

Bioprospecting for microorganisms of biotechnological importance in soils contaminated with agrochemicals

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Abstract

The aim of the present study was to isolate microorganisms from soils contaminated with agrochemicals and to evaluate their potential for biodegradation and production of bioactive metabolites. For this, microorganisms were isolated from a soil sample by the serial dilution technique using four different media: potato dextrose agar (PDA), Mueller Hinton agar (MH), malt extract agar (MEA), and Bushnell Haas agar (BH). The isolated microorganisms were identified by their macro and micromorphological characteristics and were tested for their ability to use the DMA 806 BR agrochemical, by the dichlorophenol indophenol (DCPIP) method. Biosurfactant production and antimicrobial activity were evaluated in the selected microorganisms. The emulsifying activity was evaluated by the emulsification index (IE24) technique, while the antimicrobial activity was evaluated through the solid medium assay against pathogens of clinical interest. Among the media tested, MEA yielded the highest number of isolates, as well as a greater diversity of microbial groups, with a predominance of bacteria. Of the selected microorganisms, ten had the ability to use the agrochemical. Of these ten microorganisms, five presented emulsifying activity and two presented the capacity to produce secondary metabolites. Among them, the J5 and B48 strains were distinguished by their emulsifying and antimicrobial activity.

Keywords: Microhabitat; Biotechnology; Diversity

1 Introduction

Soil is a naturally diverse habitat with highly complex biological communities, in which different forms of microorganisms, both eukaryotes and prokaryotes, interact in a dynamic and steady state environment (CARRER FILHO, 2002). Microbial transformations, as well as their different chemical reactions, can be altered by the interference of different populations that occur in them (MACHADO, et al., 2012). In addition to their role in the environment, microorganisms and their derivatives have great biotechnological potential, such as bioinoculants for agroforestry production, biological control, bioremediation, antibiotic production, enzymes, and dyes (GOI, SOUZA, 2006).

According to Costa et al., (2017) traditionally, the soil has been a receptor of substances resulting from human activities, among them being the agrochemicals. Currently, Brazil is the world's largest consumer of agrochemicals and according to a survey done by ABRASCO (Brazilian Association of Collective Health); more than 850 million liters of agrochemicals are used annually, corresponding to an average environmental/food exposure of more than 4 liters per individual.

According to Rosado et al. (1997), the diversity of soil microorganisms is relatively less understood. The microbial mass is a critical component of the natural or man-made ecosystems, since it is involved in the decomposition of organic matter, altering the availability of nutrients to the plants, and influences the soil physical properties such as aggregate stability. Given these characteristics, soil remains the most important reservoir for the isolation of microorganisms. The microbial community, in particular the number and types of microorganisms present in the soil, is heavily influenced by geographic location, soil temperature, soil type, soil pH, organic matter content, cultivation, aeration and humidity (DAVIS et al., 2005).

Brazil has about 20% of the world's biodiversity and is an important source of raw materials for many diverse sectors. However, little is known about its biological diversity, the species that are involved and their phylogenetic relationships, including microorganisms and their interactions with other beings (SOUZA et al., 2004). Microorganisms are often seen as pathogens, especially the fungi and bacteria. However, they are also useful in the production of chemical substances, such as antibiotics, antitumorals, immunosuppressants, and other natural microbial products (CONTI et al., 2012). Thus, microorganisms are important agents for the pharmaceutical industry as a source of natural products (MENEZES et al., 2012).

In Brazil, the northeast region is the main consumer of agrochemicals; among its states, Maranhão is among the top 10 states in agrochemical consumption in the country. Among the municipalities studied, the rural area of the municipality of Lima Campos has a resident population of approximately 4,630 inhabitants. The primary agricultural activity in this region is rice cultivation, mainly in family farming (IBGE, 2016). The predominant crops are rice, maize, and vegetables. DMA 806 BR is the major agrochemical used by farmers in this region. DMA 806 BR is a selective acidic herbicide of the aryloxyalkanoic acid group of toxicological classification I - extremely toxic and have in their composition a compound equivalent to 2,4-D acid, which is a member of the family of chlorophenoxyacetic herbicides. In view of this, the current research study is necessitated by the urgent need to seek new microorganisms of biotechnological interest, that are not only possible remediators of the environment contaminated with agrochemicals, but are also producers of secondary metabolites of biotechnological interest.

2 Materials and methods

2.1 Collection site

Soil samples were collected from a family agriculture settlement, where rice, corn, and vegetable cultivation are taken care of by eight families, in the settlement of São Francisco-MA, in a settlement area comprising six hectares.

2.2 Collection procedure

Ten points were delimited in an area of approximately 1 hectare, where 10 samples with 5 cm of soil were obtained; the soil had been contaminated with DMA 806 BR pesticide, with approximately 60 days of application of the product. After collection, the samples were hermetically sealed in bags, sterilized, and transported to the Laboratory of Environmental Sciences of the University Ceuma, and mixed aseptically to yield a composite sample. For comparison, physico-chemical analysis was conducted on soil that had not been contaminated with agrochemicals.

2.3 Soil physico-chemical characterization

The chemical and physical properties of the soil were analyzed from the composite sample, for organic matter content, moisture, pH, and grain size, based on the methodology recommended by the Alef (1995).

2.4 Isolation

Isolation of the microorganisms was performed according to Clark (1965), in which 25 g of soil contaminated with agrochemicals was added to 225 mL of sterile distilled water. Subsequently, serial dilutions of 1:10 were made, up to the dilution of 10^{-6} . A 100 μL sample of the 10^{-3} dilution was inoculated into the Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Mueller Hinton Agar (MH), and in the chemically-defined minimal medium Bushnell Haas Agar (BH) using the agrochemical (DMA 806 BR) as the carbon source. All experiments were performed in triplicate. The plates were incubated at 30°C for 10 days and the appearance of the colonies was observed every 24 hours. Subsequently, a screening was carried out on each plate, where the macromorphological characteristics of the colonies were observed for subsequent purification and re-plating for the test tube culturing. These colonies were stored in a refrigerator at 4°C until completely identified.

2.5 Identification of isolated colonies

The colonies were identified macroscopically based on color, texture, shape of the edges, brightness, and size. And according to the difference in these characteristics, they were selected for the microscopic identification by Gram staining in the case of bacteria, where the morphology and the arrangement of the cells were observed. For the identification of the fungi and actinomycetes, the microculture technique was used, where the vegetative and reproductive structures of the isolates were observed.

