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BIOTECHNOLOGY

Bioremediation of diesel/biodiesel contaminated sandy soil in microcosm: evaluation of fungal bioaugmentation and natural attenuation

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ABSTRACT. Biodiesel is a clean renewable fuel used as alternative energy source to diesel and it is commercialized as a minor component in diesel blends. Similarly to diesel, biodiesel spill is a source of contamination for the ecosystem making necessary to provide effective remediation strategies. Bioremediation is a technology that has been applied with success to clean up hydrocarbon-contaminated environments. In this study, fungal bioaugmentation strategy was compared with natural attenuation during bioremediation of a sandy soil contaminated with diesel, biodiesel and blends (B20 and B50). Respirometric assays simulating the contamination of soil were carried out in Bartha flasks used to measure microbial CO_2 production. *Penicillium* sp. AV4 isolated from the wastewater of a biodiesel factory has the ability to degrade the fuels and was used in bioaugmentation. After 111 days, CO_2 evolution demonstrated no significant difference in soil microbial activity between fungal augmentation and natural attenuation treatments for all fuels. The lack of influence of *Penicillium* sp. AV4 can be related to its inability to compete with soil microorganisms and/or increase its metabolic activity. During natural attenuation, B50 showed a higher CO_2 production, followed by the B100, B20 and diesel, which is less biodegradable. Therefore, from a biodegradation perspective, biodiesel could be more beneficial than diesel during bioremediation spill.

Keywords: biodegradation; hydrocarbon; respirometric assay; CO_2; *Penicillium* sp. AV4.

Biorremediação de solo arenoso contaminado por diesel/biodiesel em microcosmo: avaliação de bioenriquecimento fúngico e atenuação natural

RESUMO. Biodiesel é um combustível renovável utilizado como fonte energética alternativa ao diesel e é comercializado misturado a esse combustível. Assim como o diesel, derramamentos de biodiesel são fontes de contaminação dos ecossistemas, sendo necessário aplicar estratégias para a remediação. A biorremediação é uma tecnologia que vem sendo aplicada com sucesso para remediar ambientes contaminados com hidrocarbonetos. Neste trabalho, o bioenriquecimento fúngico foi comparado à atenuação natural durante a biorremediação de um solo arenoso contaminado com diesel, biodiesel e misturas (B20 e B50). Ensaios respirométricos foram efetuados, utilizando respirômetros de Bartha para avaliar a produção microbiana de CO_2 no solo contaminado. *Penicillium* sp. AV4, isolado de efluentes de usina de biodiesel, possui a capacidade de degradar os combustíveis e foi usado no bioenriquecimento. Após 111 dias, a evolução de CO_2 devido à atividade microbiana no solo não apresentou diferença estatística entre o bioenriquecimento fúngico e a atenuação natural, considerando todos os combustíveis. A ineficácia do fungo pode estar relacionada com sua incapacidade de competir com os microrganismos do solo e/ou expressar sua atividade metabólica degradadora. Durante a atenuação natural, B50 demonstrou maior produção de CO_2, seguido por B100, B20 e diesel, o qual é menos biodegradável. Do ponto de vista da biodegradação, o biodiesel pode ser mais facilmente biorremediado do que o diesel.

Palavras-chave: biodegradação; hidrocarboneto; ensaio respirométrico; CO_2; *Penicillium* sp. AV4.

Introduction

Fuels based on vegetable oils gained importance after indication of petroleum reserve depletion and environmental damage resulting from the exploration and use of this non-renewable fuel (Colla et al. 2014). Biodiesel, a mixture of mono-alkyl esters of fatty acids, derived from transesterification of vegetable oils and animal fats (Knothe, 2010), is a clean renewable fuel recognized for its environmental benefits. During biodiesel

combustion, carbon, sulfur and particulate material emissions are lower compared to petrodiesel combustion (Yassine, Wu, Suidan, & Venosa, 2013; Knothe & Razon, 2017). Furthermore, studies have demonstrated that biodiesel is more biodegradable in freshwater and soil (Sørensen, Pedersen, Nørgaard, Sørensen, & Nygaard, 2011; Silva et al. 2012; Lisiecki et al. 2014).

