



Application of the Biosurfactant produced by *Candida lipolytica* in the Remediation of Heavy Metals

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Soil that is accidentally contaminated with petroleum hydrocarbons can be remediated by physical, chemical, or biological methods. However, new trends in soil and water restoration avoid introducing synthetic chemicals. Among the remediation techniques available for contaminated sites, bioremediation is regarded as environmentally friendly because it preserves the soil structure, requires little energy input, and involves the complete destruction or immobilization of the contaminants, although the efficiency of biodegradation of oil pollutants is often limited by their poor water solubility. Despite their advantages, due to the high production costs biosurfactants are not extensively used. In this sense the lipopeptide biosurfactant produced by *Candida lipolytica* (UCP 0988), is an attractive alternative to the synthetic surfactants, showing properties in bioremediation of heavy metals. The biosurfactant reduced the surface tension of the production medium from 50 mN/m to 25 mN/m, with a yield of 8 g/L, after 72 h of fermentation. The biosurfactant showed a CMC of 0.03 %. The lipoprotein removed 96 % of Zn and Cu, and reduced the concentration of Pb, Cd and Fe. The optimization process of biosurfactant production, which permitted the reduction of costs, suggests its ample utilization according to commercial requirements.

1. Introduction

With the growth of industrial production, many products are improperly accumulated in the environment, contaminating soil and groundwater. There are several types of contaminants and according to Sparks (1995) water and soil contaminants include inorganic species such as nitrate and phosphate, heavy metals like cadmium, chromium, mercury and lead, organic chemicals, including hydrophobic compounds, inorganic acids and radionuclides. In the natural soil, these contaminants cannot be completely degraded and may be more or less mobile in the soil (Rufino et al., 2011).

Several physicochemical and biological methods, or a combination of both, are used to treat slurry. According to Gomes (2000), the biological process allows the biodegradation of organic compounds in slurry by the action of microorganisms that turn them into simpler substances as water, carbon dioxide and methane. The physical-chemical treatment combined with biological treatment, instead, can eliminate the undesirable leaching of chemical species in the effluent, such as heavy metals and organic compounds. This study is focused on the use of the technique of permeable reactive barrier in the treatment of the slurry generated within a landfill. Some of the most important processes include precipitation, sorption, oxidation/reduction, fixation, and degradation. These processes may occur simultaneously to absorb the pollutants from the groundwater or to convert harmful chemicals to harmless by-products (Gomes 2000).

Surfactants are chemical compounds consisting of amphipathic molecules that partition preferentially at the interface between fluid phases that have different degrees of polarity as oil/water or air/water interfaces (Rufino et al., 2011; Luna et al., 2011). These features allow surfactants to reduce surface and interfacial tension and forming microemulsions where the oil can be solubilized in water or where water can be solubilized in hydrocarbons (Muthusamy et al., 2008). These properties enable a wide range of industrial applications involving detergency, emulsification, lubrication, foaming capacity, wettability, solubilization and phases dispersion. Considering that few technologies can be used together in the treatment of organic pollutants and heavy metals, the biosurfactants, depending on their properties, can be applied not only in the removal of hydrophobic organic compounds, as well as in the removal of heavy metals. Biosurfactants act reducing the interfacial tension and solubilizing hydrocarbons in the aqueous phase or via the capture of oil droplets within their micelles, while biosurfactants of anionic nature, can also capture the metal ions through electrostatic interactions or complexation (Rufino et al. 2008; Batista et al., 2010).

The objective of this study was to investigate the applicability of a yeast biosurfactant that shows excellent surfactant properties in the treatment of contaminants from municipal solid waste contained in a soil used as slurry barrier.

2. Materials and Methods

2.1 Microorganism

The microorganism *Candida lipolytica* UCP 0988 was kindly supplied from the Culture Collection of Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Recife-PE, Brazil. The microorganism was maintained as the anamorph state at 5 °C on Yeast Mold Agar (YMA) slants containing (w/v): 0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose and 2 % agar. Transfers were made to fresh agar slants each month to maintain viability.

2.2 Growth Conditions

The production medium used for the experiments consisted of the following: 0.1 % NH_4NO_3 , 0.02 % KH_2PO_4 and 0.02 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The medium was supplemented with soybean oil refinery residue, glutamic acid and yeast extract. The refinery residue was obtained from ASA Indústria e Comércio LTDA (Recife-PE, Brazil). The composition of the refinery residue and the biosurfactant was produced as previously described by Rufino et al. (2008).

