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Application of surfactants and biosurfactants in the bioremediation of multi-contaminated soils: microcosms and bench scale bioreactor trials

Daniele Leonel da Rocha[1,2], Eliana Flávia Camporese Servulo [1], Rodrigo Gouvêa Taketani[2,3], Sandy Sampaio Videira[2], Andrea C. de Lima Rizzo[2], Cláudia Duarte da Cunha[2]

[1]Federal University of Rio de Janeiro, Escola de Química, Rio de Janeiro – RJ, Brazil.
[2]Centre for Mineral Technology, MCTIC, Rio de Janeiro – RJ, Brazil.
[3]College of Agriculture Luiz de Queiroz, University of São Paulo, Piracicaba – SP, Brazil.

Abstract**—** *In the present study, in order to rehabilitate areas multi-contaminated by oil and metals, bioremediation, coupled with bioreactor technologies, was applied. Firstly, a microcosm trial was performed using two synthetic surfactants, TRITON X-100 and TWEEN 80, and a biological one, JBR210, at two different concentrations (0.1 and 1.0 mg. g^{-1} soil) for 42 days to select the two best surfactants of different natures in order to scale up a solid-phase bench bioreactor under controlled operating conditions. The results obtained with the use of Tween 80 and biosurfactant JBR210 at a concentration of 0.1 mg. g^{-1} soil, with humidity corrected to 70% of WHC, were a 25.5% and 30.00% removal of THP (Total Petroleum Hydrocarbon), respectively, while control with humidity adjustment alone achieved 20.5% removal. The evaluation of the behavior of microbial activity through next generation sequencing (NGS) of the soil was performed before and after the different treatments in the bioreactor, where the control showed an increase of Actinobacteria population. The treatments with surfactants showed an increase of Alphaproteobacteria and Sphingobacteria in this same period, and a decrease in the abundance of Actinobacteria. The diversity measured by the Shannon index (H') showed a significant decline for the Tween 80 treatment, with no sign of recovery, unlike JBR210 biosurfactant treatment. Therefore, the application of surfactants of different natures has different effects, where both promoted the reduction of diversity, whereas the biosurfactant JBR210 showed a tendency to increase after 42 days, indicating a less intense effect, ie. it is better for the environment.*

Keywords**— Bioremediation, hydrocarbon, surfactant, biosurfactant, bioreactor.**

I. INTRODUCTION

Despite the numerous alternative sources of energy available today, oil is still considered the main source of energy and raw material for various petroleum products, including solvents, fertilizers, plastics, paints, pesticides, among others [1]. Due to the widespread use of these compounds, production, transportation, and storage activities have been identified as the main soil contamination routes, as they inevitably involve the risk of accidental spills [2,3].

Due to the harmful effects caused by the contaminants in question, many research and technologies have been developed and applied in order to minimize their negative effects on the environment and human health [4,5,6,7].

Increasing costs and limited efficiency of physical and chemical treatments have spurred the development of alternative technologies for in situ and onsite application based in particular on biological remediation (bioremediation) capacity [8,9,10,11].

In addition to the use of biostimulation and/or bioaugmentation to increase the efficiency of the bioremediation process, the use of surfactants has significantly contributed to the bioavailability, and consequent biodegradation, of contaminants [12]. These surfactants can be chemically synthesized or produced by microorganisms, called biosurfactants [13]. Biosurfactants, besides presenting lower processing costs, are considered environmentally friendly substances, since they are based on renewable and sustainable resources, and biologically degradable [14].

In general, biodegradation of oil can be optimized with the application of surfactants by maintaining controlled, process-friendly conditions [12]. One of the technologies that favor this process is the use of

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bioreactors, which promote the ideal conditions for microbial activity, due to the control of factors such as temperature, pH, agitation, aeration rates and others. The use of this type of technology seeks to shorten treatment time, as it enhances biological degradation and minimizes abiotic losses [15].