In Brazil, biodiesel represents 8% of the diesel formulation. Actually, there are 51 biodiesel factories operating in the country and the authorized production capacity is 20,930.81 m^3 day^{-1}. Mato Grosso and Rio Grande do Sul are the states with higher number of biodiesel factories, 15 and 9 respectively. The main feedstock used to produce biodiesel are soybean oil (64.8%) and animal fats (15.5%) (*Agência Nacional do Petróleo, Gás Natural e Biocombustível* [ANP], 2017).

Similarly to petroleum derivatives, production, transportation and use of biodiesel represent risks of environmental contamination, making necessary to provide effective remediation strategies. A variety of technologies are currently available to treat contaminated environments, including soil washing, soil vapor extraction, stabilization and solidification, soil flushing, thermal desorption, encapsulation, solvent extraction, air sparging, and pump-and-treat technology (Khan, Husain, & Hejazi, 2004). Many of these technologies, however, are either costly or do not result in complete destruction of contamination. On the other hand, bioremediation is a technology based on natural biological processes, in which microorganisms can degrade and even mineralize hazardous compounds with low environmental impact, costs and energy requirements (Mancera-López et al., 2008; Morais & Tauk-Tornisielo, 2009; Guarino, Spada, & Sciarrillo, 2017).

Bioremediation of petroleum derivatives has been extensively studied in recent decades (Mancera-López et al., 2008; Mariano et al., 2010; Silva et al., 2012; Wu et al., 2017). The strategies applied in bioremediation are biostimulation of the indigenous microorganisms by nutritional amendment, oxygenation, temperature and pH control, and addition of surfactant; and/or bioaugmentation, which uses specific strains of microorganisms to degrade hydrocarbons (Soares Jr., Mariano, & Angelis, 2009). Other strategy applied in bioremediation is natural attenuation that uses natural processes to reduce the amount of pollutants at contaminated sites. This means the environmental contaminants are undisturbed while natural attenuation works on them (Khan et al., 2004).

Biodiesel bioremediation studies may be unsatisfactory mainly when the incorporation of microorganisms in the contaminated environment is the chosen strategy to bioremediation. Furthermore, a deeper understanding of the biodiesel degradation process is needed to properly implement biodiesel bioremediation strategies, especially when it is found the wide range of feedstock used in biodiesel production and several possibilities to combine the blends of diesel/biodiesel.

Penicillium is a fungal genus belonging to ascomycetes. Its species occur worldwide and play important roles as decomposers of organic materials (Visagie et al., 2014). Different studies have shown that species of *Penicillium* have the ability to degrade several pollutants such as short-chain alkanes (C_{12}-C_{18}) (Omar & Rehm, 1988; Elshafie, AlKindi, Al-Busaidi, Bakheit, & Albahry, 2007), long-chain alkanes (up to C_{50}) (Yamada-Onodera, Mukumoto, Katsuyama, & Tani, 2002), polycyclic aromatic hydrocarbons (Boonchan, Britz, & Stanley, 2000; Aranda et al., 2017), waste grease (Kumari, Ahmad, Negi, & Khare, 2017), azo dyes (Saroj, Kumar, Pareek, Prasad, & Singh, 2014) and pesticides (Birolli et al., 2015). Thus, this fungal genus has great potential to be applied in bioremediation. In this study, microcosm experiments were performed in order to evaluate the bioremediation of diesel, biodiesel and blends (B20, B50) in sandy soil during bioaugmentation by the strain of *Penicillium* sp. AV4 isolated from wastewater of a biodiesel factory. The biodegradation of these fuels during natural attenuation was also evaluated.

Material and methods

Pure diesel oil was purchased from a fuel supplier in Brazil. Biodiesel, produced from soybean (85%) and cottonseed (15%), was purchased from a local supplier in Cuiabá (15° 35′ 56″ S, 56° 06′ 01″ W), Mato Grosso State, Brazil (Cooperbio - *Cooperativa de Biocombustíveis*). B20 and B50 blends were prepared in laboratory combining diesel with biodiesel. The number represents the percentage of biodiesel in the blend.