2.3 Isolation of biosurfactant

The 72 h culture was filtered through Whatman no. 1 filter paper and centrifuged at 5000 x g for 20 min. The cell-free broth was concentrated (500 m/L) by freeze drying and extracted two times with chloroform (1:1, by vol.) in a separatory funnel at 28 °C (Cirigliano and Carman, 1984).

2.4 Surface tension and Critical Micelle Concentration (CMC)

The surface tension was measured by the ring method using a DuNouy Tensiometer model Sigma 70 (KSV Instruments LTD, Finland) at room temperature. The concentration at which micelles began to form was represented as the Critical Micelle Concentration (CMC). At the CMC, sudden changes in surface tension, electrical conductivity and detergency were observed. The CMC was automatically determined by measuring the surface tensions of the purified biosurfactant in distilled water up to a constant value of surface tension.

2.5 Analytical methods

Protein in the isolated biosurfactant was estimated by using the total protein test kit from Labtest Diagnóstica S.A., Brazil. Sugars were measured by the phenol-sulphuric acid method using glucose as standard (Dubois et al., 1956). To determine the lipid content, 0.5 g of the isolated material was extracted with chloroform: methanol in different proportions (1:1 and 1:2 v/v). The organic extracts were then evaporated under vacuum and the lipid content determined by gravimetric estimation (Manocha et al. 1980).

2.6 Soil

A barrier silty soil was utilized in the experiments. The soil was collected from Abreu e Lima City, in Pernambuco state, Brazil. Samples of the soil (5 kg) were homogenized and left to stand at room temperature for four days to dry and finally stored for later use.

2.7 Soil samples preparation

The soil was air dried, passed through a 50 mesh (0.297 mm opening), and mixed with tap water to a water content of 19 % (natural soil). To simulate a contamination of the slurry and subsequent compaction of the test specimen, it was necessary to proceed with manual homogenization of the two phases. The natural soil and the mixture soil plus waste slurry were statically compacted (optimal water content of 19 and 13 %, respectively) using a standard Proctor method (ABNT, 2004) at a specific dry weight of 16.00 kN/m³, extruded from the compaction mold, and trimmed to a diameter of 98 mm using a thin walled sharp edge pipe. The trimmed soil had a height of 109 mm.

The soils chemical analyses were performed in the inflow and outflow of each test specimen percolated by the contaminant. The site of sectioning was defined as the midpoint orthogonal to its height, parallel to this stream. The description of the specimens and their proper contamination and percolates are shown in Table 1.

Table 1: Test specimens and percolates used in the experiments

Identification of test specimens	Percolate types
NS	Natural soil (soil before percolation of contaminants)
S-DW	Soil percolated by distilled water
S-SL	Soil percolated by the slurry
S-SL-B	Soil percolated by the slurry followed by the biosurfactant

2.8 Slurry

The natural slurry was obtained from the Aguazinha landfill, located in Olinda city, Pernambuco state, Brazil. The chemical characterization of the slurry was held in the Laboratories of Chemical Engineering from Federal University of Pernambuco, according to APHA (1995). The slurry was used as a soil percolate.

2.9 Heavy metals determination in the soil samples

The soil samples were analyzed for heavy metals before and after percolation of the fluids by flame atomic absorption spectrophotometry using a Varian AA-220 FS instrument.

3. Results and Discussion

The biosurfactant produced was isolated in the aqueous-phase as a brown precipitate. The average yield of precipitate was approximately 8 g/L. One of the important characteristic properties of a potent surfactant is its ability to lower the surface tension in aqueous solutions.

Surface tension reduction depends on the specific concentration of the surface-active compound, the CMC which is defined as the minimum concentration of biosurfactant required to give a maximum surface tension reduction of water and initiate micelle formation (Coimbra et al., 2009; Silva et al., 2010). Members of the genus *Candida* have been recently shown to produce highly effective biosurfactants able to reduce the surface tensions of water to low values (Rufino et al. 2008; Sarubbo et al. 2007; Batista et al., 2010; Luna et al. 2011). Such values are lower than those of synthetic surfactants in the partially purified and even in cell free broth preparations containing biosurfactants.

