African Journal of Biotechnology Vol. 8 (12), pp. 2774-2778, 17 June, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Biodegradation of biodiesel/diesel blends by *Candida viswanathii*

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Accepted 13 April, 2009

This work is aimed to assess the aerobic biodegradation of biodiesel/diesel blends (0, 2, 5, 20 and 100%, v/v) by *Candida viswanathii*. The biodegradation potential of the inoculum was assessed with the redox indicator 2,6-dichlorophenol indophenol (DCPIP) test and with respirometric experiment in biometer flasks (250 mL) used to measure the microbial CO_2 production. In the latter, the inoculum was added to a contaminated soil with the blends (addition of 50 mL of fuel/Kg of soil from a non-contaminated site). *C. viswanathii* was able to increase significantly (approximately 50% in terms of CO_2 production) the biodegradation in soil of biodiesel/diesel blends and neat biodiesel since it preferable biodegrades biodiesel. Without inoculum the biodegradation of diesel oil was higher than biodiesel and blends (47.3, 51.1, 5.7 and 22.1% in terms of CO_2 production by B2, B5, B20 and B100, respectively) presumably due to the presence of the antioxidant terc-butyl-hydroquinone (TBHQ) in the biodiesel.

Key words: Diesel, biodiesel, blends, biodegradation, bioaugmentation, TBHQ.

INTRODUCTION

The introduction of the biodiesel into the Brazilian energetic matrix was determined by a Federal law that establishes a compulsory blend of 2% of biodiesel in mineral diesel as of 2008 and 5% as of 2013. In Europe, since 2004, the diesel fuel standard EN590 allows oil companies to add up to five percent of biodiesel to diesel fuels (Owsianiak et al., 2009).

Although with the same function, biodiesel and diesel have very distinct origins and compositions. Biodiesel is composed of methyl or ethyl esters of fatty acids with low structural complexity as oleate, palmitate, estearate, linoleate, myristate, laureate and linolenate derived from different vegetable oil sources such as soybean, sun-flower, peanut, cotton, palm oil, coconut, babassu and castor oil and from animal fat (Pinto et al., 2005).

Differently, diesel oil contains 2000 to 4000 hydrocarbons, a complex mixture of normal, branched and cyclic alkanes and aromatic compounds obtained from the middle-distillate fraction during petroleum separation (Gallego et al., 2001).

Besides the recognized environmental benefits related to the biodiesel combustion (less emissions of CO_2, CO and SOx, volatile organic compounds and particulate material) (Pinto et al., 2005), the difference between the fuels compositions also influences their biodegradability.

As occurs to the diesel oil, the commercialization of biodiesel or the biodiesel/diesel blend may cause environmental damages due to spills. The clean-up of these contaminated areas can be achieved with bioremediation, a technique based on the action of microorganisms, which turn hazardous contaminants into non toxic substances such as CO_2, water and biomass.

Some studies demonstrated that biodiesel is more easily biodegraded than diesel oil and can promote and speed up the biodegradation of hydrocarbons by means of co-metabolism (Zhang et al., 1998; Makareviciene and Janulis, 2003; Pasqualino et al., 2006; Lapinskiene et al., 2006). On the other hand, DeMello et al. (2007) and Mariano et al. (2008a) observed that hydrocarbon biodegradation was not accelerated by the presence of biodiesel and Owsianiak et al. (2009) reported that the biodegradation of the mixture with 10% of biodiesel was lower than for petroleum diesel fuel.

The bioremediation of petroleum products such as gasoline and diesel oil has been tried successfully many times at the commercial scale (US National Research

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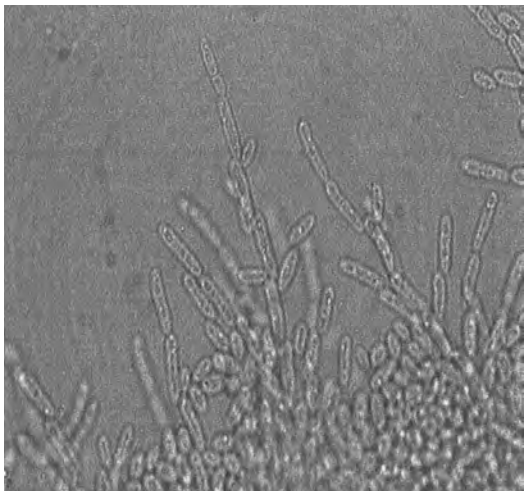

Figure 1. Morphological aspect of ***C. viswanathii*** 400x.

