The release of crude oil into the environment by oil spill is gaining attention in the world and many accidents cause soil pollution. Many techniques have been developed to cleaning up those contaminants and the biological treatments are more efficient and cheaper than chemical and physical ones. The low solubility and adsorption are two major properties of high molecular weight hydrocarbons that limit their availability to microorganisms. For this reason, the addition of surfactants enhances the solubility and removal of those contaminants, improving the oil biodegradations rates. For this purposes, this work aimed at evaluating the potentiality of the addition of two anionic surfactants, a biological and a chemical ones to improve the bioremediation rates. Some toxicity tests such as dehydrogenasic activity and seed germination with *Lactuca sativa* were conducted to evaluated the behavior of the addition of both surfactants into the soil, being evaluated different concentrations of the active ingredient of both surfactants (0.1 to 1.5g of active ingredient for each 100g of soil) to verify whether or not those concentrations could be poisonous to the soil. After the tests, it was verified that high concentrations of both were poisonous to the soil. Besides the toxicity tests, the oil biodegradation rates were evaluated by the addition of those different concentrations of both surfactants and the biodegradability tests were carried out in bioreactors containing 450 g of crude oil contaminated soil and a 3cm layer of support rock. The tests were carried out at room temperature; being used a humid airflow of 3L/h. The nutrients were corrected through the addition of NH₄NO₃ and KH₂PO₄, in a nutritional ratio of C:N:P=100:15:1, being the humidity adjusted in 50% of the liquid retention capacity. Those experiments were accomplished with different concentrations of each active ingredient of both surfactant that varied 0.1 up to 1.5% w/w, besides a control(without surfactant addition). The experiments were run for 45 days and the removal of oil and grease (O&G) were evaluated. After finishing the biodegradation tests, an increase about 50% in the oil biodegradation efficiency was verified when 0.4 and 0.6% w/w of biological surfactant were used. The chemical surfactant didn't improve the bioremediation rates.

*Key-words: Bioremediation, toxicity, biosurfactant, soil, petroleum.*
1. INTRODUCTION

In spite of the petroleum industries being more and more careful regarding the environment preservation, there are still risks of environmental accidents either by sea transport or pipelines breaking, causing negative impact in every local ecosystem. When those accidents take place, there are several techniques that can be applied to minimize the impacts of the crude oil on the environment. Among the developed technologies, it stands out the bioremediation that is a technique that bases on the property that the microorganisms have to metabolize the hydrocarbons and other compounds found in crude oil that represent a source of energy for the bacteria. When the bacteria consume the oil, they convert it in more soluble products, forming, when the conversion is complete, CO₂ and H₂O. However, the bioremediation of crude oil-bearing soil suffers some limitations, depending on the characteristics and properties of the polluted soil. Very clay soils hinder the biodegradation of crude oil, bearing in mind that as bigger the soil organic fraction, the higher the sorption and smaller the hydrocarbons availability for microbial action. A possibility to increase the availability of those hydrocarbons to the microorganisms to metabolize them would be the use of bio-surfactants. The more positive effects of using bio-surfactants, so as to increase the crude oil biodegradation is the increase of hydrocarbons solubility; desorption of hydrocarbons previously adsorbed in the soil and the favored diffusion of hydrocarbons of the solid phase for the liquid phase, increasing, therefore, the availability of the oil for the microorganisms to act. However, it is essential a more detailed study of the bio-surfactants applicability in order to stimulate the bioremediation, as for the toxicity and optimum concentration to be used, aiming at reaching low environmental impact. Therefore, the present work aimed at evaluating an optimum bio-surfactant concentration to be added to the soil, in order to reach higher biodegradability and lower environmental impact.

2. LITERATURE SURVEY

2.1- Surfactants

Surfactants are surface-active agents and they are amphipathic molecules consisting of hydrophilic polar head moiety and a hydrophobic nonpolar tail moiety. They reduce the surface tension by forming an extremely small aggregate that is called micelles. At low concentrations, surfactants are soluble in water, and increasing their concentration, the molecules of surfactants form micelles in solution. The smaller concentration at which micelles begin to form is called as the Critical Micelle Concentration (CMC). Above their CMC, surfactants have been reported to solubilize petroleum hydrocarbons in soil-water systems, but some surfactants may increase the water solubility of hydrocarbon molecules below the CMC (ARONSTEIN & ALEXANDER, 1992; ALEXANDER, 1994; DESHPHANDE, 1999). Therefore, surfactants may be useful for the biodegradation of low-solubility hydrocarbon contaminated soil. The aim of this work was to evaluate surfactants and their concentration to enhance the biodegradation of petroleum hydrocarbons contaminated soil.

