugmented tailings (Govarthanan et al., 2013). At the same time, the carbonate Pb fraction increased by 38% due to bacterially-mediated precipitation of Pb (see Figure 4b). XRD spectra showed differences in PbO and $Pb(OH)_2$ in bioaugmented mine tailings when compared with the control, indicating that MICP could effectively scavenge different species of Pb (Govarthanan et al., 2013).

Recently, urease-producing *Sphingobacterium* sp., *Enterobacter cloacae*, and *Lysinibacillus sphaericus* which showed a high Pb tolerance were isolated from soils at abandoned metal mine sites (Kang et al., 2015). These bacteria showed the presence of ureC genes which were amplified using UreC-F and UreC-R primers (Gresham et al., 2007). A high removal rate (68%) of Pb was observed within 48 h based on MICP resulting in lead carbonate precipitates of diameter ~5 μm. The MICP

process also resulted in a significant increase in enzyme activities (phosphatase 37%, dehydrogenase 14%, and urease 334%) in the treated mine tailings (Govarthanan et al., 2013). Increased urease and dehydrogenase activity in Pb-contaminated soils after adding ureolytic bacteria has also been reported by others (Achal et al., 2012c).

4.6 Radionuclide bioprecipitation by urease-producing bacteria

Radioactive contamination has been a serious problem since the development of nuclear technology. Significant amounts of radionuclides are discharged by industrial activities allied to nuclear power generation, nuclear weapons and accidental release (Pollmann et al., 2006). Soils contaminated with radionuclides, such as ^{137}Cs, ^{235}U and ^{90}Sr, pose a long-term radiation hazard to human health through exposure via the food chain and other pathways. They pose serious health impacts on humans and cause neurological disorders, infertility, birth defects, and various types of cancer (Najem & Voyce, 1990; Mossman, 2003; Das, 2012).

The concept of biomineralization in radionuclide bioremediation was introduced several years ago. Radionuclides can be immobilized through interactions between microbially-produced sulfide (White, Sharman, & Gadd, 1998; Lebranz et al., 2000) and phosphate (Macaskie et al., 1992; Boswell, Dick, & Macaskie, 1999; Jeong & Macaskie, 1999), or through bacterial iron oxidation (Banfield et al., 2000) in the general process of biomineralization (Martinez et al., 2007). Uranium phosphate precipitation has been facilitated by diverse bacterial genera including *Arthrobacter*, *BacillusI, Rahnella*, *Deinococcus, Escherichia* and *Pseudomonas* (Basnakova et al.,

1998; Powers et al., 2002; Appukuttan, Rao, & Apte, 2006). It has also been shown that *Bacillus subtilis* can immobilize U through the formation of uranyl-hydroxide, uranyl-carbonate, and calcium-uranyl-carbonate species with functional groups present on cell surfaces (Fowle, Fein, & Martin, 2000; Gorman-Lewis, Elias, & Fein, 2005). *Pseudomonas aeruginosa*, an indigenous bacterial isolate from uranium mine waste, could sequester soluble uranium in mineral form, the bioaccumulated uranium being sequestered as crystalline needle-shaped U phosphate compounds within the cell envelope, identified as $UO_2(PO_3)_2$, $(UO_2)_3(PO_4)_2 \cdot H_2O$ and $U_2O(PO_4)_2$ (Choudhary & Sar, 2011).

Biomineralization of radionuclides has been further investigated using urease-producing bacteria. The remediation of ^{90}Sr from the Snake River Plain Aquifer (SRPA), which underlies the Idaho National Engineering and Environmental Laboratory (INEEL), USA, was evaluated based on a ureolytically driven calcite precipitation approach (Fujita et al., 2004). ^{90}Sr is a significant aquifer and vadose zone contaminant at the INEEL, as well as at a number of DOE facilities across the USA (Riley & Zachara, 1992). Native ureolytic microbes were used to remediate ^{90}Sr contamination at the Hanford 100-N area in Washington where ureolytic activities of microbes were confirmed by UreC amplification (Fujita et al., 2010). Quantitative assays detected up to 2×10^4 putative ureC gene copies mL^{-1} in water and up to 9×10^5 copies g^{-1} in sediment. Further analyses indicated that the Sr was incorporated into calcite ensuring the relative stability of ^{90}Sr (Fujita et al., 2010).

