CIÊNCIA e NATURA

UFSM

Ci. e Nat., Santa Maria v.42, e6,
Special Edition: 40 anos, p. 1-27, 2020
DOI:10.5902/2179460X39667
ISSN 2179-460X

40 years - anniversary

Received: 22/08/19 Accepted: 27/09/19 Published 03/09/20

Recovery of mangrove sediment contaminated with fuel oil by endogenous microbial consortium

Recuperação de sedimentos de manguezal contaminados com óleos combustíveis por consórcio microbiano endógeno

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ABSTRACT

This work aimed to select a microbial consortium enriched with isolated microorganisms of mangrove sediment as to its capacity to recover sediment contaminated by lubricating oil. The promising microorganisms were selected using the colorimetric dichlorophenol indophenol technique (DCPIP) using lubricating oil as the carbon source, to evaluate the emulsifying and enzymatic activity of the microorganisms. The antagonism test was also used for further evaluation of the consortia. The fractional factorial experimental design methodology (2n) was used to establish the process conditions for the subsequent accomplishment of the degradation kinetics of the lubricating oil by the selected microorganisms and consortium. Eight bacteria and three fungi were evaluated, of which five were selected with a 36 h turn of the DCPIP indicator. Eleven microorganisms produce emulsifying substances and five produce enzymes. The results showed that the best consortium was B5F2F4, with a degradation rate of 95% of the phenol at 70 rpm in 250 µL of the oil. The kinetics of oil degradation showed a phenol degradation rate of 65% after 24 days of treatment. The microorganisms are suitable for the degradation of phenol, the main constituent of the oil, and can be used as a recovery model for environments contaminated with hydrocarbons.

Keywords: Mangrove; Bioremediation; Microorganisms; Consortium

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RESUMO

Este trabalho objetiva selecionar um consórcio microbiano composto por microrganismos isolados de sedimentos de mangue quanto à sua capacidade de recuperar sedimentos contaminados por óleo lubrificante. Os microrganismos promissores foram selecionados pela técnica colorimétrica de diclorofenol indofenol (DCPIP), utilizando óleo lubrificante como fonte de carbono, para avaliar a atividade emulsificante e enzimática dos microrganismos. O teste de antagonismo foi realizado para avaliação dos consórcios. O planejamento experimental fatorial fracionário (2n) foi utilizado para estabelecer as condições do processo para a realização subsequente da cinética de degradação do óleo lubrificante pelos consórcios selecionados. Foram avaliadas oito bactérias e três fungos, dos quais cinco foram selecionados com uma viragem de 36 h do indicador DCPIP. Onze microorganismos produzem substâncias emulsificantes e cinco produzem enzimas. Os resultados mostraram que o melhor consórcio foi o B5F2F4, com uma taxa de degradação de 95% do fenol a 70 rpm em 250 µL do óleo. A cinética da degradação do óleo mostrou uma taxa de degradação do fenol de 65% após 24 dias de tratamento. Os microrganismos são adequados para a degradação do fenol, o principal constituinte do óleo, e podem ser usados como modelo de recuperação para ambientes contaminados com hidrocarbonetos.

Palavras-chaves: Manguezal; Biorremediação; Microrganismos; Consórcio

1 INTRODUCTION

Petroleum is an organic compound, formed by biogeochemical processes, and its composition varies according to the chemical, physical, and biological conditions of its geographical location. The compound consists mainly of a complex combination of hydrocarbons with small quantities of non-hydrocarbon compounds such as sulfur and nitrogen (CRAPEZ et al. 2002; USBERCO & SALVADOR, 2002, CETESB, 2004;).

Containing a complex composition, petroleum consists of numerous compounds that are grouped according to their solubility in organic solvents (e.g., hydrocarbons, paraffinic, naphthenic, aromatic, resins, and asphaltenes). Because polycyclic aromatic hydrocarbons (HPA) are the main constituents of petroleum, they are recalcitrant pollutants with high molecular weights (CAPELLI et al., 2001; ABNT, 2004).

Pollution from hydrocarbons has become a global concern because they are difficult to decompose, posing a risk to the environment and human health. (MARTINS & AZEVEDO, 2012). The oil industry is often subject to accidents, such as oil spills during transportation. The elimination of these products and by-products is currently one of the most important issues in pollution control, which has led researchers to seek new and more effective techniques that aim to remove these substances from the environment (ANDRADE, 2003).

Among the affected ecosystems, the mangrove is classified as one of the most sensitive and vulnerable to oil spills with the increased port activities and the transport of oil (CURY, 2002; MONTEIRO, 2003). The mangrove is a coastal ecosystem, characteristic of tropical and subtropical regions. It creates a transition between terrestrial and marine environments and is subject to the tidal regime, presenting favorable conditions for the feeding, protection, and reproduction of many living beings (SILVA, 2005). With the external actions, it is possible to observe how human intervention affects the mangrove, acting in search of its self-preservation, degrading various pollutants, and using them as a source of nutrients (BRITO et al., 2004).

The Brazilian coast exhibits one of the largest extensions of mangroves in the world, being the third largest mangrove area. This area is a narrow strip of mangrove forest of approximately 20,000 km² that stretches from the mouth of the Oiapoque River in the state of Amapá in northernmost Brazil to the state of Santa Catarina (SANTOS, 2014).

Biodiversity is an important aspect of coastal ecosystems, especially for mangroves. The coastal Amazon contains the largest continuous area of mangroves in the world of approximately 8,900 km^2. . For the state of Maranhão, alone, 50% of the total coastal area is comprised of mangroves (DIAS et al. 2017).

It is important to seek techniques to minimize the effects of contamination. Bioremediation is a technique that uses microorganisms for the remediation of contaminated environments. This technique has the advantage potentially eliminating contaminants through mineralization, transforming the compounds into water and CO_2, while being an economically competitive and viable alternative for soil treatment.

It is possible to make use of bioremediation for on-site and ex-situ treatments. The degradation of polluting organic contaminants can be carried out by means of one or more microbial consortia, natural to that region or not, because no microbial species is capable of degrading all petroleum components alone (PEREIRA; LEMOS, 2003)

The mangrove is an ecosystem with a large biodiversity of microorganisms. Due to the complexity of the metabolic processes necessary for the degradation of hydrocarbons, there is a need for the formation of consortia consisting of fungi and bacteria of different genera and species, each specializing in degrading the different oil categories. (CRAPEZ et al., 2002).

This work aimed to select a microbial consortium enriched with isolated microorganisms found in the mangrove sediment its capacity to recover sediment contaminated by lubricating oil.

2 MATERIALS AND METHODS

2.1 Microorganisms and Fuel Oil

Eight bacteria and three fungi, previously isolated from the Mangue Seco sediment located in the municipality of Raposa on the island of Upuon Açu in Maranhão, were used, as described by Dias et al. (2017). The number of microorganisms used in this study and their morphologies, are shown in Table 1.

The fuel oil used work was acquired from mechanical workshops in the city of São Luís-MA and its hydrocarbon compounds were characterized by gas chromatography-mass spectrometry (CG/MS).

Before the chromatographic analysis, the residue was subjected to a fractionation (Clean Up technique according to the United States Environmental Protection Agency - US EPA) in a vertical separative column of glass of size 30 mm x 10.5 mm to identify the fractions of total petroleum hydrocarbons (TPH). The column was packed with activated silica at 800 °C and conditioned with 20 mL of hexane. After conditioning, 20 mg of the oily extract and 20 ml of hexane fluids

were transferred. The first fractions (here denominated F-I) were collected. The column was then treated with 15 mL 1:1 (v/v) benzene/hexane and the following fractions (F-II) were collected. The F-I and F-II fractions were mixed in the ratio 1:1, and the mixture was used for TPH analysis.

