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Growth of *Paecilomyces variotii* in B0 (diesel), B100 (biodiesel) and B7 (blend), degradation and molecular detection

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Abstract

The introduction of biodiesel to diesel may allow the fuel to be more susceptible to microorganism growth, especially during incorrect storage. To evaluate the effect of adding biodiesel in pure diesel on the growth of *Paecilomyces variotii*, microcosms containing pure diesel (B0), blend diesel/biodiesel (B7) and pure biodiesel (B100) were used. In microcosm with minimal mineral medium and B0, B7 or B100, after 60 days, the biomass (dry weight) formed at interface oil-water in B7 and B100 was significantly higher when compared to that of B0. Infrared analysis showed reduction of the carbonile fraction in B7 and B100 suggesting formation of intermediate compounds in B7. To monitor possible contamination of fuel storage tank by *P. variotii* samples were collected and analysed by specific-PCR assay for detection of *P. variotii* spores in the aqueous phase. This method was able to detect a minimum of 10^3 spores ml^{-1}, corresponding to 0.0144 ng μl^{-1} of DNA. Specificity was tested against *Aspergillus fumigatus* and *Pseudallescheria boydii*.

Keywords: biodiesel, *Paecilomyces variotii*, deteriogenic, biodegradation, infrared spectroscopy, PCR.

Crescimento de *Paecilomyces variotii* em B0 (diesel), B100 (biodiesel) e B7 (mistura), degradação e detecção molecular

Resumo

A introdução de biodiesel ao diesel pode permitir que o combustível se torne mais suscetível ao crescimento de microorganismos, especialmente durante o armazenamento incorreto. Para analisar o efeito da adição de biodiesel em diesel puro no crescimento de *Paecilomyces variotii*, avaliou-se seu desenvolvimento em microcosmos contendo diesel puro (B0), mistura diesel/biodiesel (B7) e biodiesel puro (B100). Em microcosmos com meio mineral mínimo e B0, B7 ou B100, após 60 dias, a biomassa (peso seco) formada na interface óleo-agua com B7 e B100 foi significativamente maior quando comparada com a de B0. A análise de infravermelho mostrou redução da fração carbonila em B7 e B100, sugerindo a formação de compostos intermediários em B7. Para monitorar uma possível contaminação de tanque de armazenamento de combustível por *P. variotii*, amostras foram colhidas e analisadas por um teste de PCR específico para detecção de esporos deste fungo em fase aquosa. Este método foi capaz de detectar um mínimo de 10^3 esporos ml^{-1}, correspondente a 0.0144 ng μl^{-1} de DNA. Especificidade foi testada contra *Aspergillus fumigatus* e *Pseudallescheria boydii*.

Palavras-chave: biodiesel, *Paecilomyces variotii*, deteriogênico, biodegradação, espectroscopia de infravermelho, PCR.

1. Introduction

The microbial contamination of stored fuels, mainly diesel oil, is a major problem in refineries and distribution systems (Bento and Gaylarde, 1996, 2001; Bento et al., 2004). Many factors, such as the presence of water in the bottom of the tanks during storage, have been cited as increasing microbial growth in the systems and can lead to blocking

of pipelines and filters, affecting the final quality of the fuel and corrosion of the tanks (Bento and Gaylarde, 2001; Bento et al., 2004; Bücker et al., 2011; White et al., 2011; Cazarolli et al., 2012, 2014; Zimmer et al., 2013; Passman, 2013). A concentration of only 1% water in a storage system is sufficient for the growth of aerobic and anaerobic bacteria and yeasts, as well as for the development of fungal biomass at the oil/water interface (Gaylarde et al., 1999; Chesneau, 2000; Bento and Gaylarde, 2001; Bento et al., 2004; Bücker et al., 2011; Sørensen et al., 2011; Cazarolli et al., 2012, 2014; Zimmer et al., 2013; Passman, 2013). Numerous microorganisms have been isolated from fuels (Atlas, 1981; Gaylarde et al., 1999; Bento and Gaylarde, 2001; Bento et al., 2004; Bücker et al., 2011; White et al., 2011). The fungi most frequently and considered as deteriogenics in diesel include *Hormoconis resinae*, *Aspergillus fumigatus*, *Paecilomyces variotii*, *Penicillium* sp., *Rhodotorula glutinis* and *Candida silvicola* (Bento and Gaylarde, 1996, 2001; Bento, 1999). Bücker et al. (2011) reported that *Paecilomyces* sp produced the highest biomass in biodiesel blends, while *Aspergillus fumigatus* grew best in pure biodiesel.

In Brazil, became mandatory the addition of 5% biodiesel to diesel since 2010, according to regulations of the National Petroleum Agency (ANP) (Soares Junior et al., 2009). Biodegradability of biodiesel compared to mineral diesel it is a problem specially when occurs microbial contamination during storage which may lead to blocking of pipelines and filters, affecting the final quality of the fuel and corrosion of the tanks (Bento and Gaylarde, 2001; Bento et al., 2004, 2006; Passman and Dobranick, 2005; Bücker et al., 2011; White et al., 2011; Cazarolli et al., 2012, 2014; Zimmer et al., 2013; Passman, 2013).