2.6 Selection of hydrocarbonoclastic microorganisms

The selection of the microorganisms for their ability to use the agrochemical as a nutrient source, was performed using the previously described technique (HANSON et al., 1993). The principle of this test consists of the transfer of electrons to the acceptors such as oxygen, nitrate, and sulfate during the microbial oxidation of

hydrocarbons. By incorporating an electron acceptor such as dichlorophenol Indophenol (DCPIP) into the culture medium, it is possible to determine the capacity of microorganisms to use xenobiotic compounds as a substrate, by observing the change in coloration of DCPIP from blue (oxidized) to colorless (reduced). These assays were performed in multiwell plates where each well contained 1 mL of BH medium (pH 7.0), a microbial suspension standardized at 10^8 CFU/mL, 500 μ L of the agrochemical and 500 μ L of the DCPIP indicator. All assays were performed in triplicate, in addition to the abiotic controls (indicator, hydrocarbons and BH medium) and biotic controls (microbial suspension, glucose, indicator and BH medium) and maintained at 30°C under static conditions for a period of up to seven days.

2.7 Selection of microorganisms with emulsifying activity

The ten microorganisms selected in the previous assay were tested for their emulsifying activity, for which suspensions with 10^8 cells were standardized and inoculated in 50 mL of MH or Sabouraud broth, depending on the microorganism tested. These were incubated under shaking at 150 rpm for 72 h for further evaluation of the emulsification index (IE_{24}), according to the methodology recommended by Cooper & Goldenberg (1987).

2.7.1 Selection of microorganisms that produce biosurfactants - emulsification measurement

Emulsifier activity was measured by adding 6 ml of kerosene to 4 ml of aqueous sample and vortexing at high speed for 2 min. Measurements were made 24 h later. The emulsion index (E_{24}) was calculated as shown in equation 1.

$$IE_{24} = \frac{\text{height of the emulsion layer}}{\text{total height}} \times 100 \quad \text{Equation 1.}$$

2.8 Screening for biological activity

The antimicrobial activity of the fungi and actinomycetes isolated were tested in triplicate using the plug assay technique (HOSKISSON et al., 2004) against gram-positive, gram-negative, resistant alcohol acid bacteria and fungi TCC, and clinical isolates. The tested microorganisms were inoculated in Petri dishes containing PDA medium and incubated at 30°C for approximately 10 days, after which 5 mm blocks were removed and inoculated into Petri dishes previously seeded with the standardized pathogens at 10^8 CFU/ml. The plates were then incubated at 37°C for up to 48 h to observe the formation of the inhibition zones.

2.9 Statistical analysis

Student's t-test was performed on each analysis to determine the statistical significance of the values obtained.

3 Results

Soils contaminated with agrochemicals are microniches indicated for the screening of microorganisms of biotechnological interest. These environments allow the adaptation of microorganisms to the new environmental conditions of oligotrophy, alteration of pH and humidity, causing these organisms to adapt to produce primary and secondary metabolites that were not previously required. The characterization of such soils is important for the understanding of the conditions in which these microorganisms are found. Table 1 shows the physico-chemical characterization of the soil contaminated with agrochemicals.

Table 1 – Physico-chemical characterization of the soils

Soils	Description (%)						
	Clay	Silt	Sand	Organic matter	Moisture	pH	Inorganic matter
uncontaminated	2.9	22.2	18.3	20.7	13.8	6.5	79.3
contaminated	3.9	23.2	16.5	20.9	28.6	6.4	79.1

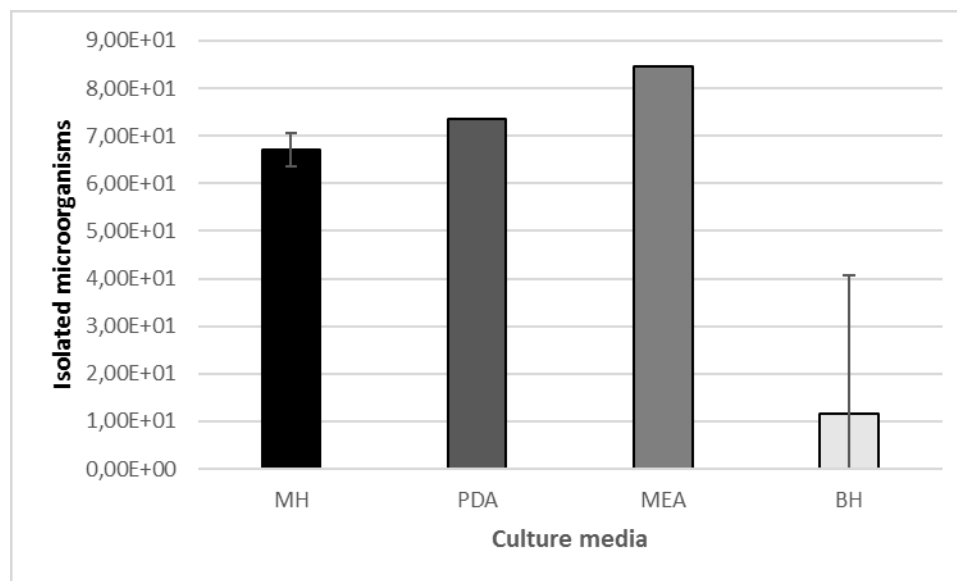
After the physico-chemical characterization of the contaminated and uncontaminated soils, it was observed that both had a very similar profile, which shows that the agrochemical had less influence on the structure and chemical characteristics evaluated. The texture of both soils is silty to sandy. Both have high inorganic than organic material, which can be explained by the presence of the agrochemical, and the pH is around neutral. Moisture is higher in the contaminated soil than in the uncontaminated soil. This can be explained by the ability of the droplets of the agrochemical diluted in water, to adhere to the soil particles, thereby promoting increased moisture. The characteristics presented by both soils are favorable to the growth of bacteria and fungi. The low organic matter content can be explained by the monoculture planting nature of the soils, which limits the microbial diversity and, thus, favors the increase of the inorganic matter that is also influenced by the application of agrochemicals. The soil texture is a factor that must be considered when insulation is desired; the microorganisms adhere to the soil particles by chemical adsorption by performing the exchange of charges and releasing micronutrients by the action of their enzymes. A sandy soil has smaller particles and favors the fixation of bacteria because they are smaller and not filamentous. On the other hand, since these soils also have a higher percolation rate, the fungi and actinomycetes can attach their hyphae and remain in the soil in the form of spores.

To isolate microorganisms from soils, an important factor is the choice of media to be used. These should provide conditions to isolate the microorganisms of interest amid the microbial diversity known to be present in the soil. Figure 1 shows the quantity of microorganisms isolated from the soil contaminated with the agrochemical DMA 806 BR.

