

PETROLEUM DEGRADATION BY FILAMENTOUS FUNGI

Judith Liliana Solórzano Lemos¹, Andrea C. Rizzo¹, Valéria S. Millioli¹, Adriana Ururahy Soriano², Maria Inez de Moura Sarquis³ & Ronaldo Santos¹.

¹ Centro de Tecnologia Mineral - CETEM, Rio de Janeiro; ² Centro de Pesquisas e Desenvolvimento Leopoldo Américo M. de Mello - CENPES, Rio de Janeiro; ³ Instituto Oswaldo Cruz, Fundação Oswaldo Cruz - FIOCRUZ, Rio de Janeiro.

ABSTRACT

It is known that bacteria and fungi are the principle petroleum degrading microorganisms. Therefore, the study of the petroleum hydrocarbon degradation was accomplished by filamentous fungi to evaluate the potential of strains, previously isolated from Guararema (SP - Brazil) soil, accidentally contaminated with crude oil. The results of such an evaluation allowed the selection of those microorganisms with ability to degrade petroleum, added as the only carbon and energy source to a mineral medium. The selected strains were submitted to identification, being the following genera detected: *Aspergillus*, *Penicillium*, *Paecilomyces* and *Fusarium*. After that, degradation tests were accomplished at 30 °C, in the contaminated soil, to select the best microorganism, or a pool of them, working under the following previously established process conditions: 50% of water holding capacity, scheduled aeration, and without nutrients supply.

Key words: degradation, petroleum hydrocarbon, fungi

INTRODUCTION

The petroleum industry is responsible for the generation of high amounts of organic residues, as well as for the pollution of soils, rivers and seas. The potentiality of the microorganisms, pointed out in literature as agents of degradation of several compounds, indicates biological treatments as the most promising alternative to reduce the environmental impact caused by oil spills.

It is known that the main microorganisms consuming petroleum hydrocarbons are bacterias and fungi. However, the filamentous fungi possess some attributes that enable them as good potential agents of degradation, once those microorganisms ramifies quickly on the substratum, digesting it through the secretion of extracellular enzymes. Besides, the fungi are capable to grow under environmental conditions of stress, for example: environment with low pH values or poor in nutrients and with low water activity. Several authors have made lists containing bacteria and fungi genera that are able to degrade a wide spectrum of pollutants, proceeding from marine atmosphere as well as the soil (1, 2, 3). In accordance with several scientific publications, can be pointed out that, amongst the filamentous fungi *Trichoderma* and *Mortierella* spp are the most common ones isolated from the soil. *Aspergillus* and *Penicillium* spp have frequently been isolated from marine and terrestrial environments. In this way, microbiology of hydrocarbons degradation constitutes a field of research under development, once microbiological procedures may be used in the decontamination processes (4).

Therefore, the objective of the present work was to identify microorganisms capable to degrade petroleum hydrocarbons with views to a future employment in the bioremediation of polluted soils.

MATERIALS AND METHODS

Soil

The soil employed in this study was collected in Guararema, SP, Brazil and was characterized as a clayey-sand. It was accidentally contaminated in December of 1998 due to a crude oil spill. Nonetheless, the soil samples used in the isolation and identification of the fungi as well as in the biodegradation tests were collected in October of 1999 and in October of 2001, respectively. An uncontaminated soil sample was collected in a boundary area.

Microorganisms Isolation

Microbial isolation was realized from two samples of Guararema polluted soil, through successive dilutions, and inoculation in Petri dishes, where 3 different media were used (Sabouraud, Potato Dextrose Agar -PDA- and Czapeck) with the purpose of offering different nutritional options for the development of several fungi species that could have been established in the samples, during the natural process of soil weathering.

Determination of the Fungi Hydrocarbons Degradation Capacity

Qualitative determination of the degradation capacity of hydrocarbons of the strains already isolated, was driven in 24 well cell culture cluster - flat bottom with lid in polystyrene, adding to each well 1,8 mL of mineral liquid medium, 10 μ L of crude oil and inoculum obtained through the delicate removal of conidia and mycelium of each isolated microorganism cultured in the tubes.

Identification

The isolated strains were identified in the Department of Mycology of Oswaldo Cruz Institute - FIOCRUZ, according to Gerlach and Nirenberg (5), using Synthetic nutrient agar - SNA (6) and PDA media by the microculture technique of Rivalier and Seydel (7) modified method. After identification the species were deposited in the Culture Collection of the referred Institute.