Council, 1993). However, it is known that intrinsic bioremediation rates are typically slow, taking several years to fully clean-up sites with high levels of concentration of hydrocarbons. In this sense, engineered bioremediation is a remediation technique based on strategies that acelerate the biological breakdown of the pollutant by stimulation of the indigenous microorganisms (nutritional amendment, oxygenation, temperature, pH control and addition of surfactants) and/or by inoculation of specific strains of organisms (bioaugmentation). Hence, the assessment of the capability of ***Candida viswanathii*** is to accelerate the biodegradation of biodiesel/diesel.

In the biotechnology field, ***C. viswanathii*** is known to be a biocatalyst for stereoselective reduction (Pankaj et al., 2006; Fatima et al., 2007; Kansal and Banerjee, 2009) and its capability to biodegrade petroleum hydrocarbons and diesel oil was reported respectively by Chaillan et al. (2004) and Yu (2003). However, to the authors' knowledge so far, there is no report assessing the biodegradation of biodiesel/diesel blends by this yeast. Therefore, contributing to the work of previous investigations and bearing in mind that the commercialization of diesel blended with biodiesel is the current scene in the fuel market, this research dealt with this issue.

MATERIALS AND METHODS

Inoculum preparation

The ***C. viswanathii*** strain used throughout this study was isolated from the waste water of a Brazilian oil refinery (Replan/Petrobras). On Sabouraud's dextrose agar, colonies were white to cream colored, smooth, glabrous and yeast-like in appearance. Microscopic morphology (Figure 1) shows the formation of pseudo-micelles.
The inoculum was prepared using the cells transferred from the storage culture tube and streaked onto the surface of Petri dishes containing Sabouraud media (Acumedia, EUA). The dishes were incubated at 28°C during 48 h and after growing, cells were harvested using sterile saline solution. The cell suspension concentration was determined in Neubauer´s counting chamber.

Fuel mixtures

Neat diesel oil (B0) and the B2 blend [diesel containing 2% (v/v) of biodiesel] were purchased from service stations respectively, BR and ALE distributor, Brazil. Biodiesel (B100) in the form of a mixture of soybean and fat animal oil methyl esters was purchased from local supplier in Brazil (Caramuru Alimentos SA). B5 and B20 blends were prepared in laboratory.

Biodegradability test – DCPIP indicator

The biodegradability of blends by ***C. viswanathii*** was verified using the technique based on the redox indicator 2,6-dichlorophenol indophenol (DCPIP) (Hanson et al., 1993). The principle of this technique is that, during the microbial oxidation of the carbon source, electrons are transferred to electron acceptors such as O_2, nitrates and sulphate. By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of the microorganism to utilize the substrate by observing the color change of DCPIP from blue (oxidized) to colorless (reduced). This technique has been employed in several works (Cormack and Fraile, 1997; Roy et al., 2002; Mariano et al., 2008a, b; Pirollo et al., 2008).

Inoculum (0.2 mL and 10^8 CFU/mL) were added to essay tubes (triplicates) that contained 10 mL sterile Bushnell-Hass (BH) medium and 1% v/v of the fuel. The concentration of DCPIP was 0.16 mg/mL. The tubes were kept under agitation 60 rpm at 28.0 ± 1.0°C. The BH medium consists of g/L, $MgSO_4$, 0.2, $CaCl_2$, 0.02, KH_2PO_4, 1.0, K_2HPO_4, 1.0, NH_4NO_3, 1.0, $FeCl_3$, 0.05 (Difco, 1984).

Respirometric experiment

Table 1 summarizes the respirometric biodegradation experiment carried out to evaluate the biodegradation of biodiesel/diesel blends in soil inoculated with ***C. viswanathii***. Bartha biometer flasks (250 mL) were used to measure the microbial CO_2 production (Bartha and Pramer, 1965; Mariano et al., 2007, 2008a). The CO_2 produced is proportional to the percentage of substrate biodegraded. Mineralization studies involving measurements of total CO_2 production can provide excellent information on the biodegradability potential of hydrocarbons (Balba et al., 1998).

CO_2 evolution measures ultimate degradation (mineralization) in which a substance is broken down to the final products, while for instance, the gas chromatography (GC) analysis measures primary degradation in which the substance is not necessarily transformed to end products.

For each experimental condition, the biometer flasks were prepared in triplicates (50 g of soil) and incubated in the dark at 27 ± 2°C during 88 days. The quantity of fuel and inoculum added to the soil was 50 and 240 mL per Kg of soil respectively. In treatments without inoculum, the same quantity of deionized water was added. Inoculum concentration was 10^9 CFU/mL.
The CO_2 produced was trapped in a 10.0 mL solution of KOH (0.2 N), located in the side-arm of the biometer. This solution was periodically withdrawn by syringe and the amount of carbon dioxide absorbed was then measured by titrating the residual KOH (after the addition of barium chloride solution (1 mL; 1.0 N) used to precipitate the carbonate ions) with a standard solution of HCl (0.1 N). During this procedure, biometers were aerated during 30 s through ascarite filters.