On comparing the four culture media used for isolation, the highest number of colonies was obtained in MEA with a mean of 84×10^3 CFU/mL. The next highest was in PDA averaging 74×10^3 CFU/mL, followed by MH with a mean of 67×10^3 CFU/mL and finally the chemically-defined BH culture medium with average of 12×10^3 CFU/mL. Among the media tested MEA is the richest in regard to macro and micro nutrients. This explains the large number of colonies obtained in this medium. Although it presented the least number of colonies, the BH culture medium presented a greater diversity of colonies, with a variety of macro-morphologically different colonies. This is mainly due to the composition of the medium. The BH is a chemically-defined minimal medium and the agrochemical DMA 806 BR was used as the sole carbon source, thus selecting for the bacterial colonies that were most adapted to soil contaminated with agrochemicals. The selective media are more suitable for the isolation of microorganisms of biotechnological interest; but it is important to use conditions which, even if selective, allow the growth of a higher quantity of microorganisms. The MEA medium is a very rich medium, in that it is composed of low molecular weight carbohydrates that are easily metabolized and absorbed, such as maltose, in addition to containing some micronutrients that would favor the growth of the Actinomycetales group. The PDA is a medium for the growth of yeast and filamentous fungi. It is composed of a potato infusion which makes it rich in starch, favoring

the growth of organisms capable of metabolizing this polysaccharide. Unlike other media, MH has defined micronutrient composition with known concentrations of CaCl_2 , MgCl_2 , and ZnCl_2 , as well as peptone and casein which impart the macronutrients to the medium, thus favoring the growth of bacteria

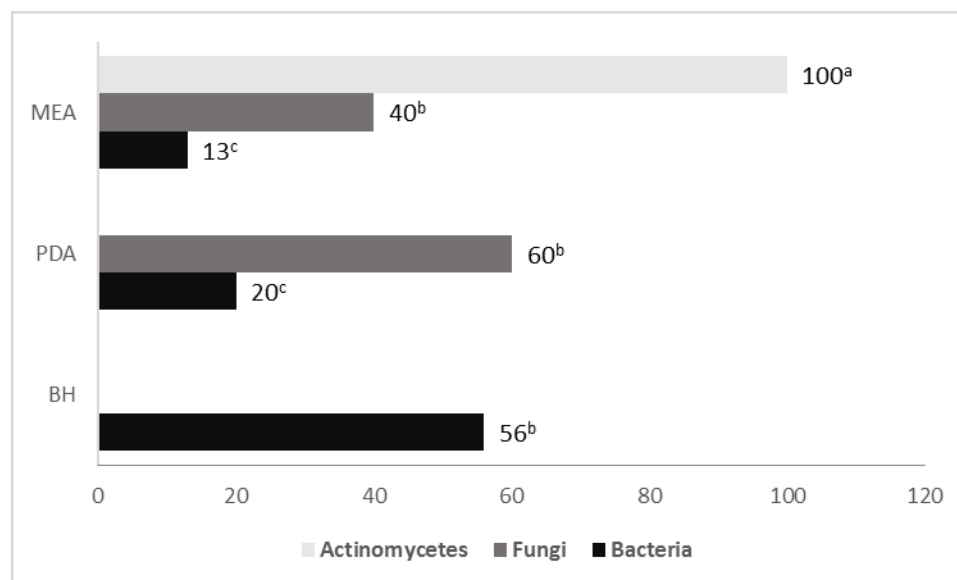
Figure 1 - Isolation of microorganisms from soil contaminated with the DMA 806 BR agrochemical using four different selective culture media



After counting the colonies on the plates, macromorphological observation and selection were performed for subsequent purification and identification of the microorganisms. The criterion used for the selection of colonies was the differences in their macromorphology. It was observed that in the MEA, despite the isolation of a large number of colonies, all had very similar macromorphology, with whitish cottony milky small colonies, with well-defined borders; here, selection was based on colonies that presented different aspects, such as yellowish or rosy coloration, irregular edges and glow in the colony. The PDA culture medium presented a number of very similar, small, whitish colonies with brightness in the colony, some of which presented characteristics of the filamentous fungi, such as the presence of mycelium and differentiated staining. The MH medium presented colonies with similar characteristics, with small, opaque, milky colonies, and some with irregular edges. The medium that presented a greater diversity of colonies was the BH, with several colonies of different colors, irregular borders suggestive of the *Bacillus* genus, and in some plates greenish colonies with fluorescence were observed. From these observations, microbial colonies were selected that would be purified to be tested later.

The identification of the microorganisms was carried out with the above selected microorganisms. The microculture technique was used to observe the hyphae and morphology of the hyphae (cenocytic or septate) and the arrangement of conidia and conidiospores of filamentous fungi. Actinomycetes, in addition to aerial mycelium formation (macromorphology), were also observed for their hyphae morphology and spore arrangement through the microculture technique. Figure 2 shows the frequency of the microbial groups in the culture media after selection based on macromorphological observation.

Figure 2 - Frequency of microbial groups selected based on their macromorphological characteristics, after isolation in the three media used

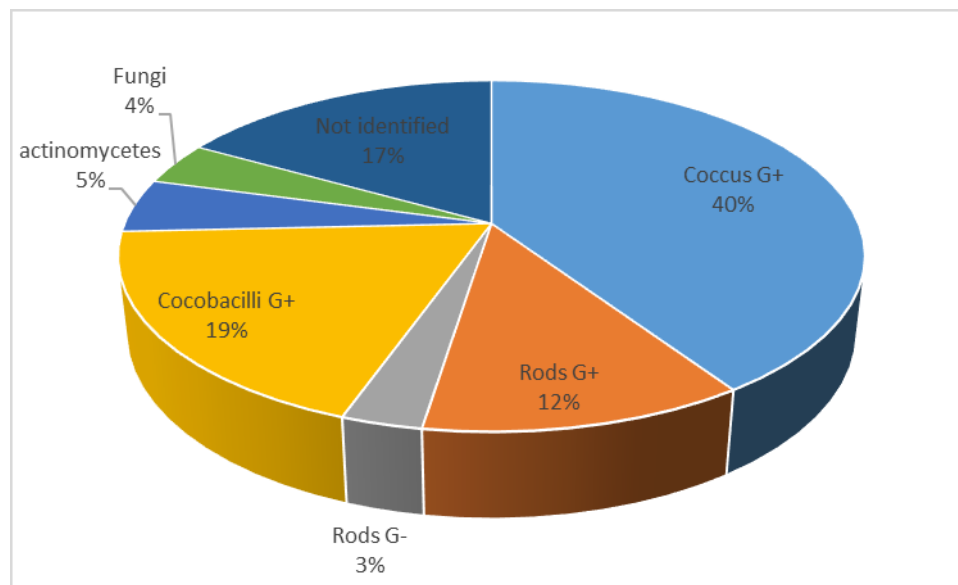


*Values with different superscripts are significantly different (p < 0.05)

