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REMOVAL OF ZINC AND CADMIUM IONS FROM CONTAMINATED SOILS WITH RHAMNOLIPID BIOSURFACTANT PRODUCED BY *PSEUDOMONAS AERUGINOSA* S7PS5

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ABSTRACT

A soil treatment process using froth flotation technique involving anionic biosurfactant (rhamnolipids) using Sodium sulfide was studied. The supernatant produced by the strain *Pseudomonas aeruginosa* S7PS5 was tested for biosurfactants production, HPLC analysis showed the presence of L-rhamnosyl- β- hydroxydecanoyl- β-hydroxydecanoate (RL1) and L-rhamnosyl L-rhamnosyl- β-hydroxydecanoyl-β- hydroxydecanoate (RL2). The influence of the collector (rhamnolipid), pulp pH, a chemical activation step (sulfidization) and process time on metal removal efficiency has been investigated to recover Zn and Cd ions from a contaminated soil. An effective CMC of 35 mg/L was obtained. A perfect Zn and Cd removal efficiency was made at pH = 12 and 4 mg/g of Na_2S during the first 5 min of soil washing process, then a longer flotation time (> 5 min) caused mechanical entrainment of Zn and Cd.

Keywords: Biosurfactants, flotation, *Pseudomonas aeruginosa*, rhamnolipids, soil washing.

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1. INTRODUCTION

The presence of low concentrations of heavy metals in the soils are known to have potential impact on environmental quality and human health *via* ground water, surface water, plants and agricultural products [1].

Soil composition, clay mineralogy, permeability, pH, cation exchange capacity, particle size and other factors such as the presence of competing ligands, the ionic strength of the soil and the simultaneous presence of competing metals and contaminants significantly affect sorption–desorption processes and leaching potential through a soil profile [2, 3].

Cadmium (Cd) and zinc (Zn) have a great interest because of their high toxicity and mobility in soil and as metals in the contaminated sites are not degraded, they must be either immobilized or removed [1].

Biosurfactants over synthetic surfactants include higher selectivity for metals and organic compounds [4], lower toxicity, higher biodegradability, higher foaming [5], better environmental compability, less expensive, more tolerant to pH, salt, and temperature variation [6].

Rhamnolipids are mostly produced by *Pseudomonas aeruginosa*, which are composed of one or two rhamnose molecules as a hydrophilic portion, and up to three molecules of hydroxy fatty acids (C8–C14) as a hydrophobic portion [7]. These surfactants have been studied in various environmental applications and are applied to the removal of Zn and Cd [1].

2. MATERIALS AND METHODS

2.1. Sampling site

The soil used for the washing tests was collected from Sidi Bel Abbess (Algeria) at the agricultural engines factory (SONACOM) (depth of 15 -100 cm). The soil samples were dried at 105 °C and sieved (< 2 mm) according to AFNOR X 31-101 standard [8], then homogenized and kept away from light.

2.2. Rhamnolipids production and purification

The used rhamnolipids were produced by *Pseudomonas aeruginosa* S7PS5 (Figure 1) (GenBank accession no. **KR349493**) isolated by Bendaha et al. [9] after a culture of 22 h at

room temperature in the following media: 100 ml of nutrient broth, 1 % of olive oil with 1 % of inoculum with shaking at 75 rpm/min [9]. Biosurfactants are recuperated in the supernatant after centrifugation at 9000 g for 15 min [10].