From Figure 1, it is evident that the the biosurfactant from *Candida lipolytica* cultivated in the vegetable fat waste was able to reduce the surface tension of water from 71 mN/m to 25 mN/m. The CMC of the crude biosurfactant was computed as 0.03 % which is within the range of CMC values reported for different types of biosurfactants produced by other *Candida* species (Sarubbo et al. 2006; Rufino et al. 2008; Luna et al. 2009; Batista et al., 2010).

Preliminary chemical characterization of biosurfactant revealed that the examined agent was a lipoprotein material which consisted of protein (50 %), lipid (20 %) and carbohydrate (8 %). Differently,

the emulsifier Liposan produced by *Candida lipolytica* grown in hexadecane as substrate is composed of 83 % carbohydrate and 17 % of protein (Cirigliano and Carman, 1985).

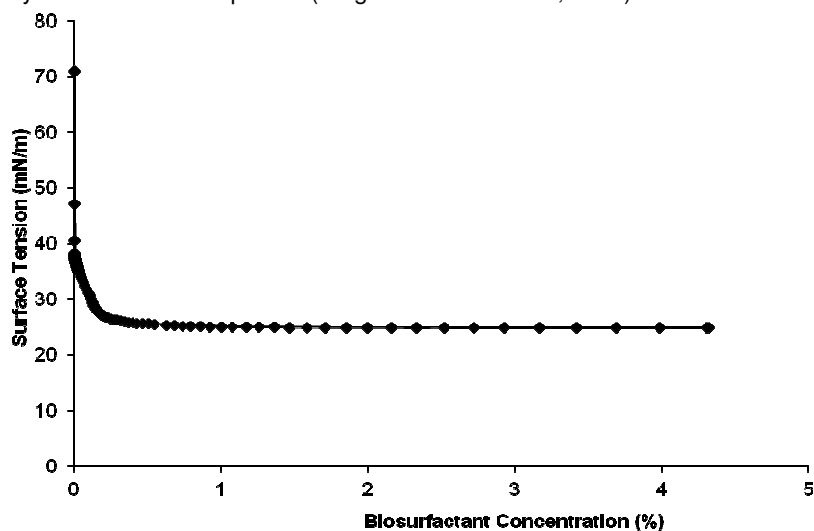


Figure 1: Critical micelle concentration of the biosurfactant isolated from *Candida lipolytica*

Others biopolymers produced in our laboratory from *C. lipolytica* seem to be polysaccharide-protein-lipid complexes, as the emulsifier obtained from glucose as substrate which contains about 47 % protein, 45 % carbohydrate and 5 % lipid (Sarubbo et al. 2001) and the emulsifier obtained from babassu oil, composed by 60 % carbohydrate, 23 % protein and 1.1 % lipids (Sarubbo et al. 1999). The biosurfactant produced by *Pseudomonas aeruginosa* UCP0992 in medium supplemented with glycerol as carbon source was characterized as glycolipids (Silva et al., 2010).

The possible mechanisms for the extraction of heavy metals by biosurfactants are ion exchange, precipitation–dissolution, and counterion binding. The polar head groups of micelles can bind metals. This makes the metals more soluble in water. Surfactant monomers likely acted to solubilize adsorbed metals through formation of dissolved complexes. In addition, some binding of the metal may occur to the anionic exterior of surfactant micelles. Surface tension will predominately influence sorption that occurs through hydrophobic interactions such as partitioning of non-ionic organic compounds rather than metals (Asçi et al., 2008). Biosurfactants used for soil treatment are also required to have minimal sorptive interactions applied to the soil system, in other words, most of the biosurfactant should remain in the aqueous phase (Asçi et al., 2008).

Table 2: Release of metals from test specimens before and after percolation of the fluids

Heavy metals	Quantification of heavy metals in the test specimens				
	Soil (g/kg)	S-SL ^a (g/kg)		S-SL-B ^a (g/kg)	
		Inflow	Outflow	Inflow	Outflow
Fe	52.500	48.075	48.080	44.500	50.985
Cd	0.0009	0.0010	0.0010	0.0005	0.0007
Zn	0.0191	0.0144	0.0144	0.3616	0.0137
Cu	0.0133	0.0076	0.0041	0.1615	0.0051
Pb	0.014	0.015	0.013	0.013	0.017

S-SL: Soil percolated by the slurry; S-SL-B: Soil percolated by the slurry followed by the biosurfactant

In this sense, the low cost lipoprotein biosurfactant produced from the cultivation of the yeast *Candida lipolytica* in soybean refinery residue, capable of reducing the surface tension of water to 25 mN/m was tested in the removal of heavy metals contained in specimens contaminated with slurry in a

permeability apparatus. The tests of flame atomic absorption spectrometry made in the natural soil and in the tested specimens S-SL and S-SL-B are shown in Table 2. All comparisons made of increasing and decreasing percentages were taken based on the levels of each metal in the natural soil.

