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Bioremediation of Soil Contaminated with Biodiesel and Glycerin - Results of Soil Microbial Adaptation Through Evidence Contaminants Removal

Diego A. Lemos, Saulo L. Cardoso, Patrícia A. Vieira* , Vicelma L. Cardoso

Faculty of Chemical Engineering, Federal University of Uberlândia, Avenue- João Naves de Ávila 2121,, Uberlândia, MG, Brazil.
patriciavieira@feq.ufu.br

Sources of contamination with Total Petroleum Hydrocarbons (TPH) in soil are related to exploration, production, storage, transportation, distribution and disposal of fuels, such as biodiesel. Therefore, this study examined the ability of bioremediation of soil contaminated with biodiesel and glycerol using mixed culture (C1) from site contaminated by petroleum products. The culture was adapted in two stages of adaptation: in the first stage to the liquid medium and later in contaminated soil contaminated by biodiesel and glycerol, called R01. For adaptation of microorganisms to the contaminated soil, the reactor R01 contained 1.5 kg of soil in which the relationship C: N: P-100 has been corrected: 10:1, as well as pH, humidity and oxygenation of the soil. It was used the technique bioaugmentation with 4 reactors called R1, R2, R3 and R4. In each of these reactors was used a total of 400g of contaminated soil with percentages of 15 %, 20 %, 25 % and 30 % by weight of soil corresponding to fractions removed from R01 source. The same operating procedures employed in R01 were applied in monitoring the 4 reactors for 111 d. The results show that after 111 days there was a reduction of TPH (%): 21, 31, 43 and 51 in reactors.

1. Introduction

In the context of environmental contamination by petroleum special attention in the scientific community has been given to the impacts of accidental spills of oil and its derivatives. There are several scientific studies that address biological treatment of soils contaminated by petroleum products (Vieira et al., 2009a, 2009b). The search for alternative sources of energy and sustainable processes in order to reduce environmental pollution and global warming has spurred the global market for clean fuels such as biodiesel, which is a renewable and environmentally safe alternative to fossil fuels. With the determination Brazilian government, in force since January 2010, which makes the addition of 5% biodiesel to diesel, increased market availability of biodiesel in its pure form or mixed with diesel can possibly generate accidental spills in the environment (soil and water) causing a potential risk of contamination.
In Brazil, production and marketing of biodiesel has important advantages due to the large availability of raw material for its production and continued growth of the vegetable oil industry and ethanol (Oliveira et al., 2006 apud Rivaldi, et al., 2008).
Investments for the production of biodiesel in the state of Minas Gerais has grown and increases concerns about possible contamination of soil and water waste generated by the process.
Glycerol is the main byproduct generated in the production of biodiesel, with approximately 10 % of the total volume of biodiesel produced correspond to glycerol (Rivaldi, et al. cited Dasari et al 2008. 2005).
In the state of Minas Gerais, research in this sector are scarce and measures to bioremediate areas contaminated by these compounds are still unknown. For the bioremediation of areas (soil or water) contaminated by these compounds (biodiesel and glycerol) can be successfully applied, a good initial planning and selection of a microorganism effective in the degradation of pollutants (soil or water) should be performed.

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Scientific studies have shown the effectiveness of certain micro-organisms such as pure cultures or mixed in the degradation of oil present in water and soil step after proper adaptation of these cultures to these pollutants (Souza et al. 2004, Marquez-Rocha et al. 2005, and Okerentugba Ezeronye, 2003). It can be noted that these cultures also have great potential for application in treatment of contaminated environments for biodiesel and glycerol. In this context, the aim of this work was to study the bioremediation of soil contaminated with biodiesel and glycerin employing the technique of bioaugmentation. The mixed culture called C1 originated from contaminated site for oil was used in this process step after adaptation to new contaminants (biodiesel and glycerin).

2. Methodology

2.1- Contaminated soil

The soil contaminated by waste generated in the production of biodiesel (biodiesel + glycerol) used in this study was donated and collected by Petrobras and sent to the biochemistry laboratory, Faculty of Chemical Engineering, Federal University of Uberlândia (NUCBIO / UFU).

2.2- Characterization physics and physical chemistry of soil

The soil was characterized according to the following parameters: cell quantification, soil humidity, water retention capacity of the soil, pH, particle size, determination of total phosphorus and total nitrogen determination - Modified Kjeldahl method.

