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Research article

Bioleaching of toxic metals from sewage sludge by co-inoculation of *Acidithiobacillus* and the biosurfactant-producing yeast *Meyerozyma guilliermondii*

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ABSTRACT

The aim of this research is to evaluate the influence of co-inoculation of *Acidithiobacillus* bacteria and the biosurfactant-producing yeast *Meyerozyma guilliermondii* in bioleaching processes. The tests were carried out using sewage sludge from UASB reactors co-inoculated with cultures of *Acidithiobacillus* and *M. guilliermondii* to promote the solubilization of Cd, Cr, Cu, Ni, Pb and Zn which were determined by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP- OES). After 10 days of incubation, 76.5% of Zn, 59.8% of Ni, 22.0% of Cu, 9.8% of Cd, 9.8% Cr and 7.1% of Pb were solubilized. It was observed that the presence of yeast accelerated the time required for Cd solubilization from 240 to 96 h and there was a 20.1% reduction in nitrogen concentration and 7.6% for phosphorus in this assay. After the bioleaching and co-inoculation assays, the product obtained reached the maximum permissible concentrations for soil disposal for all the analyzed metals in the State of São Paulo, United States and also European Community standards.

1. Introduction

One of the consequences of an increase in the population and economic activity is the exponential growth in waste generation, such as sewage sludge (Azizi et al., 2013). Sewage treatment systems aim at minimizing the environmental impacts caused by improper disposal (Du et al., 2015; Zhang et al., 2014). Therefore, sewage sludge treatment can also generate secondary waste, such as sewage sludge, which can contaminate soil and water (Mao et al., 2015).

Because of the nutrient composition of sewage sludge, it can be used as a fertilizer and it is one of the alternatives recommended for its destination, but the presence of pathogenic organisms, organic and also inorganic pollutants, especially toxic metals, can restrict this allocation. The metals cadmium (Cd), copper (Cu), chrome (Cr), nickel (Ni), zinc (Zn), lead (Pb) and metalloid arsenic (As) are the most common inorganic pollutants that can be found in this kind of material (Pathak et al., 2009).

Most of the cationic contaminants, such as toxic metals, are found in soil, sediment and sludge adsorbed in the organic fraction or in particulate material. In fact, these elements are mostly pH-dependent and are more soluble in acid conditions and more insoluble and adsorbed/complexed with other compounds in alkaline conditions. This occurs because the ligation sites are also pH-dependent (McLean and Bledsoe, 1992) and at acidic pHs, many of the functional groups that were observed in the organic matter of the sewage sludge become protonated, releasing metals and, consequently, solubilizing them. Therefore, most of the techniques to remove toxic metals from sewage sludge are based on this assumption (Camargo et al., 2016).

An emerging technique to solubilize toxic metals from sewage sludge is known as bioleaching. According to Pathak et al. (2009), in short, bioleaching is a process based on the principle that the

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metabolism of bacteria from *Acidithiobacillus* genus can naturally acidify the medium. Although there is a large body of research on metal bioleaching from soils (Chen and Lin, 2001; Kumar and Nagendran, 2007; Mulligan et al., 2001; Naresh Kumar and Nagendran, 2009), there are only a few contributions in the field of sewage sludge treatment (Fang and Zhou, 2007; Wen et al., 2013; Zhou et al., 2013a).

Previous studies have clearly shown that the bioleaching process using species of *Acidithiobacillus* genus shows better results when the experiments were conducted using the widely known acidophilic species, *A. ferrooxidans* and *A. thiooxidans*, in co-inoculation (Pathak et al., 2009).

Recent research, Zhou et al. (2013a, 2013b), have shown that adding the yeast *Galactomyces* sp. Z3 in sludge could increase the bioleaching process. This is because heterotrophic microorganisms can also consume some organic acids that can inhibit or retard the *Acidithiobacillus* growth (Karwowska et al., 2014; Ngom et al., 2014) as they produce a substance known as biosurfactant, which can improve the metal solubilization (Banat et al., 2010). These studies have greatly contributed to our understanding of how much the co-inoculation of biosurfactant producing yeasts with *Acidithiobacillus* bacteria can affect the metal solubilization and degradation of inhibitory substances in sludge. The authors showed that co-inoculation of *Galactomyces* sp. Z3 and *Acidithiobacillus* strains reduced the period required for sludge bioleaching by 4.5 days compared to *Acidithiobacillus* alone in sewage sludge (Zhou et al., 2013a) and also in pig slurry (Zhou et al., 2013b), removing about 94% of Zn and 85% of Cu in the last substrate of the co-inoculation assays. On the other hand, in the control assay (without *Acidithiobacillus* bacteria or *Galactomyces* sp. inocula), there was approximately 51% of Zn solubilization. Cu solubilization in this assay was hardly observed and the authors attributed this result to the decline in pH from 5.3 to 4.1, meaning that the pH required for Cu solubilization was lower than Zn solubilization.

Despite the acceptance of the contribution of yeasts to the bioleaching process, there are few studies addressing the use of wild strains as data already published focus on standardized strains, such as *Galactomyces* sp. Z3 (Zhou et al., 2013a, 2013b) and *Brettanomyces* B65 (Fang and Zhou, 2007).