Penicillium sp. AV4 was isolated from the wastewater of a Brazilian biodiesel factory (Cooperbio - *Cooperativa de Biocombustíveis*). It was maintained by bimonthly subculture on Sabouraud Agar and stored at 4°C after incubation at 28°C for 7 days. Identification of this fungal strain was performed by means of micromorphology analysis. Through microcultivation technique (Weber & Pitt, 2000), glass slides containing the growing fungus stained with trypan blue was obtained. Slides were

evaluated using a Primo Star microscope (Carl Zeiss, Götting, Germany), and images were captured using Axiocam ERc 5s microscope camera (Figure 1). The fungal strain was identified based on criteria from Visagie et al. (2014).

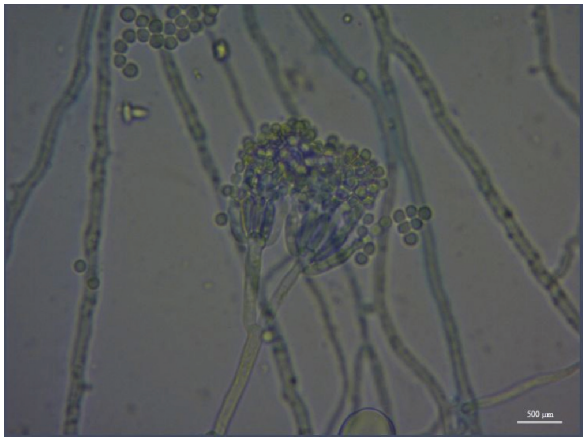

Figure 1. Conidiophores obtained by microcultivation technique.

Initially, the ability of *Penicillium* sp. AV4 to degrade the fuels (diesel, B20, B50 and B100) was verified using the technique based on the redox indicator 2,6 dichlorophenol indophenol (DCPIP) (Hanson, Desai, & Desai, 1993). The principle of this technique is that during the microbial oxidation of hydrocarbon, electrons are transferred to electron acceptors (O_2, nitrate and sulphate). Whereas DCPIP is an electron acceptor, if it is added to the culture medium, it is possible to check the ability of the microorganism to use the substrate by observing the color change of DCPIP from blue (oxidized) to colorless (reduced). This technique (Hanson et al., 1993) has been employed as a qualitative technique (Kubota, Koma, Matsumiya, Chung, & Kubo, 2008; Soares Jr. et al., 2009). In this work, however, the color change of DCPIP was monitored with a spectrophotometer (Hach 6000, λ = 600 nm) to evaluate changes in redox indicator absorbance values, so the ability of the fungus to degrade the different fuels was evaluated.

To prepare the inoculum *Penicillium* sp. AV4 used in DCPIP test, the fungus was grown on Sabouraud Agar (7 days, 28°C) and the spores were suspended in sterile saline solution (0.85%). The inoculum (2.0 mL, 10^7 spores mL^{-1}) was poured into Erlenmeyer flasks containing 100 mL of sterile Bushnell- Haas (BH) medium, 50 mg L^{-1} DCPIP and 1% (v v^{-1}) of the fuels (unsterilized diesel, biodiesel and blends). Erlenmeyer flasks (triplicate) were incubated at 28 ± 2.0°C for 6 days under static condition. Uninoculated controls were included. The absorbance was measured after centrifugation (10,000 rpm, 10 min) of aliquots. The BH medium consists, per liter, of: 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 1.0 g NH_4NO_3, 0.05 g $FeCl_3$ and pH 7.0.

The sandy soil used in microcosm bioremediation experiments was collected from the superficial layer (0-15 cm) in the Grove of Federal University of Mato Grosso. The site has not been exposed to diesel and biodiesel contamination before. Composite samples were collected and mixed in a plastic bag and stored at 4°C for a maximum of four weeks. Analyses of soil chemical and physical properties were performed according to the methodology proposed by *Empresa Brasileira de Pesquisa Agropecuária* (Embrapa, 1997) (Table1).

Table 1. Chemical and physical properties of clean soil.