In another study, a possible role of ureolytic *Halomonas* sp. was reported for the

remediation of strontium (Sr) in aquifer sand (Achal, Pan, & Zhang, 2012). The overall reactions involved in the bioremediation process included urease producing NH_4^+ and HCO_3^-, desorption of Ca^{2+} and/or Sr^{2+} from solid surfaces by NH_4^+ and HCO_3^- promoted precipitation of $CaCO_3$ and co-precipitation of ^{90}Sr (Wu et al., 2011). The hydrolysis of urea produces bicarbonate and ammonium, where bicarbonate participates directly in calcite precipitation, and ammonium can exchange for sorbed strontium, calcium, and other metals, resulting in their enhanced susceptibility to recapture via carbonate mineral formation (Fujita et al., 2010). Some possible chemical reactions can be summarized as follows (Achal, Pan, & Zhang, 2012) (Eqs. 8 and 9):

(i) Urease mediated reaction producing NH_4^+ and HCO_3^-

$$H_2N(CO)NH_2 + H^+ + 2H_2O \rightarrow 2NH_4^+ + HCO_3^- \quad (8)$$

(ii) Precipitation of calcite and co-precipitation of ^{90}Sr, promoted by HCO_3^-

$$x90Sr^{2+} + (1-x)Ca^{2+} + 2HCO_3^- \leftrightarrow Ca(1-x)^{90}Sr_xCO_3 + H_2O + CO_2 \quad (9)$$

5. BIOPRECIPITATION OF METAL(LOID)S BY FUNGAL-INDUCED CARBONATE PRECIPITATION

Fungi are ubiquitous chemoorganotrophic (heterotrophic) organisms and their importance as animal and plant symbionts and pathogens, and spoilage organisms of natural and manufactured materials is profound (Gadd, 2008). Metals, metalloids, metal radionuclides, organometals and organometalloids, and their compounds,

interact with fungi in various ways depending on the chemical speciation, organism and environmental factors (Gadd, 1993, 1999, 2007; Gadd et al., 2012). Both metabolism-independent and -dependent fungal activities can result in the precipitation of secondary organic and inorganic minerals (e.g. oxalates, oxides, phosphates and carbonates). Fungi can act as effective biosorbents for a variety of metals including U, Th, Pb, Cu, Zn, Cd and Ni, and can also affect speciation and mobility of metals and radionuclides through mineral dissolution and bioprecipitation (Gadd, 1993, 2007, 2009, 2010). The key factors that can influence the nucleation, growth and deposition of biominerals on and around fungal biomass include pH and cell wall composition as well as excretion of various organic and inorganic metabolites (Gadd, 2010). The precipitation of carbonates, phosphates and hydroxides can increase soil aggregation and cations such as Si^{4+}, Fe^{3+}, Al^{3+} and Ca^{2+} (that may be released through mineral dissolution mechanisms) may act as bonding agents for soil particles. Hyphae can also enmesh soil particles (Bronick & Lal, 2005). Apart from the biomineral examples that follow, several other carbonate minerals precipitated by fungi have been recorded (Table 2).

One mechanism commonly associated with the biomineralization of $CaCO_3$ is based on urea degradation, as in bacteria, which leads to the release of carbonate which is then precipitated by available Ca (Whiffin, van Paassen, & Harkes, 2007; Burbank et al., 2011). Li et al. (2014) used urea-hydrolysing *Neurospora crassa* grown in a urea and calcium-rich medium in order to produce ammonium (NH_4^+) and dissolved carbonate which together with increasing medium pH, resulted in calcite

bioprecipitation (Eqs. 10, 11):

$$CO(NH_2)_2\,(aq) + 2H_2O\,(aq) \xrightarrow[\text{urease}]{\text{Fungal}} 2NH_4^+\,(aq) + CO_3^{2-}\,(aq) \qquad (10)$$

$$CO_3^{2-}\,(aq) + Ca^{2+}\,(aq) \longrightarrow CaCO_3\,(s) \qquad (11)$$

It was shown that more than 90% of supplied calcium (at a concentration of 50 mM) could be precipitated as calcite by the fungus (Li, Csetenyi, & Gadd, 2014). When incubated in urea-containing medium modified with different concentrations of $CaCl_2$ and $SrCl_2$, various other minerals were deposited in the medium and around the biomass (see Figure 5) and these were identified as calcite and strontianite ($SrCO_3$) (unpublished data). Furthermore, cracks involving hyphae were observed on the surface of some of the crystals (see Figure 5a) which indicated that hyphae may act as nucleation sites for some of the calcite precipitation observed. Compared to the simpler bacterial cell form, the fungal filamentous growth habit could provide more framework support and stability for the precipitation of calcite or other biominerals. Such performance of a urease-positive fungus in urea-supplemented media suggests a promising method for calcite synthesis as well as other metal-containing carbonates. For example, 50% of supplied $CdCl_2$ (at a concentration of 0.5 M) was precipitated as pure otavite ($CdCO_3$) by the culture supernatant obtained after growth of *N. crassa* in urea-supplemented medium (Li, Csetenyi, & Gadd, 2014). Urease-positive fungi (*Pestalotiopsis* sp. and *Myrothecium gramineum*) isolated from calcareous soil were also found to precipitate $CaCO_3$ and $SrCO_3$ as well as olekminskite ($Sr(Sr,Ca)(CO_3)_2$)