2.2 – Toxicity

Ecotoxicity bioassays are analytical methodologies that allow characterizing the toxicity of chemical substances in general. The exposition of bioindicators to those substances is a valuable tool for environmental analysis. For toxicity tests in soil, the interactions between the chemical compositions and the soil should be taken into account to predict the chemical impact correctly in the atmosphere. The soil is a component-key of the environment and, depending on the type of minerals, organic matter, pH, potential redox, humidity and handling of the soil, the pollutants can be adsorbed or liberated, tends different poisonous effects (KAPANEN & ITAVAARA, 2001). Following, some toxicity tests will be described in the present work.

2.2.1 – Enzymatic Tests

The enzymatic activity can be used to describe the effects of poisonous compounds on the soil microbiological population. The enzymes used in the soil microbiological activity are the hidrolases (phosfatases and ureases) and the oxidoreductases (desidrogenases) (RATSEP, 1991). The determination of the desidrogenasic activity is the most common method used for enzymatic toxicity tests and it is the method based on the estimate of the reduction rate of TTC (hydrochloric triphenletrazolium) and TPF (triphenil formazan) in the soils after incubation at 30°C for 24 h. TTC works as final electrons acceptor, being, therefore, one of the methods more frequently used for such estimate (BITTON & KOOPMAN, 1992).
2.2.2 – Phytotoxicity

Sensitive plants to poisonous substances can be used as bioindicators. According to FLETCHER (1991), the tests with plants can be used in 5 different categories: 1) - Biotransformation: transformation of compounds by the plants; 2) - capitation of alimentary chain: amount and concentration of poisonous elements that can enter in the alimentary chain through the capitation of the plants; 3) - sentinel: monitoring of pollutant observing the toxicity symptoms exhibited by the plants; 4) - indicative: certain plants that indicate the characteristics of the soil, either physicals or chemicals; 5) – phytotoxicity: among those tests, the one of phytotoxicity has been receiving higher attention in the last years. The phytotoxicity can be determined by the germination of seeds, elongation of the root and growth of the seedling. Some species recommended by the OECD (1984b); USEPA and FDA (FLETCHER, 1991) are Radish (Raphanus sativus); Carrot (Daucus carota); Rice (Oryza sativa); Turnip (Brassica rapa); Soja (Glycine max); Oats (Avena sativa); Cabbage (Brassica campestris); Corn (Zea mays); Tomato (Lycopersicon esculentum); Bean (Phaseolus aureus; Phaseolus vulgaris); Onion (Allium cepa); Sorghum (Sorghum bicolor) and Lettuce (Lactuca sativa).

Through the literature survey it was possible to evaluate that for soils with high clay content and polluted with high concentrations of hydrophobic compounds, the bioremediation technique suffers limitations, bearing in mind that those compounds, for being absorbed at the soil matrix, are not much available for the microorganisms to act. Thus, many studies are demonstrating that the surfactants addition and/or biosurfactants help the bioremediation technique increasing the availability of hydrocarbons to begin the attack of the present microorganisms in the soil. However, the interactions between surfactants and crude oil; surfactants and the components of the soil and between surfactants and the microorganisms of the soil should be well investigated, being the investigation of the surfactants toxicity in soils is extremely important to foresee the impact of the addition of them to the environment.

3. EXPERIMENTAL

3.1 – Soil

In this work a soil of the Northeast area of Brazil was used. This soil was homogenized, sieved and further contaminated with 5% of crude oil. The chemical, physicals and biological characteristics of the soil are shown in Table 1.

Table 1: Soil characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Virgin</th>
<th>Contaminated</th>
<th>Parameter</th>
<th>Virgin</th>
<th>Contaminated</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (g/kg)</td>
<td>0.36</td>
<td>0.3</td>
<td>Particle Dens. (g/mL)</td>
<td>2.2</td>
<td>1.5</td>
</tr>
<tr>
<td>P (g/kg)</td>
<td>0.1</td>
<td>0.075</td>
<td>Aparent Density</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Silte(1)</td>
<td>14%</td>
<td>—</td>
<td>Porosity (%)</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Sand(1)</td>
<td>75%</td>
<td>—</td>
<td>Organic Matter (%)</td>
<td>4</td>
<td>9.2</td>
</tr>
<tr>
<td>Clay(1)</td>
<td>11%</td>
<td>—</td>
<td>pH</td>
<td>6.8</td>
<td>6.4</td>
</tr>
<tr>
<td>C organic (%)</td>
<td>2.3</td>
<td>5.3</td>
<td>LRC (2) (%)</td>
<td>45</td>
<td>28</td>
</tr>
<tr>
<td>T/ HBO3(3) (UFC/g)</td>
<td>9.8 X10^6</td>
<td>2.3 X10^6</td>
<td>BH (4) (cel/g)</td>
<td>2.8 X10^4</td>
<td>2.1 X10^4</td>
</tr>
</tbody>
</table>