Table 1 - Number, group, gram, and morphology of microorganisms isolated from mangrove sediment used in the study

Microbial Group	**Number**	**Gram**	**Morphology**
Bacteria	B1	+	*Streptobacillus* sp.
Bacteria	B2	-	coccus
Bacteria	B3	+	coccus
Bacteria	B4	-	coccus
Bacteria	B5	+	rod
Bacteria	B6	-	coccus
Bacteria	B7	-	rod
Bacteria	B8	+	*Bacillus* sp.
Fungi	F2	*Aspergillus* sp.	Hyphatic septae with end sporangia
Fungi	F4	*Trichoderma* sp.	Hyphae septate spores with arrangements along the hyphae
Fungi	F5	NI	Non-septate hyphae with filaments at the ends

2.2 Sediment Collection

The sediment used in the study was collected from Mangue Seco, located in Raposa (Figure 1) in the mesoregion of the northern Maranhão, 30 km from the capital, São Luís, at the coordinates 02°25'22"S, 44°06'10"W.

2.3 Qualitative selection of microorganisms

Twelve microorganism strains were evaluated for the ability to degrade fuel oil following the method of Hanson et al. (1993) using the redox indicator 2,6-dichlorophenol-indophenol (DCPIP) to determine the electron transport chain rate. Assays were performed in plates containing 250 µL Bushnell Haas (BH) medium (1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $FeCl_3$, and 0.02 g $CaCl_2 \cdot 2H_2O$, dissolved in 1 L H_2O, and adjusted to pH 7.0); a microorganism suspension standardized to 10^8 UFC/ml; 10 µL fuel oil; and 25 µL DCPIP and maintained at 30°C without shaking for five days. Changes in the DCPIP color from blue (oxidized form) to colorless (reduced) were monitored to assess the microorganisms' oil degradation. The assays were performed in triplicate alongside an abiotic control without microorganisms.

2.4 Determination of the Emulsification Index (IE_{24})

The emulsifying activity was determined by the emulsification index after 24 h (IE_{24}). In this process, 6 ml of kerosene was added to 4 mL of the aqueous sample and stirred vigorously for 2 min. Measurements were taken after 24 h of rest. The emulsifying activity, as a percentage, was evaluated by the height of the emulsion layer, divided by the total height of the layer, multiplied by 100, as shown in Equation 1. (COOPER & GOLDENBERG, 1987).

$$\%EI_{(24)} = \frac{AEmulsification\ Layer\ Height}{Total\ Layer\ Height}\ X\ 100 \quad (1)$$

2.5 Enzyme Characterization

The phenol oxidase activities of laccase, lignin peroxidase, and manganese peroxidase were assessed in the BH medium with automotive oil residue concentrations of 1%, 3%, and 5%. The enzymatic activities were measured spectrophotometrically using a Shimadzu™ 1240UV/MINI. The laccase activity was determined using *p*-nitrophenol following established protocols (BUSWELL et al. 1995). The manganese peroxidase activity was measured at 610 nm by the oxidation

of phenol in reaction mixtures containing 500 µL enzyme extract, 100 µL phenol red (0.01% w/v), 100 µL sodium lactate (0.25 M), 200 µL bovine serum albumin (0.5% w/v), 50 µl $MnSO_4$ (2 mM), and 50 µL hydrogen peroxide in a sodium succinate buffer (20 mM, pH 4.5), incubated at 30°C for 5 min then quenched by the addition of 40 µL 2N NaOH (KUWAHARA et al. 1984). The lignin peroxidase activity was determined by the oxidation of veratryl alcohol (BUSWELL et al. 1995) in reaction mixtures containing 1 mL 125 mM sodium tartrate buffer (pH 3.0), 500 µL veratryl alcohol (10 mM), and 500 µL enzyme extract; the reaction was started by the addition of 500 µL hydrogen peroxide (2mM) and the absorbance was measured at 310 nm. Results were expressed in enzyme units, defined as 1.0 µM product formed per minute under assay conditions.

2.6 Expansion of the Microbial Consortium

The efficiency of degradation is directly related to the ability of microorganisms to use different mechanisms of action. Therefore, it is necessary to use microbial consortia in degradation processes rather than a single microorganism. For this reason, consortia have been developed with isolated bacteria and fungi. To verify that there were no antagonisms between them, the test of antibiosis in a Petri dish was performed. Subsequently, the consortia were set up with non-antagonistic strains, according to Silva et al. (2015). The consortia were selected according to the methodology of Hanson et al. (1993) and modified.

The consortia were inoculated into 50 mL of BH containing 2 mL of DCPIP and 1% of burned lubricant oil residue as the carbon source. The entire reaction was carried out in a 250 mL Erlenmeyer flask and incubated at 30 °C under agitation of 150 rpm.

2.7 Determination of Treatment Conditions

The conditions of the oil's degradation by the microbial consortia were established in flasks for a period of 4 days. For this, the fractional factorial (2n) experimental design of the central composite rotational design (CCRD), consisting of

two levels (-1, +1), two axial points (-1.41 and +1.41), and three central points, totaling 11 experiments. The experimental matrix, as well as the analysis of the results, were processed with the Statistic® 8.0 software. The independent variables were the degree of agitation and the percentage of oil, and the process-dependent variable was the biological activity of the microorganisms.

With the process conditions selected, the optimal values were established using the response surface methodology and the Statistic® 8.0 software.

2.8 Evaluation of Hydrocarbon Degradation at Laboratory Scale

The experiments were carried out according to the methodology described by Savu et al. (2012), where 300 g of soil contaminated with the hydrocarbon residue was weighed and bio-increased with the previously selected consortium.

The treatment of the oil-contaminated sediment was carried out for 30 days. The oil was extracted every 3 days, and the biological activity was evaluated by of the basal respiration of the microorganisms. To ensure sufficient aeration, the soil was regularly agitated. The degradation of soil contaminant residue was analyzed by GC / MS under the same conditions as described for the characterization of the oil.

2.8.1 Biological Activity Test

After determining the best degradation condition, the soil biological activity (the response variable of the experimental design) was measured by basal soil respiration using volumetric titration, using a method proposed by Alef (1995). Ten flasks were used, and duplicate tests were performed; thus, a total of 20 flasks contained the soil. The concentration of the variants was applied to the soil. To evaluate the CO_2 demand, small containers containing 30 ml of NaOH were placed in the vials, and two bottles were selected every 3 days. The contents of the remaining containers were regularly replaced. Then, 10 ml of NaOH was obtained from the selected containers, and 10 ml $BaCl_2$ and 3 drops of phenolphthalein were added. The mixture was titrated with HCl to determine the CO_2 concentration. The respiration rate, in mg of evolved C-CO_2, was calculated by Equation 2:

$$(C - CO_2) = (B - V) \times M \times 6 \times \left(\frac{V1}{V2}\right) \quad (2)$$

where *B* is the volume of HCl used to titrate the blank, *V* is the volume of HCl used to titrate the sample, *M* is the actual molarity of HCl, *6* is the atomic mass of C (12) divided by the number of moles of CO_2 that react with NaOH (2), *V1* is the amount of NaOH used in the vials, and *V2* is the amount of NaOH used in the titration.

