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Application of a Low Cost Biosurfactant in the Removal of Heavy Metals in Soil

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Several synthetic surfactants have been used in heavy metals decontamination tests in soils. However, the need of substitution of synthetic compounds by natural tensoactives has motivated researches for the use of surfactants of microbiological nature, the so-called biosurfactants. The use of biosurfactants in bioremediation processes has stood out due to the characteristics of these compounds as biodegradability, reduced toxicity and efficiency in the removal of heavy metals from soils and waters. Thus, a biosurfactant was produced by the yeast *Candida guilliermondii* UCP 0992 in medium formulated with distilled water supplemented with 4.0% corn steep liquor, 2.5% sugar cane molasses, and 2.5% residual soybean oil. The biosurfactant was initially characterized as an anionic molecule with capacity to reduce the surface tension of the water from 72.0 to 28.0 mN/m in the concentration of 0.42%, corresponding to its critical micelle concentration (CMC). The anionic biosurfactant showed no toxic effect on plant seeds of cabbage (*Brassica oleracea*) and was able to significantly reduce the electrical conductivity of solutions containing heavy metals, demonstrating the ability to complex metal cations, and then applied the removal of metals contained in soil samples from a battery industry. Solutions of the crude and isolated biosurfactant in different concentrations were tested in the mobilization of Iron, Zinc and lead metals contained in the soil. Solutions of HCl were also tested combined with the biosurfactant. The crude biosurfactant, that is, in its most economical form of application, was able to remove 98.9% of Zn, 89.3% of Fe and 89.1% of Pb. The properties of the biosurfactant as well as the results of the metal removal experiments clearly demonstrate the feasibility of applying this biomolecule as a biotechnological additive to remediation processes that consider the preservation and reduction of environmental impacts on ecosystems, essential aspects for the maintenance of life quality and social well-being.

1. Introduction

The global accumulation of heavy metals in European and American soils already amounts to 939,000 t of copper, 783,000 t of lead, 1,372,000 t of zinc and 22,000 t of cadmium (Hazra et al., 2012). In the United States, for example, lead is present in 15% of contaminated soils, followed by chromium, cadmium and copper found in about 7 to 11% of soils. There are two technologies commonly applied to the treatment of metals contaminated soils. The first consists in immobilizing the heavy metals in a solid matrix strongly bound to the soil, minimizing the migration. This technology, however, does not consist of a definitive solution to the problem, considering the impossibility of reuse of the soil and the need for long term monitoring. The second technology promotes the mobility of the metal and its migration to the liquid phase by desorption and solubilization (Singh et al., 2007). A potential solution for the remediation of metals contaminated soils is the use of surfactants. Surfactants or detergents, as they are more commonly known, constitute a class of chemical compounds used in various industrial sectors. These compounds are formed by hydrophilic and hydrophobic moieties that tend to distribute at the interfaces between phases with different degrees of polarity (oil / water), promoting the reduction of surface and interfacial tension, giving the detergency, emulsification, solubilization and phase dispersion (Sarubbo et al., 2015).

The most important property for surfactants is surface tension, which is the attraction force between molecules of liquids. The boundary between a liquid and air is defined as the surface and the boundary between two liquids as the interface. Thus, the tensions between the air / water and oil / water phases are known as surface tension and interfacial tension, respectively (Sarubbo et al., 2015; Marchant and Banat, 2012).

The surface tension decreases as the concentration of surfactant in the aqueous medium increases, with the formation of micelles, which are amphipathic molecules aggregated with the hydrophilic portions positioned towards the outside of the molecule and the hydrophobic portions towards the inner part. The concentration of these micelles forms the Critical Micellar Concentration (CMC). This concentration corresponds to the minimum concentration of surfactant required so that the surface tension is reduced to the maximum. When CMC is reached, several micelles are formed (Santos et al., 2016).

Due to their amphipathic nature, the surfactants can be added in solutions, enabling the solubilization, dispersion and desorption of soil contaminants, allowing their reuse (Silva et al., 2014). Several synthetic surfactants have been evaluated in decontamination tests. On the other hand, the need for substitution of synthetic compounds by natural similar has motivated researches to use surfactants of a microbiological nature, the so-called biosurfactants or biosurfactants (Almeida et al., 2016).