Biodegradation Tests

Inoculum: The bioremediation essays were realized using the 9 filamentous fungi presented in Table 1. All the fungi were cultivated in slant tubes at 30°C for 6-7 days. The conidia of each strain was suspended in sterile distilled water, counted in Neubauer camera, and inoculated in the concentration of 10^7 conidia/g of soil.

Biodegradation process: The degradation of the polluted soil containing 26.26 mg/g of total petroleum hydrocarbon (TPH) was carried out in filtering flasks (kitazato) of 250mL, each one containing 50g of soil sample, with humidity corrected to 50% of water holding capacity. The experiment was conducted at 30°C during 40/42 days without pH correction (original pH 5,1). No nutritional adjustment was done. The weathered oil was used as the sole carbon source. The amounts of natural occurring nutrients were: 1.0 g/Kg of nitrogen and 0.001 g/Kg of phosphorous. Based on those data the carbon, nitrogen and phosphorous (C:N:P) ratio was calculated and the result was: 100:4.44:0.0044. The samples were evaluated in agreement with the conditions specified in Table 2.

CO₂ quantification

Biodegradation process was monitored by the evolution of CO₂, generated by the fungi as part of the cellular metabolism along 41 days using gas chromatography. The gas, confined in filtering flasks of 250 mL, hindered with rubber stoppers on the top, and a hose of latex in the lateral tube, compressed with a Hoffman tong, it was picked up in volume of 500 μ L through the suction of the internal atmosphere of the flasks headspace. The chromatographic equipment employed for this determination was HP 5890 series II, the analysis conditions are described below:

Flow of the carrier gas (He) to 17.89 mL/min

Flow of the reference gas (He) to 17.89 mL/min

Detector temperature: 220°C

Auxiliary oven temperature: 105°C

Injector temperature: 110°C

Stainless-steel column (3m/3mm) stuffed with Chromosorb 102

Based on the accumulated value of CO₂ generated at the end of the experiments, and on the information that 50% of the total carbon consumed is normally incorporated into the biomass, the biodegradation efficiency could be calculated (EB) as follows:

$$\text{Mass of carbon totally degraded} = 2 \times \text{Mass of generated CO}_2$$

$$\text{EB\%} = \frac{(\text{Mass of carbon totally degraded})}{\text{Mass of soil total organic matter}} \times 100$$

Organic matter

Analysis of organic matter were carried out through the ignition method using muffle at 1000 °C by 1 hour, with the objective of corroborating the results obtained by evolution of CO₂. Afterwards the samples were weighed after reaching room temperature.

RESULTS AND DISCUSSION

Several microorganisms were obtained in the present work through media and conventional techniques of isolation as shown in Figure 1.

Determination of hydrocarbons degradation capacity allowed the selection of the strains that actually contributed in the degradation of the petroleum hydrocarbons, diminishing the number of strains guided for identification.

The results of petroleum hydrocarbons degradation (Figure 2) along 7 days, in mineral, mineral with glucose and mineral with yeast extract (YE) media showed that the microbial action was stimulated in the media that contained other sources of carbon besides petroleum, accelerating, in that way, the degradation of the substratum in the 1st day of cultivation. That fact was well observed in the medium containing YE, that besides supplying an extra carbon source for the strains, also offered nitrogen source, vitamins and mineral salts. In spite of that, after the 7th day of cultivation, the degradation effect of the microorganisms in mineral medium were similar to those cultivated in media with glucose and YE.

A total of 9 different species were detected in Guararema soil and are presented in Table 1. All of those strains have been reported as commonly isolated from soil by other authors (8, 9, 10).

Biodegradation efficiencies of the studied conditions were calculated from CO₂ accumulated values, and the results are presented in Figure 3. In agreement with those

results, condition 5 presented the higher biodegradation efficiency (10.8%). This preliminary experiment allowed to point out *Aspergillus versicolor* as bearing the highest potential to degrade petroleum hydrocarbons when compared to *Aspergillus niger* (7.3%) responsible for the second best result, in relation to the others fungi whose biodegradation efficiency were between 7.3% and 6.6%.

Figure 4 represents the evolution of CO₂ obtained with the addition of *A. versicolor* to the polluted soil and monitored in a period of 41 days. A continuous and growing evolution of CO₂ was observed along the treatment, indicating the possibility of obtaining highest biodegradation efficiencies in an extended period of time. On the other hand, the absence of the lag phase in the degradation process evidences a previous adaptation of *A. versicolor* and the native microbes to the existent conditions in the polluted soil, as well as to the ones established for the assay.