(1) Results provided by the environmental geo-technique laboratory of COPPE.
(2) CRL = Liquid retention capacity
(3) THB = Total heterotrophic bacteria
(4) HB = Hydrocarbonoclastic bacteria

3.2 – Crude oil

The crude oil was classified as paraffinic oil, with the following characteristics as sulphur (S) = 0.44; Carbon (C) = 86.2; Hydrogen (H) = 12.3; Nitrogen (N) = 0.3 (Below the detection limit).
3.3 – Surfactants

The chosen biosurfactant was a rhamnolipid one that is thoroughly used as aiding in the bioremediation process (NOORDMAN et al. 2002; NITSCHKE & PASTORE, 2002; MULLIGAN et al. 2005). That biosurfactant is concentrated with only 10% of rhamnolipid and didn’t suffer purification processes. The chemical surfactant analyzed was the dodecyl sodium sulfate (DSS)

3.4 – Ecotoxicity Test

3.4.3 – Ecotoxicity test

These experiments were available to verify if the surfactant addition could be damage for the soil desidrogenasic activity and seed germination with Lactuca sativa.

1) Desidrogenasic activity: A lot of variations of this technique have been tested and those tests were based on ALEF& NANIPIERE (1995) method. This test was accomplished in five replicates and remained incubated for 24 h in an oven at 30°C. TPF (tri-phenyl formazan) was extracted in 40 mL of methanol, being filtered and the liquid phase sent for further reading in spectrophotometer in the range of 485 nm. 2) Germination Test: The method of seed germination and growth, suggested by YERUSHALMI et al. (2003) was applied being used lettuce seeds of the Lactuca sativa species. The extract of the soil samples was put in Petri plates; being distributed 10 seeds of L. sativa, equally spaced. The plates were incubated at 24°C during 120 hours. After this time, the number of germinated seeds was counted and the length of the roots was measured from the transition point among the hypocolite to its extremity. The germination index (%GI) could be calculated through the following formula: %GI = (% seeds germination) x (% roots growth): 100

3.5 - Tests of soil biodegradation mediated by the addition of surfactant in bioreactors

The experiments were accomplished in 20cm high and 5cm of diameter bioreactors, being used 3cm of layer of support rock. The tests were maintained at room temperature, being used a humid airflow of 3L/h as established by PALLA (2002) in previous tests. The Figure 1 shows an outline of the reaction system for the biodegradation tests. The tests were accomplished by adding different concentrations of the surfactant in different essays. The concentration of the surfactant varied from 0.1 to 1.5% p/p (g of rhamnolipid or g of DSS for 100g of soil). The nutrients were corrected through the addition of ammonium nitrate (NH4NO3) and bihydrogen potassium phosphate (KH2PO4), being used a nutritional ratio of C:N:P = 100:15:1, as established in previous tests (OLIVEIRA, 2005). The tests were carried out in 45 days. Samples were taken every each 15 days for determining oil and Grease (O&G), pH and microbial population. During the experimental period the humidity was controlled through thermogravimetric method.

![Figure 1 - Schematic representation of the aerobic fixed bed bioreactor](image-url)
3.6 – Analytical methodologies

1) Oil and grease (O&G) determination: The method described by the Standard Methods (APHA, 1992) was adapted for the soil samples. The methodology consisted of the separation of O&G from a certain mass of the soil (~ 2g). The extraction was accomplished with hexane, for 4 hours to a speed of 20 cycles per hour. After this period, the extracts were concentrated in rotavaportor and transferred for 1cm diameter tubes as described by RIZZO and RAIMUNDO (2003).

2) Quantification of the microbial population: The quantification of the total heterotrophic microbial population was done by Pour Plate technique, using a solid organic medium (TSA - Trypic Soy Agar). 5g of soil were added in 50 mL of saline solution (NaCl 0.85%) and suspended in shaker for 1:00 hour at 30 ºC. After agitation the plating operation was carried out adding 0.2 mL of the previously prepared saline suspension in the plates. That plate was incubated by 48 hours at 30 ºC and the number of colonies forming units was counted (result expressed as CFU / g of soil). The quantification of the degrading microbial population was done by the technique of the most probable number (MPN) in agreement with OBLINGER & KOBURGER (1975). The liquid mineral medium, obtained in agreement with VECCHIOLI et al. (1990), was used for the growth of the degrading microorganisms. The methodology was conducted in accordance with VOLPON (1998).