2.3- Mixed culture

The mixed culture used in this project called C1 was isolated from soil contaminated lagoon for diesel and gasoline and adapted to these contaminants. The culture from this soil was the same used in the work of Vieira et al. (2010). Some of the micro-organisms constituents were isolated and identified by Vieira et al (2007). Before the culture be adapted to contaminated soil by this glycerin and biodiesel was previously adapted mineral minimal medium (liquid medium) supplemented with yeast extract and contaminants (and glycerin biodiesel) according Cardoso et al. (2011).

2.4- Correction contaminated soil nutrients

The relationship Carbon: Nitrogen: Phosphorus (C: N: P) was corrected by adopting the 100:10:1 ratio (this ratio widely used in bioremediation experiments) with the insertion of NH_4NO_3 and K_2HPO_4 compounds as sources of N and P respectively.

2.5- Adaptation of soil contaminated by mixed culture

For acclimatization of micro-organisms in contaminated soil, was mounted a reactor inoculum - R01, with 1.5 kg of soil in a plastic container of 25 cm x 15 cm (width x height). The humidity adopted for R01 was 50% retention capacity of the soil. The humidity was monitored for 2 in 2 days, in addition to the R01 reactor was covered with perforated film role, to contribute to the circulation of air in the reactor. Soil pH was determined in the analysis of 5.2. Although the pH closer to neutral be suitable for bacterial growth, this experiment the value of 5.2 was maintained, as was also of interest to know if there was a variation in this parameter during the acclimatization period.

The oxygenation reactor R01 was made by manual turning of the soil in the reactor for incorporation into the air to contribute to bacterial growth and result in removal of contaminants.

The micro-organisms (C1 culture) were inserted into the reactor R01, weekly, with the insertion of 80 ml of biomass arising from centrifuged culture C1 samplings performed in a liquid medium contaminated with biodiesel and glycerol. In order to efficiently transfer and homogenous biomass that the contaminated soil was performed suspension of the mass, as well as nutrients (item 2.4) in water restitution humidity.

To verify the acclimatization of micro-organisms in the soil bacterial growth was monitored parameter.

2.6- Biodegradation tests

After the adaptation of microorganisms in the reactor R01 (item 2.5) was performed to remove samples of this soil aiming inoculation of 4 reactors. The reactors were designated R1, R2, R3 and R4. The settings of these reactors follow the same model described in section 2.5. It was used the technique bioaugmentation. Each reactor contained a total of 400g of contaminated soil to which 15 %, 20 %, 25 % and 30 % by weight corresponding to mass removed from the reactor R01 inoculum. The same correction procedures of nutrients, pH, moisture and oxygen were applied in monitoring (items 2.4 and 2.5) during the 111-day

process. Monitoring of bioremediation was conducted by the determinations of total petroleum hydrocarbons (TPH) and cell growth**.**

2.7- Analytical Methods

Determination of Total Petroleum Hydrocarbons (TPH) - Measurement of Biodiesel Using Extract solubilised

TPH analyzes were used to verify the biodegradation of the contaminant present in the soil in accordance with USEPA method (2001) modified. 10 g dry soil (in an oven for 24 h at 40 ° C) contaminated with different concentrations of contaminants were transferred to a 50 mL beaker tared. To this system was added distilled water to complete the beaker. Subsequently, the beaker was placed on magnetic stirrer for 30 min, after which time it was allowed to stand for 7 d (ABNT, NBR 1006). An aliquot of 30 mL of supernatant was centrifuged at 12,500 rpm (corresponding to a relative centrifugal field of 18,900 g to remove residual soil). The supernatant was transferred to a separatory funnel along with 4 ml of hexane, the system was stirred vigorously for 2 min and allowed to stand. 100 mL was collected and the supernatant was placed in a cuvette reading equipment. Promoted to forced evaporation of hexane and brought to the cuvette for reading the residue analyzer.

3. Results and discussion

3.1- Characterization soil

Table 1 shows the results of the physico-chemical and microbiological sample of soil contaminated used.

Through Table 1 shows that the soil is acidic pH of 5.2, which is characteristic of most Brazilian soils. According Quaggio (2000), approximately 70% of Brazil is composed of acidic soils. The soil analysis shows that this is still poor in nitrogen and phosphorus, limiting element in processes of biodegradation, showing the need for corrections.

