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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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A B S T R A C T

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),

Table 1
Physicochemical characteristics of anaerobic sewage sludge before and after the bioleaching assays.

Parameters	Initial concentration	Assays		
		Control	Bioleaching	Co-inoculation
pH	7.4 ± 0.0	6.1 ± 0.1	2.1 ± 0.1	2.2 ± 0.0
TS, %	10.4	8.9	9.2	9.9
TVS, % TS	59.5	36.1	36.4	32.9
SO_4^{2}, % TS	3.9	9.5	12.5	7.1
Total N, % TS	0.7	0.6	0.6	0.6
Total P, % TS	0.2	0.3	0.3	0.2
4 Cd, mg/kg	5 20.7 ± 0.0	18.4 ± 0.0	17.21 ± 0.5	18.2 ± 0.0
6 Cr, mg/kg	7619.9 ± 0.0	572.8 ± 0.1	542.56 ± 0.3	552.0 ± 0.1
8 Cu, mg/kg	9 2201.0 ± 1.8	2128.5 ± 0.0	1378.6 ± 0.3	1714.2 ± 0.2
10 Ni, mg/kg	11 356.0 ± 6.3	345.2 ± 0.1	166.9 ± 4.9	140.3 ± 0.0
12 Pb, mg/kg	1629.2 ± 0.0	442.0 ± 0.1	414.6 ± 0.0	421.6 ± 0.1
13 Zn, mg/kg	14 8216.8 ± 0.1	7955.1 ± 0.0	2438.6 ± 3.8	2008.4 ± 0.0

where 500 mL erlenmeyer flasks containing 222.5 mL autoclaved sewage sludge (121 °C for 20 min) and 12.5 mL *A. ferrooxidans* + *A. thiooxidans* as inoculum. 2.0 g/L of Fe^{2+} (as $FeSO_4 \cdot 7H_2O$, 10.0 g/L), 2.0 g/L of elemental sulfur (S^0), and 15 mL of *M. guilliermondii* (as described in item 2.2) were added to the flasks mentioned above (hereafter referred to as the co-inoculation system). Control flasks contained only the energy substances (2.0 g/L of Fe^{2+} and 2.0 g/L of S^0) and all flasks were filled with ultrapure water (03.1 MΩ cm) up to 250 mL, when necessary. All flasks were incubated at 30 °C and 150 rpm during the incubation period of 10 days, and 10 mL of sewage sludge samples were withdrawn from the flasks every 24 h to analyse the pH and metal content.

The samples were centrifuged (10.000 rpm, 4 °C, 10 min) and then the liquid phase was acidified with HNO_3 (pH < 2.0) and stored at 4 °C until used. The metals and the physicochemical analysis were carried out as described in item 2.1. All the treatments were carried out in duplicate and data presented were the mean and standard deviations of two independent samples.

2.4. Data analysis

The metal solubilization (%) in the solid phase of the sewage sludge at the end of each test was calculated by the ratio between the concentration of metal solubilized and the total concentration of the metal in the sludge.

The assay results were compared using the parametric analysis of variance (ANOVA), evaluating their relevance using Graph Pad Instat software with a 95% confidence interval.

2.5. Theoretical study of the solubility of metals at different pH values

In order to evaluate if toxic metals were mostly in soluble species or precipitated forms, the software Visual MINTEQ 3.1 (Gustafsson, 2016) was used as an alternative chemical equilibrium model. The values of total metals (Cd, Cr, Cu, Ni and Zn), total phosphorus and nitrogen and sulfate obtained in the initial characterization of sewage sludge were used (Table 1), such as the fixed values of S^0 (2.0 g/L) Fe^{2+} (2.0 g/L) and temperature (30 °C).

3. Results and discussion

3.1. Physicochemical parameters during the bioleaching process

Pathak et al. (2009) state that nitrogen and phosphorus can respectively reach concentrations between 1.5-6.0% and 0.8–11.0% of TS and in the present study, values of 0.7% of TS for nitrogen and 0.2% for phosphorus were found.

