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Abstract

+\GURFDUERQ GHJUDGLQJ \HDVWV ZHUH LVRODWHG IURP WKUHH VLWHV FRQWD originating from an oily residue treatment Brazilian company. A selective enrichment technique was used by VXSSOHPHQWLQJ PHGLXP ZLWK JDVROLQH ZKLFK UHVXOWHG LQ Rhodotorula mucilaginosa, HYHQ LVRODW one as Rhodosporidium diobovatum, four as Meyerozyma (Pichia) guilliermondii, two as Wickerhamia sp., and two as Meyerozyma sp. The strains were evaluated for their growth capacity in medium containing 1% (v/v) gasoline, kerosene or lubricating oil as the only carbon source; the largest values for cellular biomass and growth rates (µ) were observed with gasoline supplementation. The strains were tolerant to aromatic (toluene and xylene) and aliphatic (hexene and n-heptane) compounds, which are part of the composition of gasoline, at concentrations up to 30 mM toluene (0.3% v/v), 20 mM xylene (0.25% v/v), 80 mM n-heptane (1.17% v/v) and 100 mM hexane (1.33% v/v). The R. mucilaginosa S47 and Meyerozyma sp. SP1 strains showed the greatest degradation percentages of gasoline, and have the potential to be used in the bioremediation of gasoline-contaminated environments.

Keywords Yeasts; Gasoline; Bioremediation; Tolerance to hydrocarbons

Introduction

Crude oil and its derivatives have been used by humans since 5,000 years B.C. In the early 19th century, the derivatives of petroleum started to be used as fuel for vehicles, components of explosives (glycerin and toluene), and as synthetic materials for clothes, solvents, and medicines, among other uses. With the increasing use of these products, there is also an increased risk of environmental contamination. e release of gasoline into the environment is a common occurrence, arising principally from leaking storage tanks; this can result in the contamination of drinking sources by mobile gasoline components, which can migrate through the soil matrix [1].

Strategies for controlling environmental contamination with petroleum and its derivatives have been the subject of various studies over the past three decades. ere is a great diversity of microorganisms able to utilize hydrocarbons as a sole carbon source, including bacteria, yeasts and molds. Bacteria are among the best-described hydrocarbon utilizing microorganisms [2-5]. In addition, some yeast strains display an excellent capacity to degrade oil-related compounds [6-9].

e biodegradability of the most water-soluble components of gasoline, such as benzene, toluene, ethyl benzene and xylene isomers, compounds usually termed BTEX, has been clearly established using pure strains or mixed cultures [4,9,10]. However, little is known about the degradability of other gasoline components, except for some polyalkylated benzenes and some linear and branched alkanes [4,11]. In addition, the biodegradability of gasoline is not easy to assess since it consists of more than 200 identi able components; interactions may occur between individual components and may a ect, in particular, the degradation kinetics [12].

Knowledge regarding the biodegradability properties of all components of gasoline followed by an evaluation of the speci c degradative capacities of the autochthonous micro ora present at contaminated sites will allow an assessment of the prospects for natural attenuation and the potential use of bioremediation technologies. Bioremediation using selected microorganisms provides a good opportunity because it is environmentally friendly and cost e ective. Some microbial strains can degrade hydrocarbons and utilize the resulting carbon compounds as food and energy sources for growth and reproduction. Simultaneously, the hydrocarbons are hydrolyzed from toxic into non-toxic compounds and simple inorganic compounds, such as CO_2 and H_2O, along with microbial biomass accumulation, through oxidation under aerobic and anaerobic conditions. To degrade organic pollutants, microorganisms must have metabolic processes to optimize the contact between microbial cells and organic pollutants, such as the production of biosurfactants, intracellular pathways to initiate the attack on organic pollutants, usually mediated by the activation and incorporation of oxygen by oxygenases and peroxidases, as well as peripheral degradation pathways to convert organic pollutants step by step into the intermediates of central intermediary metabolism [2,13].

e objective of this study was to isolate, identify and investigate the potential of yeasts to degrade gasoline for use in the bioremediation of contaminated areas. Furthermore, yeast cells were characterized for changes in cell hydrophobicity a er culturing in medium supplemented with di erent carbon sources. Additionally, the tolerance of these yeast strains to aromatic hydrocarbons (toluene and xylene) and aliphatic compounds (n-heptane and hexene) present in di erent fractions of gasoline was assessed, as was the potential for degradation of gasoline compounds using assays in liquid medium.

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Materials and Methods

Sampling site and isolation of yeasts potentially able to degrade petroleum hydrocarbons

e yeast strains used in this study were isolated from samples collected from four sites contaminated with re ned petroleum products. e samples were obtained from a company that treats oily waste in the metropolitan region of Belo Horizonte, Brazil. ese oily wastes include gasoline, diesel oil, lubricating oil and kerosene. Samples were collected from sites that receive mixtures of oily waste (MOW), oily sludge and leachate from these sites (OL), soil from garages for trucks and heavy vehicles (S), and storage tanks for wastewater containing emulsi ed lubricant oils (EO). e samples were preserved on ice and transported to the laboratory for the isolation of microorganisms.