Monitoring of storage conditions is critical to understanding the degree of microbial contamination. Recognized institutions linked to the fuel market, such as the International Air Transport Association (IATA), the Institute of Petroleum (IP) in the UK and ASTM in the U.S.A., established methodologies and values for diagnosis of an acceptable condition and alert of microbial contamination in fuel. According to White et al. (2011) the fuel industry relies on phenotypic cultivation-based identification to monitor the level of contamination, which may lack accuracy and neglect difficult-to-culture taxa. Molecular methods like polymerase chain reaction (PCR) have been developed for detection of fungi with several advantages compared to those of conventional techniques, such as high specificity and sensitivity (Busch and Nitschko, 1999). The ribosomal DNA (rDNA) is widely used for fungal identification by PCR, consisting of interspersed stretches of highly conserved, moderately conserved and divergent sequences. The internal transcribed spacer (ITS) region is an ideal target for the development of specific primers for detection and identification of fungal species (White et al., 1990).

The aim of this study was to assess in the laboratory the effect of soy-derived biodiesel addition to diesel oil on the growth of *P. variotii* with different concentrations of spores (10^2, 10^4 e 10^6 spores mL^{-1}) as inoculum during 60 days. We also used the PCR assay for the detection of *P. variotii* spores in the aqueous phase.

2. Material and Methods

2.1. Microorganisms

For fungal growth experiments *P. variotii* isolate (PV2) was utilized (Bento and Gaylarde, 1996). This isolate was identified based on morphological studies and confirmed by molecular identification conducted at André Tosello Foundation (FAT, 2010 - Campinas, SP, Brazil). For molecular assays (PCR development) the *P. variotii* strain ATCC 16023 (PV1) was used. The filamentous fungi *Pseudallescheria boydii* (Cazarolli et al., 2012) and *Aspergillus fumigatus* (Bento and Gaylarde, 1996) were included to assess the specificity of the primers. The microorganisms were grown and maintained on 2% malt agar pH 6.5 (HIMEDIA, Mumbai).

2.2. Fuels

The fuels used were diesel oil pure (B0) with low sulfur content (S50 ppm) and biodiesel pure (B100-from soybean oil), provided by Ipiranga Petroleum Products (Canoas, Rio Grande do Sul, Brazil). The blend B7 was prepared asseptically in the laboratory at the following volume percentage composition of biodiesel/diesel: 7/93. The fuels were sterilized by vacuum filtration, using membranes of pore size 0.22 µm (Millipore), and placed in previously sterilized glass bottles, which were covered with aluminum foil to prevent photo-oxidation of the fuel.

2.3. Growth assays

Growth experiments (triplicate) were carried out in 150-mL flasks containing 20 mL minimal medium as aqueous phase and 20 mL of diesel oil (B0), biodiesel (B100) or the blend (B7) as oil phase. The mineral medium used (Richard and Vogel, 1999) contained (g L^{-1}): 0.7 KCl, 2.0 KH_2PO_4, 3.0 Na_2HPO_4, 1.0 NH_4NO_3, 4.0 $MgSO_4$, 0.2 $FeSO_4$, 0.2 $MnCl_2$, and 0.2 $CaCl_2$. The aqueous medium was sterilized by autoclaving and the sole carbon source was diesel, biodiesel, or the blends described in Section 2.2.

The procedures were carried out according to Bücker et al. (2011) with slight modifications. Briefly, moulds inocula were grown on malt agar at 28 °C for 21 days. After growth, suspensions of mould spores were prepared in sterilized distilled water. The final concentration obtained was 10^8 spores ml^{-1}, obtained by counting in a Neubauer chamber. Spore concentrations tested were 10^2, 10^4 e 10^6 mL^{-1}. Control flasks did not contain spores. The experiments were conducted at room temperature for 60 days, ranging from 16 °C to 30 °C, with the average temperature of 22±2 °C. After 7, 14, 21, 28, 42, and 60 days the biomass of moulds formed at the oil-water interface was filtered (total volume). Ten milliliters of hexane was utilized to remove the residual oil, the filter paper with

biomass was dried at 50 °C for 4 days to a constant weight, and the final weight was recorded.

2.4. Evaluation of oil degradation by infrared spectroscopy

The infrared spectra were obtained by attenuated total reflectance using a spectrophotometer Spectrum 400 (FTIR-Perkin Elmer), at wavelengths in the range of 4000-650 cm^{-1}, with 16 scans per spectrum and 4 cm^{-1} resolution. Our study focused on the region of the spectrum lying between 1750 and 1725 cm^{-1}, where a biodiesel absorption peak of carbonyl group (C=O) occurs.