For soil mixed with waste oil the percolation of the biosurfactant caused a reduction in the iron concentration around 16.5 % and in cadmium concentration of 50 % in the outflow section of the specimen. With respect to zinc, there was practically no change in the concentration of this metal. In the case of copper and lead, there was a displacement of these metals to the outflow section of the soil, thus increasing their concentrations at 20 and 17 % respectively.

For the soil percolated by the slurry, no changes were observed in the concentrations of metals on the top (inflow section), except with respect to copper and lead, which were reduced by 46 and 13 % respectively. For the soil percolated by the slurry and subsequently by the biosurfactant, there was an increase in the concentration of iron and cadmium in the base (outflow section) of the test specimen over the top, although the values found are below the respective concentrations of these metals in the natural soil. For zinc and copper, it was observed a reduction of concentrations in the base of the specimen over the top of 96.2 and 96.8 % respectively. To lead, it was observed that there was a shift of the metal to the base of the test specimen of 15.4 %.

The results showed that the biosurfactant was found to be effective for use in bioremediation mainly of zinc and copper. The concentration of the metals in the natural soil attended to the recommendations of the São Paulo State Environmental Company, Brazil, (CETESB, 2001), except for the concentration of cadmium, which exceeded the limit of 0.0005 g/kg.

The possibility of the use of biosurfactants in the removal of heavy metals has been demonstrated in some studies conducted in laboratory scale. The surfactant saponin, for example, which has a surface tension of 36 mN/m, was tested at concentrations of 0.1 and 10 %. The removal of heavy metals in the soil was proportional to its concentration (Hong et al. 2002).

Mulligan et al. (2001) demonstrated the increased removal of Zn when 2 % surfactin was used in combination with NaOH. Daharazma and Mulligan (2007) observed that the removal percentage of the heavy metals in the soil increased linearly with increasing concentration of the rhamnolipid tested. The use of 5 % of the rhamnolipid removed 37 % Cu, 7.5 % Zn and 33.2 % Ni. Two agents compatible environmentally, ie a rhamnolipid (0.5 %) with a CMC of 0.005 % (0.05 g/L) and carboxi methyl cyclodextrin (5.3 %) were tested for removal of metals adsorbed in two soil types. After ten successive washes, the rhamnolipid was able to remove 14.2 and 15.3 % of Pb contained in the soils tested, while the CMCD removed 5 and 13.4 % of this metal in the soils tested (Neilson et al. 2002).

Since there are no reports in the literature for trials similar to those used in this work with the biosurfactant from *Candida lipolytica* (UCP 0988), it becomes difficult to discuss our results, although the percentage removal obtained can be considered satisfactory when compared to those reported in other experimental conditions discussed above, since we used the cell-free broth containing the biosurfactant, ie the crude biosurfactant. The action of the surfactant was higher than the soil-contaminant interaction, since it was able to remove the heavy metals detected. Importantly, the depth of the soil significantly influenced the outcome of removal. The results show that the biosurfactant can be applied with municipal solid waste barrier in reducing soil permeability and in the simultaneous recovery of heavy metals. The environmental compatibility and the possibility of combination with conventional methods of remediation drive the development of this alternative technology.

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5. References

ABNT, Associação Brasileira de Normas Técnicas. 2004. NBR 10.005: Procedure for obtaining extract leached solid waste (In Portuguese), <www.usiferr.com.br/ABNT.asp>, Accessed 24/05/2012.