Bioremediation is already a well-established technology, successfully proven in numerous applications for the treatment of hydrocarbon-contaminated areas, but changes in bioavailability as well as the production of interfering metabolites are common. Therefore, when soil contamination occurs, identification of the microbial community can be a valuable tool for predicting the potential efficiency of bioremediation processes. High-performance molecular techniques such as new generation sequencing have now emerged to assist traditional cultivation methods, enabling a more complete assessment of biodiversity and the identification of organisms and essential metabolic pathways related to bioremediation [16,17,18]. Molecular tools applied to contaminated soils before and after treatment can be a complementary tool for verifying the effect of contaminants such as petroleum and other substances added during treatment [16,19].

Thus, the objective of the present work was to evaluate the bioremediation of a multi-contaminated soil with hydrocarbons and metals through the use of microcosm and solid-phase batch bioreactor trials, together with the application of surfactants, synthetic and biological. In the bioreactor soil samples, the microbial activity was evaluated before and after the different treatments using next generation sequencing (NGS).

II. MATERIAL AND METHODS

2.1 Soil

For the present study we used a silt soil multi-contaminated with hydrocarbons and metals, coming from a region near an oil refinery, located in southeastern Brazil. Due to the results of the chemical analysis of the soil, it was not necessary to incorporate nitrogen and phosphorus to maintain the C: N: P ratio of 100: 10: 1. The physical and chemical characteristics of the soil are presented in Table 1.

Table 1. Physicochemical properties of the contaminated soil.

Parameter	**Value**
(%) silt (weight)	58.78
(%) sand (weight)	25.74
(%) clay (weight)	15.48
Organic matter ($mg.kg^{-1}$)	6.5
Total N ($g.kg^{-1}$)	16.65
Available P ($g.kg^{-1}$)	2.13
pH	6.23
Water holding capacity (%)	44.35
CTC ($cmol_c.kg^{-1}$)	24.47
Contaminating metals ($mg.kg^{-1}$ of soil)	
Cu	Ni	Zn
310	158	2100

2.2 Surfactants

The unpurified commercial biosurfactant JBR210® (JENEIL BIOSURFACTANT COMPANY, USA), containing 10% rhamnolipid in its composition, produced by a *Pseudomonas aeruginosa* strain was the surfactant of biological origin. The synthetic surfactants used were Tween 80 and Triton X-100, both from INLAB. Surface tension measurements of surfactants were 27.1 $mN.m^{-1}$ for JBR210®, 34.9 $mN.m^{-1}$ for Tween 80 and 35.33 $mN.m^{-1}$ for Triton X-100 by the Du Noüy Ring Method at room temperature (25ºC) using a Krüss K10T Tensiometer.

2.3 Microcosm Trials

With humidity corrected to 50 and 70% of WHC (values defined in preliminary assays), 250 mL flasks containing 50g of multi-contaminated soil were used for microcosm trials. For the tests with synthetic and biological surfactants, two different concentrations (0.1 and 1 $mg.g^{-1}$ soil) were used. All assays were duplicated for 42 days to assess the removal of total petroleum hydrocarbons (TPH). The system was aerated with an injection of compressed air at a flow rate of $20mL.min^{-1}$ for 2 minutes and homogenized three times a week. The humidity content was corrected to the value corresponding to each system three times a week.

2.4 Bioreactor trial

In the larger scale experiments, a bench type U (13L) bioreactor containing 4 kg of soil was developed by CETEM in partnership with PETROBRAS [20]. Based on the results obtained in the microcosm experiments, three trials were conducted: (1) humidity adjusted to 70% of WHC, and the addition of a synthetic surfactant at a concentration of 0.1 $mg.g^{-1}$ soil; (2) humidity adjusted to 70% of WHC, and the addition of a biological surfactant at a concentration of 0.1 $mg.g^{-1}$ soil; (3) humidity adjustment to 70% WHC (no surfactant added; control). All assays were duplicated for 42 days, with weekly

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humidity correction. Twice a day, the systems were aerated with an injection of compressed air at a flow rate of 20 L.min^{-1} for 30 minutes and homogenized for 15 minutes at 4 rpm. Samples were taken at beginning and after 42 days for evaluation of TPH removal by infrared spectrometry. For molecular analysis samples were taken weekly.