2.5. Detection of P. variotii by PCR

To determine the minimum number of *P. variotii* spores can be detected in the aqueous phase by PCR, serial dilutions (10^2 to 10^7 spores mL^{-1}) were prepared from a concentrated suspension of 10^8 spores ml^{-1}. For DNA extraction, spore containing tubes were centrifuged for 5 min at 6.700 g and the supernatant discharged. The sediment was allowed to dry at 37 °C and the spores were ground with pestle, followed by DNA extraction according to technique described elsewhere (Ferreira and Grattapaglia, 1996). DNA quantification was carried out by fluorometry (Qubit 2.0 Fluorometer - INVITROGEN). The same procedure was applied to DNA extraction of *Aspergillus fumigatus* e *Pseudallescheria boydii*, from a suspension of 10^7 spores ml^{-1}.

The primers selected 5'-CGAAGACCCCTGGAACG-3' and 5'-GTTGTTGAAAGTTTTAATTGATTGATTGT-3' were developed by Haugland et al. (2004). Information about the sequence is deposited in GenBank under accession number AY373941. The calculated Tm for the primers was 65°C. The primers amplified a sequence located on ITS region and the amplified fragment has 73 base pairs (bp).

For amplification, methodology described by Haugland et al. (2004) was utilized with few modifications. The solution was prepared with 4.95 mg of bovine serum albumin, 1.5 U Taq DNA Polymerase, 1.5 mM MgCl2, reaction buffer (10 mM Tris-HCl and 50 mM KCl), 12.5 pmol of each primer, 200 mM dNTP and 10 µg of DNA template and distilled water for a total volume of 25 µl. All reagents mentioned were supplied by Ludwig Biotec (2010), except primers (Oligos GBT, Argentina). The conditions for amplification were those described by Haugland et al. (2004) in Eppendorf Mastercycler Personal equipment. PCR products were analyzed on 2.5% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) and compared to a molecular weight marker (Lambda / Hind III 0.1 mg/µl - Ludwig Biotec, 2010, Brazil).

2.6. Statistical analysis

Experiments were carried out using three independent replicates. Data were submitted to analysis of variance and the averages were compared by the Tukey multiple range test at the 5% level of significance. All analyses were performed with the statistical software Assistat program version 7.4.

3. Results and Discussion

The growth of *P. variotii* in B0, B7 and B100 with different inoculum concentrations over a period of 60 days is shown in Figures 1, 2 and 3. *P.variotti* evaluated in this study were able to grow using diesel (B0), biodiesel (B100) and the blend B7 as the sole source of carbon and energy. During the first 7 days of incubation increased biomass was detected, regardless of the quantity of microorganisms inoculated or the type of fuel used. In B0 and B7, however, biomass production was significant ($p<0.05$) only with the 10^6 inoculum, differently from B100 where all inocula showed significant growth during this initial phase. These results differ from those of Bücker et al. (2011), who found no significant growth of *P. variotii* in B0. This could be explained by the occurrence of a lag phase related to the change in growth conditions since the inoculum was prepared on malt agar, a rich medium, and inoculated immediately into bottles without a prior incubation period

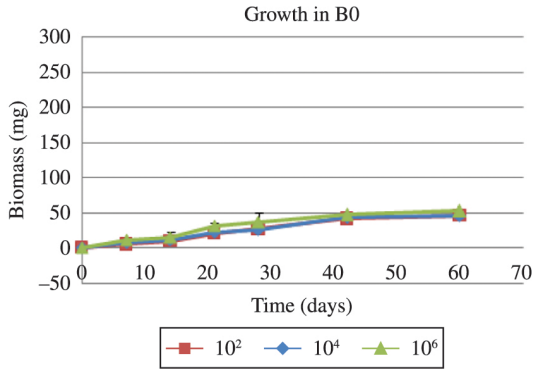

Figure 1. Biomass produced at interface oil-water of *P. variotii* in B0 with differents concentrations of spores inoculated (10^2, 10^4 and 10^6 spores mL^{-1}). Each point on the curve represents the mean of three determinations.

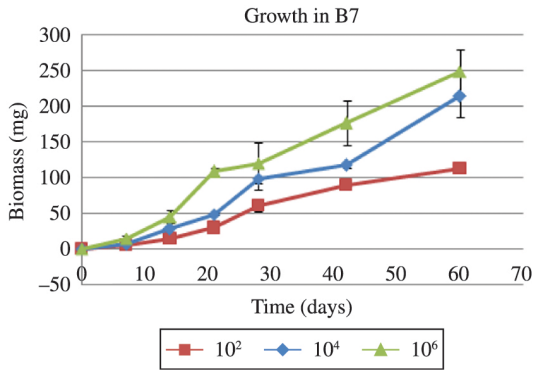

Figure 2. Biomass produced at interface oil-water of *P. variotii* in B7 with differents concentrations of spores inoculated (10^2, 10^4 and 10^6 spores mL^{-1}). Each point on the curve represents the mean of three determinations.

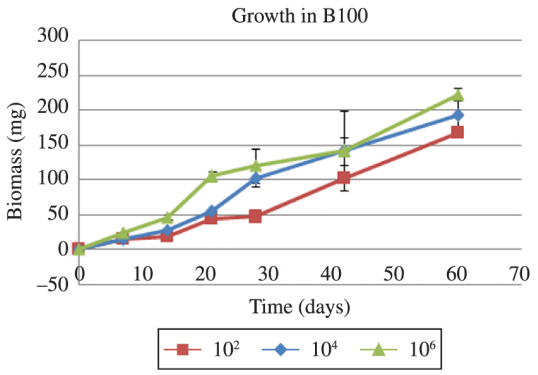

Figure 3. Biomass produced at interface oil-water of *P. variotii* in B100 with differents concentrations of spores inoculated (10^2, 10^4 and 10^6 spores mL^{-1}). Each point on the curve represents the mean of three determinations.