Four culture media were used to isolate the microorganisms from soil contaminated with agrochemicals. After macromorphological selection, a bacterial prevalence was observed in the three media tested. The appearance of colonies of filamentous fungi was observed in two of the media tested, the PDA and the MEA, and the Actinomycetales group was present only in the MEA medium. The culture media tested here are used for the isolation of microorganisms in general, which explains the appearance of bacteria in the three media. This microbial group possess a faster generation time than that by the other groups, besides having a broader nutritional need, thus, facilitating the growth in a greater variety of substrates. The PDA culture medium is rich in starch, which favors the growth of filamentous fungi. It is known that this microbial group has individuals producing the amylase enzyme and is very useful because of its nutritional content. Among all the four media tested, the MEA has the most nutrients, and this fact explains the appearance of actinomycetes in this medium. Even with the appearance of other heterotrophic, non-filamentous bacteria, this medium was conducive to the appearance of Actinomycetales due to their nutritional richness and culture conditions like pH. After identification of the isolated microorganisms, a predominance of gram-positive cocci bacteria was observed, up to 41% of the total of purified microorganisms. Gram-positive coccobacilli were the second most frequent group of microorganisms constituting 19% of the total; gram-negative rods were 12%, actinomycetes 5%, fungi 5% and 17% of the purified microorganisms were not identified, as can be visualized in Figure 3.

The identification shows a prevalence of bacteria gram-positive cocci. Among the cocci, different morphologies such as, isolated cocci, diplococci and coccobacilli were observed. Bacteria are the predominant group in soils, and according to the literature $10^8 - 10^{10}$ CFU can be isolated from 1 g of soil; this explains the prevalence of this group after isolation. Gram-positive bacteria have a greater adaptive capacity in contaminated environments due to their thick cell wall, with several layers of peptidoglycan, and many have the capacity to form endospores.

Figure 3 - Percentage of microorganisms selected, purified, and identified after isolation



They have a tendency to organize by the mechanism of quorum sensing, which gives this group a greater capacity for adaptation. Actinomycetes and fungi, although small in number, were also isolated and identified. Among the isolated microorganisms, four actinomycetes were selected for their macromorphological characteristics, of which three were identified micromorphologically as *Streptomyces* spp. (2) and *Streptosporangium* sp. (1), and one was not identified. Fungi were also identified in three different genera, the *Penicillium* sp., *Trichoderma* sp. and *Aspergillus* sp. Fungi and actinomycetes are important constituents of the soil. They are organisms that participate in the decomposition process because they produce a variety of enzymes, which may explain their adaptive capacity in contaminated soil. In addition, both fungi and actinomycetes are responsible for the production of secondary metabolites of clinical interest. In order to select the microorganisms that were most adapted to the conditions of the contaminated soil, the selection test was performed using the redox indicator DCPIP. This methodology consists of a colorimetric test where the organisms that have the ability to metabolize the agrochemical will transform the culture medium from blue to colorless. The microorganisms with the greatest capacity to metabolize the DMA 806 BR agrochemical can be visualized in Table 2, where the discoloration of the BH culture medium can be observed by the action of the microorganisms with up to 72 h of incubation.

Seventy-eight microorganisms were selected by macromorphological characteristics and identified by micromorphology, of which ten were selected because they could discolor the BH medium within 72 hours. Of these 7 were bacteria (B30, B44, B47, B53, B59, and B72), 2 were actinomycetes (6PN and J5) and one was a filamentous fungus (F5). Among the ten microorganisms that discolored the culture medium, the bacteria showed the best results, discoloring the medium within 48 h. The bacteria B30 and B53 were the ones that presented better adaptive capacity by discoloring the medium within 24 h of incubation. Bacteria B44, B48, B59, B72 and the actinomycetes 6PN and J5 discolored the medium within 48 h of incubation; the F5 filamentous fungus and the B47 bacterium discolored the culture medium within 72 h of incubation. It is to be noted that all the microorganisms started the process of discoloration beginning from 12 to 14 h of incubation, being that the bacteria discolored more quickly.

Table 2 - Selection of microorganisms based on their potential to utilize the agrochemical as a carbon source, as measured through the DCPIP technique

Microorganisms	Identification		Indication Redox (h)		
	Gram	Morphology	24	48	72
B30	+	coccus	+++		
B44	+	coccorods	++	+++	
B47	+	coccus	+	++	+++
B48	+	coccus	++	+++	
B53	+	rods	+++		
B59	+	rods	++	+++	
B72	+	coccus	++	+++	
6PN		<i>Streptomyces</i> sp.	++	+++	
J5		<i>Streptomyces</i> sp.	++	+++	
F5		<i>Aspergillus</i> sp.	+	++	+++

+ - slightly discolored; ++ - medium colorless; +++ - totally discolored

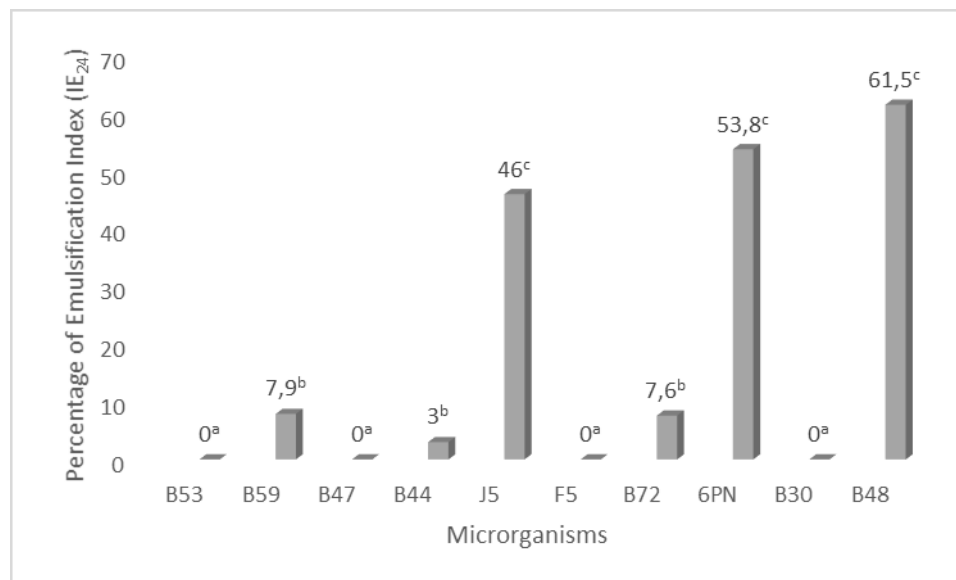
The mechanism of biodegradation of bacteria and fungi are well described in the literature; although different, they complement each other and can be used with the objective of mineralizing complex molecules such as agrochemicals. Although both microbial groups have a predilection for initiating degradation of polycyclic-aromatic compounds, fungi are the main producers of the enzymes mono, dioxygenase, and phenoloxidases. Whereas, bacteria are surfactant-producing agents that help to approximate specific groups of organisms that can contribute in the degradation processes of the molecule and to break down the unsaturated aliphatic compounds. These mechanisms are only possible because the surfactants are amphipathic molecules that surround the chemical compounds forming micelles and allow the approach of more specific organisms. One of the indirect ways of determining whether certain organisms produce biosurfactants is to measure the emulsification index after 24-h incubation of the cell-free fermented liquid in contact with some oily compounds. The formation of micellar lamina is an indication of the production and secretion of surfactants in the specific culture medium.