and Sr-containing vaterite ($(Ca_xSr_{1-x})CO_3$) (Li et al., 2015). The soil fungus *Paecilomyces javanicus* was found to mediate the transformation of metallic lead into lead secondary minerals: plumbonacrite ($Pb_{10}(CO_3)_6O(OH)_6$), hydrocerussite ($Pb_3(CO_3)_2(OH)_2$) and a new lead hydroxycarbonate (Rhee, Hiller, & Gadd, 2015). The roles of fungi in the environmental fate of toxic metals is of considerable interest although biologically-induced calcium carbonate precipitation has received little attention as a potential remediating strategy for contaminated environments or for element biorecovery (Pan, 2009; Achal et al., 2012a, b). Many free-living fungi are capable of urea degradation (Li *et al*., 2014, 2015). Most ammonia fungi as well as ectomycorrhizal fungi also show strong abilities of urea degradation (Yamanaka, 1999; Barua *et al*., 2012). Ammonia fungi are an abundant group of soil fungi which flourish when additional nitrogenous substances are present, such as urea, the degradation of which leads to soil alkalinization to pH 9-10 (Navarathna et al., 2010).

6. CONCLUSIONS

One of the primary objectives of bioremediation of contaminated soil is to reduce the bioavailability of metals. The urease driven MICP process may offer a promising option for immobilizing heavy metals. Since urea-hydrolysing microorganisms show the ability to precipitate Ca as $CaCO_3$, this means they can also be applied to other toxic metals to form other metal carbonates. During the precipitation of calcite, toxic metal ions may be incorporated into the $CaCO_3$ by substituting for Ca^{2+} or may also co-precipitate within the $CaCO_3$ lattice structure. Although the total toxic metal

concentration in soil remains unchanged during MICP, a significant majority of the contaminant may be removed from the soluble-exchangeable fraction to the carbonate-bound fraction. Microbial metal carbonate precipitation is also relevant to detoxification of contaminated process streams and effluents, as well as the synthesis of novel metal carbonates and biorecovery of metals and radionuclides that form insoluble carbonates.

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Table 1 Some examples of the application of ureolytic bacteria for immobilization of metal(loid)s by MICP.

Name of bacteria	Metal(loid)	Bioremediation efficiency	Reference
Sporosarcina ginsengisoli	As	96% removal from aqueous media (ic = 10 mg L^{-1}) 96% removal from exchangeable soil fraction (ic = 500 mg Kg^{-1})	Achal et al. (2012a)
Exiguobacterium undae	Cd	84% removal from aqueous media 90% in exchangeable soil fraction	Kumari et al. (2014)
Lysinibacillus sphaericus	Cd	99.95% removal from aqueous media (ic = 2 g L^{-1})	Kang et al. (2014)
Terrabacter tumescens	Cd	99% removal from aqueous media (ic = 2 g L^{-1})	Li et al. (2013)
Kocuria flava	Cu	96% removal from exchangeable soil fraction (ic = 340 mg Kg^{-1})	Achal et al. (2011)
Bacillus sp.	Cr(VI)	>68% removal from Cr slag	Achal et al. (2013)
Enterobacter cloacae	Pb	68% removal from aqueous media (ic = 7.2 mg L^{-1})	Kang et al. (2015)
Sporosarcina koreensis	Pb	99% removal from aqueous media (ic = 2 g L^{-1})	Li et al. (2013)
Halomonas sp.	Sr	86% removal from quartz sand (ic = 100 mg Kg^{-1})	Achal et al. (2012c)
Sporosarcina sp.	Zn	99% removal from aqueous media (ic = 2 g L^{-1})	Li et al. (2013)

*ic: initial concentration of metal(loid)

Table 2 Fungal species reported for the biomineralization of various metal carbonates.