(24 hours at 28 °C) of the pre-inoculum. Bento and Gaylarde (2001), evaluating the ability of various fungi to grow in metropolitan diesel and Bushnell-Haas mineral medium, showed that *P. variotti* did not grow significantly on pure diesel. Owsianiak et al. (2009) and Schleicher et al. (2009) conducted biodegradation experiments for diesel/biodiesel blends in liquid culture with microorganisms. These studies revealed that there was an almost linear relation between biodiesel content and biomass production.

In general, the biomass was higher where more biodiesel was present in the blend. In our experiments the lowest biomass recorded was approximately 50 mg in pure diesel (B0) with all spore concentrations at 60 days (Figure 1). Differently, in B7 and B100 the biomass increased substantially from 21 days onwards reaching the highest values at the end of 60 days, especially with 10^4 and 10^6 inocula (Figures 2 and 3). Thus, biodiesel had positively influenced its growth. These results agree with those reported previously by our group, with slight differences (Bücker et al., 2011). Zimmer et al. (2013) noticed that the biomass formed in B100 (control) after 60 days was on average 3.5 times higher than that formed in blends B7 and B10 and in pure diesel (B0), using an uncharacterized inoculum (as suggested in ASTM E1259-10) (ASTM, 2010).

In some cases especially with higher inocula, we found less biomass in B100 than in B7, which might indicate growth benefits by having a blend instead of pure biodiesel. It would be interesting to test also a B20 blend to evaluate the biomass growth. The production of biomass, as a direct measurement of growth depends mainly of nature of substrate and production of enzymes for the microbial comunity.

The presence of fatty acid esters, which can be recognized by microorganisms makes biodiesel more biodegradable than fossil fuels (Schleicher et al., 2009; Sorensen et al., 2011). The diesel from petrol is composed by aliphatic hydrocarbons like alkanes (normal, alicyclic and cyclic), naphthenic and aromatic that can be more recalcitrant (Bücker et al., 2011; Cazarolli et al., 2014; Passman, 2013). However, some studies consider a synergic effect of biodiesel in biodegradation of diesel and biodiesel/blends due to co-metabolism (Pasqualino et al., 2006; Schleicher et al., 2009; Sorensen et al., 2011). Zhang et al. (1998) reported that the rate of diesel biodegradation in water can be three times greater in the presence of biodiesel.

Studies with Brazilian diesel type B containing 5% of biodiesel in its formulation have shown the increase of susceptibility to microbial contamination during storage (Bücker et al., 2011; Zimmer et al., 2013).

Spectroscopy is not only selective but also allows quantifying the amount of biodiesel in the mixture with concentrations as low as 0.1% (Guarieiro et al., 2008). Abbas et al. (2008) concluded that the FTIR analysis indicates stages of degradation and structural modifications of compounds. Genov et al. (2008), using the same method, reported different degrees of oil hydrocarbons degradation while Liao et al. (2009), studying asphaltene, found that the absorption of C=O tends to decrease with increasing biodegradation.

In the present work there was a reduction in the absorption peak of the carbonyl fraction present in the fatty acid methyl-esterified biodiesel in B7 and B100. According to the spectra shown in Figure 4, the peaks related to C=O remained constant after 28 days with all inocula and after 60 days with inocula 10^2 and 10^4, without secondary peaks. The C=O of ester constituents present in the mixture of biodiesel B7 decreased after 60 days with an initial inoculum of 10^6 spores ml^{-1}. Taking together, these results suggest biodegradation of B7 under the experimental conditions tested. The appearance of a secondary peak in the region of 1710 cm^{-1} suggests the formation of carbonyl compounds intermediates, as this region is related to carbonyl group (Silverstein et al., 1991). The principal component analysis (PCA) shown in Figure 5 indicates the proximity of the B7 replicates after 60 days of inoculation with 10^6 spores/mL, as well as a distance in relation to other groups enabling differentiation among them. The IR analysis of B100, however, did not allow conclusive results on degradation (data not shown). Further studies will be necessary to adopt this method for evaluation of diesel/biodiesel blends degradation.

The samples when subjected to the PCR generated products corresponding to the expected target fragment of 73 bp as shown in Figure 6. It is possible to visualize amplified fragments with increasing intensity from 10^3 spores ml^{-1} dilution (column 3), considered the detection limit of this technique within the conditions tested in this study. The corresponding DNA concentration was 0.0144 ng μl^{-1}.