2.8.2 Degradation Analyses by Mass Spectrometry Coupled Gas Chromatography (GC / MS)

The hydrocarbon fractions of the natural residue, after having undergone the degradation process (under the established conditions for 60 days), were analyzed by GC-MS, using the Shimadzu ™, GC-MS Model: 17A / QP5050A with a 30 m x 25 µm size column, 0.25 mm VB-5, and helium as a carrier gas. The oven temperature was programmed to increase linearly from 50 °C to 300 °C increasing by 6 °C/ min and maintained for 20 min, the interface temperature was 280°C with a flow of 1 mL/min. The main compounds were detected by the mass spectrometer in scan mode, and the analyzed mass ranges were 40 to 350 m/z in 63.67 min of analysis. For the analysis of degradation, the area of the major peak of the chromatogram was automatically calculated and integrated automatically.

2.9 Statistical Analysis

The results were analyzed statistically through analysis of variance (ANOVA) to verify an association between the variables.

3 RESULTS

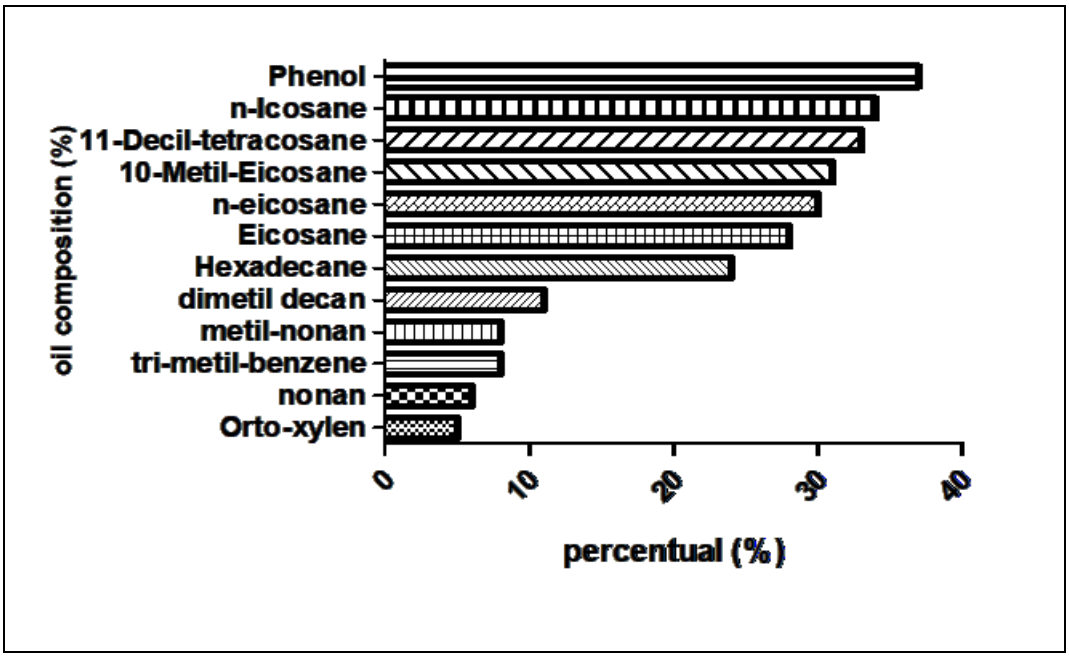
3.1 Oil Fuel Description

The characterization of the residue for TPH by GC-MSis shown in Figure 1. The predominant compound is observed to be phenol constituting 40% of all residue, followed by hydrocarbon compounds consisting of secondary bonds of 10 or more

carbon atoms. The aromatic compounds, constituted of benzene rings, present smaller percentages in the residue's composition. The presence of these heavy compounds with large and aromatic carbon chains is due to the oil previously undergoing a thermal process when it was used in an automobile engine.

Among all constituents of the residue, it is important to highlight the toxicity of phenol, the predominant compound. Because its chemical structure consists of a benzene ring attached to two hydroxyls, this compound is extremely stable and difficult to break down by most biological organisms. In addition to phenol, the other aromatic compounds present in the waste contribute to its high toxicity.

Figure 1 - Composition of the fuel oil used in this work



3.2 Qualitative selection of microorganisms

The results of the qualitative selection test of the microorganisms show that 45% of the microorganisms (B2, B5, B7, F2, F4) were able to completely change the substrate color from blue to colorless. While of the isolated microorganisms (B1, B3, B6, F5) have undergone a partial change, , only 20% of the isolated microorganisms (B4 and B8) were invariant, failing to perform the microbial oxidation of the

hydrocarbons. With this result, subsequent tests were performed with the microorganisms that completely discolored the BH medium.

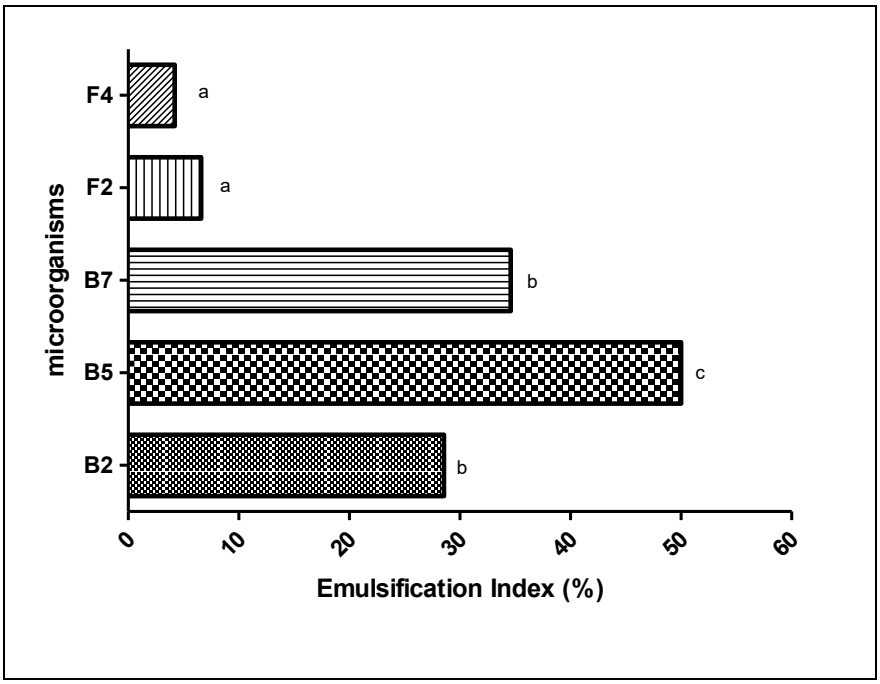
Among the most promising microorganisms, three were bacteria and two were filamentous fungi. This result demonstrates the ability of the two microbial groups to metabolize hydrocarbon compounds under the experimental conditions tested.

3.3 Determination of the Emulsification Index (IE_{24})

The result of the IE percentage (24) can be seen in Figure 2. Bacteria B2, B5, and B7 presented an emulsification index of 28.5%, 50%, and 34.6%, respectively, while the F4 and F2 fungi obtained 4.28% and 6.66%, respectively.

When the statistical analysis between the IE24 values was performed, the value obtained by bacterium B5 presented p <0.001, being statistically significant and in relation to the others, while the values obtained by the bacteria B2 and B7 showed no statistically significant difference. In relation to the fungi, the obtained values did not show a significant statistical difference in each other.