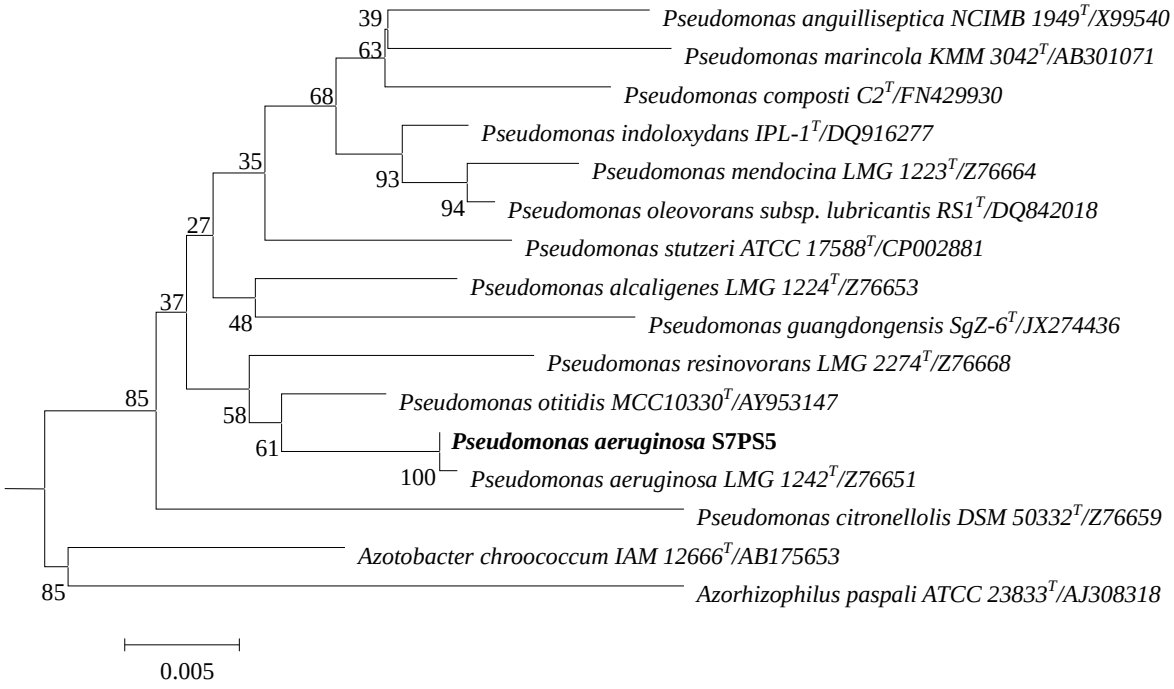

Fig. 1. Phylogenetic tree based on sequence analysis of 16S rDNA and showing the relationships between *Pseudomonas aeruginosa* S7PS5 and the other similar species.

Note: The numbers at the nodes indicate "bootstrap" levels (As a percentage of 1000 re-sampling). The bar indicates 0.005 substitutions per nucleotide position [9]

2.3. Biosurfactants producing tests

The drop collapsing was conducted using the test of collapse described by Jain et al. [11], supernatant of the culture that led to the collapse of the drop is shown as a positive result, and the drops remaining with the beads are marked as negative results [9].

15 µL of crude oil are placed on the surface of 40 ml of sterile distilled water for the oil displacement test, then; 10 µL of supernatant was slightly put on the oil film surface. After 30 s, diameter of the clear halo is measured under visible light [12].

For the emulsification activity was done according to Paraszkiewicz et al. [13]. The emulsification index (E_{24}) was estimated after 24 h, as follows:

$$E_{24}(\%) = \frac{H_{EL}}{H_S} \times 100$$

H_{EL}: Height of emulsion layer, H_S: Height of total liquid column.

The surface tension measurement was carried out according to the Du Nouy ring method described by Zajic et al. [14] and the surface activity is expressed as a reduction percentage of the surface tension reduction by the following equation:

$$\% \text{ surface tension reduction} = \frac{(\gamma_m - \gamma_c)}{\gamma_m} \times 100$$

γ_m is the surface tension of the medium as prepared, γ_c is the surface tension of the supernatant.

2.4. Rhamnolipids purification

Supernatant was collected after centrifugation at 9000 g for 15 min, rhamnolipids were then precipitated by acidification at pH = 2 using 1 N HCl [10]. A second centrifugation was done at 9000 g for 20 min and the precipitate was extracted with ethyl acetate at room temperature [10]. The organic phase was transferred to a round bottom flask connected to a rotary evaporator (RE300 Stuart), allowing to remove solvent from viscous honey-colored rhamnolipid product [15]. The crude biosurfactant was purified and kept at 4 °C for 24 h.

2.5. Measurement of critical micelle concentration (CMC)

Biosurfactants solutions were prepared in ultra-pure water from a solution of rhamnolipids of 1 g/L with pH adjusted to 7 prior to use. The CMC determination was done by plotting the surface tension versus concentration of biosurfactant in the solution, with a curve having a downward slope to the CMC then becomes constant [16].

2.6. HPLC analysis

HPLC analyses of the rhamnolipids compounds were performed on a Shimadzu-system (Prominence i. LC-2030C 3D) equipped with Ascentis Express C18 column (15 cm X 4.6 mm) id packed with 2.7 µm partially porous particles (Supelco, Bellefonte, PA, USA). The binary mobile phase consisted of water/acetic acid (solvent A) and methanol/acetic acid (solvent B), in a linear gradient mode: 0 min, 5 % B; 10 min, 40 % B; 30 min, 70 % B; 60 min, 100 % B; 65 min, 100 % B; 67 min, 2 % B. The mobile phase flow rate was 0.7 ml/min.

Pure rhamnolipids Rha C10-C10 and Rha-Rha C10-C10 were used with the testing sample.

2.7. Soil preparation

The soil washing process used in this work was designed as described by Dermont et al. [17] in order to increase the degree of liberation of metal mineral phases (Figure 2). To obtain a soil with a particle-size range of 0–250 µm, a procedure combining successive sieving and crushing/grinding steps was done [18].