The sensitivity of PCR for detection of fungi varies depending on the material and the conditions of the test. Williams et al. (2001), in experiments to evaluate methods for detection of fungal spores in air samples, obtained DNA amplification from 10^3 spores mL^{-1} of *Penicillium roqueforti*. Breaking spores using beads, the sensitivity increased to 10^2 spores/mL and using nested PCR it was possible to detect DNA from only one spore/mL. Ruiz and Brown (2011) were able to amplify fragments by PCR from 10^4 spores mL^{-1} of *Hormoconis resinae*.

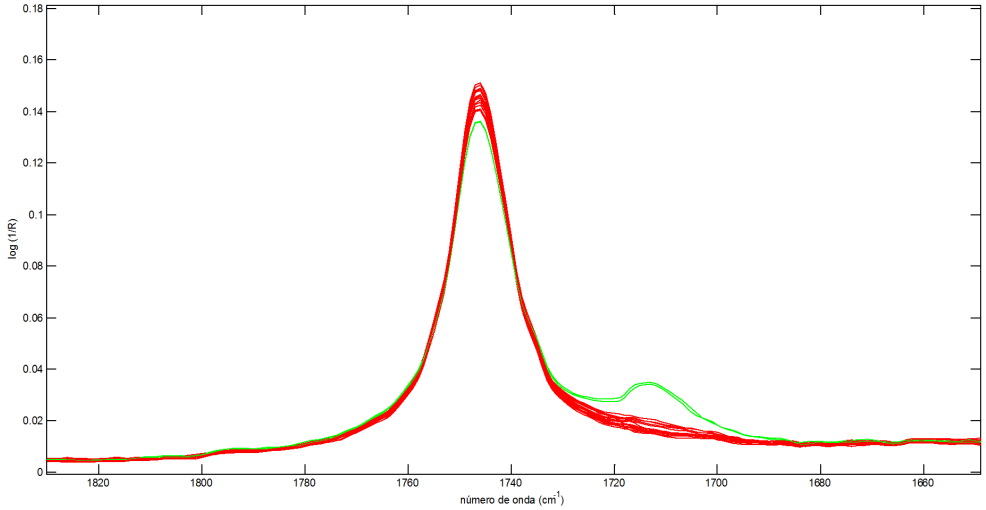

Figure 4. IR spectra of B7 corresponding to carbonyl present in biodiesel during 60 days of experiment. **Red**: B7 at the beginning of the experiment, 28 days after inocula 10^2, 10^4 and 10^6 spores mL^{-1} and after 60 days with inocula 10^2 and 10^4 spores mL^{-1}. **Green**: B7 after 60 days with inoculum of 10^6 spores mL^{-1}.

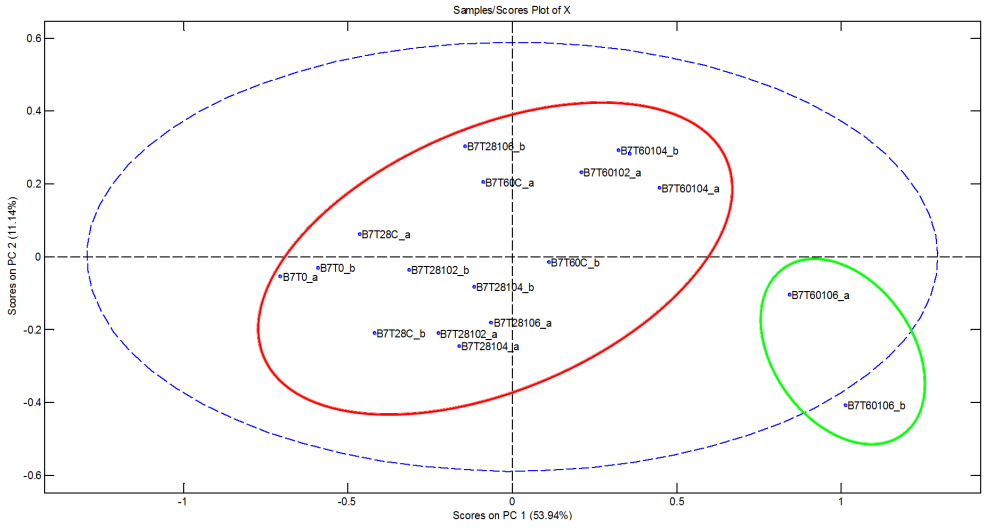

Figure 5. Scores (PCA) of systems containing the mixture B7 with inocula 10^2, 10^4 and 10^6 spores mL^{-1} at baseline, after 28 days and after 60 days. Characters such as "_a" and "_b" indicate replicates.

Traditional methods of microbial detection are based on filtering techniques monitored by microscopy and/or culture but are time consuming, especially for fungi, which are slow growing (Gaylarde et al., 1999). MicrobMonitor 2 (Echa Microbiology Ltd.), a test devised to give a faster result than the standard laboratory procedures, is based on culturing for microbial colony forming units and has been used in the aviation industry. Other methods, like Hy-Lite Jet A-1 (Merck), are based on ATP detection. Because these methods are based on different principles and measure different parameters, results obtained will not always be comparable. Lopes and Gaylarde (1996) developed an immunofluorescent test for aviation kerosene samples using polyclonal antibodies against *Hormoconis resinae*. In spite of reducing time in relation to traditional techniques this assay may produce cross reactions. Methods based on chemical reactions (MicrobMonitor Sig Rapid WB Test-Echa Microbiology Ltd.) or immunochromatography (Conidia Biosciences) are available commercially, but they lack specificity or detect only one species of microorganism.