Therefore, considering the serious problems caused by contamination of heavy metals, the possibility of applying biosurfactants as an emerging technology in soil remediation as found in the literature on the potential use of yeast biosurfactants in the removal of heavy metals, the present manuscript had as a main objective to use a biosurfactant with potential of industrial and mainly environmental application through the decontamination of soil coming from a battery industry containing heavy metals. It is also justified to present a product generated with the development of new technologies, enabling the application of an effective biotechnological compound, not only in environmental decontamination, as well as in the several industrial segments of the country with economic and social advantages, which will improve the quality of life and social well-being.

2. Material and Methods

2.1 Soil, Microorganism, and Culture Media

Samples of soil materials were collected in the municipality of Belo Jardim, an automotive battery industry region, located in the northern region of the State of Pernambuco, Brazil.

The yeast *Candida guilliermondii* (UCP 0992) was used for producing the biosurfactant. Cultures of the yeast were kept in sloped test tubes under refrigeration at 5 °C and were repeated every 30 days.

Yeast maintenance was carried out in Yeast Mold Agar (YMA) medium, composed of 0.3% yeast extract, 0.3% malt extract, 1% glucose, 0.5% tryptone and 5% Agar (pH 5.0). The growth of the inoculum was performed in YMB (Yeast Mold Broth) medium, which has the same composition as the YMA medium, excluding agar. A low-cost production medium formulated with distilled water supplemented with 2.5% soybean frying oil, 2.5% sugar cane molasses and 4% corn steep liquor was used for the production of the biosurfactant, as described by Sarubbo et al. (2016). The soybean frying oil was provided by a restaurant located in the city of Recife-PE, while sugarcane molasses and corn steep liquor were obtained from local industrial Plants. The media were autoclaved at 121 ° C for 20 minutes.

2.2 Inoculum Preparation and Production of Biosurfactant

For the production of the biosurfactant, the required cell concentration was obtained from the yeast inoculum, which was standardized by transferring the culture to a tube containing the YMA medium in order to obtain a youthful culture of the microorganism. The sample was then transferred to vials containing 50 mL of YMB medium and incubated under 150 rpm shaking at 28°C for 24 hours. After this time, dilutions were performed until a concentration of 106 cells/mL was obtained. The fermentations for the production of biosurfactant were carried out in 1 L Erlenmeyer flasks containing 300 ml of production medium incubated with the inoculum cell suspension, obtaining the final concentration of 10^4 cells/ml. The flasks were kept under 200 rpm orbital shaking for 144 hours at 28 °C (Brasileiro et al., 2015; Sarubbo et al., 2016).

2.3 Surface Tension and CMC Determination

The measurement of the surface tension was carried out on the cell-free broth obtained by centrifuging the cultures at 5000 g for 20 min by the ring method using a Sigma 700 Tensiometer (KSV Instruments Ltd., Finland) at room temperature. The critical micelle concentration (CMC) was determined by measuring the surface tensions of dilutions of isolated biosurfactant in distilled water up to a constant value of surface tension.

2.4 Biosurfactant Isolation and Composition

The cell-free broth was acidified with 6 M HCl to pH 2.0 and precipitated with two volumes of methanol. After 24 h at 4 °C, samples were centrifuged at 5000 g for 30 min, washed twice with cold methanol and dried at 37°C for 24–48 h. The yield in isolated biosurfactant was expressed in g/l. Known amounts of crude precipitate were resuspended in distilled water and used for measurement of the critical micelle concentration (CMC) (Sobrinho et al., 2008).

Protein concentration in the isolated biosurfactant was estimated by using the total protein test kit from Labtest Diagnóstica S.A., Brazil. The total carbohydrate content was estimated by the phenol–sulphuric acid method (Dubois et al.,1956). The lipid content was determined according to Manocha et al. (1980): 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.