Soil sampling and characterization

The soil used in the respirometric experiment was collected from the superficial layer of a non-contaminated site. Until performing the

Table 1. Respirometric experiment.

Treatment	Components
1	soil control[a]
2	soil + B0
3	soil + B0 + inoculum
4	soil + B2
5	soil + B2 + inoculum
6	soil + B5
7	soil + B5 + inoculum
8	soil + B20
9	soil + B20 + inoculum
10	soil + B100
11	soil + B100 + inoculum

[a]Without addition of contaminants and inoculum.

biodegradation experiments, the sample was stored at 5°C. Table 2 summarizes some of the soil physicochemical characteristics. The analyses were performed according to the methodology proposed by Embrapa (1997). Values of heavy metals concentrations were not above the more restricted levels set by the Cetesb (Sao Paulo Environmental Agency - Brazil) and by the Dutch list (Cetesb, 2005).

RESULTS AND DISCUSSION

The results of the redox indicator experiment (Table 3) show that ***C. viswanathii*** preferably biodegrades biodiesel, since the higher the concentration of biodiesel in the blend, the lower the time to decolorization of the DCPIP indicator. Thus, the following order of biodegradability was found: B100 > B20 > B5 > B2 > B0. The cumulative CO_2 production of the respirometric experiment is shown in Figures 2 and 3 respectively, neat fuels and blends. While for neat diesel (B0) the addition of inoculum did not result in different CO_2 productions (Anova, p = 0.05), the biodegradation of B2, B5, B20 and neat biodiesel B100 benefited from the inoculum. The CO_2 production increased significantly in these treatments 48.3, 56.8, 38.8 and 55.4%, respectively.

Comparing the treatments without inoculum, B0 had a higher CO_2 production than the blends and B100 (47.3, 51.1, 5.7 and 22.1% respectively, B2, B5, B20 and B100). On the other hand, with the addition of inoculum, only the CO_2 production of B2 was lower than B0. The other blends and B100 were not statistically different from B0. Thus, this comparison also demonstrates that the efficiency of the inoculum was dependent on the presence of biodiesel.

One question that has been debated for a decade (Zhang et al., 1998; Makareviciene and Janulis, 2003; Pasqualino et al., 2006; Lapinskiene et al., 2006; DeMello et al., 2007; Mariano et al., 2008a; Owsianiak et al., 2009) is if diesel blended with biodiesel is more biodegradable than neat diesel. Zhang et al. (1998) claim that biodiesel is more easily metabolized than diesel because the former is a natural product consisting of pure fatty acids that are hydrocarbon chains with two oxygen atoms attached at one end, which are very biologically active, being recognized and attacked immediately by enzymes such as acetyl-CoA dehydrogenase.

The biodegradation of diesel, which consists of a large amount of alkanes (hydrocarbon chains from C10 - 20) without oxygen attached, demands adapted microorganisms able to produce enzymes that recognize these molecules. Moreover, the presence of aliphatic cyclic

Table 2. Soil sample characteristics.

Parameter	Value
pH ($CaCl_2$)	4.0
Organic matter (g/dm^3)	8.0
Residual phosphorus (mg/dm^3)	8.0
K (mmolc/dm^3)	0.6
Ca (mmolc/dm^3)	1.0
Mg (mmolc/dm^3)	1.0
Total bases (mmolc/dm^3)	2.7
Al (mmolc/dm^3)	1.0
Cation exchange capacity (mmolc/dm^3)	27.7
Grain size distribution (%)	
Sand	86.0
Silt	4.1
Clay	9.9
Micronutrients (ppm)	
S	10
Na	3.0
Fe	25
Mn	0.6
Cu	0.9
Zn	0.9
B	0.2
Heavy metals (ppm)	
Ba	14.4
Cd	< 0.01
Cr	11.9
Ni	< 0.01
Pb	< 0.01

Table 3. Time to decolorization of the DCPIP indicator.

Blend	Time (h)
B0	29
B2	24
B5	18
B20	17
B100	8

During the experiment, no decolorization of the substrate controls (without inoculum) or of the inoculum controls (without fuel) was observed.

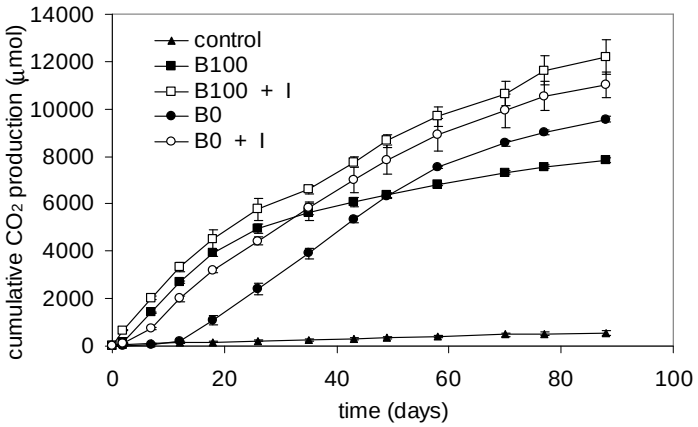

Figure 2. Cumulative total amounts of CO_2 produced in the respirometric experiment (neat fuels).