Figure 7 shows the absence of bands in the region where is located the amplified fragment of *P. variotii*, indicating specificity of the primers. Under the experimental conditions of this preliminary study PCR displayed a reasonable specificity and sensitivity as it was able to detected DNA equivalent to 10^3 spores mL^{-1} in aqueous phase. Some modifications can be introduced, as different methods of DNA extraction, or the use of real time or nested PCR,

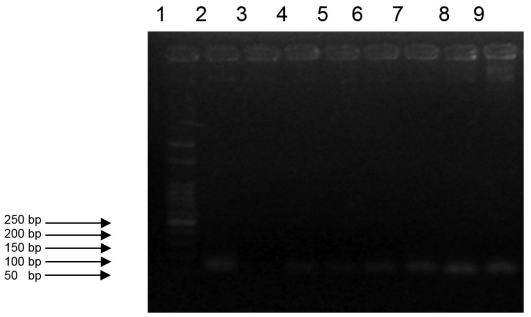

Figure 6. Concentration of PCR products from DNA extracted from suspensions of spores in increasing concentrations. Lane 1: molecular weight marker (50-1000 bp); Col 2: positive control (10 ng of template DNA); Col 3: 10^2 spores mL^{-1}; Column 4: 10^3 spores mL^{-1}; Column 5: 10^4 spores mL^{-1}; Column 6: 10^5 spores mL^{-1}; Column 7: 10^6 spores mL^{-1}; Column 8: 10^7 spores mL^{-1}; Column 9: 10^8 spores mL^{-1}.

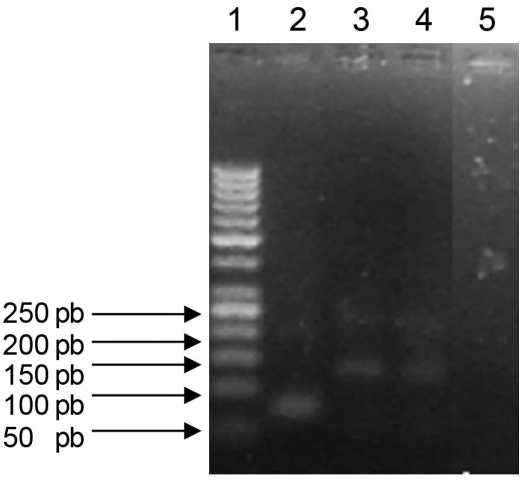

Figure 7. Specificity of the primers. Lane 1: molecular weight marker (50-1000 bp); Column 2: *P. variotii*; Column 3: *P. boydii*; Column 4: *A. fumigatus*, Column 5: control.

for improving the technique sensitivity and thus making possible its use for monitoring the degree of contamination in biodiesel blends.

4. Conclusions

Experiments carried out in this study showed the significant production of biomass when the oily phase was blend B7 and with pure biodiesel, due to the ability of *P. variotii* to develop especially when biodiesel (esthers) is present. After 60 days incubation the FTIR analysis suggests fuel degradation, which depends on the initial amount of microorganisms (inoculum) and exposure time, with possible formation of intermediate compounds. Taken together, our results and those of others discussed here confirm the hypothesis that the addition of biodiesel to conventional diesel increases the susceptibility to microbial growth and fuel biodegradability, especially during the storage process. We also tested a protocol to detect *P. variotii* spores in the aqueous phase by PCR which displayed reasonable specificity and sensitivity and makes this technique very promising to assess the degree of contamination of biodiesel blends.

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References

ABBAS, O., RÉBUFA, C., DUPUY, N. and KISTER, J., 2008. FTIR-multivariate curve resolution monitoring of photo-Fenton degradation of phenolic aqueous solutions. Comparison with HPLC as a reference method. *Talanta*, vol. 77, no. 1, p. 200-209. http://dx.doi.org/10.1016/j.talanta.2008.06.008. PMid:18804621.

American Society for Testing and Materials – ASTM, 2010. *ASTM Standard E 1259. Standard practice for evaluation of antimicrobials in liquid fuels boiling below 390°C*. West Conshohocken: ASTM. http://dx.doi.org/10.1520/E1259-10.

ATLAS, RM., 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiological Reviews*, vol. 45, no. 1, p. 180-209. PMid:7012571.

BENTO, FM., 1999. Micro-organismos e o armazenamento de óleo diesel. *Revista Petro & Química*, vol. 211, p. 70-77.

BENTO, FM. and GAYLARDE, CC., 1996. Microbial contamination of stored diesel oil. *Brazilian Journal of Microbiology*, vol. 27, p. 71-75.

BENTO, FM. and GAYLARDE, CC., 2001. Biodeterioration of stored diesel oil: studies in Brazil. *International Biodeterioration & Biodegradation*, vol. 47, no. 2, p. 107-112. http://dx.doi.org/10.1016/S0964-8305(00)00112-8.