MPN assays to determine endogenous *Acidithiobacillus* and *Thiobacillus* from sewage sludge indicated that *A. ferrooxidans*, *A. thiooxidans* and *T. thioparus* were already present in the material analyzed. An endogenous bacterium of sewage sludge (*T. thioparus*) is related to the bioleaching process besides acidophilic bacteria (Chen and Lin, 2001) and is even one of the main bacteria responsible for natural acidification of sewage sludge (Blais et al., 1993).

The sewage sludge was submitted to FT-IR analysis to determine the functional groups. The results are presented in the frequency of 4.000–400 cm^{-1} and the analyses of the functional groups corresponding to the bands were performed according to Silverstein and Webster (1981) (Fig. 1). A broad band of approximately 3.500 cm^{-1}, characteristics for stretching hydroxyl (OH) groups of carboxylic acid, water, alcohols and/or phenols and amides and amines (N-H) were observed. It is known that hydroxylic and carboxylic groups can adsorb the metals, forming chelates that are considerably stable (Pinheiro, 2007).

In the portion between 2.960 and 2.850 cm^{-1}, there are aliphatic hydrocarbon groups (CH_3 and CH_2). These groups can be found in hydrocarbons and cellulose, for example, which are very common in sewage sludge. Bands around 1.000 cm^{-1} may be related to silicates and/or to sulfoxide groups (S=O). Identifying functional groups becomes less representative after the region known as

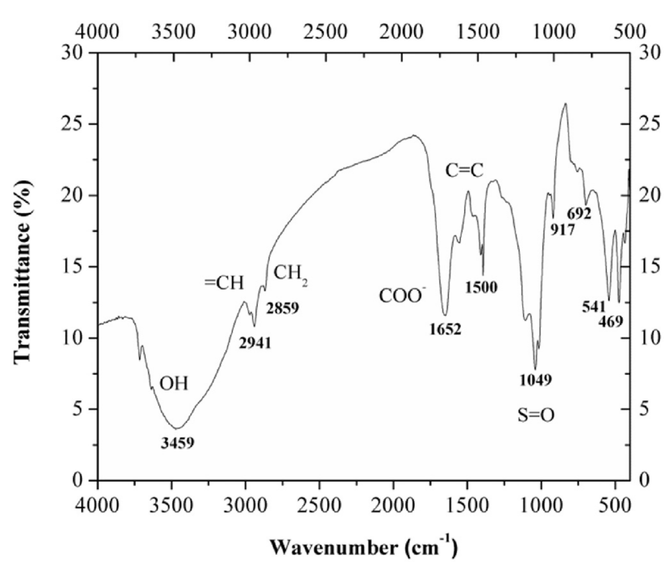

Fig. 1. Fourier Transform Infrared Spectroscopy (FT-IR) of anaerobic sewage sludge. Spectrogram generated by the OriginPro8 program.

"fingerprint" (<900 cm^{-1}), however bands in this region may suggest the presence of cycloaliphatic structures, aromatic structures, halogenated derivatives and also compounds containing phosphorus (Pedroza et al., 2011).

The characteristic bands of C-OH groups in phenols at 1.270 cm^{-1} were not observed, and nor were the characteristic bands of C=O stretches of the COOH group, aldehydes and ketones at 1.710 cm^{-1}, which may suggest both their non-existence as the link between these groups and the metals present in the sewage sludge. In addition, the band at approximately 1.600 cm^{-1} may suggest the presence of C=C of aromatics, especially due to their intensity, but the presence of an asymmetric stretching band of COO at approximately 1.650 cm^{-1} may also suggest the formation of metal complexes (Polak et al., 2005).