2.5 Determination of Ionic Character

The ionic charge of the biosurfactant was determined using the agar double diffusion technique (Meylheuc et al., 2001). Two regularly spaced rows of wells were made in an agar of low hardness (1% agar). Wells of one row were filled with the biosurfactant solution and wells of the other were filled a pure compound of known ionic charge. The anionic substance chosen was sodium dodecyl sulphate (SDS) 20 mM and the cationic one was barium chloride, 50 mM. The appearance of precipitation lines between the wells, indicative of the ionic character of the biosurfactant, was monitored over a 48 h period at ambient temperature.

2.6 Toxicity Test on Plant Seeds with Biosurfactant

The laboratory control phytotoxicity test used for seeds of *Brassica oleracea* L. var. *Capitata* L. (cabbage), where solutions of the biosurfactant were applied in concentrations of 1/2xCMC, CMC and 2xCMC. After 5 days of incubation in dark environment, germination of seeds with root growth (≥ 5 mm) and germination index (≥ 80%) were calculated (Tiquia et al., 1996).

2.7 Conductivity of the Biosurfactant

The biosurfactant and the chemical surfactants sodium dodecyl sulphate (SDS), Tween 20 and Tween 80 were used. Tween 80 or polysorbate has CMC of 0.0124% (w/v), being able to reduce the surface tension to 43.7 mN/m, while tween 20 has CMC of 0.00736% (w/v), being able to reduce the surface tension to 43.5 mN/m and SDS has CMC of 0.234% (w / v) and surface tension of 37.0 mN/m.

The conductivity of chemical surfactants and the biosurfactant were measured using a conductivity metre (TEC-4MP, Tecnal LTDA., Brazil). Biosurfactant solutions at the ½ ×CMC, at the CMC and at twice the CMC were then added separately to 500 ppm solutions of lead nitrate and cadmium nitrate. The metal-biosurfactant precipitate was removed and conductivity of the resulting solution was measured. The conductivimeter was calibrated with deionized water before measuring conductivity of each sample. All tests were per-formed in triplicate (Das et al., 2009).

2.8 Identification and Quantification of Heavy Metals in Soil

For the identification of the metals contained in the soil, a portable X-ray fluorescence system ED-XRF was used. The system consists of an X-ray tube, a Si detector, multi-channel and amplifier. The X-ray machine (brand MOXTEK, model 40 kV MAGNUM) has a tungsten target and weighs 450 grams, with a length of 5.53 cm and a width of 2.86 cm.

The analysis of the content of heavy metals in the soil collected in the city of Belo Jardim was performed by atomic absorption spectrophotometry after acid extraction method according to the Standard Methods (1995).

2.9 Soil Treatment with Biosurfactant Solutions

The soil washing process was performed using the crude biosurfactant (cell-free broth) and with the isolated biosurfactant (1/2xCMC, CMC and 2xCMC). For the selection of the best biosurfactant concentration for the removal of heavy metals, 5 g of soil were placed in Erlenmeyer flasks, and 50 mL of the biosurfactant solution was added at the different concentrations described above. The soil sample was treated in parallel with distilled water in order to determine the removal of the heavy metals by physical mixing (control). Solutions of 0.7% HCl as well as combinations of solutions of the isolated biosurfactant and the crude biosurfactant with 0.7% HCl as additive were also used. The samples were shaken for 24 hours and centrifuged at 5000 rpm. The supernatants were filtered and the concentration of metal determined by atomic absorption spectrophotometry. The percentage of metal removal was determined based on the initial metal content in the soil and the results were presented as percent removal of the metal.

3. Results and Discussion

3.1 Properties of the Produced Biosurfactant

The yield of the biosurfactant produced by *C. guilliermondii* was 2.15 g/L and the surface tension of the cell-free broth was reduced from 50.0 mN/m to 28.0 mN/m. The isolated biosurfactant produced exhibited excellent surface tension reduction ability, since the surface tension of the water was reduced from 72.0 mN / m to 28.0 mN/m with a concentration of 0.42% of biosurfactant (Figure 1). From this point, the increase of the concentration of the biosurfactant solution did not cause greater reductions in the surface tension of the water, indicating that the CMC had been reached in that concentration.

