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An outlook on microbial behavior: Mimicking a biodiesel (B100) spill in sandy loam soil

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ABSTRACT

Although different types of biodiesel are marketed worldwide, studies on their environmental impact are scarce. Therefore, we selected pure biodiesels (B100) from rapeseed (BR), sunflower (BF), and soybean (BS) to mimic their contamination of sandy loam soil and then studied their biodegradation by the soil microcosms. All the biodiesel samples presented significant values ($p < 0.05$) of CO_2 release. The fatty acid profiles revealed that they all had high rates of degradation. After 56 days of study, the BR- and BF-contaminated samples had the most altered microbial profile, presenting a similarity of 75% with the uncontaminated sample. The results showed the high biodegradable potential of these three types of biodiesel, indicating that their compositions favored the degradation process. However, the high CO_2 level released by the mineralization of these biocomposites and the alteration in the soil microbial profile can be worrying factors. This is the first work to evaluate the biodegradability of these three types of biodiesel, the microbial profile of soil contaminated by each type, and their emission of greenhouse gases.

1. Introduction

Owing to a shortage of fossil fuels and the environmental problems caused by them, there is a need for fuels that emit less greenhouse gases when burned [1–5]. In this context, biodiesel has been presented as one of the main sources of such biofuels [6–9], where its environmental advantage lies in the reduced emissions of atmospheric pollutants, such as sulfur compounds, carbon monoxide, carbon dioxide (CO_2), hydrocarbons, and particulate matter [2,4,5,10–12].

Biodiesel can be produced using different types of raw materials, such as soybean, canola, sunflower, palm plant, cotton, animal fat, residual oil, and algae [13]. This diversity in raw materials favors production in different locations, even within the same country [3,10,13]. The biodiesel produced by transesterification is composed of a mixture of fatty acid alkyl ethers that vary according to the composition of the raw material [14–16]. The presence of fatty acid ethers favors the degradation of biodiesels by microorganisms [16]. However, the composition of fatty acids and the saturation index of fatty acid esters interfere with the degradation process [17–21]. Oils with a higher percentage of unsaturated fatty acids (oleic, linoleic, linolenic) are more unstable than oils containing a higher percentage of saturated fatty acids (palmitic, stearic), which favors the degradation of these compounds [3,22].

Countries such as the USA, Brazil, and Germany have been prominent in the production and commercialization of this biofuel [13]. Biodiesel is used in different countries in its pure form (B100) or is mixed with diesel (BX, where X corresponds to the percentage of biodiesel added to diesel). In 2005, Brazilian Law No. 11,097 established the mandatory use of biodiesel in the energy matrix in Brazil. Biodiesel began to be commercialized and was mixed into diesel at 2% (B2). Currently, the percentage stands at 8% (B8), and the Brazilian government will increase this to 10% (B10) in the year 2018.

In a previous study, Silva et al. [1] reported that B100 caused a reduction in the microbial profile in Atlantic Forest soil contaminated with soybean biodiesel. Recent studies have also reported that the use of biodiesel may lead to an increase in CO_2 emissions, in contrast to many other published reports [23,24]. Therefore, the need for more studies is evident since the fuel is already commercialized. The

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Table 1
Chemical and physical characteristics of the soil used in the investigation of rapeseed, sunflower, and soybean biodiesel biodegradation.

Characteristics							
P (mg/Kg)	K (mg/Kg)	Ca (mg/Kg)	Mg (mg/Kg)	Al (mg/Kg)	pH (H_2O)	N total (g/Kg)	O.C. (g/Kg)
11	75	816	85	77	6.3	3.1	37
Ca/Mg	Ca/K	Mg/K	C/N	CTC effective ($coml_c/dm^3$)	CTC pH 7 ($cmol_c/dm^3$)	Organic matter (g/Kg)	Textural classification
9.4	10.9	1.9	12	5.7	9.4	33	Sandy loam

CTC – Cation exchange capacity.
O.C. – Organic carbon.

objectives of this work were to evaluate the microbial behavior in soil after in vitro spillage of three different types of biodiesel (viz., biodiesel from soybean (BS), rapeseed (BR), and sunflower (BF)), the biodegradability of these biodiesels, and their emission of greenhouse gases.

2. Materials and methods

2.1. Soil and fuel samples

Soil samples were collected at a depth of 10 cm from an area without a history of contamination in the Atlantic Forest, a rainforest near the Universidade Estadual de Santa Cruz (UESC, 14°47′48.874″S, 39°10′20.166″W), Ilhéus, Bahia, Brazil. The samples were sieved through a 2 mm mesh and immediately mounted. Portions of the samples were subjected to physical and chemical analyses (Table 1) by the Laboratory of Agronomic and Environmental Analysis of Fullin, Linhares, ES, Brazil. The three different types of biodiesel used in this work, namely, BR, BF, and BS, were supplied by the Bioenergy and Environment Laboratory of UESC, Ilhéus, Brazil. The biodiesels were produced by the transesterification of the corresponding oils with methanol, using sodium hydroxide as the homogeneous catalysis. The methanol/oil molar ratios were 6: 1, with 1% sodium hydroxide relative to the mass of the oil. The temperature and reaction time were 45 °C and one hour, respectively.