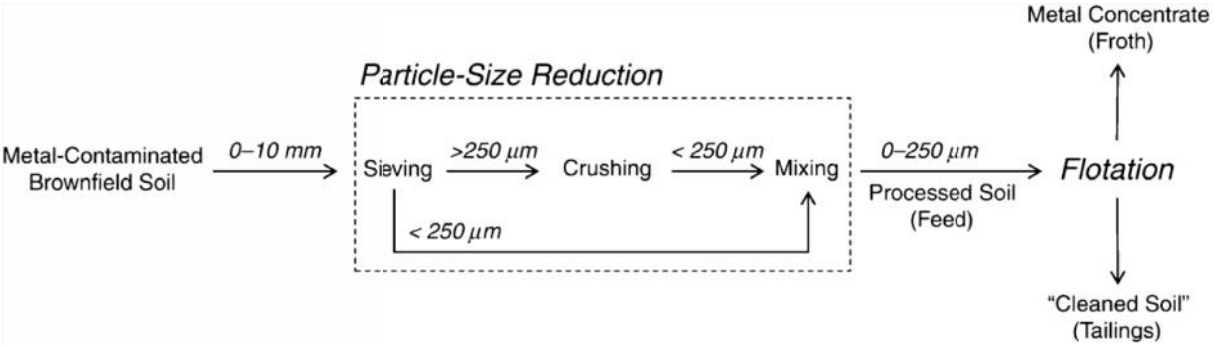

Fig.2. The schematic soil washing process [17]

2.8. Rhamnolipids sorption

Rhamnolipids are anionic biosurfactants that have less sorbance in soil [19].

For soil washing process, an effective CMC must be used, because in case a surfactant is sorbed in the soil, its effectiveness can be reduced.

Sorption experiment was carried out in a 250 ml Erlenmeyer flask with 10 % of soil in distilled water for 24 h. Selected biosurfactant concentration was the same as the CMC.

The surfactant fraction attached to soil is obtained by the following formula:

$$\text{Adsorbed } \% = \frac{C_0 - C_r}{C_0} \times 100$$

With:

C_0 : Initiale concentration of rhamnolipids (mg/L).

C_r : Residual concentration of rhamnolipids (mg/L).

2.9. Metals distribution and physico-chemical characterization of the soil

The total concentration of each element (Fe, Mn, Zn, Cu, Cd, Co, Cr, Ni and Pb) was measured after a complete decomposition by mixed acid digestion using an atomic absorption spectrophotometer (AAS) Perkin–Elmer model AA300, in a certified laboratory, namely the

"*Labo Bio Qual*" of Blida, Algeria, where the quality control of the analytical method is ensured.

The physicochemical characterization of soil was conducted using AFNOR standard techniques [8]. These analyzes were conducted at the *National Institute of Agricultural Research of Algeria* (INRAA), Lamtar's Station, Sidi Bel Abbess, Algeria.

2.10. Flotation method

The flotation cell used in this study was developped in the *Laboratoire de Bioconversion, Génie Microbiologique et Sécurité Sanitaire* (LBGMSS), University of Mascara, Algeria.

The pulp pH influences the mobilization of heavy metals in surfactants presence [17], the different pH values used in this study are 8, 9, 10, 11 and 12.

The experiences were realized using a laboratory flotation cell (Figure 3) with a mechanical agitator motor **(**Ultra-Turrax T25). Separation principle is based on the affinity of hydrophobic surfaces of particles for injected air bubbles in the soil suspension. The collector is a surfactant agent that attaches on the mineral surface (*via* physical adsorption or chemisorption) in order to produce a hydrophobic surface or to enhance the hydrophobic character of the mineral phase to be floated [17]. To have 10 % of solids in the pulp [17], 50 g of soil (0–250 µm) were mixed with the appropriate amount of distilled water in the flotation cell. The frothing agent (rhamnolipid) was added in the last minute of the conditioning step. The pulp was then conditioned for 10 min with the collector agent in the flotation cell with an agitation speed of 8000 rpm. After the conditioning step, the air valve was opened and flotation test was conducted for the given duration. The froth layer was continuously removed during the flotation process using a vacuum pump and collected in glass containers. After completion of the flotation stage, the various produced fractions were filtered and the concentration factor (CF) of Zn and Cd were calculated using the equation given by Dermont et al. [17]:

$$\text{Element CF} = \frac{[\text{Element}]_{\text{Froth}}}{[\text{Element}]_{\text{Feed}}}$$

With:

$[\text{Element}]_{\text{Froth}}$: Concentration of element in froth.

$[\text{Element}]_{\text{Feed}}$: Concentration of element in feed.