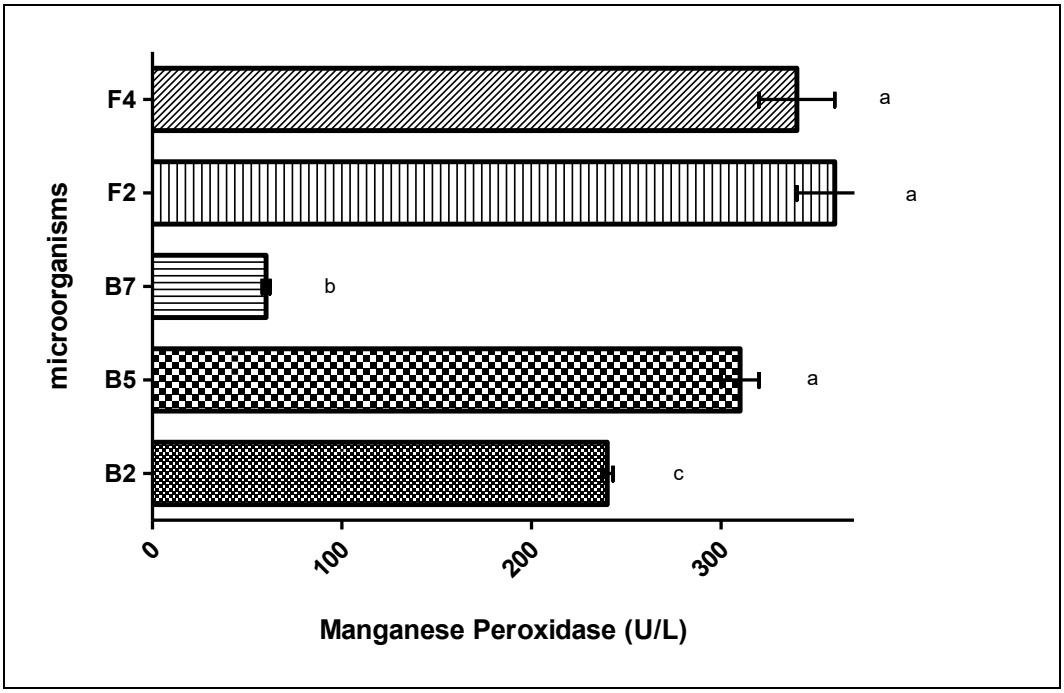
Figure 2 - Index of Emulsification (EI_{24}) of the selected microorganisms regarding the capacity to use the oil as carbon source. a=$p < 0.01$; b=$p < 0.05$; c=$p > 0.05$



3.4 Enzyme Characterization

The results of the enzymatic activity can be observed in Figure 3. After the enzymatic activity against the phenol oxidase enzymes, there was no production of the enzymes laccase and lignin peroxides by the microorganisms isolated under the conditions tested.

Figure 3 - Activity of Manganese Peroxidase (U / L) Enzyme produced by microorganisms isolated from mangrove soils. a=$p < 0.01$; b=$p < 0.05$; c=$p > 0.05$



Of the five selected microorganisms, four presented the production of the enzyme manganese peroxidase. Among these, the filamentous fungi F2 and F4 showed the higher activity of the enzyme with values of 360 U/L and 340 U/L, respectively. However, it is important to emphasize the production of the enzymes by the bacteria B5 showing 320 U/L activity. This bacterium has a Gram-positive rod morphology suggesting that it is genus *Bacillus* sp.

Bacteria B2 and B7 did not show a statistical difference between the values obtained. However, the bacteria B7 and fungus F2 e F4 showed statistically significant values in relation to the others.

3.5 Elaboration of the Microbial Consortium

Prior to the preparation of the microbial consortia, the antagonistic potential of the microbial strains selected in the previous tests was evaluated. No microorganisms possessed antagonism, allowing the expansion of seven microbial consortia (B5F2, B5F4, B7F2, B7F4, B5F2F4, B5B7F2, and B5B7F4).

With these consortia, a new selection was made using the DCPIP methodology recommended by Hanson et al. (1993). The consortiums B5F2 and B5F2F4 were the most promising as the blue-to-colorimetric shift occurred with 24 h incubation, while the other consortia occurred at 36 h (B5F2, B7F2) and at 48 h (B7F4, B5B7F2, and B5B7F4).

3.6 Determination of Treatment Conditions

To optimize the conditions of the process, a CCRD experimental design was used for each microorganism. Eleven experiments were carried out for the B5F4 consortium and 11 experiments for the B5F2F4 consortium, totaling 22 experiments. To analyze the degradability of the oily residue, the degradation was evaluated considering the reduction of the major compound (phenol) by the microorganisms. For this, the microorganisms were inoculated in a BH mineral medium, using the hydrocarbon residue as a substrate in different concentrations with various degrees of agitation. Samples were collected after 4 days. The experimental design is a statistical methodology whose main objective is the reliability of the results with a minimum amount of experiments that guarantees the desired degree of freedom.

Table 1 shows the percent degradation of the major component (phenol) of the oily residue used in the 11 experiments carried out for the two microbial consortia studied. In most of the tests there was a proportional difference of degradation between the consortia. Additionally, for the test performed with 0.5% (250 µL) of the oil at a 75 rpm agitation rate for the two consortia, the compound B5F2F4 had a degradation rate that was 95% higher than the rate for the consortium composed of B5F4, which was 75%. To assert that the rate of degradation of the residue by the B5F2F4 consortium was greater than the rate of degradation obtained by the B2F4

consortium, a two-way ANOVA analysis was performed, comparing the two values, and it was verified that p <0.0001.

Table 1 – Matrix of factorial experimental design of the central composite rotational design (CCRD) with the dependent and independent variables of the process