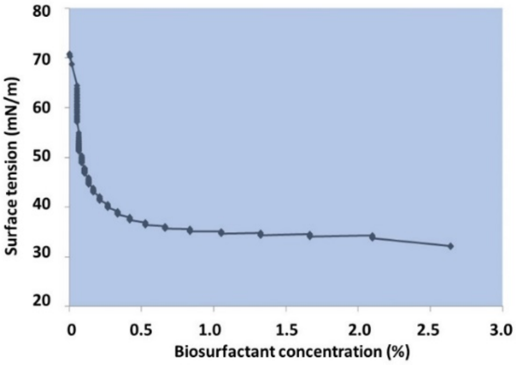

Figure 1: Critical Micelle Concentration (CMC) of the Biosurfactant Produced by Candida guilliermondii

The preliminary analysis of the biosurfactant identified the presence of 12.4% of lipids, 52.3% of proteins and 10% of carbohydrates. The anionic character of the biosurfactant was determined from the appearance of precipitation lines around the wells containing the cationic compound Barium chloride ($BaCl_2$), which was not observed for the anionic compound Sodium Dodecyl Sulfate (SDS).

The germination index (GI), which combines the measures of relative seed germination and relative root growth, was used to evaluate the biosurfactant toxicity to cabbage seeds (*Brassica oleracea* var. *Captata* L.), indicating that the biosurfactant at the concentrations tested showed no toxicity.

3.2 Conductivity Test

The solution with the isolated biosurfactant at the concentration of 2xCMC (0.42%) showed a conductivity of 13 μS, whereas the solutions of the synthetic surfactants in the same concentration (2xCMC) presented values between 0.84 and 8.70 μS. On the other hand, the conductivity of the solutions containing the heavy metals cadmium (Cd) and lead (Pb) decreased in the presence of the biosurfactant. This reduction was proportional to the increase of the biosurfactant concentration due to precipitation of the positively charged metals after being captured by the biosurfactant, reducing the amount of metal ions available in the metal solution and consequently the conductivity values of the solution.The conductivity of the metal solutions decreased from 510 to 6.5 μS (solution containing Cd) and from 675 to 6.5 μS (solution containing Pb) in the presence of the *C. guilliermondii* biosurfactant. As for the synthetic surfactants, the higher the concentrations used, the higher the conductivities of the solutions containing the metals. Similar results were observed by Das et al. (2009) where the synthetic surfactants Tween 20, Tween 60 and Tween 80 also did not change the conductivity of the metals, regardless of the concentrations used.

3.3 Application of Biosurfactant in the Removal of Heavy Metals in Soil

The soil used for the tests was characterized by the presence of lead (Pb), iron (Fe) and zinc (Zn) at concentrations of 3038, 1877 and 1470 mg/l, respectively. These values demonstrate the high contamination rate of the soil removed from the battery producing industry.

3.4 Soil treatment with biosurfactant solutions

The possible mechanisms for the extraction of heavy metals by biosurfactants include ion exchange, precipitation-dissolution and association. It is considered that the metals are removed by the formation of complexes with the surfactant, on the surface of the soil, being detached from the ground by the reduction of interfacial tension and associating, consequently, to surfactant micelles (Sarubbo et al., 2015). Anionic surfactants provide satisfactory results since the cations of metals have affinity for negatively charged surfactants, and it is also possible that the biosurfactant allows greater removal due to its ability to reduce interfacial tension (Asçi et al., 2007).

Solutions of the isolated biosurfactant at different concentrations determined on the basis of the CMC (0.42%), were tested in the mobilization of heavy metals, through the treatment of the soil. Removal of the metals by the crude biosurfactant was also evaluated. The possibility of increasing percentages of metal removal was tested by the combination of surfactant with hydrochloric acid (HCl) as an additive, while distilled water was used as a control. The results for soil treatments with the different solutions tested are shown in Table 1.