Fungal species	Carbonate minerals	Reference
Acremonium strictum	Calcite ($CaCO_3$)	Li and Gadd, unpublished
Cephalosporium sp.	$CaCO_3$	Gadd and Raven (2010)
Cephalotrichum (syn *Doratomyces*) sp.	Calcite ($CaCO_3$)	Burford et al. (2006)
Fusarium oxysporum	Calcite ($CaCO_3$)	Ahmad et al. (2004)
Morchella sp.	Calcite ($CaCO_3$)	Masaphy et al. (2009)
Myrothecium gramineum	Calcite ($CaCO_3$), vaterite ($(Ca_xSr_{1-x})CO_3$), strontianite ($SrCO_3$),	Li et al. (2015)
Neurospora crassa	Calcite ($CaCO_3$), otavite ($CdCO_3$)	Li et al. (2014)
Neurospora crassa	Strontianite ($SrCO_3$), $CoCO_3$, nickel carbonate, $La_2(CO_3)\cdot 8H_2O$	Li and Gadd, unpublished
Paecilomyces javanicus	Hydrocerussite ($Pb_3(CO_3)_2(OH)_2$), plumbonacrite ($Pb_{10}(CO_3)_6O(OH)_6$)), lead hydroxycarbonate	Rhee et al. (2015)
Penicillium corylophilum	$CaCO_3$	Gadd and Raven (2010)
Penicillium simplicissimum	Hydromagnesite ($Mg_5(CO_3)_4(OH)_{2.4}\cdot H_2O$)	Burford et al. (2003)
Pestalotiopsis sp.	Calcite ($CaCO_3$), strontianite ($SrCO_3$), olekminskite ($Sr(Sr, Ca)(CO_3)_2$), $(Ca,Sr)CO_3$, vaterite ($CaCO_3$)	Li et al. (2015)
Serpula himantioides	Calcite ($CaCO_3$)	Burford et al. (2006)
Trichothecium sp.	Calcite ($CaCO_3$)	Ahmad et al. (2004)
Verrucaria spp.	$CaCO_3$	Easton (1997)
Verticillium sp.	$CaCO_3$, $BaCO_3$	Rautaray et al. (2004)

Legends to figures

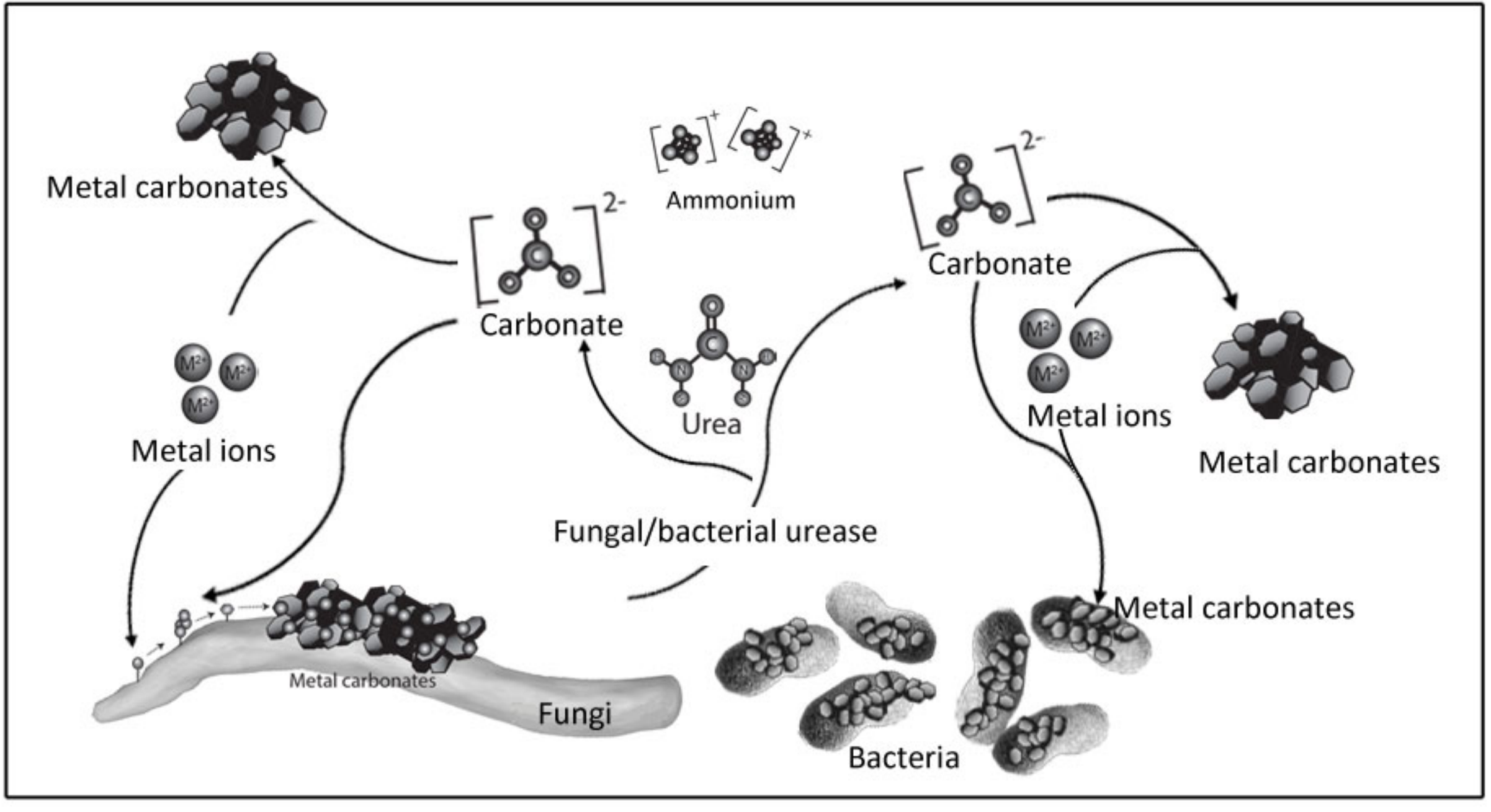
Figure 1 Diagram of precipitation of metal carbonates by urease-producing microorganisms. M^{2+} represents a divalent metal cation. Adapted from Li *et al.* (2014).