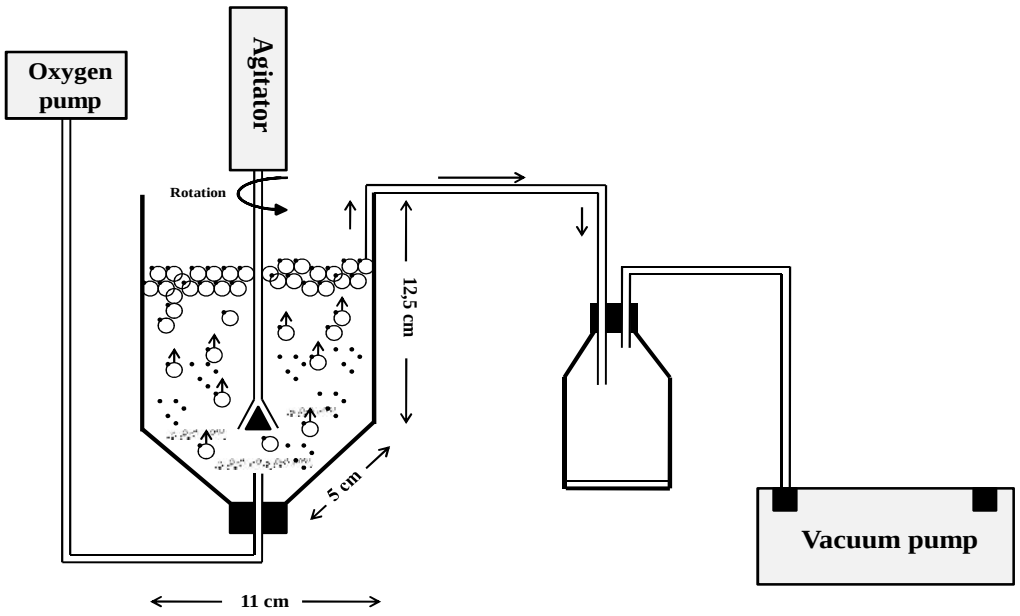

Fig.3. Process and instrumentation diagram of the flotation unit

2.11. Influence of the chemical activation of the pulp

The effect of chemical activation of the pulp was tested by sulfudization with sodium sulfide (Na_2S) which is generally used as a sulfurizing agent [20, 21]. The sulfudization step was performed on 10 % of soil contained in the cell for 20 minutes at room temperature [17]. Three concentrations of Na_2S were tested: 2, 3 and 4 mg/g. The activation by HS^- ions modifies the particle surfaces to make them more disposed to the biosurfactant action.

2.12. Influence of flotation time

Flotation time was assessed by measuring the concentration factor (CF) and the surface activity of rhamnolipids in the pulp (10 % of soil) of Zn and Cd at four different times: 2.5, 5, 7.5, and 10 min.

3. RESULTS AND DISCUSSION

3.1. Biosurfactants producing tests

The drop collapsing test show biosurfactants presence in the tested supernatant, interfacial tension between water droplet and hydrophobic surface is reduced resulting in the spread of

the water drop on hydrophobic surface [22]. Clear halo diameter, E_{24} (%) and reduction of the surface tension (%) results are presented in figure 4.

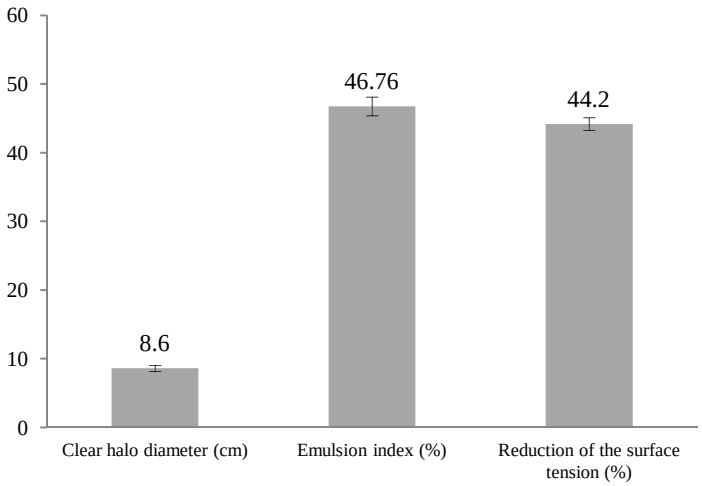

Fig.4. Biosurfactants producing tests (oil displacement, emulsion index E_{24} and reduction of the surface tension) for *Pseudomonas aeruginosa* S7PS5

Biosurfactants have less density than water and two different polarities, so they float to the surface for the oil displacement test and will be in competition with the latter to the surface occupation [9].

Micelles are formed when hydrophobic portions unable to form hydrogen bonds in aqueous phase, unite and move towards the center leaving the hydrophilic portions outward, oil molecules were trapped in a pseudohydrophobic phase formed by micelles caused by biosurfactants which increase the solubility of hydrophobic compounds [23].

Surface tension of the supernatants was measured function to the concentration of biosurfactants excreted in order to have the surface activity of *Pseudomonas aeuginosa* S7PS5. Surface tension of the nutrient broth (5.66 mN/m) is rapidly decreased because of the high biosurfactants.

3.2. Critical micelle concentration (CMC)

Figure 5 represents the surface tension in function of biosurfactant concentration. The surface tension decreases exponentially until reaching a minimum of 40.06 mN/m, for a biosurfactant concentration greater than or equal to 35 mg/L. The CMC value is consistent with the reported values (27 - 54 mg/L) for rhamnolipid [24].

For the CMC, rhamnolipids concentration from which the surface tension value begins to be stable is 35 mg/L.