Table 1: Removal of Heavy Metals Contained in the Soil from Belo Jardim City by the Biosurfactant

Treatments	Removal (%)		
	Zinc (Zn)	Iron (Fe)	Lead (Pb)
Distilled water	13.0	10.0	12.0
Crude biosurfactant (cell-free broth)	98.9	89.3	89.1
Crude biosurfactant (cell-free broth) + HCl at 0.7%	89.7	89.6	89.5
Isolated biosurfactant solution at 0.21% (1/2CMC)	99.8	96.3	86.6
Isolated biosurfactant solution at 0.21% (1/2CMC) + HCl at 0.7%	99.8	95.3	56.4
Isolated biosurfactant solution at 0.42% (CMC)	99.9	98.6	93.8
Isolated biosurfactant solution at 0.42% (CMC) + HCl at 0.7%	99.9	95.7	81.5
Isolated biosurfactant solution at 0.84% (2xCMC)	99.9	96.8	93.5
Isolated biosurfactant solution at 0.84% (2xCMC) + HCl at 0.7%	99.9	95.5	62.1

Comparing the three concentrations of the isolated biosurfactant solutions tested, it was observed that increasing the concentration from 0.21% (1/2 CMC) to 0.84% (2xCMC) favored the removal of metals, especially in the case of Fe. Removals of 99.9, 96.8 and 93.5% were obtained for Fe, Pb and Zn, respectively, when the 0.8% surfactant solution was used, although the metabolic liquid removed percentages comparable to the solutions of the isolated surfactant (98.9% Zn, 89.3% Fe and 89.1% Pb). Physical treatment with distilled water as a control removed about 10% of the metals present in the soil.

In relation to the use of the HCl additive together with the surfactant solutions, it was observed that HCl did not potentiate the removal percentage of the metals by the crude biosurfactant and the biosurfactant solutions at 0.21, 0.42% and 0.84%, indicating that the removal occurred due to the electrostatic interaction between the molecules of the biosurfactant and the metals. It is important to note that, although acids and bases are applied in soil washing for metal removal, treatment with these compounds reduces fertility and causes changes in soil chemical composition (Sarubbo et al., 2015).

Mulligan et al. (1999) demonstrated the applicability of biosurfactants in the removal of heavy metals contained in soils. Initially, the sophorolipid of yeast *Torulopsis bombicola* grown in glucose and soybean oil with a CMC of 0.8 /L (0.08%) and capacity to reduce surface tension to 34 mN / m was tested. The solution of 4% sophorolipid did not remove the Zn contained in the soil, removing only 3% of Cu. The most significant removal of Cu and Zn occurred with the use of 0.7% HCl and 4% of the sophorolipid, reaching a removal of 16 and 37% of Zn and Cu, respectively. This percentage was increased by performing a series of five washes, reaching percentages of removal of 100 and 50% of Zn and Cu by the combination of 0.7% HCl and 4% of the sophorolipid. However, the *Pseudomonas aeruginosa* rhaminolipid produced in mineral medium containing 4% glucose, with a CMC of 0.003% and surface tension of 26 mN/m, allowed the removal of 20 and 35% of zinc and copper, respectively, for a high concentration of 12%, since the 2% rhaminolipid solution removed only 5% and 10% of zinc and copper, respectively.

One of the most promising field of technology emerging in the last decade is the biological techniques employing biosurfactants as heavy metal removal tools. The efficiency and success of biosurfactants in facilitating removal of heavy metal contaminants from soil systems, however, depends largely on the soil texture, structure, clay content, predominant clay type, cation exchange capacity, permeability, ionic strength, etc. The economics of this process will need to be determined to compare with existing technologies. However, with more development, the use ofbiosurfactants can be an effective, non-toxic process of remediating dredged sediments contaminated with heavy metals. For this purpose, much work remains needed to optimise biological and engineering processes. As demonstrated here, biosurfactants are candidates for the replacement of synthetic surfactants, especially in the petroleum industry. Thus, greater investments are needed in strategies aimed at optimizing cost-effective and viable large-scale production, downstream processing and utilisation of biosurfactants.

4. Conclusions

The biosurfactant produced by *C. guilliermondii* grown in low cost medium showed attractive tensoactive characteristics and absence of toxicity. The low-cost medium has shown to be promise for industrial use, and the biomolecule produced can be considered an efficient agent for the control of environmental pollution caused by heavy metals. The properties of the biosurfactant as well as the results of the metal removal experiments clearly demonstrate the feasibility of applying this biomolecule as a biotechnological additive to remediation processes that consider the preservation and reduction of environmental impacts on terrestrial ecosystems.