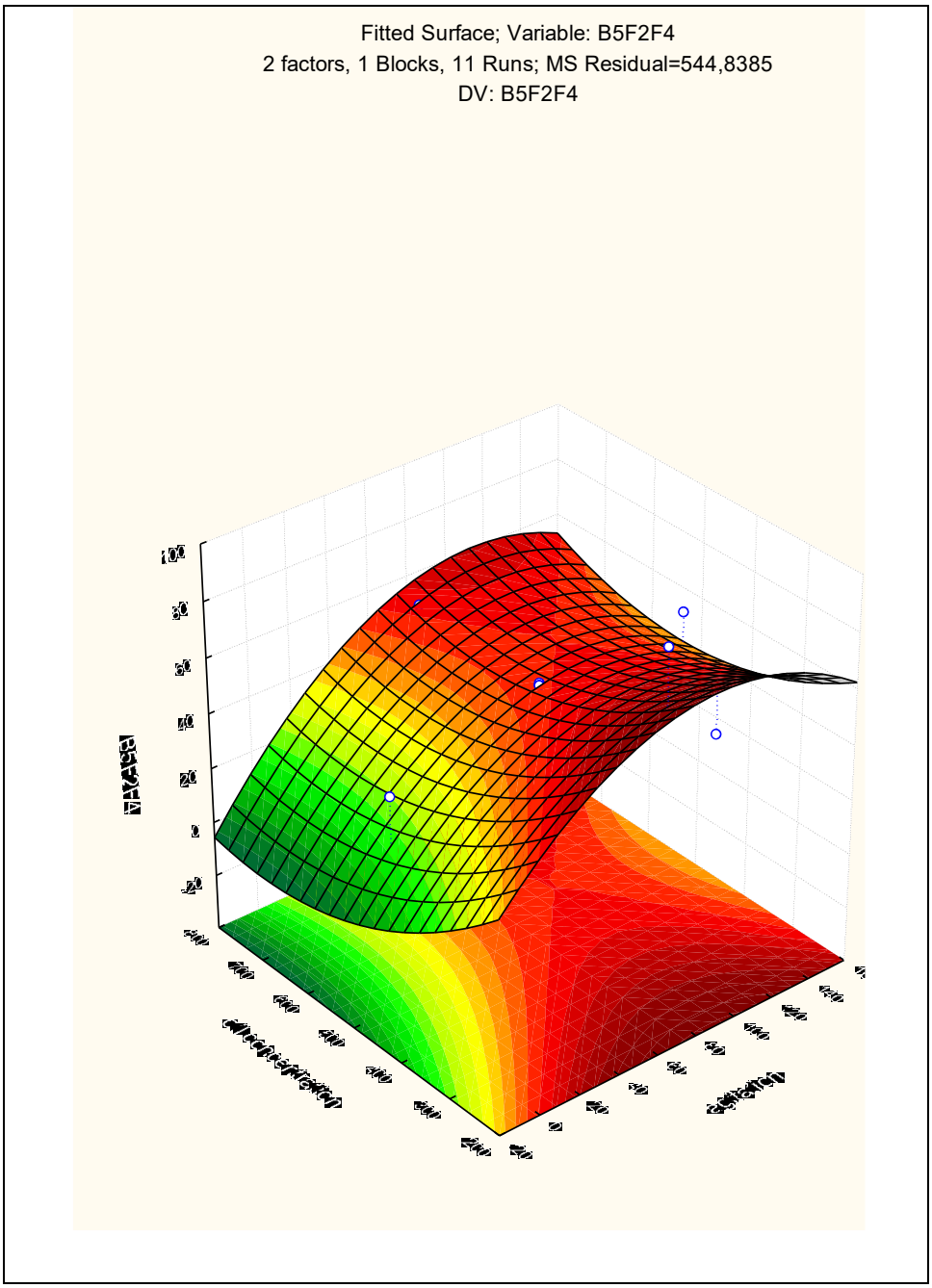
Assays	Independents Variables				Dependent Variables	
	Encoded values		Real values		Degradation Percentual (%)	
	Agitation (RPM)	Oil (%)	Agitation (RPM)	Oil (%)	B2F4	B5F2F4
1	-1	-1	21,8	0,7	12,	15,
2	+1	-1	128,2	0,7	21,	37,
3	-1	+1	21,8	1,3	9,	12,
4	+1	+1	128,2	1,3	35,	44,
5	-1,41	0	0	1	27,	38,
6	+1,41	0	150	1	50,	58,
7	0	-1,41	75	0,5	72,	95,
8	0	+1,41	75	1,5	48,	56,
9	0	0	75	1	45,	55,
10	0	0	75	1	46,	54,
11	0	0	75	1	45,	54,

Considering the results obtained in the experiments with the two consortia, it was decided to follow the analysis and experiments with only the consortium B5F2F4.

To know which independent variable influenced the process, an effect table was generated, showing that the interaction between the variables and the agitation in their linear form had a significant influence on the process, with p = 0.000037 and p = 0.041234, respectively. From these data, a model using Equation 3 was proposed.

$$\%\ degradation\ B5F2F4 = 54.50 + 27.13\ agitation \quad (3)$$

Figure 4 - Graph surface response obtained from the phenol degradation model by the B5F2F7 consortium



From the model, the response surface graph of the percentage degradation variable was generated, indicating the trend of the optimum oil degradation values. When the percentage response variable for degradation is considered, the trend can be seen in Figure 4. There is a region with a high concentration of these two parameters, indicated as the dark red areas, and a region of low concentration in the light red areas. There are optimum regions for high degradation values at 80 rpm of

agitation and near 20 rpm, indicated by a ring-shaped band in the light red hue. These values indicate that the best conditions for subsequent experiments are 250 µL of oil with a range of 20 to 80 rpm.

3.7 Evaluation of Hydrocarbon Degradation at a Laboratory Scale

Before the experiment, an evaluation of soil degradation with the concentration of oil obtained in the planning and in a static condition was carried out. To evaluate if the selected consortium had the capacity to degrade the phenol, the major compound selected to analyze the degradation, degradation kinetics was performed for 30 days where the samples were taken every 3 days. The biological activity and the degradation obtained during the 30 days of the experiment, as shown in Figure .5.

Figure 5 - Biological activity of soil microflora versus phenol degradation over 30 days of treatment of oil-contaminated sediment

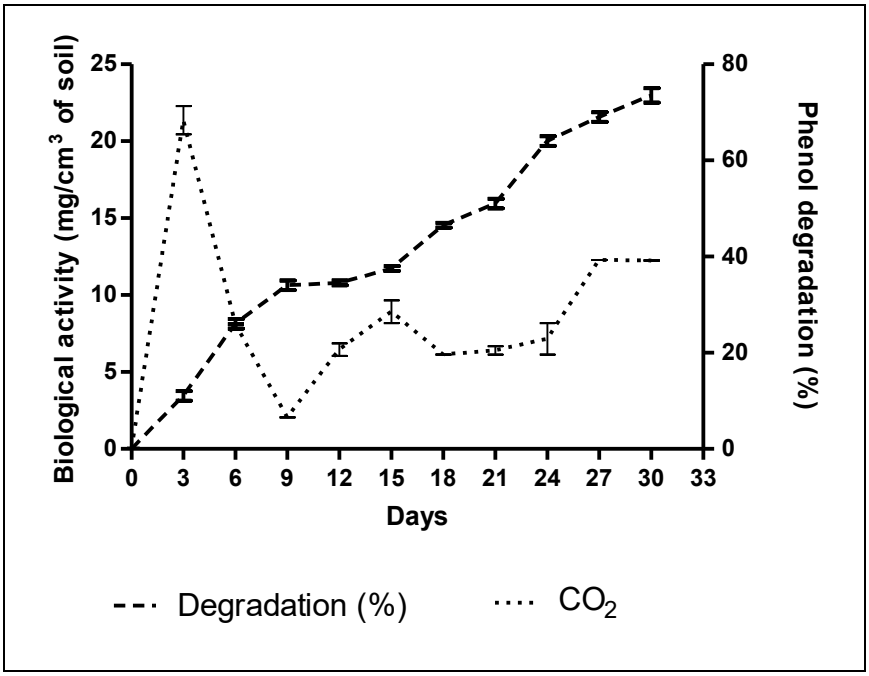


Figure 5 shows the daily production, in the sampled times, of the CO_2 generated by the microbial communities versus the rate of degradation of the phenol during the 30 days of the experiment. It is possible to verify that the microorganisms underwent an adaptive phase in the initial period of the experiment, between days 3 and 9, when

from then the production of CO_2 began to grow. This adaptation phase coincides with the phase where phenol degradation begins, reaching the ninth day with a 34% degradation. After the period of 15 days, there was a decrease in daily CO_2 production, a fact that may have been caused by the carbon source transition. At this stage, an increasing rate of degradation of the phenol can be observed, thereby generating new hydrocarbon fractions which may be more readily biodegradable and requiring adaptation of the microorganisms to more recalcitrant hydrocarbons. (MARIANO, 2006).