- APHA, Standard methods for examination of water and wastewater. 1995. American Public Health Association, 19th ed. Washington, United States.
- Asçi Y., Nurbas M., Açikel Y.S., 2008, A comparative study for the sorption of Cd(II) by soils with different clay contents and mineralogy and the recovery of Cd(II) using rhamnolipid biosurfactant, *Journal of Hazardous Materials*, 154, 663-673.
- Batista R.M., Rufino R.D., Luna J.M., Souza J.E.G., Sarubbo L.A., 2010, Effect of Medium Components on the Production of a Biosurfactant from *Candida tropicalis*. Applied to the Removal of Hydrophobic Contaminants in Soil. *Water Environment Research*, 82, 418-425.
- Calvo C., Manzanera M., Silva-Castro G.A., Uad I., González-Lopéz J., 2009, Application of bioemulsifiers in soil oil bioremediation processes. Future prospects, *Science of the Total Environment*, 407, 3634-3640.
- CETESB. Companhia Ambiental do Estado de São Paulo, 2001, Report on establishment of guiding values for soils and groundwater in the state of Sao Paulo (In Portuguese). São Paulo, Brazil.
- Coimbra C.D.; Rufino R.D.; Luna J.M.; Sarubbo L.A., 2009. Studies of the cell surface properties of *Candida* species and relation with the production of biosurfactants for environmental applications. *Current Microbiology*, 58, 245-249.
- Gomes L.P., 2000, Generation, features and processes related to manure generated by the degradation of municipal solid waste. *Revista do Centro de Ciências Exatas e Tecnológicas da UNISNOS. São Leopoldo, Brazil.*
- Hong K.J., Tokunaga S., Kajiuchi T., 2002, Evaluation of remediation process with plant-derived biosurfactant for recovery of heavy metals from contaminated soils, *Chemosphere*, 49, 379-387.
- Luna J.M., Sarubbo L.A., Campos-Takaki G.M., 2009, A new biosurfactant produced by *Candida glabrata* UCP1002: characteristics of stability and application in oil recovery. *Brazilian Archives of Biology and Technology*, 52, 785-793.
- Luna J.M., Rufino R.D., Albuquerque C.D.C., Sarubbo L.A., Campos-Takaki G.G., 2011, Economic Optimized Medium for Tensio-Active Agent Production by *Candida sphaerica* UCP0995 and Application in the Removal of Hydrophobic Contaminant from Sand. *International Journal of Molecular Sciences*, 12, 2463-2476.
- Mulligan C.N., Yong R.N., Gibbs B.F., 2001, Surfactant-enhanced remediation of contaminated soil: a review. *Engineering Geology*, 60, 371–380.
- Muthusamy K., Gopalakrishnan S., Ravi T.K., Sivachidambaram P., 2008, Biosurfactants: properties, commercial production and application. *Current Science*, 94, 736-747.
- Neilson J.W., Artiola J.F., Maier R.M., 2002, Characterization of lead removal from contaminated soils by non-toxic soil-washing agents. *Journal of Environmental Quality*, 32, 899–908.
- Rufino R.D., Sarubbo L.A., Campos-Takaki G.M., 2008, Enhancement of stability of biosurfactant produced by *Candida lipolytica* using industrial residue as substrate. *World Journal of Microbiology and Biotechnology*, 23, 729-734.
- Rufino R.D., Rodrigues G.I.B., Campos-Takaki G.M., Sarubbo L.A., Ferreira S.R.M., 2011, Application of a Yeast Biosurfactant in the Removal of Heavy Metals and Hydrophobic Contaminant in a Soil Used as Slurry Barrier. *Applied and Environmental Soil Science*, 2011, 1 - 7.
- Sarubbo L.A., Marçal M.C., Neves M.L.C., Silva M.P.C., Porto A.L.F., Campos-Takaki G.M., 2001. Bioemulsifier production in batch culture using glucose as carbon source by *Candida lipolytica*. *Appl. Biochem. Biotechnol.*, 95, 59-67.
- Sarubbo L.A., Farias C.B.B., Campos-Takaki G.M., 2007, Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*, *Curr. Microbiol.*, 54, 68-73.
- Sarubbo L.A., Luna J.M., Campos-Takaki G.M., 2006, Production and stability studies of the bioemulsifier obtained from a new strain of *Candida glabrata* UCP 1002. *Eletronic J. Biotechnol.*, 9, 400-406.
- Silva S.N.R.L., Farias C.B.B., Rufino R.D., Luna J.M., Sarubbo, L.A., 2010, Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. *Colloids and Surfaces. B, Biointerfaces*, 79, 174-183.
- Sparks D.L., 1995, *Environmental soil chemistry*. Academic Press, San Diego, United States, 267 p.