Parameter	Value
pH ($CaCl_2$)	5.4
Organic matter (g dm^{-3})	61.0
P (mg dm^{-3})	6.1
K (mg dm^{-3})	193
Ca ($cmol_c$ dm^{-3})	7.3
Mg ($cmol_c$ dm^{-3})	2.7
Al (cmol dm^{-3})	0.0
Cation exchange capacity ($cmol_c$ dm^{-3})	19.1
Base saturation (%)	55.1
Particle size distribution (%)	
Sand	67.5
Clay	24.7
Silt	7.8

Bioremediation of fuel-contaminated sandy soil was determined in microcosm by measuring CO_2 in tightly closed Bartha flasks (respirometers) (Montagnolli, Lopes, & Bidoia, 2009; Morais & Tauk-Tornisielo, 2009; Silva et al., 2012). For each experimental condition, the flasks were prepared in triplicates (3 x 50 g of soil and 5% of fuel) and incubated at 28 ± 2°C for 111 days. Flask with only soil was prepared as control. The CO_2 produced during mineralization was captured in a 10.0 mL solution of KOH (0.2 N), located in the side-arm of the respirometer. Periodically, this solution was substituted for another containing no CO_2 to prevent saturation with this gas. Quantification of the CO_2 was done by titration of KOH removed from the flask, after the addition of 1.0 mL barium chloride solution (1.0 N) used to precipitate the carbonate ions and two drops of phenolphthalein, with a standard solution of HCl (0.1 N).

The proposed study was composed of ten triplicate assays (Table 2) to evaluate bioremediation of fuels in sandy soil.

Table 2. Respirometric assay.

Assay	Components
1	soil control[a]
2	soil control + fungus[b]
3	soil + diesel[c]
4	soil + diesel + fungus[b]
5	soil + B20[c]
6	soil + B20 + fungus[b]
7	soil + B50[c]
8	soil + B50 + fungus[b]
9	soil + B100[c]
10	soil + B100 + fungus[b]

[a]No addition of fuel; [b]Bioaugmentaion (1.0 mL inoculum, prepared as previously described); [c]Natural attenuation.

Data were presented as means from triplicate experiments. Analysis of variance (ANOVA) was performed with Tukey multiple comparisons test. Readings were considered significant when p was ≤ 0.05.

Results and dicussion

Fuel biodegradation by *Penicillium* sp. AV4 was examined during redox indicator DCPIP experiments (Figure 2). AV4 preferably biodegrades biodiesel and blends since the order of biodegradability was: B100 > B50 > B20 > Diesel. The microbial preference for fuels containing biodiesel can be attributed to the presence of fatty acid methyl esters, which are a better source of carbon to support microbial growth when compared to petroleum hydrocarbons (Sørensen et al., 2011). The characteristics of wastewater from biodiesel factory, from which the fungus was isolated, can also impact its preference for biodiesel since this effluent contains high levels of oils and its microbiota can metabolize these compounds.

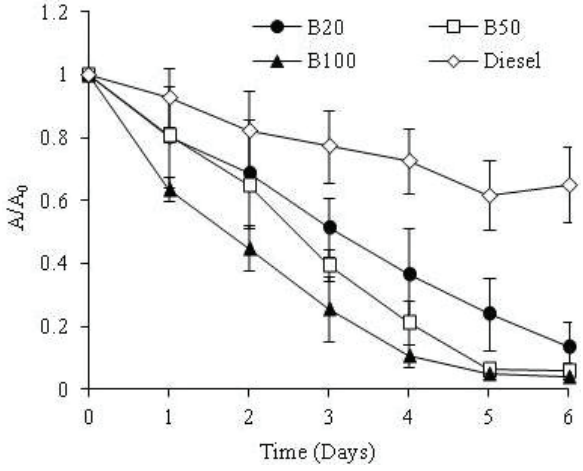

Figure 2. DCPIP reduction quantified at 600 nm absorbance during diesel and biodiesel biodegradation by *Penicillium* sp. AV4. A_0 = initial absorbance; A = absorbance at time t (day).

The ability of fungi to biodegrade petroleum hydrocarbons has been extensively reported (Mancera-López et al., 2008; Soares Jr. et al., 2009; Winquist et al., 2014). On the other hand, biodiesel biodegradation studies have been conducted especially using bacteria (Yassine et al., 2013; Colla et al., 2014; Lisiecki et al., 2014, Souza et al., 2016), although fungi present high bioremediation potential.

DCPIP experiments without inoculum (control) were also conducted (Figure 3). The results showed DCPIP reduction indicating degradation of fuels. This degradation can be due to abiotic or biotic processes, since unsterilized diesel, biodiesel and blends were used. B100 and B50 were more biodegradable than B20 and diesel.