Aliquots of 100 μL from serial dilutions (10^{-1} to 10^{-7}) were plated on Sabouraud Dextrose Agar (SDA; Difco, USA), modi ed with (per liter): 10.0 g casein peptone, 20 g glucose, 5 g of yeast extract and 20 g agar, and in mineral medium for fungi (MMF) containing (per liter): 3.4 g K_2HPO_4, 4.3 g KH_2PO_4, 0.3 g $MgCl_2.2H_2O$, 1.0 g $(NH_4)_2SO_4$, 0.05 g yeast extract, 20 g agar and 5 mL of a solution of trace elements (in mgL^{-1}: $MnCl.4H_2O$, 1; $FeSO_4.7H_2O$, 0.6; $CaCl_2.H_2O$, 2.6; $Na_2MoO_4.2H_2O$, 6). Both types of medium were supplemented with chloramphenicol at a concentration of 200 mg/L and 1% gasoline. e gasoline was added to the media a er they were autoclaved and homogenized in a sterile blender before being distributed onto the plates. e plates were incubated at 28´(e ;knSknSkrio)1i8.m rio(l)lvi6(ev)19(td3(a)-5(m s)-8(erm)19(pu)12(ti-6(h t)-n o)12(n)19(t)6(o t)-6(h)4(e p)7(t a)9(n)4(5)13(

Microbial adhesion to hydrocarbons

Changes in cell surface hydrophobicity during growth in liquid medium supplemented with glucose or gasoline was assessed by the microbial adhesion to hydrocarbon method (MATH) described by Rosenberg et al. [18], with modi cations. Yeast cells were grown in 60 mL asks containing 10 mL of MMF supplemented with 1% (w/v) yeast extract and 1% (v/v) gasoline or 1% (w/v) glucose, under the same conditions as the biodegradation experiments. A er growth, the cells were washed twice with a PUM bu er ($g\ L^{-1}$): K_2HPO_4, 19.7; KH_2PO_4, 7.26; H_2NCONH_2, 1.8 and $MgSO_4 7H_2O$, 0.2, suspended in the bu er to an optical density of 0.7 O.D. at 600 nm (A0). Next, 500 µL of hydrocarbons were added to 2 mL of the microbial suspension, vortexed for 2 min and equilibrated for 30 min. e bottom aqueous phase was carefully removed with a Pasteur pipette and the O.D. at 600 nm was measured (A1). e degree of hydrophobicity was calculated as [(A0 - A1)/A0] X 100%.

Gasoline degradation assays

e yeasts were cultured for 7 days in 60 mL asks containing 10 mL of MMF plus 1% (v/v) gasoline at 28°C, with agitation at 180 rpm. All bottles were sealed with rubber stoppers and sealed with plastic lm to prevent the evaporative loss of hydrocarbons. A er this period, the residual gasoline was extracted from the medium for analysis. All tests were performed in triplicate. e control for these experiments consisted of MMF containing gasoline without an inoculum, and the asks were subjected to the same incubation conditions of the trial test.

Extraction of gasoline constituents by the Solid Phase Micro-Extraction (SPME) method

To extract the gasoline constituents and quantify the aromatic hydrocarbons during the biodegradation process, an SPME ber coated with a 100 µm polydimethylsiloxane layer (Supelco) was pierced through the Te on-silicone septum of the asks with samples and pushed down into the middle of the headspace created by heating the asks at 90°C in a heater block (Reacti- erm III, Pierce). e ber was exposed to the gaseous phase for 5 mioethrs

GC-MS (Shimadzu™, Model 17A/QP5050A) using a PTE™-5 (Supelco chromatographic column with a size of 30 m x 0.25 mm x 0.25 µm. e temperature was programmed to vary linearly from 40°C to 270°C at a rate of 7°C min^{-1} and maintained for 22 min. Helium was the carrier gas with a ux of 50 mL min^{-1}, and the interface temperature was 280°C. e injection of samples and the control into the GC-MS system was carried out in triplicate. e data were obtained using CLASS 5000 so ware (Shimadzu) and analyzed using AMDIS so ware (Automated Mass Deconvolution and Identi cation System, version 2.1, National Institute of Standards and Technology (NIST), USA). e individual hydrocarbons were identi ed using the NIST Mass Spectral Search so ware, based on similarities between their mass spectra and those provided by the NIST/EPA/NIH compound library, version 2.0. Control peak areas were used as a point of reference for the remaining compounds (100%) in the untreated system. Sample peak areas were reported as a percentage of the control peak area. e gasoline degradation potential of yeasts was expressed as the reduction in the total area of the chromatograms and of the peaks corresponding to gasoline compounds, in relation to the control sample. In the chromatographic analysis, we used a solution of hexadecane in ethyl acetate (1:1,600,000) as the internal standard for the normalization of data. e compound used as the internal standard, as well as its concentration, were previously standardized. For this, 1 µL of this solution was added to the samples with a 10 µL syringe (Hamilton # 701), immediately before SPME extraction.