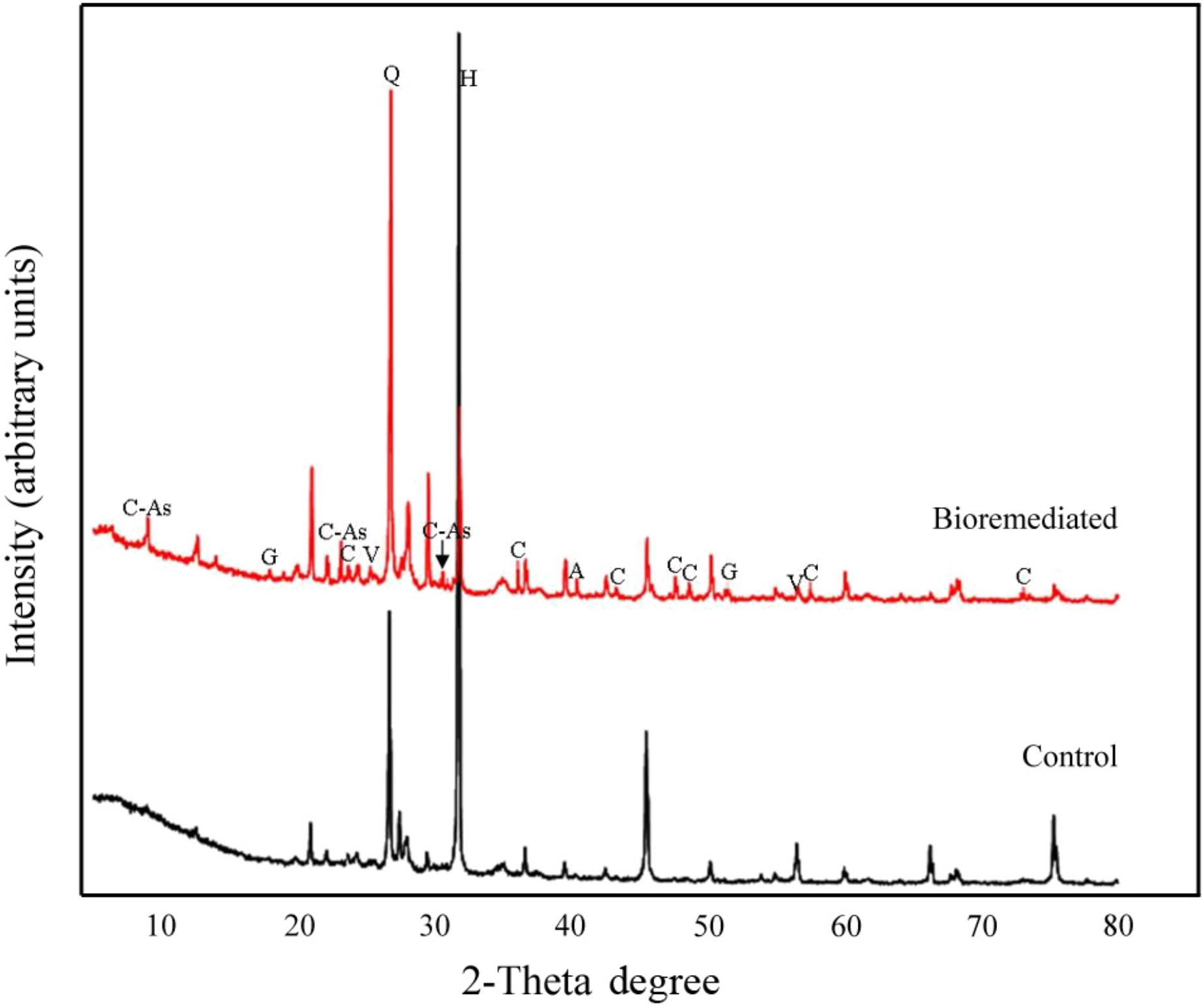
Figure 2 XRD spectra conforming biomineralization products in soil induced by *Sporosarcina ginsengisoli* CR5 (C= calcite, A= aragonite, C-As= calcite- arsenite precipitate, V= vaterite, G= gwihabaite, Q= quartz, H= halite). Adapted with permission from Achal *et al*. (2012a)