2.2. Experimental systems: Basal respiration

We prepared microcosms that were hermetically sealed 500 mL flasks containing 300 g of soil with 70% humidity, 10 mL of minimal medium (0.1% w/v) KH_2PO_4, 0.1%, w/v K_2HPO_4, 0.1% w/v NH_4NO_3, 0.05% w/v $MgSO_4$, 0.01% w/v $FeSO_4$, and 0.0% (w/v) $CaCl_2$, pH 7.0) to maintain soil nutritional conditions, and 1% v/w B100 (BR, BF, or BS) as the soil contaminant were added. A non-sterile control sample and a sterile control were prepared similarly, but with uncontaminated soil. The microcosms, prepared in triplicate, were kept in the dark at room temperature and analyzed over a period of 56 days. For the measurement of basal respiration, one tube containing 10 mL of sodium hydroxide (NaOH, 0.5 M) was added to each of the flasks and measured by titration as described by Stotzky [25], but with modifications. Titration of the NaOH aliquots withdrawn from each of the flasks (total volume with replacement) was performed with HCl (0.5 M) on days 0, 7, 14, 21, 28, 35, 42, 49, and 56 after addition of phenolphthalein as indicator. The amount of acid needed to neutralize the base in each sample was used to determine the quantity of CO_2 released in mg/100 g of soil.

2.3. Microbial count

Soil heterotrophic microbial counts were performed using serial dilutions on days 0, 28, and 56. One gram of each soil sample was diluted in 9 mL of saline (0.85%) solution, and the samples were then plated at dilutions of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in nutrient agar medium (3.0 g/L meat extract, 5.0 g/L peptone, and 15.0 g/L agar). The number of culturable microorganisms was determined by measurement of the colony forming units per gram of soil (CFU/g) after incubation at 30 °C for 48 h in a bacteriological incubator (Eletrolab®, São Paulo, Brazil).

2.4. Biodiesel degradation

A 20 g sample of soil contaminated with BR, BF, or BS was collected on days 0, 28, and 56 and subjected to extraction with hexane (F. Maia Indústria e Comércio Ltda, Cotia, Brazil) as the solvent. The soil samples containing hexane were then treated by ultrasound (Ultrasonic Cleaner, Maxiclean 1600; Unique Indaiatuba, Brazil) for 30 min, and the supernatant was then collected. The extraction procedure was repeated three times. The supernatant samples were combined and concentrated by rotary evaporation (RV06-ML IKA®; Werke GmbH, Staufen, Germany). The process had an extraction efficiency of 80%. The extraction rate was calculated as the ratio of the peak area of the test sample to the peak area of the control sample (solvent + oil) × 100. The concentrate was then diluted 1:100 with hexane, and 1 µL of each sample was analyzed by gas chromatography (GC 2010; Shimadzu, Kyoto, Japan), using an RTX-WAX column (30 m × 0.25 mm; 0.30 µm film; Restek, Bellefonte, PA, USA) and nitrogen as the carrier gas. The temperatures of the injector and detector were both 250 °C. The column temperature was programmed as follows: 80 °C for 1 min, heating rate of 15 °C/min up to 230 °C, and hold at 230 °C for 20 min. The percentage degradation of the compounds was calculated according to the difference between the areas of the peaks of the test samples and the control samples, where the peak area of the control was 100%.

2.5. Soil DNA extraction, polymerase chain reaction, and denaturing gradient gel electrophoresis analysis

Total DNA was extracted from the soil samples using a PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA was subjected to electrophoresis on 1% (w/v) agarose (Invitrogen, Carlsbad, CA, USA) gels, stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA), and visualized under ultraviolet light (Kodak EDAS 290 Electrophoresis Documentation and Analysis system). The V3 region fragment of the bacterial 16S rRNA gene was amplified using primers F385 (5′-GCAC TCCTACGGGAGGCAGCAG-3′) and R518 (5′ ATTACCGCGGCTGC TGG-3′) [26,27]. The polymerase chain reaction (PCR) was carried out using a mixture containing 1.25 U of Taq DNA polymerase (Invitrogen), 5 µL of 10× reaction buffer, 200 mM deoxyribonucleotide, 3.0 mM $MgCl_2$, 1 µL of DNA, and sterile Milli-Q water to make up a final volume of 25 µL. Amplification was performed using an automated thermal cycler (Mastercycler Personal; Eppendorf, Hamburg, Germany). The amplified rDNA gene sequences were analyzed on 8% polyacrylamide gels (w/v; 37.5:1 acrylamide:bis-acrylamide) composed of a denaturing gradient of 30–50%. The gels were run in 0.5× TAE buffer (20 mM Tris acetate, pH 7.4, 10 mM sodium acetate, and 0.5 mM EDTA disodium) at a constant voltage of 60 V for 18 h at 60 °C. A mutation detection system (MAXFILL; BioAgency, New York, NY, USA) was used for the denaturing gradient gel electrophoresis (DGGE) analysis. The bands were visualized by staining with silver nitrate (Sigma-Aldrich). Each band