After the acclimation period, the maximum perceived production was from day 27, and the fact may be related to the characteristics of the contaminant. During this period 72% was obtained, reaching 80% degradation at the end of the 30 days.

4 DISCUSSION

The characterization of the polluting oil is essential for proposing correct strategies for the recovery of contaminated environments. The oil used as a pollutant in this work was characterized by gas chromatography coupled to mass spectrometry according to its 11 major compounds. In this characterization, a composition with phenol, alkanes, alkenes, and aromatics was observed.

The oil for the study is waste oil from auto repair shops that has already been used in car engines. This oil proved to be toxic because nearly 40% of its composition was phenol because it was subjected to the engines' high temperatures. According to Brazil's National Petroleum Agency (ANP), lubricating oils decrease their viscosity at temperatures above 100 °C. Jabbar et al. (2019) reports that, as the car engine reaches a maximum temperature of 96 °C, there is no change in viscosity. However, volatile compounds evaporate at this temperature, and there is a slight change in chemical composition, with phenol prevailing as a major compound.

After the characterization of the oil, the qualitative selection of the microorganisms was performed. The colorimetric test using DCPIP is a valid technique

to select the microorganisms that can potentially be used in the bioremediation of hydrocarbon residues. This technique was first described as efficient for the selection of microorganisms by Hanson et al. (1993). The authors reported the use of this indicator with the BH medium and crude oil as a carbon source in multi-well plates to select bacteria with a biodegradation capacity and consists of the use 2,6-dichlorophenol-indophenol as an indicator. When using a hydrocarbon as the nutritional source, microorganisms that have hydrocarbonoclastic characteristics will cause the indicator to change color from blue to colorless (BIDOIA et al. 2010).

More recently, the authors continue to advocate for this as an efficient technique for the selection of microorganisms. The potential of microorganisms isolated from an environment contaminated with petrodermated residues in degrading hydrocarbons was evaluated using the technical discoloration indicated by the DCPIP. The authors report that the selection of microorganisms showed potential degradation within 24 h of testing (MIRANDA et al. 2007; GOMES et al. 2009).

Currently, the technique is widely used. Almeida et al. (2017) used the DCPIP redox indicator technique to select the best consortium capable of degrading marine fuel MF-8. The authors reported that they obtained discoloration by a pool of various bacteria within 24 h. Marchand et al. (2017) reported the use of the DCPIP methodology to select bacteria and fungi with the potential for degradation of crude oil. The authors emphasized the efficiency and speed of the technique. Abbas et al. (2018) used the DCPIP technique to estimate the capacity of *Aspergillus* spp. in the degradation of crude oil. The authors reported that the color change from blue to colorless in 14 days at 30 °C incubation determined that the fungi had the ability to degrade the oil. Peixoto et al. (2019) evaluated the degradation of petroleum by a *Bacillus toyonensis* strain isolated from a petrochemical-contaminated environment using the indicator technique 2,6,dichlorophenol-indophenol.

The Emulsification Index assessment is an important tool that indicates the ability of microorganisms to produce emulsifying substances that assist in the degradation processes of oily substances. The bacterium that obtained the highest Emulsification Index value (50%) was a Gram-positive rod, a bacterium of the genus

Bacillus sp., that assisted in the oil degradation processes**.** The bacterium B7 was the one that obtained the second highest value of Emulsification Index (34.7%) has the Gram-negative rod morphology. Many promising bacteria in the production of surfactants present this morphology as *Pseudomonas* sp. Bezerra et al. (2012) reported the production of a biosurfactant by *Pseudomonas aeruginosa* GVII-A, using an agro-industrial waste as a carbon source. For the initial selection, an IE_{24} test was performed, where the bacteria were fermented in a minimal salts medium with agro-industrial residues, and from that, the emulsification tests were carried out for the subsequent production of the surfactant.

Oliveita et al. (2010) advocated the use of a *Bacillus subtillis* LAMI005 strain for the production of surfactants from the addition of assorted sugars. Silva et al. (2014), in a literature review, cites several species of bacteria of the genus Bacillus as potential producers of emulsifying substances used to aid in oil degradation in aqueous environments. Araujo et al. (2019) reported the production of pulmilacidine by *Bacillus safensis* CCMA-560 and its use to reduce the surface tension in oil-contaminated reactors.

Another mechanism used by microorganisms to degrade oil and recover contaminated environments is the production of hydrolytic enzymes. Among the different microbial groups, fungi are recognized as major enzyme producers. As described in the literature, isolated mangrove sediment fungi were more efficient in enzyme production than bacteria. The fungus F2 of the genus *Aspergillus* sp. showed a greater ability to produce the enzyme manganese peroxidase. Corroborating with the authors, the oil used as a pollutant in this work has its considerable phenol composition, which may explain the enzyme production. Bonugli and Santos et al. (2010) reported the production of the enzyme manganese peroxidase by the fungus *Aspergillus sclerotiorum* using phenol substrate.

Other authors demonstrate the relationship of oxidative enzymes to the degradation of recalcitrant compounds. Khelil et al. (2015) evaluated the production of cellulases and manganese peroxidase by the *Bacillus subtilis R2* bacteria for use in the textile industry's effluent discoloration. The authors reported a 3 U/L enzyme

production within 48 h. Zhang et al. (2016) reported the ability of the fungus *Trametes vilosa* to degrade polycyclic aromatic hydrocarbons using the enzymes manganese peroxidase. In this work, other than phenol, the oil was composed of aromatic compounds that may have contributed to the enzyme production. Mallerman et al. (2019) reported the elimination of approximately 75% of a phenol-based recalcitrant compound by the fungus *Hypholoma fasciculare* using solid-phase fermentation. The authors attributed the ability to degrade this compound to the production of manganese peroxidase. Vaidya et al. (2019) highlighted the production of 16,676 IU/L of manganese peroxidase by the fungus *Trametes vilosa* using oil as a substrate.

The use of microbial consortia to improve the degradation of oily compounds and other wastes has been widely studied. In this work, it was possible to elaborate seven microbial consortia, combining bacteria and fungi. Of these, the most promising were those that contained both groups of microorganisms. This feature is common in consortia because the mechanisms used by the microorganisms are different, thus facilitating the remediation of the contaminated site. Sampaio et al. (2019) used a bacterial consortium containing a bacterium of the genus *Bacillus* sp., isolated from the earthworm digestive tract. The consortium was able to degrade 57% of petroleum waste within 24 days of processing. Kumar et al. (2019) used a bacterial consortium to degrade crude oil with the addition of $(NH_2)_2$ and K_2HPO_4. Bacteria isolated from deep-sea areas were able to completely degrade 1% of oil added to the culture medium in 10 days of the experiment.

To establish the best conditions for decreasing oil concentration in a contaminated environment, the experimental planning methodology was used, varying the oil concentration and agitation. The two variables were chosen with the possibility of environmental simulation in later experiments. Nakayama et al. (2019) used the experimental design methodology to optimize the biodegradation conditions of aliphatic compounds in seawater. As in this work, the authors used the variables agitation and oil concentration as the process-optimization parameters. Baskaran and Rajamanickam (2019) studied trichloroethylene (TCE) degradation by a microbial consortium in Turkey and used the experimental design methodology to optimize the

process conditions. The authors reported that, under the best treatment conditions, a 95% degradation efficiency of the TCE compounds was possible. The authors also reported that the strain responsible for the degradation of most compounds was *Psedomonas guguanensis.*

Agitation was the statistically significant variable that generated the model that originated the surface response graph because the degradation rate is higher under aerobic conditions when microorganisms use O_2 as the final electron acceptor. The effect of agitation on the degradation of hydrocarbon compounds has been studied for several years. Stroud et al. (2009) reported that the highest phenanthrene degradation rates in oil-contaminated soil were at 100 rpm agitation, while hexadecane was better degraded at 75 rpm for 10 days. Umar et al. (2018) used the experimental design methodology to evaluate the parameters that influenced the degradation of the fenatrene and pyrene constituents of the soil-contaminating oil. As in this paper, the authors obtained agitation as an influencing factor of the process. The authors report that the influence of agitation as an O_2 supplier is important for the degradation efficiency.