BENTO, FM., CAMARGO, FAO., GAYLARDE, CC., VISCARDI, SL., MENENDEZ, A. and DARODA, R., 2006. Suscetibilidade do óleo diesel com 2 e 5% de biodiesel à contaminação microbiana durante a estocagem. *Revista Biodiesel*, vol. 4, p. 24-26.

BENTO, FM., ENGLERT, GE., GAYLARDE, CC. and MULLER, IL., 2004. Influence of aqueous phase on electrochemical biocorrosion tests in diesel/water systems. *Materials and Corrosion*, vol. 55, no. 8, p. 577-585. http://dx.doi.org/10.1002/maco.200303772.

BÜCKER, F., SANTESTEVAN, NA., ROESCH, LF., JACQUES, RJS., PERALBA, MCR., CAMARGO, FAO. and BENTO, FM., 2011. Impact of biodiesel on biodeterioration of stored Brazilian diesel oil. *International Biodeterioration & Biodegradation*, vol. 65, no. 1, p. 172-178. http://dx.doi.org/10.1016/j.ibiod.2010.09.008.

BUSCH, U. and NITSCHKO, H., 1999. Methods for the differentiation of microorganisms. *Journal of Chromatography. B, Biomedical Sciences and Applications*, vol. 722, no. 1-2, p. 263-278. http://dx.doi.org/10.1016/S0378-4347(98)00369-7. PMid:10068145.

CAZAROLLI, JC., BÜCKER, F., MANIQUE, MC., KRAUSE, LC., MACIEL, GPS., ONOREVOLI, B., CARAMÃO, EB.,

CAVALCANTI, EHS., SAMIOS, D., PERALBA, MCR. and BENTO, FM., 2012. Suscetibilidade do biodiesel de sebo bovino à biodegradação por *Pseudallescheria boydii. Revista Brasileira de Biociências*, vol. 10, p. 251-257.

CAZAROLLI, JC., GUZATTO, R., SAMIOS, D., PERALBA, MCR., CAVALCANTI, EHS. and BENTO, FM., 2014. Susceptibility of linseed, soybean, and olive biodiesel to growth of the deteriogenic fungus *Pseudallescheria boydii*. *International Biodeterioration & Biodegradation*, vol. 95, p. 364-372. http://dx.doi.org/10.1016/j.ibiod.2013.09.025.

CHESNEAU, HL., 2000. The silent fuel killers (Stability and Microbiologicals). In *Proceedings of the International Joint Power Generation Conference*, 2000. Miami Beach, Florida. New York: American Society of Mechanical Engineers. p. 1-8.

FERREIRA, ME. and GRATTAPAGLIA, D., 1996. *Introdução ao uso de marcadores moleculares em análise genética*. 2nd ed. Brasília: Embrapa Cenargen. 220 p.

Fundação Andre Tosello – FAT, 2010. *FAT: e-solutions*. Campinas: FAT. Available from: <www.fat.com.br>. Access in: 03 Mar 2012.

GAYLARDE, CC., BENTO, FM. and KELLEY, J., 1999. Microbial contamination of stored hydrocarbon fuels and its control. *Revista de Microbiologia*, vol. 30, no. 1, p. 1-10. http://dx.doi.org/10.1590/S0001-37141999000100001.

GENOV, GY., NODLAND, E., SKAARE, BB. and BARTH, T., 2008. Comparison of biodegradation level and gas hydrate plugging potential of crude oils using FT-IR spectroscopy and multi-component analysis. *Organic Geochemistry*, vol. 39, no. 8, p. 1229-1234. http://dx.doi.org/10.1016/j.orggeochem.2008.04.006.

GUARIEIRO, NL., PINTO, CA., AGUIAR, FP. and RIBEIRO, MN., 2008. Metodologia analítica para quantificar o teor de biodiesel na mistura biodiesel:diesel utilizando espectroscopia na região do infravermelho. *Quimica Nova*, vol. 31, no. 2, p. 421-426. http://dx.doi.org/10.1590/S0100-40422008000200041.

HAUGLAND, RA., VARMA, M., WYMER, LJ. and VESPER, SJ., 2004. Quantitative PCR analysis of selected Aspergillus, Penicillium and Paecilomyces species. *Systematic and Applied Microbiology*, vol. 27, no. 2, p. 198-210. http://dx.doi.org/10.1078/072320204322881826. PMid:15046309.

LIAO, Y., GENG, A. and HUANG, H., 2009. The influence of biodegradation on resins and asphaltenes in the Liaohe Basin. *Organic Geochemistry*, vol. 40, no. 3, p. 312-320. http://dx.doi.org/10.1016/j.orggeochem.2008.12.006.

LOPES, PTC. and GAYLARDE, C., 1996. Use of immunofluorescence to detect *Hormoconis resinae* in aviation kerosine. *International Biodeterioration & Biodegradation*, vol. 37, no. 1-2, p. 37-40. http://dx.doi.org/10.1016/0964-8305(95)00110-7.