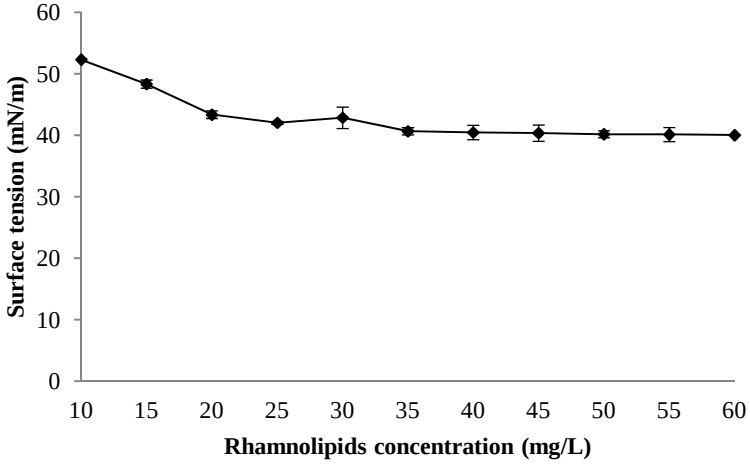

Fig.5. Surface tension vs. concentrations of rhamnolipids

3.3. HPLC analysis

After HPLC and comparison with the standards (Figure 6), the two major detected rhamnolipids correspond to monorhamnolipid (Rha C10-C10) and dirhamnolipid (Rha-Rha C10-C10). As reported by Wei et al. [10] and Maier and Soberon-Chavez (2000) [25], L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL1) and L-rhamnosyl L-rhamnosyl-β-hydroxydecanoyl-β- hydroxydecanoate (RL2) are the two most types of rhamnolipids produced by *P. aeruginosa* species.

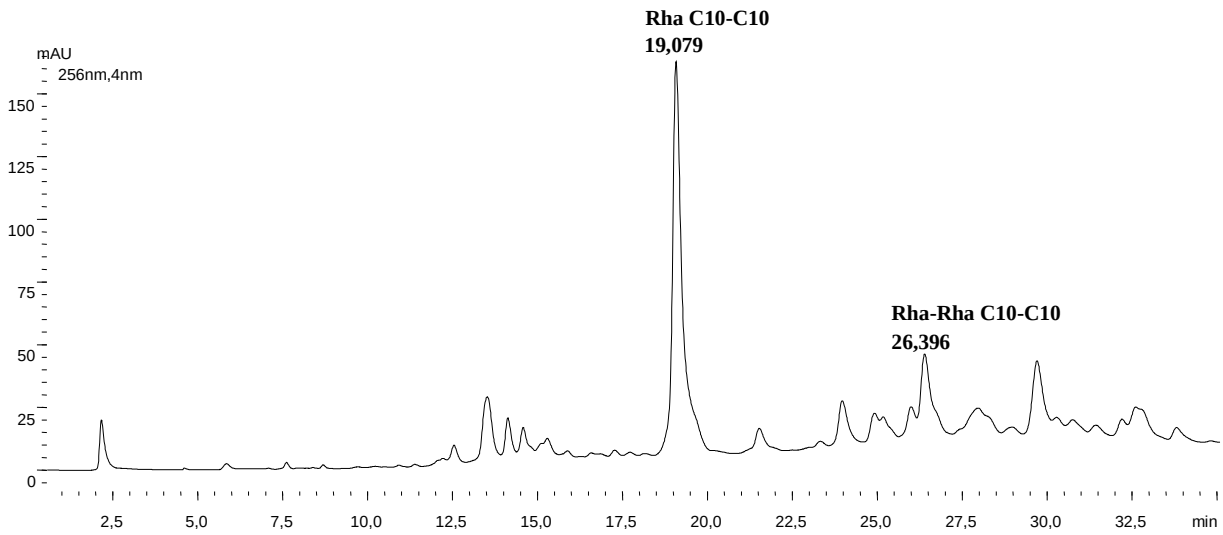

Fig.6. HPLC chromatogram of rhamnolipids from *Pseudomonas aeruginosa* S7PS5

3.4. Rhamnolipids sorption

The effective CMC is slightly greater than that obtained CMC because once the biosurfactant brought into contact with soil, the surfactant adsorbs to the matrix and thus becomes less effective, the monomers will tend to sorb to soil rather than to form micelles. The surfactant sorbed fraction after 24 h was 14.28 %. In fact, loss of biosurfactant was estimated to be 5 mg/L during the study period, the adsorption was carried out at a rhamnolipids concentration equal to 35 mg/L which corresponds to the biosurfactant CMC from which micelles are formed and therefore the effective CMC is 40 mg/L.

3.5. Metals distribution and physico-chemical characterization of the soil

The characteristics of the used soil are summarized in table 1. Concentrations of Cu, Ni and Pb are undetectable by AAS because of their scarce presence, Cd ions presents 0.83 µg/kg and Zn with 4.02 mg/kg presents the highest metal concentration level in this soil.

The pH value shows that it is a neutral soil. Particle size analysis shows that the predominant fraction is slit (40 %), sand and clay are 30 % for each fraction. The soil is classified as sandy clay loam as the textural triangle [26]. This allows to better assess of mechanisms involved in geochemical distribution of heavy metals such as adsorption on the surface of clay minerals, precipitation with carbonate or complexations with organic matter.

The dosage of the exchangeable cations led to highlight the predominance of calcium

(86.85 %), indicating that the soil tends to be neutral or alkaline, which is verified by measuring the pH (7.16).