2.5 Quantification of Total Petroleum Hydrocarbons (TPH)

The quantification of TPH in the soil samples was conducted by Infrared spectrometry using an InfraCal TOG / TPH analyzer, HART-T model (Wilks Enterprise), according to the protocol described by Rizzo et al. [20]. The oil concentration (mg.kg^{-1}soil) present in each sample was calculated using a standard curve previously obtained from reading different known oil concentrations.

2.6 *rrs* Gene Sequencing

In order to evaluate changes in microbial diversity in the bioreactor treatments, large-scale sequencing experiments of the gene that encodes ribosomal rRNA from the Bacteria Domain were performed. After DNA extraction using the FastDNA spin kit for soil (MPBio) commercial DNA extraction kit, following the manufacturer's instructions, primers 967f and 1193r were used to amplify the genes encoding Bacteria 16S rRNA [21,22]. High throughput sequencing libraries were prepared using the Ion PGM Template OT2 400bp kit according to the manufacturer's protocol (Life Technologies). Sequencing was performed using the ion torrent PGM system instrument with the Ion PGM sequencing kit 400bp on 316v2 chip. The sequences obtained were analyzed using the Qiime platform [23] following the protocol previously presented [24]. The sequences are available on the MG-RAST website under the mgp375 project.

2.6.1 Data analysis

Non-normalized data were used to calculate Shannon's diversity index (H') by the Vegan package [25]. Comparisons between treatments by ANOVA followed by Tukey HSD post hoc test were performed in the agricolae package [26]. Data transformed in relative abundance were used for the analysis of the principal coordinates, distance-based Bray-Curtis using the phyloseq package [27]. Differences between communities, treatment effects, and time were compared by PERMANOVA of the vegan package [25] with 9999 permutations. The samples were considered significantly different when the test presented p value <0.05. All graphics were generated in RStudio® using ggplot2 [28].

III. RESULTS AND DISCUSSION

3.1 TPH removal in microcosm and bioreactor assays

The results of TPH removal in the 42-day microcosm trials are shown in Table 2. The initial value of the oil concentration of the soil was 40,000 mg.kg^{-1} soil or 4%(w.w^{-1}). All the studied conditions presented a reduction of the initial oil content in the soil, the best conditions being obtained with the use of synthetic surfactants Tween 80, Triton x-100, both in the 0.1 mg.g^{-1} soil, presenting oil concentration values of 28,610 and 30,100 mg.kg^{-1}soil, corresponding to 28.50% and 24.75% of removal. These values were higher than those found in the control trials and in the presence of the JBR biosurfactant, which reached 20.75% and 19.25%, respectively, under the best conditions.

Several works positively address the use of the same synthetic surfactants. In a study by Ramamurthy & Memaryan [29], different concentrations of Triton X-100 and Tween 80 were tested for the treatment of soil contaminated by engine oil. However, they observed that higher concentrations of Triton X-100 decreased the removal values, indicating a possible toxicity to the microorganisms present in the soil, as verified in the present study.

For the control trials, the treatment with humidity correction for 70% of the WHC presented higher removal values, compared to the tests with a lower humidity content (50%), corroborating the results obtained by Taketani et al. [31], including testing this higher level operation of the solid phase bioreactor. Increasing humidity content may contribute positively to the removal of hydrocarbons, as water is critical to microorganisms, affecting osmotic pressure and intracellular metabolism [30,31].

Table 2. TPH results for the bioremediation tests in microcosms during 42 days.

Treatment Condition	Time (day)	TPH concentration (x10^4) (mg.kg^{-1}soil)	TPH removal (%)
Moisture 50% WHC	42	3.47± 0.10*	13.25
Moisture 70% WHC	42	3.17 ± 0.50*	20.75
Tween 80-0,1 mg.g^{-1} 70%WHC	42	2.86± 0.00*	28.50

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Tween 80-1 mg.g^{-1} 70%WHC	42	3.29 ± 0.20*	17.75
Triton X100-0,1mg.g^{-1} 70%WHC	42	3.01± 0.00*	24.75
Triton X100-1mg.g^{-1} 70%WHC	42	3.62± 0.10*	9.50
JBR 210 -0,1 mg.g^{-1} 70%WHC	42	3.31± 0.50*	17.25
JBR 210- 1 mg.g^{-1} 70%WHC	42	3.23± 0.00*	19.25