3.2. Concentrations of metals during the bioleaching process

The metal characterization of sewage sludge was again performed after the incubation period of the reactors (10 days) (Table 1). The pH was quite stable in the control reactor remaining around 6.0 (Fig. 2-A). Changes in the pH throughout the incubation period can be possible attributed to iron hydrolysis and chemical/aerobic decomposition of organic matter (Zhou et al., 2013b).

The pH of the medium is an important parameter because it can directly influence sorption/desorption reactions, precipitation/dissolution, complex formation and oxidation-reduction reactions (McLean and Bledsoe, 1992).

Gradual acidification of bioleaching and co-inoculation assays can be attributed to the presence of *Acidithiobacillus* inoculum (Chen and Lin, 2001; Pathak et al., 2009). Both assays reached a final pH close to 2.0 after the incubation period and this value is typical of the final stage of the bioleaching process (Zheng and Zhou, 2011).

The concentration of all evaluated metals gradually increased over the incubation period in the bioleaching and co-inoculation assays, but to a lesser extent in the co-inoculation assay, while the pH decreased similarly.

The increase in the concentration of metals in the solution occurs due to the acidification of the medium as the cationic elements in interaction with the solid phase of the sludge are adsorbed or complexed in the particulate or organic matter of the sludge, while the pH is close to neutrality. Although adsorption can be pH dependent, acidification of the medium decreases the negative sites present in the organic matter, such as carbonates, clay minerals, iron, manganese oxides, among others, reducing the possibilities of adsorption sites for cationic elements while increasing competition between these ions and also among the other cationic ions present in the solution, such as Al^{3+} and H^+ (McLean and Bledsoe, 1992).

As expected, the concentration of metals in the solid phase of the sludge tends to decrease as the concentration in the liquid phase tends to increase. All the elements presented a higher percentage of solubilization in the bioleaching assays than in the control assays, due to the acidification of the latter through the activity of acidophilic bacteria of the genus *Acidithiobacillus* (Fig. 2).

According to the data obtained and shown in graphs in Fig. 2-B-F, all metals evaluated reached their maximum concentration in the liquid phase at the end of the treatment, coinciding with the more acidic pH reached by the reactors, of approximately 2.0 exceptions: Cd, which reached the maximum concentration at 216 h in the bioleaching assay and 96 h in co-inoculation assay, and Cu, which reached its maximum concentration in the co-inoculation assay at 168 h.

In the co-inoculation assay, Cd presented a final result lower than the final result of the bioleaching assay (Fig. 2-D), however this assay reached its highest solubilization of metals in 96 h. Furthermore, at this point, the solubilization of this metal was higher than the final result of the bioleaching assay. Possibly the presence of yeast favored the solubilization of this element (Zhou et al., 2013b).

All treatments presented a statistically significant difference in relation to the control assay ($p < .001$), except for Cr in the co-inoculation assay ($p > .05$). There was also a significant statistical difference between bioleaching and co-inoculation assays, except for Cu ($p > .05$) and Ni ($p > .05$).

The metal solubilization Cu (37.4%), Ni (53.1%) and Zn (70.3%) in the co-inoculation assay presented the best results compared to the control assay, where the solubilization percentage was only 3.4; 5.18 and 3.2%, respectively, while for Cd (17.9%), Cr (12.6%) and Pb (8.1%), there was also greater solubilization in relation to the control assay, where 12.7; 10.0 and 3.8% solubilization was obtained, respectively.

Ni and Zn presented a solubilization percentage of 59.8 and 76.5% for the co-inoculation assay and 53.1 and 70.3% for the bioleaching assay, respectively. The co-inoculation presented results of solubilization of metals higher than the control assay. These data are not in agreement with the results obtained for analyzing the metals in the liquid phase at the end of the tests. According to these data, it was expected that at the end of the incubation period, the bioleaching assay would be more efficient for all the metals studied, without exceptions, because at the end of the incubation period, there was more solubilization of these elements in the co-inoculation assay (Fig. 3).