Clays are silicates which have a laminated structure (phyllosilicates), or fibrous (such as sepiolite or palygorskite). There are three main families of clay which are classified by the number of tetrahedral and octahedral layers that make up their sheets. The interstices between the layers may contain water molecules or ions. Clay minerals have large surface areas and large cation exchange capacity. The clays can therefore retain a significant amount of heavy metals by adsorption. Carbonate dissolution plays an important role in controlling the soil pH. A high carbonate content raises the soil pH. Carbonates may incorporate metal cations in their crystal lattice [27].

Table 1. General characteristics of the studied soil

Parameters	**Range**
Major mineral elements (mg/kg)	
Fe	0.65
Mn	1.07
Zn	4.02
Cu	< 0.05
Minor mineral elements (µg/kg)	
Cd	0.83
Co	0.71
Cr	0.7
Ni	< 0.01
Pb	< 0.01
Other parameters (%)	
Soil pH	7.16
Total carbon	2.46
Total limestone	22.4
Active limestone	17.5
Silt (%)	40
Clay	30
Sand	30
Exchangeable cations (Meq/100 g)	
Ca^{+2}	31.72
Mg^{+2}	3.48
Na^{+2}	1.1
K^{+2}	0.22

The surface hydroxyl groups can be formed by hydration, this allows the adsorption of metal cations [28]. Being good adsorbents, oxides and hydroxides metal in soils also play an important role in the retention of metal ions. The amount of adsorbed ions strongly depends on the pH of the medium.

3.6. Influence of the pulp pH

Washing soil by flotation was studied for the removal of Zn and Cd from polluted soil after grinding size fractions greater than 250 µm. This mechanical preparation allow the treatment of all contaminated fractions of any size, by obtaining an appropriate particle size for the flotation process and increase liberation degree of mineral float's phases [29].

The collector is a surfactant (rhamnolipid) which attached the surfaces of minerals (by physical adsorption or chemo-sorption) and produce a hydrophobic surface or enhance the hydrophobicity of the mineral phase to allow its flotation. The choice of using an anionic biosurfactant (rhamnolipids) is based on the results achieved by Aşçı et al. [1] and Dermont et al. [17] who shown that the use of an anionic surfactant gives better rate of metal removal. The anionic biosurfactant such as rhamnolipid carries a negative charge, so when the molecule encounters a cationic metal such as Zn or Cd that carries a positive charge, an ionic bond is formed. This bond is stronger than the metal's bond with the soil [30].

Flotation tests were carried out at alkaline pH as the collectors improved better stability at high pH [31].

Figure 7 shows Zn remediation by flotation using rhamnolipids produced by *Pseudomonas aeruginosa* S7PS5, characterized by proportional change of CF of Zn depending on its concentration in the foam which was 8.06 (CF) at pH = 8 and which increased by 15.26 % (CF = 9.29) at pH = 12. A proportional change of CF of Cd depending on its concentration in the foam which was 7.56 (CF) at pH = 8 and which increased by 180.42 % (CF = 21.2) at pH = 12 (Figure 8).