Could the co-inoculation of a wild strain of an acidophilic biosurfactant-producing yeast with *Acidithiobacillus* bacteria increase the bioleaching process of the metals Cd, Cr, Cu, Ni, Pb and Zn in sewage sludge? Is it possible to use the final product as a source of nitrogen and phosphorus, i.e., a fertilizer for farmland? The main aim of this paper is to investigate and answer these questions by co-inoculating a *M. guilliermondii* wild strain with the *Acidithiobacillus* (*A. ferrooxidans* and *A. thiooxidans*) bacteria in bioleaching processes in anaerobic sewage sludge.

2. Methods

2.1. Sludge sample and its characteristics

Anaerobic sludge was collected from a municipal wastewater treatment plant (WWTP) in Porto Feliz (São Paulo, Brazil), of the biological treatment step of the wastewater, directly from the upflow anaerobic sludge blanket reactor (UASB), and stored at 4 °C until used. The 1060 method from the Standard Methods for the Examination of Water and Wastewater was adopted to collect and store the sludge (APHA/AWWA/WEF, 2012).

The pH was measured immediately by directly immersing a previously sterilized electrode (formaldehyde 5%), while total solid (TS) content and volatile total solids (VTS) were measured according to the 2540B and 2540E methods, respectively (APHA/AWWA/WEF, 2012). The total nitrogen (N) and total phosphorus (P) were analyzed according to the 4500-N_{org} and 4500B methods, respectively (APHA/AWWA/WEF, 2012). Sulfate (SO_4^{-2}) was determined using the SulfaVer® kit (Permachem Reagents®, Hach®) following the manufacturer's recommendations based on the Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF, 2012). For the qualitative identification of the functional groups, Fourier Transform Infrared Spectroscopy (FT-IR) was used in the range of 4000-400 cm^{-1} and 32 scans using 1% potassium bromide tablets (KBr). The crude sludge was dried in an oven at 40 °C until a constant weight was reached.

The microbial behavior of bacteria of the genus *Acidithiobacillus* (*A. thiooxidans* and *A. ferrooxidans*) and *Thiobacillus* (*T. thioparus*) was evaluated according to the CETESB L5.217 standard (CETESB, 2004) using the most probable number (MPN) technique.

The content of toxic metals was analyzed in the solid phase according to the 3050B method (USEPA, 1996) and in the liquid phase according to the 3030E method (APHA/AWWA/WEF, 2012) after sludge centrifugation (10.000 rpm, 4 °C, 10 min). The metals Cd, Cr, Cu, Ni and Zn were quantified by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Table 1 shows the selected physicochemical properties of the sludge.

2.2. Microorganisms and inoculum preparation

The species *A. ferrooxidans* (ATCC 23270) and *A. thiooxidans* (ATCC 19377), provided by the Hydrometallurgy Laboratory from São Paulo State University (UNESP) "Julio de Mesquita Filho", *campus* Araraquara, were cultivated in modified 9 K liquid medium ($(NH_4)\cdot 2SO_4$ 3.0 g; KCl 0.1 g; K_2HPO_4 0.5 g; $MgSO_4\cdot 7H_2O$ 0.5 g; H_2O 1000 mL, pH 2.8) and modified T&K liquid medium ($(NH_4)_2SO_4$ 2.5 g; KH_2PO_4 0.45 g; $MgSO_4\cdot 7H_2O$ 2.5 g, H_2O 1000 mL, pH 1.8), respectively (Fang et al., 2011). Both media were autoclaved at 121 °C for 20 min and the 9 K medium was spiked with 44.2 g/L of 0.22 μm membrane-filtered $FeSO_4\cdot 7H_2O$ and the T&K medium was spiked with 10.0 g/L of elemental sulfur (S^0) as energy sources. The pH was adjusted with H_2SO_4 1 M and NaOH 1 M. The inoculum was prepared by growing the two-bacterial species in 250 mL erlenmeyer flasks each containing 100 mL of the 9 K or T&K medium at 150 rpm and 30 °C. The *A. ferrooxidans* and *A. thiooxidans* cell numbers were about 10^8 cells/mL at the end of their exponential phase of growth (about 48 h or 15 days after inoculation), separately (Zhou et al., 2013a).

The *Meyerozyma guilliermondii* yeast was originally isolated from soil contaminated by diesel and it is available at the Laboratory of Environmental Microbiology collection at the Federal University of São Carlos, Sorocaba *campus* (São Paulo, Brazil). It was identified using the ribosomal RNA gene sequence (Genebank access number KX455848, http://www.ncbi.nlm.nih.gov). The strain can grow in acid conditions (pH around 2.0) and produce biosurfactant/glycolipids similar to sophorolipids (unpublished data).

As previously described in Zhou et al. (2013a) for *Galactomyces* sp. Z3, the *M. guilliermondii* strain was cultivated for in Czapek medium ($NaNO_3$, 2.0 g; K_2HPO_4, 1.0 g; $MgSO_4\cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4\cdot 7H2O$, 0.01 g; sucrose 30.0 g; distilled water 1 L; pH 4.0 ± 0.2) before use. The yeast inoculum was obtained by growing the cells in 500 mL Erlenmeyer flasks, containing 250 mL of the described Czapek medium on a gyratory shaker at 150 rpm and 30 °C, and the *M. guilliermondii* cell numbers were about 10^7 cells/mL at the end of their exponential phase of growth (about 48 h after inoculation).

2.3. Effect of M. guilliermondii on the sludge bioleaching

Sewage sludge bioleaching experiments were carried out according to the modified method described in Zhou et al. (2013b),