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References

Almeida D.G., Soares da Silva R.C.F., Luna J.M.; Rufino R.D., Santos V.A., Banat I.M., Sarubbo L.A, 2016, Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances, Frontiers in Microbiology, 7, 1718.

Asçi Y., Nurbas M., Açikel Y. S., 2007, Sorption of Cd (II) onto kaolin as soil component and desorption of Cd (II) from kaolin using rhamnolipid biosurfactant, Journal of Hazardous Materials, 58, 50-56.

Brasileiro P.P.F., Almeida D.G., Luna J.M., Rufino R.D., Santos V.A., Sarubbo L.A., 2015, Optimization of biosurfactant production from *Candida guiliermondii* using a Rotate Central Composed Design, Chemical Engineering Transactions, 43, 1411-1416.

Das P., Mukherjee S., Sen R., 2009, Biosurfactant of marine origin exhibiting heavy metal remediation properties, Bioresource Technology, 100, 4887-4890.

Dubois M., Gilles K.A., Hamilton J.K., 1956, Colorimetric method for determination of sugar and related substances, Analytical Chemistry, Easton, 28, 350-356.

Hazra C., Kundu D., Chaudhari A., 2012, Biosurfactant-Assisted bioaugmentation in bioremediation. In: Microorganisms in Environmental Management: Microbes and Environment. Eds.: Satyanarayana T.; Johri B.N.; Prakash A. Springer, New York, 631-664.

Manocha M.S., San-Blas G., Centeno S., 1980, Lipid composition of *Paracoccidioides brasilienses*: possible correlation with virulence of different strains, Journal of General Microbiology, 117, 147-154.

Marchant R., Banat I.M., 2012, Microbial biosurfactants: challenges and opportunities for future exploitation, Trends in Biotechnology, 11, 558-565.

Meylheuc T., Van Oss C.J., Bellon-Fontaine M.N., 2001, Adsorption of biosurfactants on solid surfaces and consequences regarding the bioadhesion of *Listeria monocytogenes* LO28, Journal of Applied Microbiology, 91, 822-832.

Mulligan C.N., Yong R.N., Gibbs B.F., 1999, Metal removal from contaminated soil and sediments by the biosurfactant surfactin, Environmental Science and Technology, 33, 3812-3820.

Silva R.C.F.S., Almeida D.G., Luna J.M., Rufino R.D., Santos V.A., Sarubbo L.A, 2014, Applications of biosurfactants in the petroleum industry and the remediation of oil spills, International Journal of Molecular Sciences, 15, 12523-12542.

Singh A., Van Hamme J.D., Ward O.P., 2007, Surfactants in microbiology and biotechnology: Part 2. Application aspects, Biotechnology Advances, 25, 99-121.

Santos D.K.F., Luna J. M., Rufino R.D., Santos V.A., Sarubbo L.A., 2016, Biosurfactants: multifunctional materials of the XXI century, International Journal of Molecular Sciences,17, 1-31.

Sarubbo L.A., Luna J.M. Rufino, R.D., Brasileiro P.P.F., 2016, Production of a Low-cost Biosurfactant for Application in the Remediation of Seawater Contaminated with Petroleum Derivates, Chemical Engineering Transactions, 49, 523-528.

Sarubbo L.A., Rocha Junior R.B., Luna J.M., Rufino R.D., Santos V.A., Banat I.M., 2015, Some aspects of heavy metals contamination remediation and role of biosurfactants, Chemistry and Ecology, 31, 707-723.

Sobrinho H.B.S., Rufino R.D., Luna J.M., Salgueiro A.A., Campos-Takaki G.M., Leite L.F.C., Sarubbo L.A., 2008, Utilization of two agroindustrial by-products for the production of a surfactant by *Candida guilliermondii* UCP0995, Process Biochemistry, 43, 912-917.

Standard Methods for the examination of water and wastewater. 19ed. England: AWWA WEF APHA, 1995.

Tiquia S.M., Tam N.F.Y., Hodgkiss I.J., 1996, Effects of composting on phytotocicity of spent pig-manure sawdust litter, Environmental Pollution, 93, 249-256.