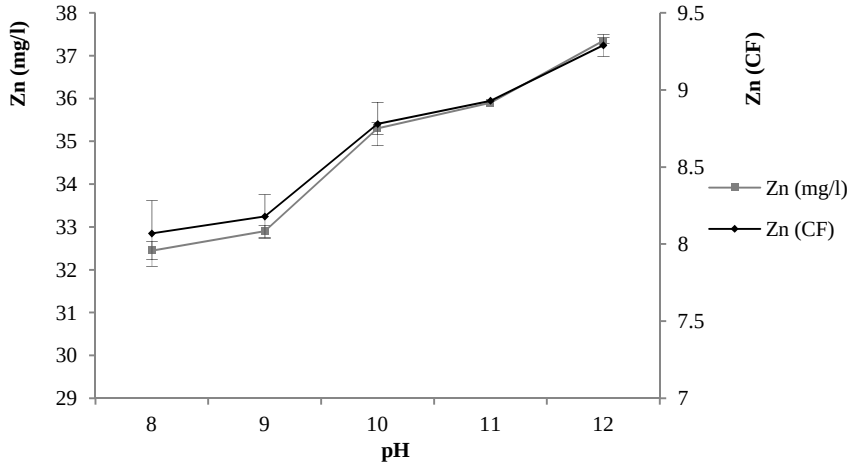

Fig.7. Influence of the pulp pH on Zn flotation using rhamnolipids

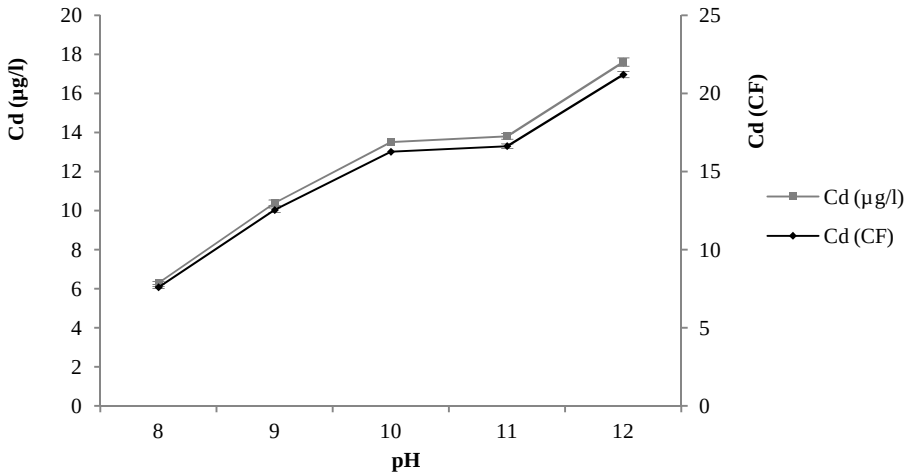

Fig.8. Influence of the pulp pH on Cd flotation using rhamnolipids

The effect of pH (8 to 12) of the pulp was evaluated using 0.40 g/L of rhamnolipids, showing its influence on the flotation process. Thus, an important mobilization rate of Zn and Cd was observed which increases with increasing pH from 8 to 12. The results of mobilization rate according to the increase of pH (8 to 12) obtained in this study join those obtained by Dermont et al. [17] with a higher Zn and Cd recovery at pH 8–11.

3.7. Influence of sulfidization step

The base-oxide minerals (or oxidized minerals) like zinc and Cd are more difficult to float, than their sulfide mineral form [32, 21]. Sulfidizing agents such as Na_2S are frequently used

to produce a sulfide surface. The combination of sulfidization and flotation has been investigated for soil washing applications, because of the heterogeneity of mineralogical forms of metal contaminants in the contaminated soil [17].

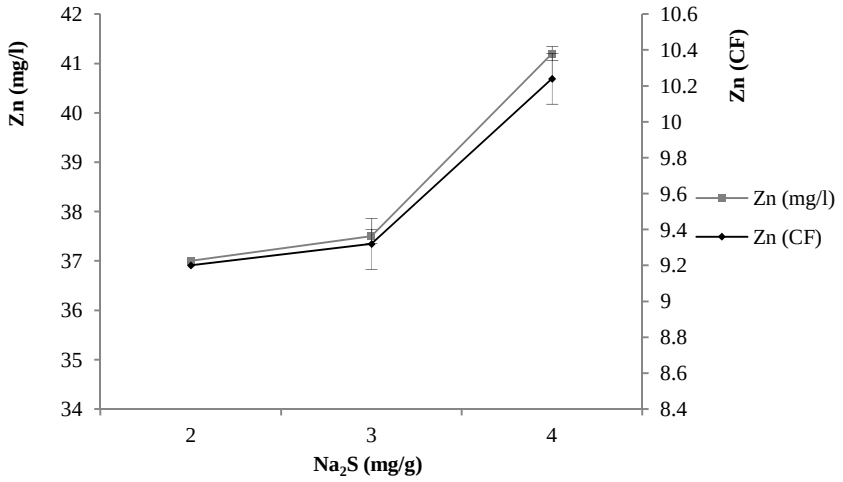

Fig.9. Na_2S effect on Zn flotation at pH = 12 in rhmanolipids presence

As shown in figure 9, the pre-treatment by sulfidization (Na_2S) increased Zn remediation, and the proportional CF of Zn depending on the concentration of Zn in the foam shows a mobilization rate of Zn which increases with the elevation of Na_2S concentration (2 to 4 mg/g), which was 9.2 (CF) at [Na_2S] = 2 mg/g and increased by 11.30 % (maximum CF = 10.24) at [Na_2S] = 4 mg/g. These experimental results could be mainly explained by Zn presence in sulfide forms (ZnS).

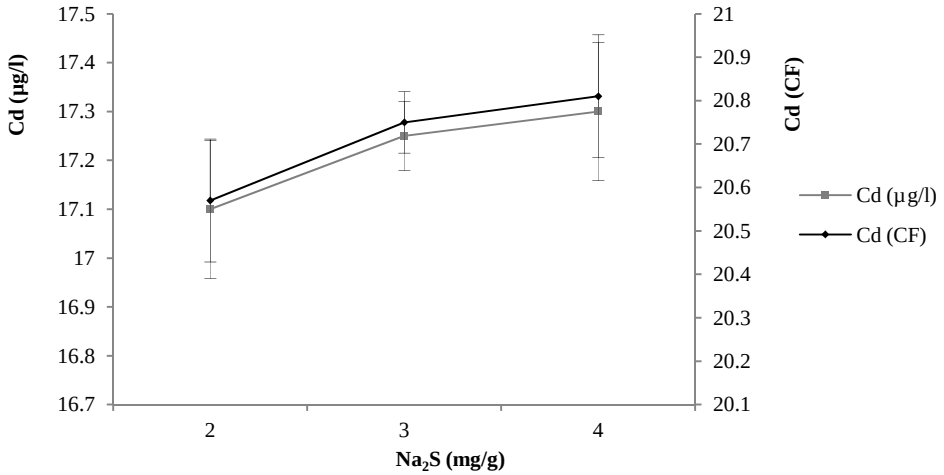

Fig.10. Na_2S effect on Cd flotation at pH = 12 in rhmanolipids presence

The addition of a sulfidization step did not cause a significant change in Cd recovery (Figure 10). Na_2S addition seems theoretically attractive, but in practice it suffers from several disadvantages: the different oxide minerals respond differently to sulfidization [33, 34]. Furthermore, the metal removal efficiency using a sulfidization step is low, compared to flotation process, this without chemical activation [35, 36].