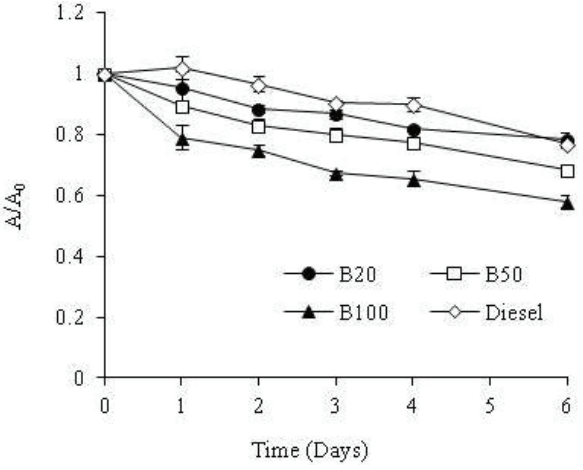

Figure 3. DCPIP reduction quantified at 600 nm absorbance in experiments without inoculum. A_0 = initial absorbance; A = absorbance at time t (day).

The accumulated CO_2 production in the respirometric experiments is shown in Figure 4 (pure fuels) and 5 (blends). The addition of *Penicillium* sp. AV4 caused no benefits to the biodegradation of fuels. In respirometers with B20, B50 and B100, the CO_2 production was lower when this fungus was added, when compared to respirometers without inoculum, nevertheless it was found no statistical difference ($p > 0.05$) between the treatments. In respirometer with diesel, there was almost no difference in CO_2 production between treatments with and without inoculum.

The influence of bioaugmentation in pollutant removal and site remediation has been debated in scientific circles. Morais and Tauk- Tornisielo (2009) used a mixed-culture inoculum (bacteria and fungus) to degrade oil sludge in soil, but detected no increase in the biodegradation rate. Similarly, Kauppi, Sinkkonen, and Romantschuk (2011) did not achieved success in bioremediation of diesel-contaminated soil using bioaugmentation. Unlike,

Soares Jr. et al. (2009) demonstrated the ability of *Candida viswanathii* to significantly increase (approximately 50%) the biodegradation in soil of biodiesel/diesel blends and pure biodiesel. Other authors also applied bioaugmentation with positive results (Mancera-López et al., 2008; Colla et al., 2014; Safdari et al., 2018).

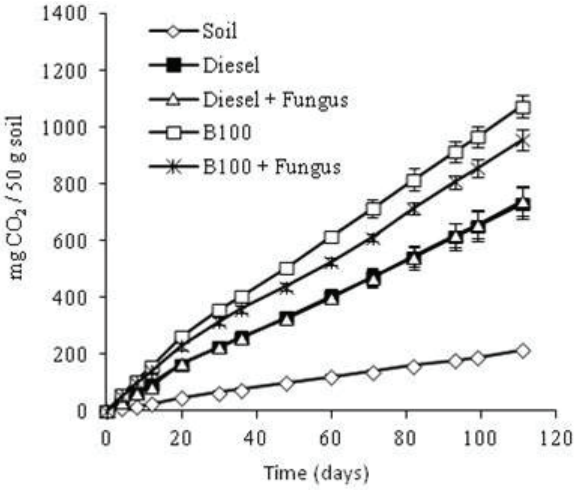

Figure 4. Accumulated CO_2 production in the respirometric experiments (pure fuels).

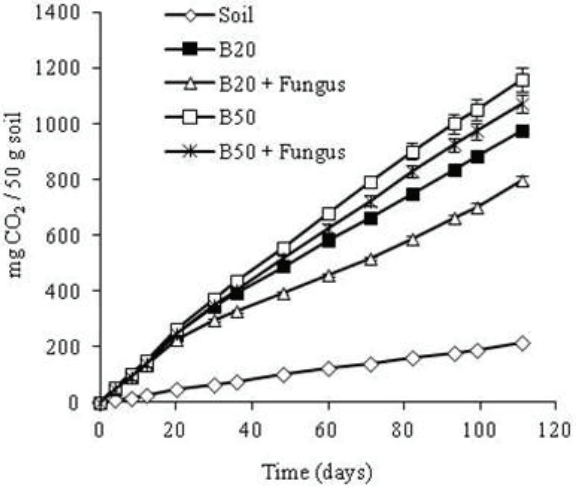

Figure 5. Accumulated CO_2 production in the respirometric experiments (biodiesel/diesel blends).