The hygroscopicity that soil moisture is low, 2.45 %, indicating the need for correction of moisture during the biodegradation process. It is known that the optimum moisture content for biodegradation of hydrocarbons is between 25 and 85 % of water holding capacity, therefore there is need for correction during the process.

Determination of the water retention capacity is important when one wants to work with solid-phase processes, because it allows knowing the range of moisture content that should be used in the study. In this work the water holding capacity was 30.7%.

It is seen in Table 1 that the soil contains a population of heterotrophic bacteria 5.0 x 10^3 CFU / g soil. This result was lower than that found in other soils, such as 4.0 x 10^4 CFU / g soil to sandy soil (Oliveira, 2001) and 2.7 x 10^5 cfu / g soil into the soil sandy clay (Trinity 2002). This indicates that the contaminated soil used in this study is poor in nutrient concentration when compared to the organic load. The concentration of fungus (1.5 x 10^4 cfu / g soil) could be considered high.

The nutritional relationship present in the soil can be calculated through the values of organic carbon (C), total nitrogen (N) and phosphorus assimilated (P). It is found that this ratio is below contaminated soil relations suggested for the bioremediation process, making it necessary correction, by addition of nitrogen and phosphorus sources.

The C: N ratio is limiting in the process of bioremediation. Oliveira (2001) evaluated the best C: N ratio in sandy soil contaminated with oil Arabic, using the following C: N ratios 100:01, 100:05 and 100:10. The author concludes that the higher C: N ratios studied were suitable for the first 14 days to process. And the C: N ratio (100:10) promoted the highest percentage removal of accumulated heavy oil fraction, 61 % in 56 days to process.

The fact was attributed to providing adequate nitrogen to biodegradation process oil for a longer period of time procedure and / or selection of strains able to degrade most of the contaminant in question. In the same study the authors confirmed through thermogravimetric analysis and gas chromatography, we conducted experiments with the C: N ratio of 100:10 was greater removal of asphaltenes and paraffins. So we adopted in this paper the carbon / nitrogen ratio of 100:10:1 to correct nutritional soil in the reactor with the contaminated soil.

By granulometric analysis performed soil can be classified as sandy due to its higher proportion of sand compared the composition of clay and silt.

Table 1: Characterization of soil

physical chemistry	values	microbiological	values	inorganic	values
pH	5.2 ± 0.10	Aerobic bacteria (UFC/mL)	5.0×10^3	$P_{assimilable}$ (g/kg solo)	0.002 ± 0.10
Optimum moisture handling (visually)	25.0 %	Fungi (UFC/ mL)	1.5×10^4	$C_{organic}$ (g/kg solo)	91.1 ± 0.01
Water retention capacity (funnel method)	30.7 ± 0.15 %			N_{total} (g/kg solo)	7.0 ± 0.10
moisture (dry weight)	2.45 ± 0.25 %			Relation C:N:P	100:7.6:0.0022

3.2- Adaptation of soil contaminated by mixed culture

After adjustment of the mixed culture C1 contaminants (biodiesel and glycerol) in a liquid medium according to Cardoso et al. (2011), began adapting the culture to contaminated soil.

Soil pH was determined in the analysis of 5.2. Although the pH closer to neutral to be suitable for bacterial growth, this experiment the value of 5.2 was maintained, as was also of interest to know if there was a variation in this parameter during the acclimatization period.

The pool of micro-organisms already adapted to the contaminants to the liquid medium for inoculation in the soil R01 reactor was 10^9 CFU / mL. After 1 week, the microbial concentration was approximately 10^2 CFU / mL. This reduction is due to the onset of microbial adaptation in an environment where the diffusion of nutrients (essential for the synthesis and function of normal cellular components) is much more complex compared to liquid medium.

After 56 days of proceedings, with moisture monitoring and implementation of soil oxygenation through its revolving in reactor R01, cell concentration was determined in the order of 10^{10} CFU / mL for bacteria and fungi. Table 2 presents this monitoring.

Table 2: Monitoring the adaptation of culture in contaminated soil (biodiesel and glycerol 8.0% v / v), using a pool of mixed culture in the order of 109 CFU / mL

microorganisms	amount (UFC/mL) 7 d	amount (UFC/mL) 56 d
Total Heterotrophic Bacteria	$2x10^2$	$5.0x10^{10}$
Fungi	$8.5x10^6$	$1.0x10^{10}$

Table 2 also shows the quantification of fungi of the order of 10^6 CFU / ml after 1 week with considerably increased after 56 days to process 10^{10} CFU / mL. Increasing the amount of fungi was consistent with the pH of the soil presented at the end of 3 weeks - 4.76, lower than the initial pH of the contaminated soil used for initial assembly of the reactor R01.