Therefore, three conditions were selected to perform a scale-up of bench bioreactors to compare the application of a synthetic surfactant and a biological surfactant, as well as the control, in order to increase the removal of TPH: (1) with humidity corrected to 70 % of WHC, where there is no cost of incorporating surfactants (control); (2) with humidity corrected to 70% of WHC and addition of Tween 80 synthetic surfactant at the lowest concentration (0.1 mg.g^{-1} soil) and (3) with humidity corrected to 70% of WHC and added to the JBR210 biosurfactant at a concentration of 0.1mg/g of soil. The lowest concentration of these products was used to reduce costs in both systems incorporating surfactants, since the differences in TPH removal obtained with the use of biosurfactant were too small to justify a 10 x greater incorporation of the product in an enlarged scale.

Table 3 shows the TPH concentration values obtained in soils treated in the bench bioreactors after 42 days, emphasizing that the initial oil concentration value of the soil was 40,000 mg.kg^{-1} soil or 4% (w.w^{-1}).

In 42 days, we observed that all conditions presented lower TPH concentration values in the soil, being 28,020, 29,900 and 31,880 mg.kg^{-1} soil, using the biological surfactant JBR210, the synthetic surfactant Tween 80, and control assay, respectively. Consequently, the oil removal values in the control test (20.50%) and with the addition of Tween 80 synthetic surfactant (25.25%) were very close to the values obtained in microcosm. At a concentration of 0.1 mg.g^{-1} of soil, the biological surfactant JBR210 was higher with an increasing scale (an approximately 10% increase compared to microcosms), with an average TPH removal of 30%, showing the efficiency of the solid phase bioreactor used. In a study by Li et al. [32], the removal of hydrocarbons in the presence of rhamnolipid biosurfactant was compared with the Tween 80 surfactant, where the highest removal values observed were achieved when the biosurfactant was used. In a solid-phase bioremediation study, the application of the rhamnolipid biosurfactant at a concentration of 0.1 mg g^{-1} of soil was studied over a period of 35 days, reaching mean values of 72.4% removal, while an average removal of 15.6% was achieved for the control, demonstrating the efficiency of biosurfactant application [33]. However, the actual efficiency depends on the type of soil, oil, and technology applied. According to Vandana and Singh [34], the use of biosurfactants is advantageous over the application of surfactants of synthetic origin, since they are environmentally friendly, easily biodegradable, and non-toxic.

Table 3. TPH results for the bioremediation tests in bench scale solid phase bioreactor during 42 days.

Treatment Condition	Time (day)	TPH concentration (x10^4) (mg.kg^{-1}soil)	TPH removal (%)
Moisture 70% da WHC	42	3.18 ± 0.10*	20.50
Tween 80-0,1mg.g $^{-1}$ 70% WHC	42	2.99 ± 0.10*	25.25
JBR 210-0,1mg/g 70% WHC	42	2.80 ± 0.40*	30.00

Corroborating the results obtained by Taketani et al. [31], the use of a solid phase bioreactor was promising, especially with the appropriate humidity adjustment to favor the microbial soil and to improve the remediation of contaminated soil. As stated above, all types of contaminated soils should be studied in advance in order to analyze the best technique / technology to be applied. Consideration should also be given to by-products formed after treatment in order to assess toxic effects on the environment. Since the TPH removal values obtained were interesting and demonstrate potential of Tween 80 synthetic surfactant and rhamnolipid JBR210 biosurfactant in bioremediation of contaminated soils, in addition to adjusting the humidity content, evaluation of changes in soil microbial community structure is necessary to infer the efficient application of this technology.