4 - RESULTS AND DISCUSSION

4.1 – Evaluation of the surfactants concentrations in the soil after biodegradation test

Before the biodegradation essays some ecotoxicity test of both surfatants were evaluated as dehydrogenase activity and index of germination, whose the objective was to evaluate which the best concentration to be added to the soil to enhance the biodegradation rates of the petroleum contaminated soil

4.1.1 – Dehydrogenize Activity

The soil dehydrogenase activity reflects the total oxidative activity of the microbiota and can act as a good indicator of the microbial activity present in the soil (GARCIA et al., 1997). The Figure 2 shows the results of the dehydrogenase activity in µg/g of soil against the biosurfactant (rhamnolipid) and DSS addition. It can be observed that all values are above of the non-contaminated soil (4.2 µg/g of TPF), characterizing a tolerance of the soil native microbiota with the addition of these surfactants. In relation to the biosurfactant, the best result was relative of the addition of the 0.4%w/w of active ingredient (~ 74 µg/g of TPF) and after it concentration was observed decrease of the activity, but, the concentration didn’t reach values below the activity of the soil without surfactant addition. However, it was observed an accentuated drop of the activity in relation to the concentration 1.5% w/w of biosurfactant (active ingredient – rhamnolipid). The activity of chemical surfactant (DSS) was increasing little by little with the addition of this surfactant.

![Figure 2: Dehydrogenize activity in the both surfactants.](image-url)
4.1.2 – Phytotoxicity

The advantages of the germination tests are due to its low cost and simplification and for that reason, to those tests are very used as phytotoxicity tests. In this study it was verified that both surfactants reduced the germination index with the addition of its concentration (Figure 3). The seed germination tests indicated with the lowest concentration (0.1% w/w) there was an increased of the index of germination above to the control, suggesting that low concentrations of these surfactants can increase the availability of some nutrients to improve the soil fertility and among the concentrations 0.4 and 0.8 the germination index was practically constant for both surfactants. Between the concentrations 1 and 1.5% w/w there was a negative effect with about 70 to 80% of inhibition in relation to the control, indicating that those concentrations are damage for germination of the *L. sativa*.

![Germination Index of both surfactants](image)

**Figure 3**: Germination Index in of both surfactants.

4.2 - Evaluation of crude oil biodegradation in soil in different surfactant concentrations.

The Figure 4A shows the results of oil and grease content in the soil before and after biodegradation tests with different addition of rhamnolipid concentrations and a control test (without rhamnolipid addition). It was verified that after 45 days of treatment, the conditions that presented the best oil and grease removal were 0.4 and 0.6% w/w, removing about 50% of oil and grease. However, the condition with 0.4% of rhamnolipid there was a tendency to reach a little more biodegradation index after 45 days of treatment. All results presented an oil and grease removal superior to the control, but with addition of 1.0 and 1.5%w/w of active ingredient the results were below or similar to the control. Comparing these results with the seed germination tests and with dehydrogenase activity, those concentrations inhibited the germination of *L. sativa* and it was not beneficial for dehydrogase activity, suggesting that they were more negative in the crude oil contaminated soil. In fact, the concentration of 1.5% w/w of rhamnolipid contributed for inhibiting the oil biodegradation process and that concentration (1.5%) decreased the population of hydrocarbonoclastics (HM) microorganisms (capable to degrade the crude oil) (Data not shown). The Figure 4B shows the results of oil and grease content in the soil before and after biodegradation tests with different addition of DSS concentrations and a control test (without DSS addition). It was verified that during the experiments and after 45 days of treatment, the results didn’t present difference neither of the different additions of surfactant nor the control essay, suggesting that this surfactant was not available for the microorganisms, probably because it was solid, needing to be better dissolved with water before use for biodegradation essays.
5. CONCLUSIONS

The seed germination using the Lactuca sativa (lettuce) species indicated that as the concentration of both surfactants increased, there was an inhibiting effect in the germination, bearing in mind that the concentrations of 1 and 1.5% of active ingredient contributed for the diminishment of the germination index.

Six different treatments with addition of each surfactant were accomplished and the obtained results, by evaluating the oils and greases removal, have shown that the rhamnolipid concentrations of 0.4 and 0.6% (w/w) presented good crude oil removal with percentage about 50%, after 45 days of biodegradation tests. The addition of the dodecyl sodium sulfate (DSS) didn't improve the biodegradations rates, but the results of biodegradation didn't show this tendency, in other words, as larger as the concentration better should be the biodegradation, but these results couldn't be observed probably because this surfactant needs to be more diluted before being added to the soil for the biodegradation tests.

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7. REFERENCES


MULLIGAN, C.N. Environmental applications for biosurfactants. Environmental Pollution. vol.133, pp.183-198. 2005


RIZZO,A.C.L; RAIMUNDO,R. (2003). Determinação de óleos e graxas, em solo, por gravimetria empregando método de extração com ultrassom. IT2003-001-00 – Instrução de Trabalho elaborada para o CETEM.

ROJAS-AVELIZAPA, N.G.; RODRIGUEZ-VALQUEZ, R.; SAVAL-BOHORQUEZ, S. ALVAREZ, P.J.J.;