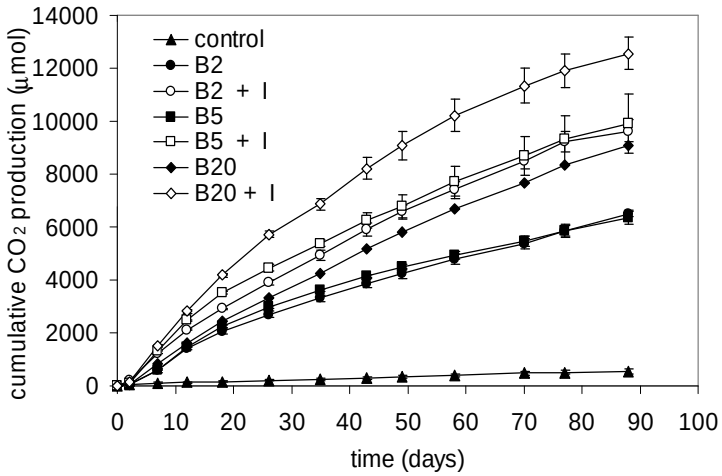

Figure 3. Cumulative total amounts of CO_2 produced in the respirometric experiment (biodiesel/diesel blends).

hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and alkylbenzenes, as well as their derivatives such as toluene, xylenes and PCBs (phenyl and biphenyls) gives the diesel a composition much more chemically complex. Other works (Makareviciene and Janulis, 2003; Pasqualino et al., 2006; Lapinskiene et al., 2006; Mariano et al., 2008a) also reported biodegradation gains obtained with the addition of biodiesel to the mineral diesel. On the other hand, Owsianiak et al. (2009), testing the biodegradation of biodiesel/diesel blends by a microbial consortium isolated from crude oil contaminated site (***Pseudomonas alcaligenes***, ***Ochrobactrum intermedium***, ***Sphingobacterium*** sp., ***Pseudomonas putida***, ***Klebsiella oxytoca***, ***Chryseobacterium*** sp. and ***Stenotrophomonas maltophilia***), found that the biodegradation of the mixture with 10% of biodiesel was lower than for diesel fuel. They claim that this might be because of the specification of the chosen microorganisms and their intrinsic capability of degradation of petroleum hydrocarbons.

In our previous work (Mariano et al., 2008a) similar respirometric experiments were carried out, however without addition of inoculum. The biodegradation of the blends was quantified in soil from a gas station and water from river. In these cases, the biodegradation of B20 and B100 was higher than neat diesel. Unlike the biodiesel employed in these experiments, in the present work the biodiesel contains the antioxidant terc-butyl-hydroquinone (TBHQ).

This substance has been added to the biodiesel produced in industrial scale in order to extend the storage time, since a significant problem associated with the commercial acceptance of biodiesel is its poor oxidative stability (Tang et al., 2008). TBHQ is also one of the most used antioxidant in vegetable oils (Masuchi et al., 2008). Antioxidants are a prerequisite to secure the stability of the oil during its shelf life, especially with the increased use of PET packaging. Furthermore, TBHQ is also known due to its antimicrobial properties (Davidson et al., 1980;

Oliveira et al., 2007).

Thereby, it is likely that the addition of antioxidants has been responsible for the decrease of the biodiesel biodegradability, as verified in the present study and others. The redox indicator experiment (Table 3) showed that ***C. viswanathii*** preferable biodegraded biodiesel. Presumably this microorganism was resistant to the antimicrobial properties of TBHQ and consequently was able to improve the biodegradability of the blends in soil.

Conclusion

This study investigated the aerobic biodegradation of biodiesel/diesel blends by ***C. viswanathii***. Based on laboratory experiments, the following conclusions were made:

(i) ***C. viswanathii*** showed to be able to increase significantly (approximately 50%) the biodegradation in soil of biodiesel/diesel blends and neat biodiesel since it preferable biodegrades biodiesel.
(ii) Without inoculum the biodegradation of diesel oil was higher than biodiesel and blends presumably due to the presence of the antioxidant TBHQ in the biodiesel.
(iii) Further research is needed to determine which specific constituents of diesel and biodiesel are biodegraded by ***C. viswanathii***, and the effects of the biodiesel with TBHQ on the microbial community structure.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Agência Nacional do Petroleo, Gas Natural e Biocombustiveis (ANP) (PRH-05) for the financial support.

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