Figure 5 shows the removal of organic matter results obtained through the analysis of organic matter by ignition of the initial and final samples. This experiment displays the same tendency of that obtained through the chromatographic analyses showing an accentuated decline in conditions 3 and 7. Besides, this experiment allowed to corroborate *A. versicolor* degrading potential through the largest removal of organic matter (11.37%), strengthening the previous results.

CONCLUSION

In conclusion the results showed that representatives of the filamentous fungi corresponded to 4 genera and 9 species, being the species as follows: *Aspergillus niveus*, *Aspergillus niger*, *Aspergillus versicolor*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Penicillium corylophilum*, *Parcilomyces variotti*, *Paecilomyces niveus* and *Fusarium sp.* *A. versicolor* presented the highest biodegradation efficiency (10.8%) as well as the organic matter removal (11.4%), and may be pointed out as a potential fungus to degrade petroleum hydrocarbons, especially those spilled out over Guararema soil.

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Table 1. Identified strains

Strains
<i>Aspergillus terreus</i>
<i>Aspergillus fumigatus</i>
<i>Aspergillus versicolor</i>
<i>Aspergillus niveus</i>
<i>Aspergillus niger</i>
<i>Penicillium corylophilum</i>
<i>Paecilomyces variotti</i>
<i>Paecilomyces niveus</i>
<i>Fusarium sp</i>

Table 2. Fungi and samples conditions in biodegradation test

Identification	Samples
1	Uncontaminated soil
2	Contaminated soil
3	Contaminated soil + <i>Aspergillus terreus</i>
4	Contaminated soil + <i>Aspergillus fumigatus</i>
5	Contaminated soil + <i>Aspergillus versicolor</i>
6	Contaminated soil + <i>Aspergillus niveus</i>
7	Contaminated soil + <i>Aspergillus niger</i>
8	Contaminated soil + <i>Penicillium corylophilum</i>
9	Contaminated soil + <i>Paecilomyces variotti</i>
10	Contaminated soil + <i>Paecilomyces niveus</i>
11	Contaminated soil + <i>Fusarium sp</i>



Sabouraud



PDA



Czapeck

Figure 1. Microbial growth in Petri dishes containing Sabouraud, PDA and Czapeck media.