The oil-degradation kinetics were evaluated in the mangrove sediment, in the area where the intercropping microorganisms were isolated. For this evaluation, phenol was the selected compound. Several authors report the use of phenol as a nutrient by several isolated or intercropping microorganisms. Silva et al. (2019) reported the ability of *Candida tropicales* ATCC 750 yeast to degrade phenol. The authors reported that the yeast was able to degrade the compound at a stirring of 150 rpm, pH 6.0, and an initial concentration of 1100 mg/L. Kamali et al. (2019) evaluated phenol degradation in a sequential reactor by a mixed culture. The authors reported a decrease from 500 mg/L to 100 mg/L of phenol in 60 days at a temperature of 18 °C. Dionisi & Etteh (2019) investigated phenol degradation in soil using a sequential batch reactor and reported a 45% phenol degradation within 45 days of the process. The authors used the bioaugmentation technique by adding peptone to the soil containing the medium.

5 CONCLUSIONS

The results show that, although the used automotive oil from mechanic workshops is very toxic with a high phenol content, the microbial consortium with the microorganisms isolated from the mangrove sediment, is able to remediate the contaminated soil by different mechanisms such as the production of emulsifying substances and oxidative enzymes. Thus, this consortium could be used in processes of treatment of environments contaminated with an oily residue.

ACKNOWLEDGMENT

The authors are grateful for the financial support from Foundation for Research Support of the State of Maranhão (FAPEMA) under concession term [UNIVERSAL-01271/17.].

REFERENCES

ABBAS AH., MOHAMMED DB, ABED EH, Bidegradation of PAHs by Consortium Microorganisms solated from Oil Contaminated Soi. World jornal of Pharmaceutical Research, 2018, 7, (11), 43-50.

ABNT NBR 10004. Resíduo sólido classificação. 2004. Associação brasileira de normas técnicas. Rio de Janeiro.

ALEF, K. Soil respiration. In: ALEF, K.; NANNIPIERI, D. Methods in applied soil microbiology and biochemistry. London: Academic Press, 1995. p. 214-216.

ALMEIDA DG, SILVA MGC, BARBOSA RN, Silva DSP, SILVA RO, LIMA GMS, ET AL. Biodegradation of marine fuel MF-380 by microbial consortium isolated from seawater near the petrochemical Suape Port, Brazil, International Biodeterioration & Biodegradation, 2017, v. 116, 73-82.

ANDRADE JA. Otimização da reação de Fenton visando aplicações na remediação *in-situ* e *ex-situ* de águas subterrâneas. [dissertation], Campinas, Universidade Estadual de Campinas, UNICAMP, 2005. 249p.

ARAÚJO, LL, SODRÉ LG, BRASIL LR, DOMINGOS DF, OLIVEIRA VM, DA CRUZ GF, Microbial enhanced oil recovery using a biosurfactant produced by *Bacillus safensis* isolated from mangrove microbiota-Part I biosurfactant characterization and oil displacement test. Journal of Petroleum Science and Engineering, 2019, 180, 950-957.

BASKARAN D, RAJAMANICKAM R. Aerobic biodegradation of trichloroethylene by consortium microorganism from turkey litter compost. Journal of Environmental Chemical Engineering, 2019, 7, (4), 103260.

BEZERRA MS, HOLANDA VCD, AMORIM JA, MACEDO GR, SANTO ES, Produção de Biotensoativos [Utilizando *Pseudomonas aeruginosa* (P.A.) e Resíduo Agroindustrial (Maniqueira) como Substrato] Portuguese,. HOLOS, 2012, 1, 14-27.

BIDOIA ED, LOPES PRM, MONTAGNOLLI RN, DOMINGUES RF, Toxicity and Biodegradation in Sandy Soil Contaminated by Lubricant Oils. Bull Environ Contam Toxicol, 2010, 84, 454–458.

BONUGLI-SANTOS RC, DURRANT LR, da SILVA M, SETTEUM LD, Production of laccase, manganese peroxidase and lignin peroxidase by Brazilian marine-derived fungi. Enzyme and Microbial Technology, 2010, 46, (1), 32–37.

BRITO, N. N. et al. Utilização de fungos na remediação de efluentes industriais. In: FÓRUM DE ESTUDOS CONTÁBEIS, 4., 2004, Rio Claro. Anais... Rio Claro: Faculdades Integradas Claretianas, 2004.

BUSWELL, JK, CAI YJ, CHANG ST Effect of nutrient nitrogen on manganese peroxidase and lacase production by *Lentinula* (Lentinus) edodes. FEMS Microb. Lett, 1995, 128: 81–88.

CAPELLI SM; BUSALMEN JP, SANCHEZ SR. Hydrocarbon bioremediation of a mineral-base contaminated waste from crude oil extraction by indigenous bactéria. International Biodeterioration & Biodegradation. 2001, 47, 233-238,

CETESB, Aspectos Físico-Químicos do Petróleo, Disponível em: http://www.cetesb.sp.gov.br/emergencia/acidentes/vazamento/oleo/a_fisicos.asp. Acesso em: 10 out. de 2004.

COOPER DG, GOLDENBERG BG, Surface-Active Agents from Two Bacilllus Species, Applied ans Environmetal Microbiology, 1987, p.224-229.

CRAPEZ MAC, BORGES ALN, BISPO MGS, PEREIRA D. Biorremediação: tratamento para derrames de petróleo. Ciência hoje, [portuguese]2002, 30: 179.

CURY JC. 2002. Atividade microbiana e diversidades metabólica e genética em solo de mangue contaminado com petróleo. [dissertation], Piracicaba – São Paulo, 2002.

DIAS LRL, BASTOS DKL, LIMA NS, SILVA MRC, Miranda RCM, Bioprospecção de Microorganismos de Interesse Biotecnológico Isolados em Ecossistema de Manguezal. Revista de Investigação Biomédica 2017, 9, 24-30.

DIONISI D, ETTEH CC. Effect of Process Conditions on the Aerobic Biodegradation of Phenol and Paracetamol by Open Mixed Microbial Cultures, Journal of Environmental Chemical Engineering, 2019, 7, (5), 103282.

GOMES EB, SORIANO AU, MIRANDA RCM, SOUSA MFVQ, PEREIRA Jr N. (2009) Biodegradation of Stored jet Fuel by a *Nocardia sp.* Isolated from Contaminated Soil, BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY, Vol.52, n. 5: pp.1279-1284.

HANSON KG, DESAI J, DESAI AJ, Rapid and Simple Screening Technique for Potential Crude Oil Degrading Microorganisms, Biotechnology Techniques, 1993, 7, 10, pp 745-748.