Figure 3 Environmental scanning electron microscopy (ESEM) of (a) Ni-containing minerals precipitated by *Terrabacter tumescens*, (b) Cu- (c) Pb-containing minerals precipitated by bacterial isolate UR47, (d) Co- (e) Zn-containing minerals precipitated by bacterial isolate UR31 and (f) Cd-containing minerals precipitated by *Terrabacter tumescens*. Scale bars: (a, b, c, e, f) = 10 μm, (d) = 40 μm. Adapted with permission from Li *et al*. (2013)

Figure 4 X-ray diffractograms of (a) *Bacillus* sp. KK1 before and after incubation with lead nitrate, (b) mine soil samples before and after bioaugmentation (C, calcite; A, aragonite). Adapted with permission from Govarthanan *et al*. (2013)

Figure 5 Scanning electron microscopy (SEM) of mineral deposition by *Neurospora crassa* grown in different media. (a, b) AP1 media amended with 40 mM urea and 50 mM $CaCl_2$, b is a higher magnification image of the area indicated by the square in a, scale bars: a =10 µm, b = 1 µm, (c) AP1 media amended with 40 mM urea, 25 mM $CaCl_2$ and 25 mM $SrCl_2$, scale bar = 10 µm, (d) AP1 media amended with 40 mM urea and 50 mM $SrCl_2$, scale bar = 10 µm. All samples were incubated for 12 days at 25^{o}C in the dark. Typical images are shown from many similar examples (Li and Gadd, unpublished data).


Metal carbonates
Ammonium
Carbonate
Carbonate
Metal ions
Urea
Metal ions
Metal carbonates
Fungal/bacterial urease
Metal carbonates
Metal carbonates
Fungi
Bacteria