3.8. Influence of flotation time

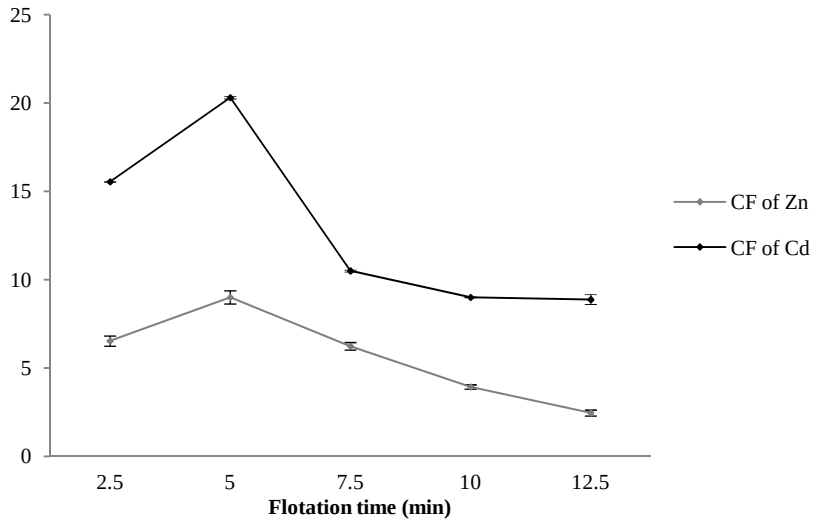

Fig.11. Concentration factors of Zn and Cd vs. flotation time. Flotation parameters: 40 mg/L of rhamnolipids, 4 mg/g of Na_2S and pH 12

Figure 11 shows that the concentration factor of Zn and Cd increased until 5 min since the beginning of the flotation, with a raise from 6.54 to 9 between 2.5 min and 5 min (an increment of 37.61 %). For the last period (5–12.5 min), CF of Zn decreased from 9 to 2.46 (a loss of 72.66 %).

A raise from 15.54 to 20.3 of the CF of Cd was observed between 2.5 min and 5 min (an increment of 30.63 %). For the last period (5–12.5 min) (Figure 11), the CF of Cd decreased from 20.3 to 8.88 (a loss of 56.25 %).

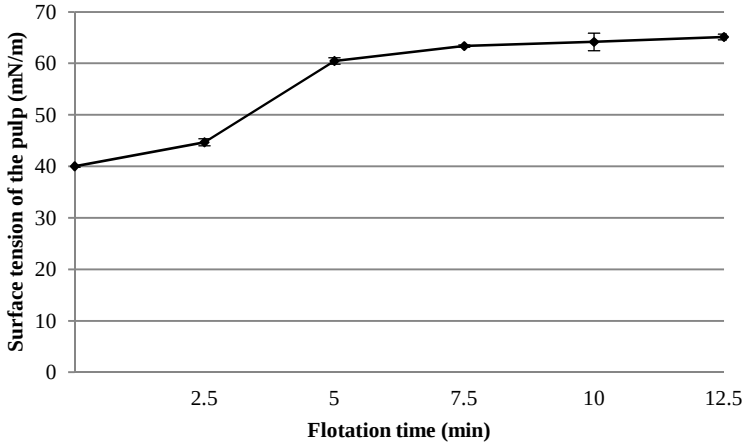

Fig.12. Surface tension (mN/m) of the pulp vs. flotation time

Figure 12 illustrates an increase of the surface tension of pulp from T_0 to 5 min (40 to 60.46 mN/m) followed by a constant surface tension for the flotation time above 5 min, which can be explained by rhamnolipids loss (movement from the pulp to the froth), thus Zn and Cd recovery from the contaminated soil by flotation (after 5 min) seems to be entirely due to mechanical entrainment, which is also reliable with Vanthuyne and Maes [36] and et al. findings [17].

4. CONCLUSIONS

The efficiency of Zn and Cd ions removal by rhamnolipids from soil systems will depend largely on the soil texture, structure, clay content and cations exchange capacity. Soil-washing technology provides rhamnolipids as a reliable solution for the remediation of heavy metal impacted soils. During this study, the chemical activation and the pH of the pulp significantly influenced the Zn ions recovery. However, the chemical activation does not caused significant Cd recovery. Effectiveness of this batch operation using rhamnolipids is strongly affected by the flotation time. In conclusion, the rhamnolipids produced by *Pseudomonas aeruginosa* S7PS5 were a kind of preferable surface-active substance, having potential application in bioremediation of various soil contaminants.

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