The solubilization results obtained are lower than those presented in other research regarding some metals (Wen et al. (2013) for Zn, Cu and Pb (88, 79 and 50%, respectively)). However, it is important to note that the incubation period used by these authors was longer (12 days) and this can increase the chances of nutrient loss (Pathak et al., 2009). Furthermore, the results obtained from the control experiment (sterilized sewage sludge + energy source) are also better when compared to this study (80.2% for Zn, 21.8% for Cu and 10.9% for Pb). That is, in spite of their final solubilization values being higher, these authors obtained only 7.8% more solubilization for Zn than in their control, while in the conditions evaluated in the present study, there is 67% more solubilization of Zn in the bioleaching assay in relation to the control assay.

When there are no significant differences between the control and bioleaching assays, the leaching of metals is attributed mainly to the chemical leaching and the bioleaching becomes secondary (Wen et al., 2013). Solubilization varies according to the evaluated metal as each element has its own adsorption/complexation characteristics in specific pH ranges (McLean and Bledsoe, 1992).

Similarly, Wong et al. (2004) also obtained final solubilization values higher than those obtained in the present study. They were 99% for Zn, 65% for Cr, 74% for Cu, 58% for Pb and 84% for Ni, while in its control assay (sludge + energy source) obtained 94%, 12%, 21%, 32% and 38% for these same metals, respectively. Thus, it can be stated that the solubilization was only 5% higher for Zn, 62% for Cr, 53% for Cu, 26% for Pb and 46% for Ni. Zn and Ni presented higher values of solubilization in the present study and were 67% and 47.9%, respectively. It is important to note that the incubation time used by Wong et al. (2004) was also higher (16 days).

Based on the data obtained and shown in Fig. 2, it can be seen that the co-inoculation assay was less efficient compared to the bioleaching assay for all metals, except for Cd, as discussed above. It was expected that, as described by Zhou et al. (2013b) when adding yeasts of the genus *Galactomyces* to the reactors, the addition of *M. guilliermondii* could also promote greater solubilization of the other metals and in a shorter incubation time. However, this was not observed for Cr, Cu, Ni and Zn. In Zhou et al. (2013b), the co-inoculation of *Galactomyces* and *Acidithiobacillus* increased the