With an aim to investigate the production of surfactants, the emulsification index of the ten microorganisms selected in the DCPIP test was measured. In Figure 4, it can be observed that of the ten microorganisms tested, six (60%) presented emulsifying activity, of which three showed an activity above 40%, which is considered a high activity. Of the five microorganisms that presented emulsifying activity, B48 bacteria had the highest IE₂₄ of 61.5%, followed by the actinomycete 6PN with 53.8% and the other actinomycete J5 with 46%. Bacteria B59, B44 and B72 presented a minor emulsifying activity, with low values of IE₂₄. The values obtained by these bacteria were 7.9%, 3% and 7.6% respectively. Filamentous fungus J5 and bacteria B53 and B30 showed no emulsifying activity.

The production of biosurfactants by bacteria is widely reported in the literature. In this study the bacteria that obtained the highest values of IE₂₄ presented in the micromorphological identification as one being a gram-positive diplococcus and two actinomycetes (J5 and 6PN). Gram-positive bacteria, especially those of the *Bacillus* spp., are the major producers of surfactants of the lipopeptide class. For this, numerous substrates have been used, from glucose to agroindustrial residues. The bacteria that presented lesser activities were also gram-positive, two cocci (B44 and B72)

and one bacillus (B59). Future studies will be carried out by modifying the substrate and the conditions of production, in an attempt to increase the emulsifying activity of these bacteria.

Figure 4 - Emulsification index (IE₂₄) of the microorganisms selected using the DCPIP test



*Values with different superscripts are significantly different ($p < 0.05$).

With an aim to evaluate the antimicrobial potential of the ten selected microorganisms, they were tested for their antimicrobial activity against ATCC pathogens and clinical isolates by means of the solid medium antimicrobial activity assay. This test aims to evaluate the production capacity of secondary metabolites by the microorganisms isolated in solid culture medium. Of the ten microorganisms tested, two showed antimicrobial activity against more than one pathogen, as can be seen in Table 3.

It can be seen in Table 3 that among the ten microorganisms selected and tested for antimicrobial activity, two presented positive results against gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Corynebacterium diphtheriae* ATCC27010, *Corynebacterium diphtheriae* ATCC27012 and *Mycobacterium abscessus*) and fungus (*Cryptococcus neoformans*). The bacterium gram-positive B48 and actinomycete J5 were distinguished by their antimicrobial activity against ATCC pathogens and clinical isolates. The bacterium B48 was active against gram-positive bacteria *S. aureus* ATCC 6538, *C. diphtheria* ATCC 27010, *C. diphtheria* ATCC 27012 and *M. abscessus* (clinical isolate), with inhibition halos showing averages of 22.3, 36, 34 and 22 mm respectively; while for the clinical isolate *C. neoformans* it presented a inhibition zone of 33.6 mm. Actinomycete J5 showed a similar result; assays with the gram-positive bacteria *S. aureus* ATCC 6538, *C. diphtheria* ATCC 27010, *C. diphtheria* ATCC 27012 and *M. abscessus* (clinical isolate), with inhibition halos having averages of 22.6, 43, 34.3 and 22.3 mm, respectively are shown; for the fungus *C. neoformans* (clinical isolate) it presented a inhibition zone of 37.6 mm. Bacteria isolated from the soil contaminated with agrochemicals showed activity against gram-positive bacteria, which suggests that the metabolite produced by them acts on the cell wall. On the other hand, the same bacteria showed activity against the yeast-like fungus *C. neoformans* of clinical interest, which may suggest the production of more than one type of metabolite by these bacteria.

Table 3 - Antimicrobial activity of isolated microorganisms against ATCC and clinical pathogens

Pathogens	Microorganism isolates									
	B30	B44	B47	B48	B53	B59	B72	6PN	J5	F5
	-	-	-	-	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i> ATCC0023	-	-	-	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC0026	-	-	-	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC 0030	-	-	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 6538	-	-	-	22 ^a ±1.5	-	-	-	-	23 ^a ±0.5	-
<i>Corynebacterium diphtheriae</i> ATCC27010	-	-	-	36 ^b ±1.7	-	-	-	-	43 ^b ±1	-
<i>Corynebacterium diphtheriae</i> ATCC27012	-	-	-	34 ^b ±1	-	-	-	-	34 ^c ±0.5	-
<i>Mycobacterium abscessus</i>	-	-	-	22 ^a ±3.2	-	-	-	-	22 ^a ±1.1	
<i>Cryptococcus neoformans</i>	-	-	-	34 ^b ±0.5	-	-	-	-	38 ^c ±0.5	
<i>Cryptococcus neoformans</i> ATCC24067	-	-	-	-	-	-	-	-	-	-
<i>Cryptococcus gattii</i> ATCC24065	-	-	-	-	-	-	-	-	-	-

Mean ± standard deviation in mm (n = 3). Means in same column with different superscripts are significantly different (p < 0.05).

The statistical analysis performed with the isolation results showed that there was a significant statistical difference of the BH medium (p = 0.034) in relation to the other culture media used. This can be explained by the composition of the medium; only in the BH a xenobiotic was used the carbon source, favoring the growth of the microorganisms that are metabolically adapted to the environment contaminated with this agrochemical. All other media tested, in addition to being nutritionally rich, had traditional carbon sources in their composition. When the statistical analysis was performed between the microbial groups, it was observed that in MEA, there was a significant statistical difference between the three groups isolated in this medium, with p = 0.025. The same observation was made in the PDA medium where there was a statistically significant difference between the two groups of isolated microorganisms with p = 0.048. When the analysis was performed between the media, a significant statistical difference was observed between the MEA medium and the other two media used with p = 0.032 (PDA) and p = 0.028 (BH). There was no significant statistical difference when the group of microorganisms isolated were fungi and the actinomycetes were only isolated in the MEA medium.