3.2 Molecular Analysis

The structure of microbial communities is shaped by the interaction between environmental and biological factors [35,36]. One of the most analyzed effects is community interaction with pollutants, their remediation,

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and how different environmental factors may affect the course of this process [31,37]. The application of adjuncts to the hydrocarbon degradation process can alter how certain populations perceive the presence of a particular substance favoring or not the desired metabolism [37]. The sequencing analysis of the gene encoding the 16S rRNA from samples indicated that there was little difference in the richness present in these samples (Fig. 1). The ANOVA indicated that although there was some variation in the Chao1 richness estimator, the observed variation was not significant. However, the diversity measured by the Shannon index (H ') showed significant variation. The Tween 80 treatment showed a significant decline in these indices and showed no sign of recovery, unlike the JBR210 biosurfactant treatment. Thus, the application of surfactants of different natures can have different effects. As observed by the analysis of Shannon's diversity index, both the bio-substance (JBR210) and the synthetic-substance (Tween 80) led to a decrease in diversity, although the biosurfactant JBR210 showed a tendency towards increased diversity at the end of the experiment. This result indicates the possibility that this biosurfactant has a less intense effect, or is better for the environment as indicated in previous work [38].

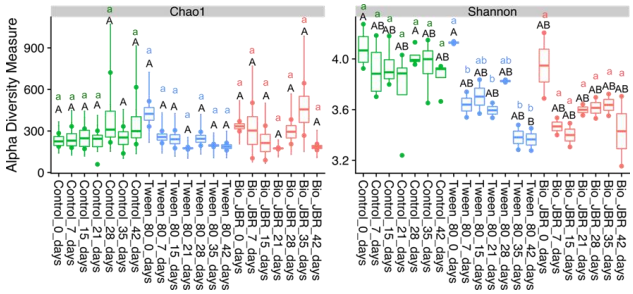

Fig.1. Values of the taxonomic unit richness index (Chao1) and Shannon index (H') obtained from the sequencing data. Capital letters on the samples indicate significant differences by Tukey HSD post hoc test between all samples. The lower case color letters of the treatments on the samples indicate significant differences by Tukey's test within each treatment. Control- humidity corrected to 70 % of WHC; Tween80- humidity corrected to 70% of WHC and addition of Tween 80 synthetic surfactant at a concentration 0.1 mg.g^{-1}soil; JBR210- humidity corrected to 70% of WHC and added to the JBR210 biosurfactant at a concentration of 0.1mg.g^{-1} soil.

The taxonomic affiliation of the sequences showed that these soils are mainly formed of the classes Actinobacteria, Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria (Fig. 2). However, all treatments varied over time. The control showed a decline in relative abundance of Betaproteobacteria and Anaerolineae between the beginning and the end of the experiment while there was an increase in Actinobacteria. The treatments that received surfactants showed an increase of Alphaproteobacteria and Sphingobacteria during the same period, while there was a decrease in the abundance of Actinobacteria. However, when looking at the taxonomic affiliation of the OTUs found in the samples, great stability is observed in the control treatment community. This indicates that a considerable part of the community has been affected by the addition of different surfactants. Increased Proteobacteria in surfactant-containing samples has been observed previously and correlated with increased pollutant removal [39,40]. This increase was due to increased hydrocarbon contact with hydrocarbonoclastic populations [39]. However, a PCoA analysis indicated that Sphingobacteria had a stronger association with surfactant containing samples than the Proteobacteria OTUs.

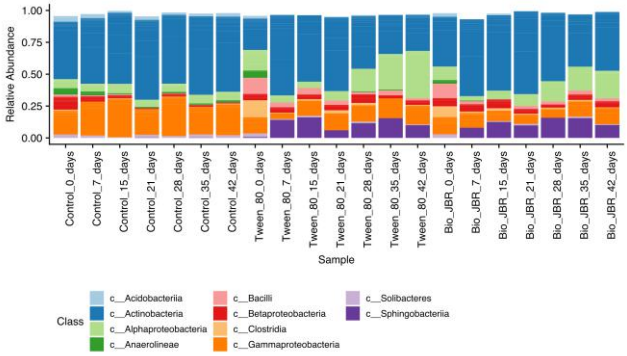

Fig.2. Taxonomic classification of sequences from the V3-V4 region of the 16S rRNA coding gene. Classification is represented at Class level. The data represent the averages between repetitions. Control- humidity corrected to 70 % of WHC; Tween80- humidity corrected to 70% of WHC and addition of Tween 80 synthetic surfactant at a concentration 0.1 mg.g^{-1}soil; JBR210- humidity corrected to 70% of WHC and added to the JBR210 biosurfactant at a concentration of 0.1mg.g^{-1} soil.