In this work, despite the ability of *Penicillium* sp. AV4 to degrade diesel/biodiesel, the bioaugmentation not worth the effort; the addition of the fungus had no effects on fuel degradation. According to Gentry, Resing, and Pepper (2004) and Fantroussi and Agathos (2005), microorganisms are major players in site remediation, however, their efficiency depends on many abiotic and biotic factors, including the chemical nature and the concentration of pollutants, their availability to microorganisms, and the physical and chemical characteristics of the environment (temperature, pH, water content, nutrient availability). Competition and antagonistic interactions with indigenous organisms and predation by protozoa and bacteriophages are also barriers imposed to the added microorganisms. These abiotic and biotic factors can have decreased the survival and activity of *Penicillium* sp. AV4 during bioaugmentation.

Several strategies are currently being explored to enhance the persistence and activity of exogenous microorganisms introduced into the environment (Gentry et al., 2004; Fantroussi & Agathos, 2005; Colla et al., 2014): encapsulation of exogenously added microorganisms to generate protective barriers; immobilization of microorganisms; combination of bioaugmentation with surfactant addition; successive addition of microorganisms; rhizosphere bioaugmentation where the microbial inoculant is added to the site along with a plant that serves as a niche for the inoculant growth.

The isolation of microorganisms from the same ecological niche of the contaminated areas is other strategy to overcome the above abiotic and biotic barriers. Once isolated, these microorganisms should be stored under laboratory conditions very similar to their native environment to maintain their existing characteristics and prevent the loss of metabolic activity related to biodegradation (Morais & Tauk-Tornisielo, 2009).

The treatments without inoculum showed higher production of CO_2 compared to the uncontaminated soil (control) ($p < 0.05$) (Figure 4 and 5). This result indicates that the soil microbiota can metabolize diesel, B20, B50 and B100 (natural attenuation). However, differences in fuel mineralization were clearly affected by the amount of biodiesel presented in the tested blends. In natural attenuation and bioaugmentation, the B50 showed a greater CO_2 production, followed by the B100, B20 and diesel, which is less biodegradable. As previously reported, biodiesel consists of fatty acids and the enzymes responsible for their breakdown are widely present in organisms. On the other hand, diesel consists of molecules that are not biologically active, thus demanding adapted microorganisms able to produce enzymes that recognize these compounds (Zhang, Peterson, Reece, Möller, & Haws, 1998).

Similarly to our work, Silva et al. (2012) verified that CO_2 production in soil contaminated with biodiesel was higher than in diesel-fuel-

contaminated soil and a higher concentration of biodiesel in blends favors the biological breakdown of diesel. Ng et al. (2015) also evaluated the biodegradation of petrodiesel in the presence of different biodiesel (palm, soybean, *Jatropha*) using CO_2 evaluation test. The experimental results showed positive synergistic effects for all cases. Nonetheless, there is no consensus in the literature on the biodegradability of diesel/biodiesel. Owsianiak et al. (2009) showed that the addition of biodiesel in amount of 10% decreases biodegradation compared to diesel fuel. Based on soil respiration, Horel and Schiewer (2014) found that diesel fuels were degraded at higher average rates than fish biodiesel.

Conclusion

This study investigated the bioremediation of sandy soil contaminated with diesel, biodiesel and blends using fungal bioaugmentation and natural attenuation. Although *Penicillium* sp. AV4 has the ability to biodegrade the fuels, there was no improvement in biodegradation of fuels in the soil. A possible explanation for the lack of influence of the inoculum is that the fungus was unable to compete with soil microorganisms and/or increase its metabolic activity. Meantime, it is noteworthy that, despite these results, several bioaugmentation strategies have worked. During natural attenuation, biodiesel and blends were more degraded than diesel. From a biodegradation perspective, biodiesel could be more beneficial than diesel during bioremediation spill. Nevertheless, more research is needed to study biodegradation of different types of biodiesel and their blends in different impacted environments.

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