4 Discussion

The physico-chemical characterization of the soil is extremely relevant to understand the dynamics that occur in the habitat, and to indicate the viability of the soil for the presence of microorganisms. The soil characterization performed in this work demonstrated that the texture is between silt and sandy; is higher in inorganic matter and therefore is an organically poor soil; the pH is around neutral and has high humidity. Some papers corroborate the results found in this study. The physico-chemical characterization of soils is described in the literature as a way to better understand this habitat. Jang et al. (2010) characterized four different soil types to understand the degree of weathering that these soils suffered over time due to anthropogenic actions. Contrary to the results found in this work, the authors observed a more clayey texture for soils rich in organic matter and acidic pH for the four soil types tested. The authors stated that the weathering caused by anthropic actions were responsible for the soil acidity. Anda et al. (2015) characterized a soil of rice plantation in Indonesia. The authors collected soil from various parts of the Indonesian islands and observed a similar profile found in this work, soil with sandy texture and low organic matter content. The only parameter that diverged was the pH the authors reported was acidic. Corroborating with our results, the authors also attributed the physico-chemical characteristics to the use of chemical compounds in the soil. Miguel et al. (2017) characterized a soil contaminated with iron, manganese, and fluorine from a miner, and observed that the characteristics were also very similar to those found in this work. Sandy soil, around neutral pH and poor in organic matter. The authors related all these characteristics to the action of humans through the extraction process of the minerals, besides the contamination with chemical substances used throughout the process. Dias et al. (2017) characterized soil collected from mangrove ecosystem for the isolation of microorganisms of biotechnological interest. From the five sites collected, all presented a sandy profile, two were poor in organic matter and the pH of soil from the five sites collected was from neutral to alkaline. The authors attributed these characteristics to the soil being of an estuarine environment, rich in vegetation, which explains the high organic matter, and as the collection was made in low tide period the sediment presents a sandy profile.

Isolation by classical techniques is the best method for obtaining microorganisms of biotechnological interest. It was observed that the isolation with the MEA culture medium was the highest, presenting colonies of all the microbial groups. According to Previati et al. (2012), the evaluation of population densities in the microbial community in soils includes plate culture technique, direct microscopic examination, and enrichment technique, and that in the bacterial community there is also variability between bacterial populations in general and actinomycetes in regard to the use of nutrients manifested in the culture medium. This profile can be observed in this work, where the MEA medium stood out among the methods used to present growth of the three isolated microbial groups. Stroze et al. (2013) with the objective of finding fungi with bioactive potential against nematodes, isolated filamentous fungi from several soil samples, using the serial dilution technique with plating in BDA medium, and obtained values similar to those obtained in this work. The authors state that choosing a suitable selective medium is critical to the success of the isolation. Kurniati et al. (2014) used fungi isolated from forest soils to test their potential for bioremediation of environments contaminated with mercury. The authors used micromorphological techniques to identify these fungi and reported the genus *Aspergillus* sp., similar to that obtained in this work. This same principle guided the work of Jesus and Rodrigue (2015); in order to isolate lignolytic bacteria from several soil samples collected, they used selective media using cellulose or lignin as the sole source of carbon. The authors obtained a total of 52 isolates, 4 in the LB medium, 24 in the lignin medium and 24 in the cellulose medium. Dias et al. (2017) with the objective of obtaining microorganisms of biotechnological potential isolated microorganisms from soil of the mangrove ecosystem. Unlike the results obtained in

this work, the authors reported a much lower quantitative of microorganisms in the selective medium used for the isolation. However, the predominant groups of microorganisms obtained in the isolation were similar to those obtained in this work. Gram-positive bacteria were the most abundant, followed by gram-negative bacteria and finally by fungi. The authors attributed the prevalence of bacteria to their adaptive capacity and biofilm formation. The isolation of microorganisms in soil with some type of contamination has been an alternative chosen by several authors. Costa et al. (2017) isolated microorganisms from soil contaminated with pesticides and obtained results very similar to those obtained in this work. The authors aimed to isolate microorganisms from soil contaminated with pesticides following the seasonality of dry and rainy seasons and observed that they had a higher quantity of microorganisms in the dry period. The microbial groups reported by the authors were the same as those obtained in this study, although the means were different. The microorganisms were identified for their macro and micromorphological characteristics and a prevalence of gram-positive bacteria, followed by filamentous fungi was observed. The authors reported that, in contrast to this work, most of the isolated bacteria had a rod morphology, however the identification of fungi showed the presence of the genera *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp., the same as obtained in this work.

The selection of microorganisms with the potential to use agrochemicals as a nutrient source can be evaluated in several ways. Some quick and practical alternatives to make this evaluation are the colorimetric techniques, which are based on reactions of discoloration of the medium due to the release of electrons by the organisms studied; this allows not only the evaluation of their potential, but also the speed of metabolization. Several authors use these techniques to select potential microorganisms, especially when they have a very large number of microorganisms to be tested. One of the techniques widely reported in the literature is the technique of DCPIP. This technique was first described as efficient for the selection of microorganisms by Hanson et al. (1993) when the authors reported the use of this indicator with BH medium and crude oil as the carbon source in multiwell plates for selection of bacteria with biodegradation capacity.

Bidoia et al. (2010) mention that the DCPIP technique is widely reported, which is based on reducing the oxidized form and changing the color from blue to colorless. Since then, several authors have used the technique as an alternative for the selection of microorganisms for biodegradation. The potential of microorganisms isolated from environment contaminated with petroderived residues in degrading hydrocarbons was evaluated using the discoloration technique indicated by the DCPIP. The authors report the selection of microorganisms that presented degradation potential with up to 24 hours of testing (MIRANDA et al. 2007; GOMES et al. 2009). Currently the technique is still widely used. Varjani and Upasani (2016) reported crude oil degradation by a lineage of *Pseudomonas aeruginosa* NCIM 5514. The authors showed the potential for degradation of the bacteria using the DCPIP indicator selection test, achieving the discoloration within 24 h with an increase in the microbial biomass in the plate as evaluated by absorbance technique. Almeida et al. (2017) used the DCPIP redox indicator technique to select the best microbial consortium capable of degrading the MF-8 marine fuel. The authors reported that they obtained discoloration by a consortium composed of different bacteria, in 24 h. Marchand et al. (2017) reported using the DCPIP methodology to select bacteria and fungi with potential for degradation of crude oil. The authors emphasized the efficiency and speed of the technique. Adnan et al. (2018) used the DCPIP technique to estimate the capacity of fungi of the genus *Penicillium* spp. in degrading crude oil. The authors reported that changing of the coloration from blue to colorless in 14 days at 30°C incubation determined that the fungi had the ability to degrade the oil.