Intensity (arbitrary units)
Q
H
C-As
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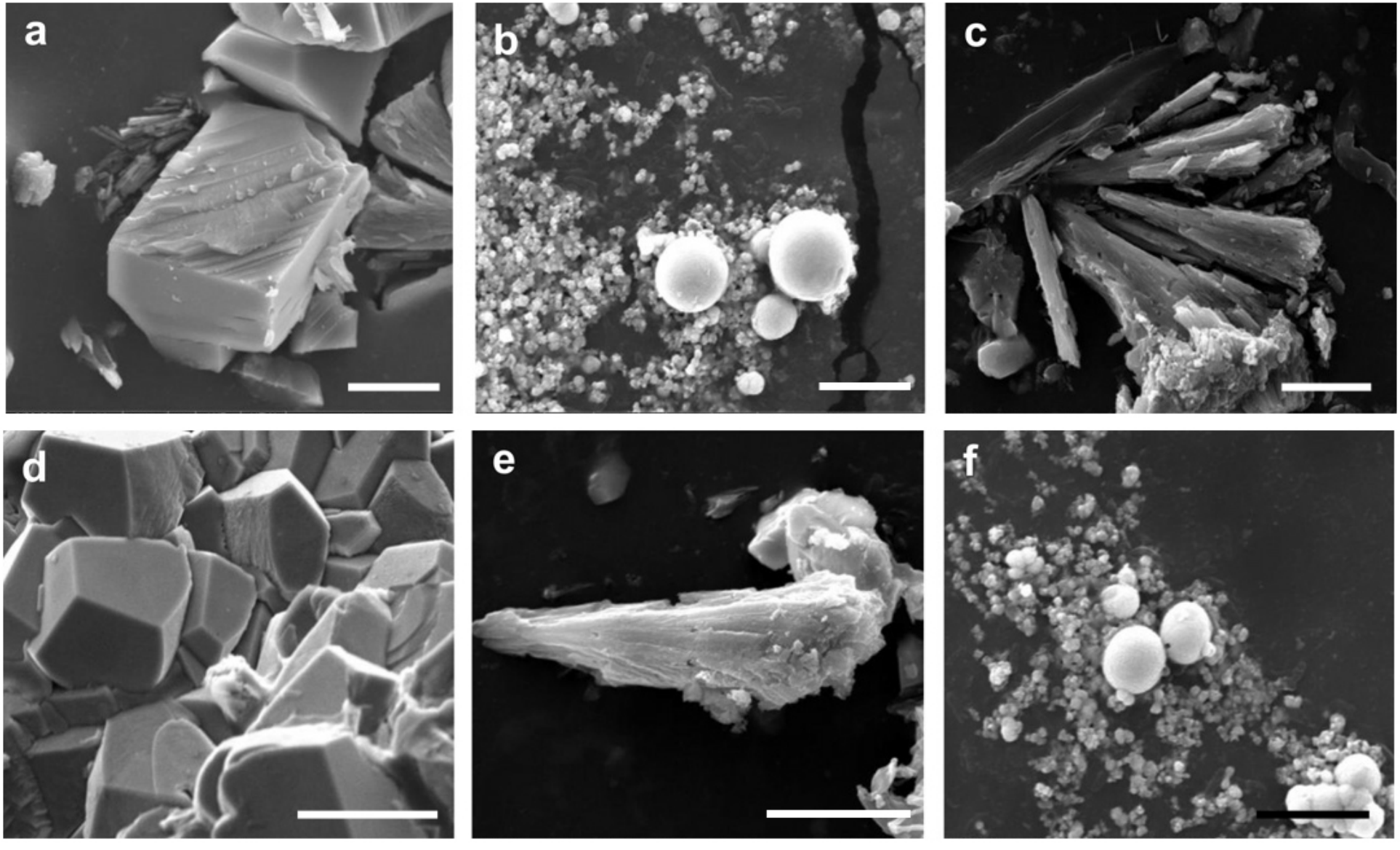
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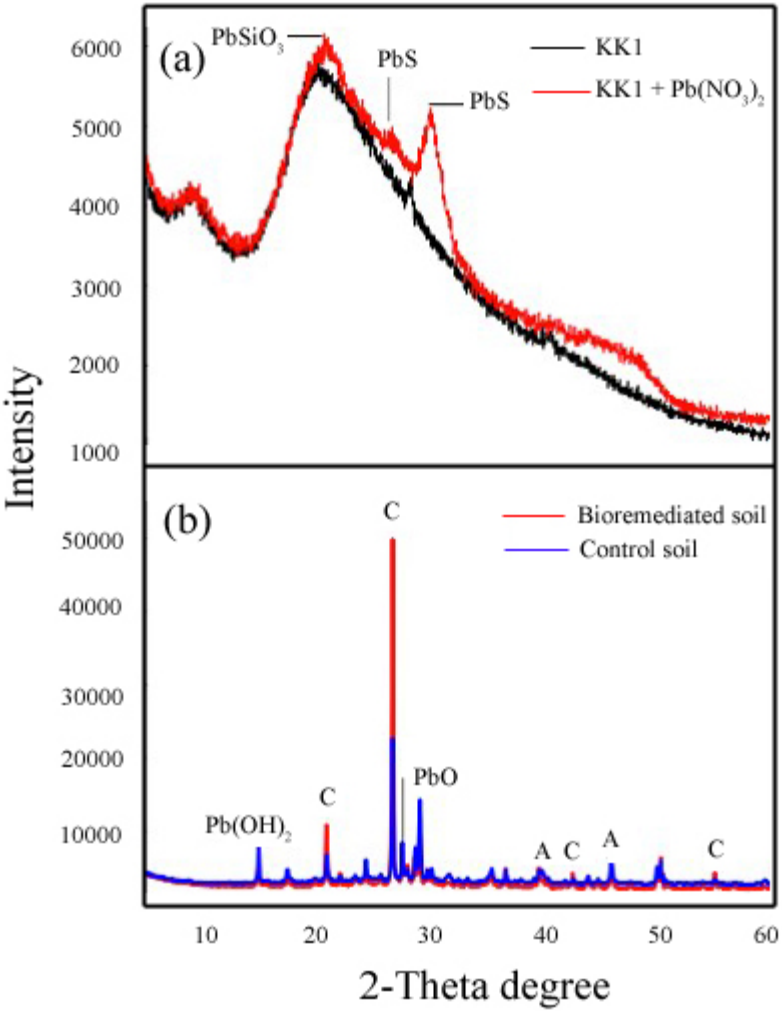
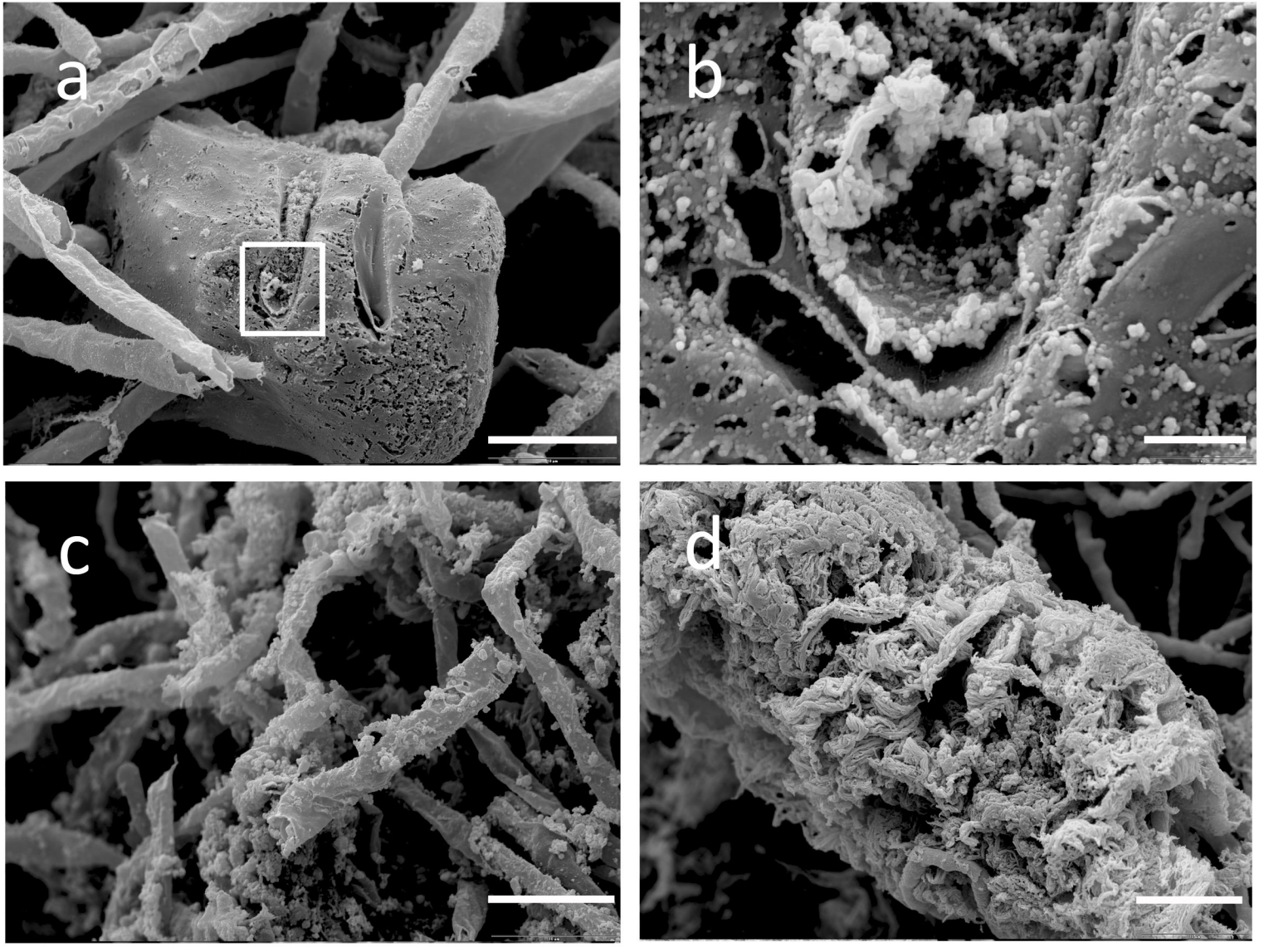

(a)
PbSiO3
PbS
PbS
KK1
KK1 + Pb(NO3)2
6000
5000
4000
3000
2000
1000
Intensity
(b)
C
Bioremediated soil
Control soil
50000
40000
30000
20000
10000
PbO
C
Pb(OH)2
A
C
A
C
10
20
30
40
50
60
2-Theta degree


a
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