Results

Isolation, characterization and identi cation of yeasts with degradation potential of oil hydrocarbons

In total, eleven morphotypes were isolated from three of the four sites contaminated with oil at cell densities varying from 10^2 to 10^6 CFU mL^{-1} or CFU g^{-1} of the residues. Two isolates were from sites that received a mixture of oily waste, eight from the oily sludge and leachate from these sites and the soil of a garage for trucks and heavy vehicles and one from the top of a wastewater storage tank containing emulsi ed lubricant oils (Table 1).

e morphological and biochemical properties and rDNA sequence analysis of the 11 selected strains of yeast indicated that six strains belong to the genus Meyerozyma, two to the genus Rhodotorula,

Detection of biosurfactant/bioemulsi er production

a LogP value greater than 4, i.e. more hydrophobic, were well-tolerated by microorganisms. It has been suggested that less hydrophobic solvents (such as toluene and xylene) can better penetrate the cell leading to the denaturation of enzymes and a reduction in metabolic activity. ese authors tested the tolerance of Saccharomyces cerevisiae type II (Sigma) to ethanol, sec-butyl alcohol, butyl acetate, n-hexane, n-heptane, n-octane, n-decane and dodecane, and found lower tolerance of microorganisms to ethanol and sec-butyl alcohol, and greater tolerance to dodecane, which was the most hydrophobic of the tested compounds. In our study, yeasts were less tolerant to toluene and xylene, with LogP values of 3.0 and 2.5, respectively, and more tolerant to hexene and n-heptane, with LogP values of 3.4 and 4.0, respectively.

Hughes et al. [29] evaluated the tolerance of lamentous fungi and bacteria isolated from Antarctica to 10 hydrocarbons by measuring cell growth, and observed that the fungi were more tolerant than bacteria. e authors observed that aromatic hydrocarbons inhibited growth more than aliphatic hydrocarbons, which was also observed in the present study. Yeasts and bacteria have shown tolerance values depending on the organism studied and the site of isolation. Zahir et al. [30] tested the toluene tolerance of bacteria isolated from soil contaminated with hydrocarbons and from rhizospheric soil. Growth was observed in the presence of toluene concentrations ranging from 20 to 100 mM, which corresponded to concentrations from 0.21 to 1% (v/v); greater tolerance was observed for the microorganisms isolated from contaminated soils. ese isolates showed greater tolerance to toluene than the yeasts evaluated in the present study, which tolerated up to 30 mM toluene (0.3% v/v). Segura et al. [9] isolated ve marine bacteria that were tolerant to 0.1% v/v toluene and benzene, but sensitive to 0.1% ethyl benzene or xylene. In our study, yeasts tolerated up to 0.3% toluene and 0.25% xylene.

In the assays to assess the hydrophobicity of cells grown in MMF supplemented with gasoline or glucose, higher values were observed for cells grown in gasoline, suggesting that hydrophobicity may have been induced by the hydrocarbons found in this fuel. ese results are consistent with those obtained by Chrzanowski et al. [8], who evaluated the in uence of phenol (hydrophilic) or n-alkane (hydrophobic) on the cell surface hydrophobicity of Candida maltosa, Meyerozyma (Pichia) guilliermondii and Y. lipolytica; the authors observed that, in cells grown in phenol, hydrophobicity varied between 25 and 40%, whereas for cells grown in the presence of alkanes, hydrophobicity reached 90%. A strain of Y. lipolytica degrader of hydrocarbons showed cell surface hydrophobicity greater than 90% a er growth in the presence or absence of hydrocarbons [31].

In addition to the production of biosurfactants, cell surface hydrophobicity can be considered an important factor in controlling the assimilation of hydrocarbons. It has been suggested that cells with greater hydrophobicity are more likely to adhere to hydrophobic compounds than those with lower hydrophobicity, and are better at assimilating hydrocarbons [13,31,32]. Under the growth conditions used here, the yeasts did not produce biosurfactants. Several studies have reported the in uence of medium composition on the yield of biosurfactants, mainly concerning sources of carbon and nitrogen. Batista et al. [33] reported that bacteria grown in medium containing

used h671(hig)-7(-4e co) h671(hig)-7(h)4(er u5)-3

e ability of isolated yeasts to utilize gasoline as a carbon source was investigated by GC-MS analysis. All hydrocarbon classes evaluated, i.e. aromatic compounds, linear, branched (o)11(und2C001D

the sole carbon source. Moreover, the simultaneous degradation of iso- and n-alkanes provided further evidence for the high ef ciency of the isolates compared with micro ora of other origins, whereas the degradation of iso-alkanes took place only a er total exhaustion of n-alkanes [38,39]. In general, the residual components of gasoline

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