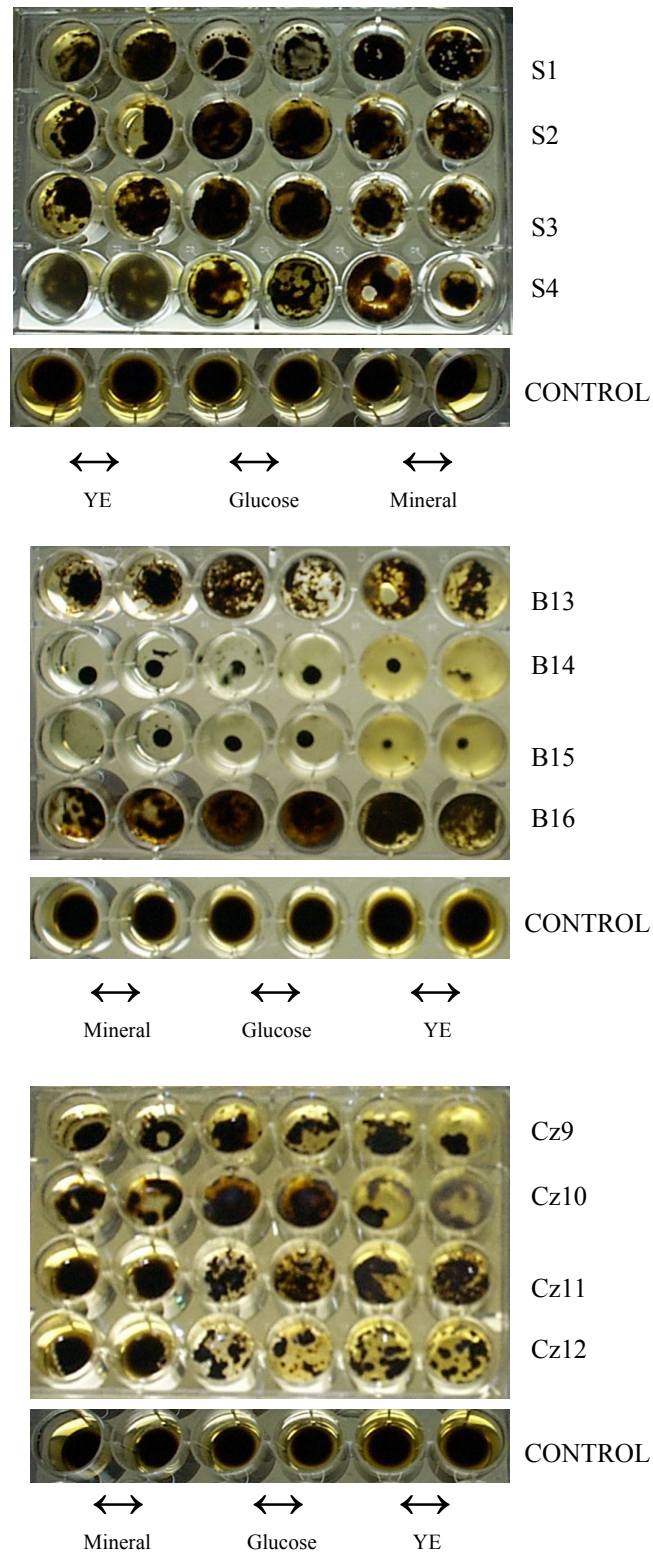


Figure 2. Degradation of petroleum hydrocarbons in mineral, mineral with glucose and mineral with extract of yeast media, of some of the strains isolated in Sabouraud, PDA and Czapeck solid media.

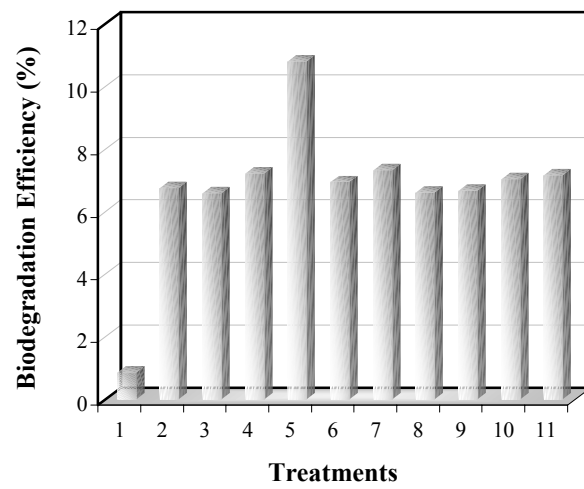


Figure 3. Biodegradation efficiency of fungi

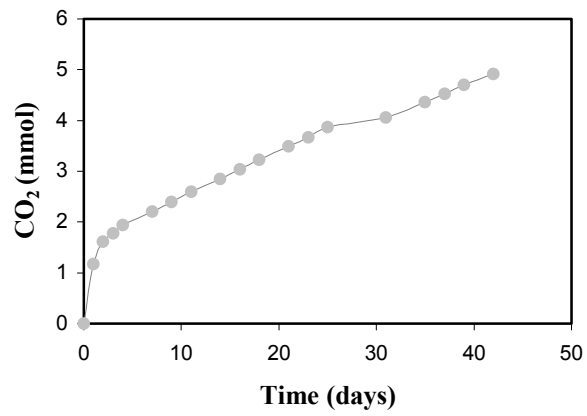


Figure 4. Production of CO₂ by *Aspergillus versicolor*.

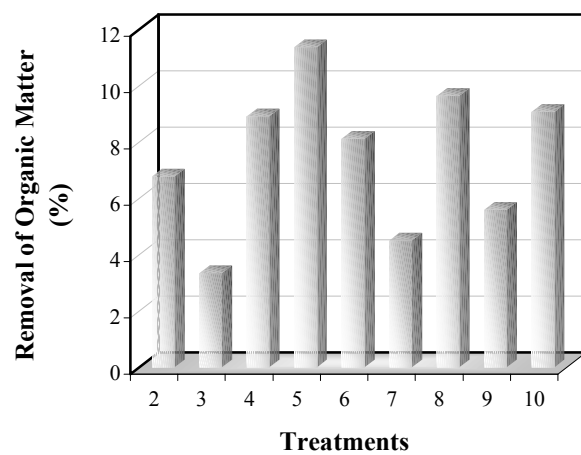


Figure 5. Efficiency of removal of organic matter