Ludwig Biotec, 2010. Available from: <www.ludwigbiotec.com.br>. Access in: 03 Mar. 2012.

OWSIANIAK, M., CHRZANOWSKI, Ł., SZULC, A., STANIEWSKI, J., OLSZANOWSKI, A., OLEJNIK-SCHMIDT, AK. and HEIPIEPER, HJ., 2009. Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: effect of the type of blend and the addition of biosurfactants. *Bioresource Technology*, vol. 100, no. 3, p. 1497-1500. http://dx.doi.org/10.1016/j.biortech.2008.08.028. PMid:18815027.

PASQUALINO, JC., MONTANÉ, D. and SALVADÓ, J., 2006. Synergic effects of biodiesel in the biodegradability of fossil-derived fuels. *Biomass and Bioenergy*, vol. 30, no. 10, p. 874-879. http://dx.doi.org/10.1016/j.biombioe.2006.03.002.

PASSMAN, F. and DOBRANICK, JK., 2005. Relative biodegradability of B-100 biodiesel and conventional low sulfur diesel fuels. In *Proceedings of 9th International Conference on Stability, Handling and Use of Liquid Fuels*, 2005. Sidges. Sidges: IASH. p. 18-22.

PASSMAN, FJ., 2013. Microbial contamination and its control in fuels and fuel systems since 1980 e a review. *International Biodeterioration & Biodegradation*, vol. 81, p. 88-104. http://dx.doi.org/10.1016/j.ibiod.2012.08.002.

RICHARD, JY. and VOGEL, TM., 1999. Characterization of a soil bacterial consortium capable of degrading diesel fuel. *International Biodeterioration & Biodegradation*, vol. 44, no. 2-3, p. 93-100. http://dx.doi.org/10.1016/S0964-8305(99)00062-1.

RUIZ, ON. and BROWN, NA., 2011. Advances in microbial mitigation of aviation fuels: characterization of the antimicrobial activity of DIEGME in fuel byquantitative Real-Time PCR. In *Proceedings of Proceedings of 12th International Conference on Stability, Handling and Use of Liquid Fuels*, 2011. Sarasota. Sarasota: ICSH. p. 16-20.

SCHLEICHER, T., WERKMEISTER, R., RUSS, W. and MEYER-PITTROFF, R., 2009. Microbiological stability of biodiesel-diesel-mixtures. *Bioresource Technology*, vol. 100, no. 2, p. 724-730. http://dx.doi.org/10.1016/j.biortech.2008.07.029. PMid:18793842.

SILVERSTEIN, RM., BASSLER, GC. and MORRIL, TC., 1991. *Spectrometric identification of organic compounds*. 5th ed. New York: John Wiley & Sons.

SOARES JUNIOR, JS., MARIANO, AP. and ANGELIS, DF., 2009. Biodegradation of biodiesel/diesel blends by *Candida visawanathi*. *African Journal of Biotechnology*, vol. 8, no. 12, p. 2774-2778.

SØRENSEN, G., PEDERSEN, DV., NØRGAARD, AK., SØRENSEN, KB. and NYGAARD, SD., 2011. Microbial growth studies in biodiesel blends. *Bioresource Technology*, vol. 102, no. 8, p. 5259-5264. http://dx.doi.org/10.1016/j.biortech.2011.02.017. PMid:21376581.

WHITE, J., GILBERT, J., HILL, G., HILL, E., HUSE, SM., WEIGHTMAN, AJ. and MAHENTHIRALINGAM, E., 2011. Culture-independent analysis of bacterial fuel contamination provides insight into the level of concordance with the standard industry practice of aerobic cultivation. *Applied and Environmental Microbiology*, vol. 77, no. 13, p. 4527-4538. http://dx.doi.org/10.1128/AEM.02317-10. PMid:21602386.

WHITE, TJ., BRUN, T., LEE, S. and TAYLOR, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols. A guide to methods and applications*. San Diego: Academic Press. p. 315-320.

WILLIAMS, RH., WARD, E. and MCCARTNEY, HA., 2001. Methods for integrated air sampling and dna analysis for detection of airborne fungal spores. *Applied and Environmental Microbiology*, vol. 67, no. 6, p. 2453-2459. http://dx.doi.org/10.1128/AEM.67.6.2453-2459.2001. PMid:11375150.

ZHANG, X., PETERSON, C., REECE, D., HAWS, R. and MOLLER, G., 1998. Biodegradability of biodiesel in the aquatic environment. *Transactions of the ASAE. American Society of Agricultural Engineers*, vol. 41, no. 5, p. 1423-1430. http://dx.doi.org/10.13031/2013.17277.

ZIMMER, AR., CAZAROLLI, JC., TEIXEIRA, RM., VISCARDI, SLC., CAVALCANTI, ESH., GERBASE, AE., FERRÃO, MF., PIATNICKI, CMS. and BENTO, FM., 2013. Monitoring of efficacy of antimicrobial products during 60 days storage simulation of diesel (B0), biodiesel (B100) and blends (B7 and B10). *Fuel*, vol. 112, p. 153-162. http://dx.doi.org/10.1016/j.fuel.2013.04.062.