JABBAR NM, ALAA KM, ESTABRIQ HK. Bioremediation of Petroleum Hydrocarbons Contaminated Soil using Bio piles System. Baghdad Science Journal, 2019, 16.1, 185-19.

MALLERMAN J, ITRIA R, BABAY P, SPARRAT M, LEVIN L, Biodegradation of nonylphenol polyethoxylates by litter-basidiomycetous fungi. Journal of Environmental Chemical Engineering, 2019, 7, 5, 103316.

KAMALI M, GAMEIRO, COSTA ME, CAPELA I, AMINABHAVI TM. Enhanced Biodegradation of Phenolic Wastewaters with Acclimatized Activated Sludge-A Kinetic Study. Chemical Engineering Journal, 2019, 378, 15, 122186.

KHELIL O, CHOUBANE S, CHEBA BA. Co-production of Cellulases and Manganese Peroxidases by *Bacillus* sp. R2 and *Bacillus Cereus* 11778 on Waste Newspaper: Application in Dyes Decolourization. Procedia Technology, 2015, 19, 980-987.

KUMAR AG, RAJAN NN, KIRUBAGARAN R, DHARANI G. Biodegradation of crude oil using self-immobilized hydrocarbonoclastic deep sea bacterial consortium. Marine Pollution Bulletin, 2019, *146*, 741-750.

KUWAHARA M, GLENN JK, MORGAN MA, GOLD MH. Separation and characterization of two extracellular H2O2 dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Left., 1984,169, 247-250.

MALLERMAN J, ITRIA R, BABAY P, SAPARRAT M, LEVIN L. Biodegradation of nonylphenol polyethoxylates by litter-basidiomycetous fungi. Journal of Environmental Chemical Engineering, 2019, 7, 5, 103316.

MARCHAND C, ARNAUD MS, HOGLAND W, BELL TH, HIIJR M, Petroleum biodegradation capacity of bacteria and fungi isolated from petroleum-contaminated soil. International Biodeterioration & Biodegradation, 2017, 116, 48- 57

MARTINS, P. F.; AZEVEDO, R. A. Perspectivas do uso de microrganismos na biorremediação. Disponível em: http://www.genetica.esalq.usp.br/pub/seminar/PFMartins-200701-Resumo.pdf. Acesso em: 16 abril. 2019.

MIRANDA RCM, SOUZA CS, GOMES EB, LOVAGLIO RB, LOPES CE, SOUSA MFVQ. Biodegradation of Diesel Oil by Yeasts Isolated from the Vicinity of Suape Port in the State of Pernambuco –Brazil, Brazilian Archives of Biology and Technology, 2007, v.50, n.1, pp.147-152.

MONTEIRO, A. G. 2003. Metodologia de avaliação de custos ambientais provocados por vazamento de óleo, o estudo de caso do complexo REDUC-DTSE. Tese de Doutorado. Universidade Federal do Rio de Janeiro.

NAKAYAMA A, YAMANO N, KAWASAKI N. Biodegradation in seawater of aliphatic polyesters. Polymer Degradation and Stability.ed hydrocarbonoclastic deep sea bacterial consortium. Marine Pollution Bulletin, 2019, 146, 741-750.

OLIVEIRA DWF, SOUSA JR, FRANÇA IWL, FELIX AKN, MARTINS, JJL, GONÇALVES LRB. Kinetic Study of Biosurfactant Production by *Bacillus subtilis* LAMI005 grown in Clarified Cashew Apple Juice. Journal of Biotechnology, 2010, 150, 421–422.

PEIXOTO FBS, PEIXOTO JC, MOTTA DCL, PEIXOTO ATM, PEREIRA JO, ASTOLFI FILHO S, Assessment of petroleum biodegradation for *Bacillus toyonensis* by the using redox indicator 2, 6 dichlorophenol indophenol. Acta Scientiarum. Biological Sciences, 2018, *40*.

PEREIRA LTC, LEMOS JLS. Os fungos Filamentosos, uma opção de estudo para Biorremediação I. in: XI Jornada de Iniciação Científica, Centro de Tecnologia Mineral – CETEM/MCT, 2003.

SAMPAIO CJS, SOUZA JRB., CARVALHO, GC, QUINTELLA CM, de ABREU, ROQUE MR. Analysis of petroleum biodegradation by a bacterial consortium isolated from worms of the polychaeta class (Annelida): Implications for NPK fertilizer supplementation, Journal of environmental management, 2019, 246, 617-624.

SANTOS LCM, MATOS HR, SCHAEFFER-NOVELLI Y, CUNHA LIGNON M, BITENCOURT MD, KOEDAM N, DAHDOUH-GUEBAS, F. Anthropogenic activities on mangrove areas (São Francisco River Estuary, Brazil Northeast): A GIS-based analysis of CBERS and SPOT images to aid in local management. Ocean & Coastal Management, 2014, 89, 39-50.

SAVU R, SILVEIRA JV, FLACKER A, VAZ AR, JOANNI E, PINTO AC, MOSHKALEV SA. Micro-reactors for characterization of nanostructure-based sensors, Review of Scientific Instruments, 2012, 83, 5, 055104.

SILVA DSP, CAVALCANTI DL, MELO EJV, SANTOS PNF, PACHECO ELL, GUSMÃO NB, SOUSA MFVQ, Bio-removal of diesel oil through a microbialconsortium isolated from a polluted environment. Int. Biodeterior. Biodegrad, 2015, 97, 85-89.

SILVA, M. A. B.; Bernini, E.; Carmo, T. M. S. Características estruturais de bosques de mangue do estuário do rio São Mateus, ES, Brasil. Acta Botanica Brasilica, 2005, v.19, n. 3, p. 465-471.

SILVA R, ALMEIDA D, RUFINO R, LUNA J, SANTOS V, SARUBBO L. Applications of Biosurfactants in the Petroleum Industry and the Remediation of Oil Spills. International Journal of Molecular Sciences, 2014, 15, 7, 12523–12542.

SILVA NCG, de MACEDO AC, PINHEIRO ÁDT, ROCHA MVP. Phenol biodegradation by *Candida tropicalis* ATCC 750 immobilized on cashew apple bagasse. Journal of Environmental Chemical Engineering, 2019, 7, 3, 103076.

STROUD JL, PATON GI, SEMPLE, KT. The effect of agitation on the biodegradation of hydrocarbon contaminants in soil slurries. Chemosphere, 2019, 77, 1, 123-128.

USBERCO, J.; SALVADOR, E. Química - volume único. 5ª ed. reform.— São Paulo: Saraiva, 2002.

UMAR ZD, AZWADY AAN, ZULKIFLI SZ, MUSKHAZLI M. Effective phenanthrene and pyrene biodegradation using *Enterobacter* sp. MM087 (KT933254) isolated from used engine oil contaminated soil. Egyptian journal of petroleum, 2018,27, 3, 349-359.

VAIDYA V, CAROTA E., CALONZI D, PETRUCCIOLi M., D'ANNIBLE A. Production of lignin-modifying enzymes by *Trametes ochracea* on high-molecular weight fraction of olive mill wastewater, a byproduct of olive oil biorefinery. New biotechnology, 2019, 50, 44-51.

ZHANG H, ZHANG S, HE F, QIN X, ZHANG X, YANG Y. Characterization of a manganese peroxidase from white-rot fungus *Trametes* sp.48424 with strong ability of degrading different types of dyes and polycyclic aromatic hydrocarbons. Journal of Hazardous Materials, 2016, 320, 265–277.