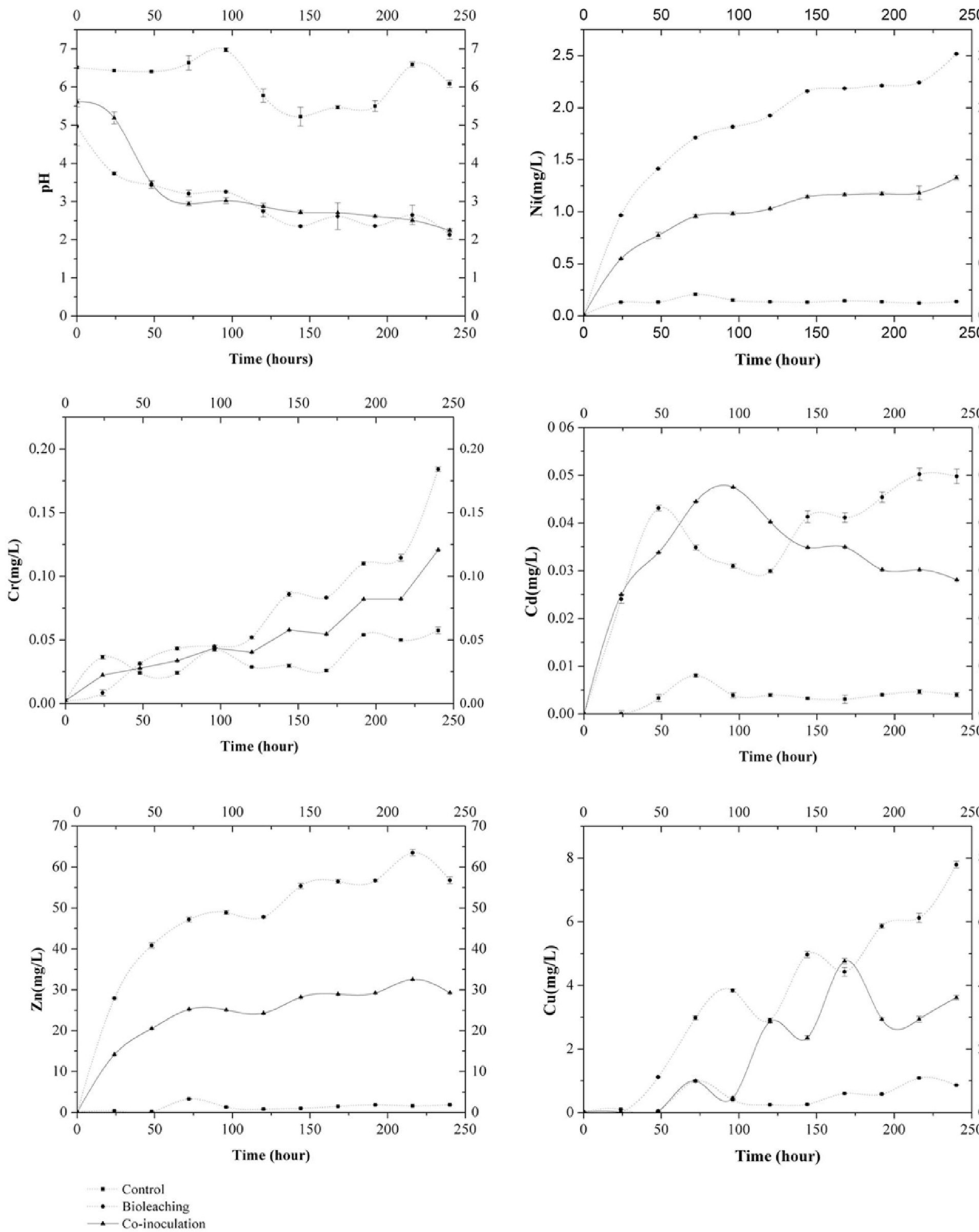

Fig. 2. Variation of acidity (pH) of the reactors (A) and concentration (mg/L) of Ni (B), Cr (C), Cd (D), Zn (E) and Cu (F) in the liquid phase of the sludge over the 240 h period.

bioleaching efficiency in sewage sludge, with solubilization obtained from 82% for Cu and 92% for Zn in the co-inoculation assays, and 64% for Cu and 84% for Zn in the same range, 132 h in the assays containing only *Acidithiobacillus*.

The results obtained show that adding yeast promoted a delay that was not expected in the solubilization of the metals (Fig. 2A). One possible explanation is that this species could be a bioaccumulator of metals, or also that its cell wall can act as a good adsorbent for these elements (Bishnoi and Garima, 2005), therefore more studies on the interaction between these elements and these microorganisms would be needed. Previous studies have demonstrated the possibility of using other genera of yeasts, such as

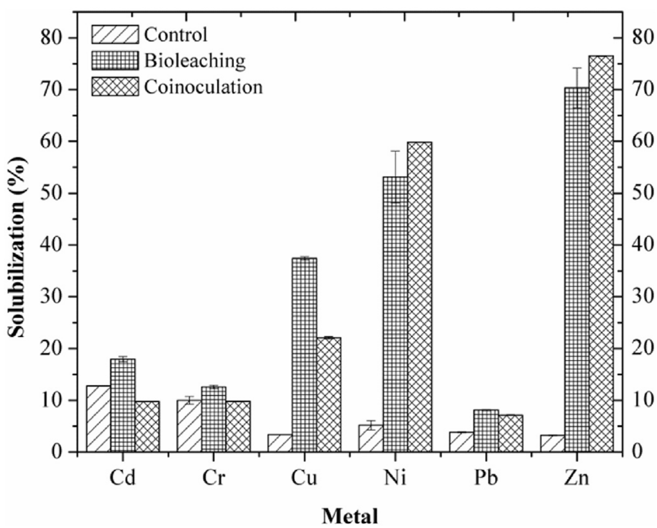

Fig. 3. Solubilization (%) of potentially toxic metals from the solid phase of sewage sludge after different assays. Error bars are relative to two independent samplings.