From the selected microorganisms in this study, the biotechnological potential was tested. As microorganisms isolated from environments contaminated with pesticides, one of the mechanisms of action of these organisms to adapt

to the environment is the production of biosurfactants; hence, the emulsifying capacity of these organisms was evaluated through the emulsification index (IE₂₄). The investigation of microorganisms producing emulsifying substances is widely reported in the literature. Bezerra et al. (2012) reported the production of biosurfactant by *Pseudomonas aeruginosa* GVII-A using agroindustrial waste as a carbon source. For the initial selection, an IE₂₄ test was performed, where the bacteria were fermented in minimal salts medium with agroindustrial residues and from that the emulsification tests were carried out for the subsequent production of the surfactant. Several authors use the technique of IE₂₄ to report the potential of bacteria of the genus *Bacillus* to produce bio-active compounds such as biosurfactants. The authors demonstrate the ability of this genus to produce surfactants in medium with different carbon sources. The genus is able to produce surface-active substances in glucose-containing medium with an inert carbon source or oily source as hydrocarbon residues (COOPER and GOLDENBERG, 1987; BARROS et al. 2008; MORAIS et al. 2015). Lins et al. (2017) evaluated the ability of the fungus *Cunninghamella phaeospora* UCP 1303 to produce biosurfactant from soybean oil and corn liquor by controlling the temperature. The authors reported EI₂₄ values similar to those obtained in this study, inferring the ability of the fungus to produce bioactive compounds. Pereira et al. (2017) evaluated the EI₂₄ using kerosene as an emulsifier source. The authors isolated and cultured the Amazonian fungi in liquid medium supplemented with soybean oil and incubated under agitation. The IE₂₄ values were similar to those obtained in this work with the same emulsifier source.

Morais et al. (2014) state that most of the products currently used are derived from microbial fermentation or obtained from chemical modifications of a microbial product, and can be used in agriculture, livestock and for human consumption. However, not all microorganisms are also capable of producing secondary metabolites. This capacity, in turn, is restricted to specific groups of bacteria and eukaryotic microorganisms. In the group of prokaryotes this includes actinomycetes filaments, myxobacteria, pseudomonas and cyanobacteria and in the eukaryotic group, it is mainly the filamentous fungi. The evaluation of the antimicrobial activity of selected isolates is a way to investigate the potential of these organisms to produce secondary metabolites. Some authors already report this activity in microorganisms isolated from environments. Feitosa et al. (2014) reported the ability of tap water-isolated fungi to inhibit the growth of pathogens of clinical interest. The fungi *Pestalotiopsis palustris*, *Cladosporium cladosporioides*, *Trichoderma pseudokoningii*, *Curvularia lunata* (50) and *Penicillium* sp. (45) were tested against *S. aureus* UFPEDA 01, *Pseudomonas aeruginosa* UFPEDA 39, *Mycobacterium tuberculosis* UFPEDA 71 and three resistant *S. aureus* (*S. aureus* ORSA UFPEDA 701, 730, and 733) using the diffusion method. Among the fungi tested, *Penicillium* sp. showed activity against *S. aureus* 730 ORSA. With the appearance of microorganisms resistant to antimicrobial drugs used in the clinic, the search for microorganisms with potential to produce secondary metabolites that have activity against clinical pathogens, continues today. Synytsya et al. (2017) extracted bioactive mycelial compounds from fungi using different solvents and observed that they were more active against gram-positive bacteria except for a fungus that showed activity against gram-negative bacteria. More recently Lotfy et al. (2018) isolated 445 microbial genera from a cave, of which 33 were evaluated and tested for their ability to produce secondary metabolites against *S. aureus*, *Salmonella typhimurium* and *Candida albicans*. Of all the bacteria tested, seven showed antimicrobial activity against the pathogens used. Ulaganathan et al. (2018) isolated 40 fungi from the arctic environment and tested against gram-positive and gram-negative bacteria using the plug assay. Most of the fungi showed high activity against gram-positive bacteria, a result that corroborates this work.

5 Conclusions

The search for microorganisms with biotechnological potential in microniches and inhospitable environments, has been increasing. Microorganisms were isolated from soil with high humidity, neutral pH, and sandy characteristics, in three selective media, and colonies were isolated from the three microbial groups only in the MEA medium. Among the isolates, 78 were selected based on the macromorphological characteristics of the colonies and micromorphologically identified, resulting in a predominance of gram-positive cocci. The appearance of three genera of fungi and actinomycetes was also observed. When the 78 microorganisms were tested for their adaptation to the agrochemical, ten showed to be more adapted for discoloring the culture medium within less time. These were tested for their ability to produce bioactive substances and secondary metabolites. Of the ten microorganisms tested, two were distinguishable because they had the capacity to produce biosurfactants and presented activity against gram-positive bacteria and pathogenic fungi. These results show that the microniches in the contaminated environments that have the characteristics of uncontaminated soil, are potentially interesting in the search for microorganisms with biotechnological potential.

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References

- ADNAN B, HAWASH AL, JAWADAYN T, HAYDER A, ABBOOD A, ZHANG J, ET AL. Isolation and characterization of two crude oil-degrading fungi strains from Rumaila oil field, Iraq, *Biotechnology Reports* 2018, <https://doi.org/10.1016/j.btre.2017.12.006>
- 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
- ANDA M, HUSNAIN ES, SUBARDJA D. Strategy to reduce fertilizer application in volcanic paddy soils: Nutrient reserves approach from parent materials. *Soil & Tillage Research*, 2015 (150),10-20.
- BARROS FFC, QUADROS CP, PASTORE GM, Propriedades emulsificantes e estabilidade do biossurfactante produzido por *Bacillus subtilis* em manipueira, *Ciênc. Tecnol. Aliment.*, 2008, 28(4), 979-985,
- BEZERRA MS, HOLANDA VCD, AMORIM JA, MACEDO GR, SANTO ES, Produção de Biotensoativos Utilizando *Pseudomonas aeruginosa* (P.A.) e Resíduo Agroindustrial (Maniueira) como Substrato. *HOLOS*, 2012, v.1, p 14-27
- BIDOIA ED, MONTAGNOLLI RE, LOPES PRM, Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study. *Current Research Technology and Education Topics in Applied Biotechnology and Microbial Biotechnology*. A. Mendez Vilas, 2010.
- CARRER Filho R, Actinomicetos como Agentes d Biocontrole de Doenças e como Promotores de Crescimento do Tomateiro. *Viçosa*, 78p. 2002
- Clark FE Agar-plate method for total microbial count. 1965, Pp. 1460-1466.
- CONTI R, GUIMARÃES DO, PUPO MT, Aprendendo com as interações da natureza: microrganismos simbiotes como fontes de produtos naturais bioativos. *Cienc Cult* 2012, 64(3), 43-47.