The amount of organic carbon found in the initial contaminated soil had 91.1 g C / kg, after 56 d the value of TOC in the reactor increased to 36.2 g / kg, which means a reduction of 60.2% removal of C soil.

3.3- Biodegradation tests

Figure 3 and 4 show the behaviour of cell growth and removal of TPH over the 111 d of the procedure.

It can be seen from Figure 3 that the initial amount of micro-organisms is related to the fraction of inoculum added to the contaminated soil. Where larger fractions of inoculum provided greater amounts of microorganisms initially showing that the procedure adopted was suitable for inoculation. Furthermore, the profile shown in this same figure it is observed that after the inoculation (0 day) the concentration of microorganisms has undergone changes over the operation process in four reactors (R1 to R4) with increases and decreases in the concentration of micro-organisms. This behaviour suggests that occurred during the upgrading of micro-organisms to new environmental conditions.

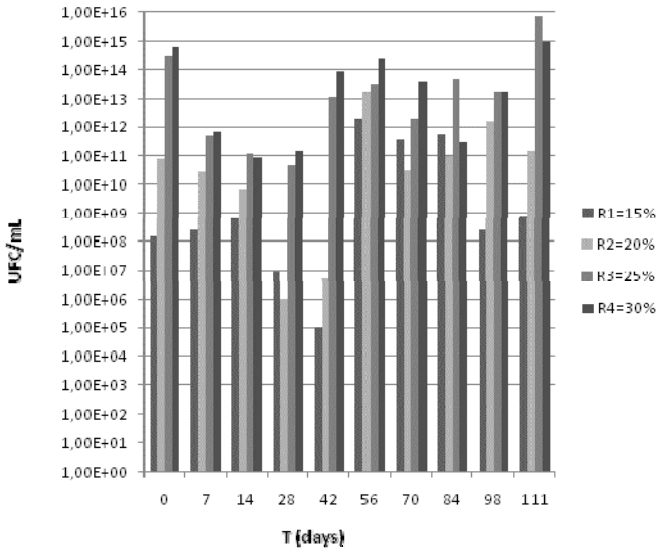

Figure 3: Profile of cell growth along the 111-d process.

Figure 3 shows that the major fraction of inoculum employed early in the process promoted increased concentration of micro-organisms to Final 111 d. This can be seen by the results presented by reactors R3 and R4. Figure 4 shows that the highest removal were observed in these reactors (R3 and R4) with removal of 45 and 51 %.

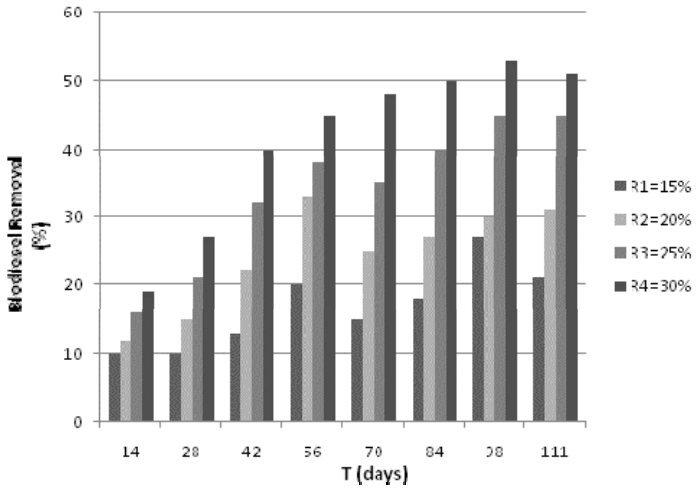

Figure 4: TPH removal profile over the 111 d of the process.

4. Conclusions

After analysis of the results the following conclusions can be listed:

• The methodology for adjusting the C1 mixed culture in soil was adequate;

• The mixed culture had the ability to biodegrade the mixture of contaminants: biodiesel and glycerol present in the soil;

• Higher levels of inoculum (adapted soil) used in bioaugmentation technique provided greater inoculum concentrations and higher TPH removals at the end of the process (111 d).

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