Saccharomyces cerevisiae, as sorbent material capable of removing metal ions such as Cd (II) from river water (Biscaro et al., 2007; Menegário et al., 2010) and bioaccumulation of Cu(II) by *Pichia guilliermondii* (teleomorph *Meyerozyma guilliermondii*) (De Silóniz et al., 2002; Menegário et al., 2007), which may explain the decrease in the solubilization of this metal in the co-inoculation assays (Fig. 3).

It is important to note that the sorptive affinity of the metal depends on its properties, surface type and also experimental conditions (McLean and Bledsoe, 1992), therefore it is important that differences in the solubilization potential of metals of different treatments are compared for the same matrix, especially in the case of sewage sludge, which varies greatly in composition and physicochemical properties.

The correlation coefficient analyses between the metal solubility and pH (Table 2), the highest correlations between pH and solubilization of metals were observed for Ni and Zn metals in the bioleaching assay and for metals Cd, Ni and Zn in the co-inoculation assay. These data show that, in general, although the bioleaching assay presented a greater solubilization of metals, in the co-inoculation assay this solubilization seems to be more related to the decrease in the pH for a greater number of metals.

3.3. Theoretical study of the solubility of metals at different pH values

The indirect evaluation of the solubility of different metals (Cd, Cr, Cu, Ni, Pb and Zn) at the initial (7.4) and final (2.0) pH of the sewage sludge after the bioleaching tests was done using the Visual MINTEQ 3.1 software (Gustafsson, 2016) in order to predict the formation of precipitates that could interfere with analysing the solubility of metals.

Based on the analyses, it was observed that all elements would be predominantly in the form of soluble species under the conditions analyzed, except for Pb, since at pH 7.4, approximately 13.5% of this element was present in the $PbSO_4$ species and 1% as Pb $(SO_4)_2^{2-}$, both insoluble. Similarly, at pH 2.0, 23.6% of the total Pb would be in the form of $PbSO_4$ and 1.4% in the form of Pb $(SO_4)_2^{2-}$. Thus, the results of the analyses carried out in the bioleaching experiments for this element may be underestimated due to the formation of precipitates.

According to Pathak et al. (2009), although sulfur was added in an elemental form as an energy source at the beginning of the tests, it is understood that there is no formation of sulphides throughout the treatments, since due to the oxidizing atmosphere, as well as the microbial metabolism, the tendency would be the formation of H_2SO_4.

3.4. Potential of bioleached sewage sludge applied on farmland

Key points to be verified for sewage use in agricultural land are: contain a low concentration of contaminants regarding metals, the maximum concentrations of metals allowed for this use and their cumulative effects (Tsutiya, 2015).

The sewage used seems to be a promising option on low income countries. Indeed, according to Nogueira et al. (2013), the use of sewage sludge as fertilizer to increase the concentration of nutrients in soils can also increase the concentration of many metals in the agricultural systems, however it poses no hazard to the environment, even in Brazilian soils, which can be quite different from temperate soils. Thus, the economic potential of using sewage sludge as a substitute for commercial fertilizers should be considered.

In the State of São Paulo, Brazil, the maximum permitted metal limits in biosolids were based on the maximum limits allowed in the United States and defined in Standard P4.230 (CETESB, 1999). Furthermore, its definition was based on risk analysis, whereas the definition of the European Community and Canadian standards, for example, used the concept of non-degradation of the soil and the environment (Tsutiya, 2015).