COOPER DG, GOLDENBERG BG, Surface-Active Agents from Two *Bacillus* Species, Applied and Environmental Microbiology, 1987, p.224-229.

COSTA MS, ARAÚJO TRR, MARTINS AJ, SILVA QHS, COSTA CH, Miranda RCM, Isolamento de microrganismos de interesse biotecnológico oriundos de áreas contaminadas com agroquímico. Rev. Investig, Bioméd, 2017, 9:17-23.

DAVIS KER, JOSEPH SJ, JANSSEN PH, Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria. Appl Environ Microbiol, 2005,71,826-834.

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.

DONADIO S, MONCIARDINI P, ALDUINA R, MAZZA P, CHIOCCHINI C, CAVALETTI L, et al. Microbial technologies for the Discovery of novel bioactive metabolites. J Biotechnol, 2002, 99(3),187-198.

FEITOSA TR, ARRUDA, FVF, CAVALCANTI FILHO JRN, BAPTISTA, NMQ, CALLOU, MJA, SILVA T, MIRANDA RCM, GUSMÃO NB. Antimicrobial Activity of Fungi Isolated from the Water of the Sky Height, Recife-PE, Supply against Bacteria of Clinical Interest System, African Journal of Microbiology Research, 2014, v8, (32), pp 2999-3007.

GOI SR, SOUZA FA, Diversidade de microrganismos do solo. Floresta e Ambiente, 2006, v.13,n.2,p. 46-65.

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, v 7, n10, pp 745- 748.

IBGE (2016) <https://www.ibge.gov.br/novo-portal/pesquisa>, Acessado em maio de 2018

JANG JU, XU REN-KHOU, ZHAO ANN ZHEN. Comparison of the surface chemical properties of four soils derived from Quaternary red earth as related to soil evolution. Catena, 2010, 154-161.

JESUS MS, RODRIGUES TB. Isolamento e purificação de bactérias associadas a amostras de solo do Bioma Caatinga com potencial celulolítico e lignolítico. Revista de Inovação, Tecnologia e Ciências, 2015, v.1, n.1, p. 1-11.

KURNIATI E, ARFARITA N, IMAI T, HIGUCHI T, KANNO A, YAMAMOTO K, SEKINE M, Potential bioremediation of mercury-contaminated substrate using filamentous fungi isolated from forest soil. Journal of Environmental Sciences, 2014, 26,1223–1231.

LINS AB, BIONE AP, FONSECA TCS, SILVA TC, SILVA PH, MORANT KV, ANDRADE RFS, TAKAK GMC, Biosurfactant Production by *Cunninghamella phaeospora* UCP 1303 Using Controlled Temperature Through of Arduino. Int.J.Curr.Microbiol.App.Sci, 2017, 6, 2708-2715.

LOFTY MM, SUEF B, University Journal of Basic and Applied Sciences 2018, <https://doi.org/10.1016/j.bjbas.2018.02.002>

PEREIRA DDF, DUVOISIN JR S, ALBUQUERQUE PM, O Estudo da Produção de Biossurfactantes por Fungos Amazônicos, The Journal of Engineering and Exact Sciences, 2017, v.3, 0688-0695

MACHADO DM, SCHOSSLER TR, ZUFFO AM, de ANDRADE FR, PIAULLINO AC. Atividades Microbianas e as Transformações no Ciclo dos Elementos no Solo. Enciclopédia Biosfera, 2012, v.8,n.15, p. 180 – 195.

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

MENEZES CBA, SILVA BP, SOUSA IMO, RUIZ ALTG, SPINDOLA HM, CABRAL E, et al. *In vitro* and *in vivo* antitumor activity of crude extracts obtained from Brazilian *Chromobacterium sp* isolates. *Braz J Med Biol Res* 2013, 46(1),65-70.

MIGUEL MG, BARRETO RP, PEREIRA SY, Study of a tropical soil in order to use it to retain aluminum, iron, manganese and fluoride from acid mine drainage. *Journal of Environmental Management*, 2017, 204, 563-570.

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.

MORAIS KAD, SOARES RS, ARAÚJO MVF, OLIVEIRA BRF, VIEIRA JDG. Produção de Biossurfactante por *Bacillus sp.* em Meio Mínimo Contendo Glucose, *Enciclopédia Biosfera*, 2015,v.11 n.22; p.3085 – 3094.

MORAIS JF, YOSHIMOTO M, RHODEN SA, PAMPHILE JA, Bioprospeção de Microrganismos Produtores de Compostos Bioativos com Atividade Antitumoral, *UNIGÀ*, 2014 v.17, pp.27-34.

PREVIATI R, da Silva JRR, de SOUZA CR, JANKE L. Isolamento e Quantificação das Populações de Bactérias em Geral e de Actinomicetos Presentes no Solo, *Arq. Ciênc. Vet. Zool.* 2012, v.15, n.2, p 155-160.

ROSADO AS, DUARTE GF, SELDIN L, ELSAS JD. Molecular microbial ecology: a minireview. *Microbiology*, 1997,28,135-147.

SOUZA AQL, SOUZA ADL, ASTOLFI S, BLÉM MLP, SARQUISM MIM, PEREIRA JO. Atividade antimicrobiana de fungos endofíticos isolados de plantas tóxicas da Amazônia: *Palicourea longiflora* (audl) rich e *Strychnos cogens* bentham. *Acta Amazônica*, 2004, 34(2),185-195.

STROZE CT, SANTIAO DC, BAÍDA FC, ISOLAMENTO, CARACTERIZAÇÃO E AVALIAÇÃO DO POTENCIAL DE FUNGOS NEMATÓFAGOS NO PARASITISMO DE *MELOIDOGYNE JAVANICA* “*IN VITRO*”. *NEMATROPICA*, 2013, v.43, n.1,17-23.

SYNYSTSYA A, MONKAI J, BLEHA R, MACURKOVA A, RUMIL T, AHN J, CHUKEATIROTE E, Antimicrobial activity of crude extracts prepared from fungal mycelia *Asian Pacific Journal of Tropical Biomedicine*, 2017, 7, 257–261.

ULAGANATHAN Y, Weber JFF, CONVEY P, RIZMAM-IDID M, ALIAS, SA. Antimicrobial properties and the influence of temperature on secondary metabolite production in cold environment soil fungi, *Polar Science*, 2017, doi: 10.1016/j.polar.2017.09.005.

VARJANI S.J, UPASANI VN, Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM 5514, *Bioresource Technology*, 2016, 222, 195–201.