Principal coordinate analysis (Fig. 3) indicated that the control samples formed a group that included the initial samples (0 days) that were treated with surfactants, however, by seven days the samples that received surfactant presented a large alteration of the community observed (Fig. 3-A). Thus, the samples (Fig. 3-A) were correlated with the OTUs belonging to the five most abundant orders in the samples (Fig. 3-B), and the result indicated that these two sample groups correlated with OTUs of different orders. The control has a strong

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correlation with Xanthomonadales while surfactant samples correlate with Sphingobacteriales. While correlating with both, most Actinomycetales OTUs are related to the control treatment. Bacteria of this group, although often capable of degrading hydrocarbons [41], are not commonly found in remediation of this type of pollutant.

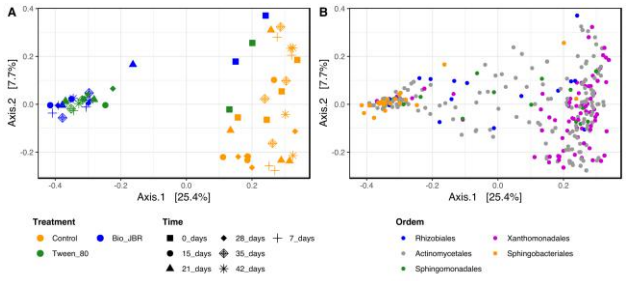

Fig.3. Principal coordinate analysis (PCoA) obtained from sequencing data. A - PCoA representing relationships between samples; B - PCoA representing the relationships between operational taxonomic units (OTU) found in the samples. The treatments are separated by colors according to the subtitle as the times are separated by shapes according to the same subtitle. OTU's are colored according to their taxonomic classification by order of legend. Values in parentheses indicate the percentage of variance represented by the axis. Control- humidity corrected to 70 % of WHC; Tween80- humidity corrected to 70% of WHC and addition of Tween 80 synthetic surfactant at a concentration 0.1 mg.g^{-1}soil; JBR210- humidity corrected to 70% of WHC and added to the JBR210 biosurfactant at a concentration of 0.1mg.g^{-1} soil.

The data of the two-way PERMANOVA indicates that there were significant differences between treatments (p = 0.001) and time (p = 0.003); however, there was no effect of interaction between the two. There was no effect of time on the control treatment, but there was an effect on both treatments containing surfactants (p = 0.001). Surfactant treatments were significantly different from the control (p = 0.001) but not among themselves.

IV. CONCLUSION

In the present study, the bioremediation of a tropical, silty, multi-contaminated soil with oily sludge and metals from a large scale application of a batch solid-phase bioreactor technology was promising, achieving removal results of approximately 20% in 42 days, only adjusted with a humidity content of 70% WHC. When combined with the incorporation of surfactants of synthetic origin (Tween 80) and of biological origin (biosurfactant, JBR210) at the concentration of 0.1 mg.g^{-1} of soil, the removal value increased to approximately 25 and 30%, respectively, which are better results than those obtained in microcosms.

All soil treatments in bioreactors showed variation in the microbial community over time. Due to the taxonomic affiliation of the OTUs found in the samples, there was a strong stability in only the control treatment community (with humidity adjustment to 70% WHC), with an increase of Actinobacteria population. This proves that much of the community was affected by the addition of different surfactants, which promoted an increase of Alphaproteobacteria and Sphingobacteria, and a decrease in Actinobacteria abundance, with Sphingobacteriales showing a stronger association with surfactant-containing samples as opposed to Proteobacteria OTUs.

Therefore, the application of surfactants of different natures has different effects, where both promote the reduction of diversity, although the biosurfactant JBR210, after 42 days, showed a tendency of increasing diversity, indicating a less intense effect, which is better for the environment.

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