Table 3 shows a comparison between the maximum permissible concentrations of metals for agricultural use in different localities, the initial concentrations of metals in the anaerobic sewage sludge and the concentrations obtained in the solid phase of the sewage sludge after the bioleaching tests and co-inoculation. The product obtained after the bioleaching and co-inoculation tests reached the maximum permissible concentrations for Zn in the State of São Paulo/United States. In addition, it is within the limits for other metals according to the requirements of the European Community (Tsutiya, 2015).

Table 2
Correlation coefficient and P value. Data obtained by Graph Pad Instat software for parametric ANOVA test with 95% confidence interval.

Metal	Control	Bioleaching	Co-inoculation
Cd	0.0356; p = .9761	−0.8941; p = .0002	−0.9630; p < .0001
Cr	0.2259; p = .50−42	−0.8248; p = .0018	−0.7720; p = .0054
Cu	0.1586; p = .6414	−0.8651; p = .0006	−0.7150; p = .0134
Ni	−0.3745; p = .2563	−0.9558; p < .0001	−0.9776; p < .0001
Zn	0.0131; p = .9695	−0.9453; p < .0001	−0.9853; p < .0001

Table 3
Comparison between the maximum permissible concentrations of metals in the biosolids for agricultural use in different localities, the initial concentrations of metals in the anaerobic sewage sludge and the concentrations obtained from the sewage sludge after the bioleaching and co-inoculation tests.

Local	Maximum concentration allowed, mg/kg					
	Cd	Cr	Cu	Ni	Pb	Zn
Bioleaching	17.2	542.5	1378.6	166.9	414.6	2438.6
Co-inoculation	18.2	552.0	1714.2	140.3	421.6	2008.4
Initial concentration	20.7	619.9	2201.0	356.1	451.2	8216.8
São Paulo	85	–	4300	420	840	7500
United States	85	–	4300	420	840	7500
European Community	40	–	1750	400	1200	4000
Canada	20	–	–	180	500	1850

It can be observed that the sewage sludge used in this study was at the beginning of the experiments within the limits of the maximum permitted concentrations in the State of São Paulo and in the United States, except for Zn, where the maximum permissible concentration is 7500 mg/kg and the result obtained after the initial characterization of the material was 8216.8 mg/kg. However, when countries with less lenient and more environmentally friendly laws are taken into account, such as the European Community and Canada, it is understood that all metals analyzed, with the exception of Pb, would be above the maximum limits allowed.

4. Conclusions

After 10 days of incubation at 30 °C, besides a reduction of the nitrogen concentration by 20.1% and phosphorus by 7.6%, the co-inoculation of *M. guilliermondii* and *Acidithiobacillus* was able to solubilize 76.5% of Zn, 59.8% of Ni, 22.0% of Cu, 9.8% of Cd, 9.8% Cr and 7.1% of Pb. *M. guilliermondii* inoculation accelerated the solubilization of Cd, reducing the required time from 240 to 96 h.

Since the co-inoculation assays presented lower solubilization for the other studied metals, it is possible to infer that the addition of the yeast strain promoted a delay in the solubilization of these metals, suggesting that the specie *M. guilliermondii* could be a bioaccumulator of metals, or also that its cell wall can act as an adsorbent for these elements. Therefore, more studies on the interaction between these elements and these microorganisms would be needed.

After the bioleaching and co-inoculation tests, the product obtained reached the maximum permissible concentrations for soil disposal for all the analyzed metals in the State of São Paulo and in the United States standards and also with requirements of the European Community. It is important to note that even if current methods cannot generate an end product that can be used as fertilizers, treating sewage sludge should not be neglected because its inadequate disposal is environmentally harmful. Furthermore, it should be considered that its application can improve the physical characteristics of the soil, although it does not influence its nutritional characteristics.

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