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The effect of tert-butylhydroquinone (TBHQ) on biodiesel bioremediation in soil samples inoculated with bacterial cells

Dorota Wieczorek [a, *], Olga Marchut-Mikolajczyk [a], Bartosz Strzelecki [a], Malgorzata Gajewska [a], Arkadiusz Polewczyk [b], Tadeusz Antczak [a]

[a] Institute of Technical Biochemistry, Technical University of Lodz, Stefanowskiego 4/10, 90-924, Lodz, Poland
[b] Institute of Food Chemistry, Technical University of Lodz, Stefanowskiego 4/10, 90-924, Lodz, Poland

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ABSTRACT

The study aimed to determine the effect of tert-butyl-hydroquinone (TBHQ) on the activities of dehydrogenases and esterases, free fatty acid level, early plant growth of *Sinapis alba, Lepidium sativum*, and *Sorghum saccharatum*, pH, degradation of biodiesel and the rate of FAME assimilation during bioremediation process with two selected bacterial strains (*Achromobacter xylosoxidans G21* or *Sarcina* spp.). Biodiesel with or without TBHQ (210 mg l^{-1}) in 5% (w/w) concentration were introduced into soil. Based on the obtained results, inhibition of esterases and dehydrogenases activity, reduction of FAME assimilation and a marked decrease in FAME degradation was found when TBHQ was added to biodiesel. Obtained results provide evidence that the presence of TBHQ in biofuel impacts the course of bioremediation of soil contaminated with biodiesel. Too high TBHQ concentrations in the fuel may reduce the effectiveness of soil clean-up processes.

1. Introduction

Biodiesel fuel differs in physicochemical properties from the conventional diesel (Hare et al., 2008) that may cause problems during exploitation of engines powered with biodiesel. The oxidative instability of biodiesel and relatively high melting temperature can lead to clogging of fuel filters, sediment deposition in fuel tanks, and changes in the appearance - from clear to cloudy (Sendzikiene et al., 2006). Products of autoxidation of unsaturated fatty acids or hydrolysis of the esters (free fatty acids) have a strong corrosive activity. To improve physical and chemical properties of the fuel, biodiesel is supplemented with special additives such as organic, inorganic and organometallic compounds. They act as corrosion inhibitors, oxidation inhibitors, depressants, antifoaming additives, detergents, cetane improvers, metal deactivators or de-emulsifiers (Groysman, 2014). Because of historic stability problems with natural antioxidants, blenders now typically use synthetic antioxidants (De Guzman et al., 2009). TBHQ (tert-butylhydroquinone), PP (pentachlorophenol), PG (propyl galate), BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are most frequently used (Ryu, 2009; Fazal et al., 2014; Larson and Marley, 2011). Synthetic and natural antioxidants delay and inhibit oxidation (Tang et al., 2008). These are radical scavengers that readily donate hydrogen atoms to radicals, generating stable products and terminating the chain reaction mechanism of oxidation (Christensen and McCormick, 2014).

EU legislation does not require the type and amount of antioxidants in biofuel to declare. Researchers and biofuels producers inform the concentration of antioxidants on the level of 0,1-1,5% as adequate for appropriate biodiesel stabilization (Dinkov et al., 2009; Chen and Luo, 2011; Rizwantul Fattah et al., 2014). However too high concentration of TBHQ or another antioxidants in biofuel may be obstacles for effective bioremediation. These compounds may also have antibacterial properties. Their antibacterial activity depends on microbial species, structure of the antioxidant and its concentration (Gutiérrez-Larraínzar et al., 2013). These compounds may inhibit microbial growth through interrupting DNA, RNA, proteins and lipids synthesis, disturbing the energetic cellular metabolism as well as changes in the performance and composition of the cell membranes. The synthetic antioxidants may also suppress the growth of filamentous fungi, yeasts, protozoa and viruses (Tseng and Tseng, 1995; Jemes et al.,

* Corresponding author. Institute of Technical Biochemistry, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Stefanowskiego 4/10, 90-924, Lodz, Poland.
E-mail address: dorota.wieczorek@edu.p.lodz.pl (D. Wieczorek).

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2005; Ooi et al., 2013; Eskandani et al., 2014).

Reports on bioremediation of soil contaminated with biodiesel are limited (Peterson and Reece, 1994; Lapinskiene et al., 2006; Mariano et al., 2008; Corseuil et al., 2011; Cyplik et al., 2011; Soares et al., 2009; Cruz et al., 2013; Matos et al., 2015; Meyer et al., 2015). Impact of additives contained in the biofuel, which can reduce the susceptibility of biodiesel to biodegradation is rarely discussed in the literature (Aluyor et al., 2009; Soares et al., 2009; Tamada et al., 2012; Bücker et al., 2011).

Chemical analyses may be insufficient to evaluate the effects of contamination on the environment. Therefore various techniques were established to assess the efficiency of biodegradation processes precisely (Aichberger et al., 2005; Bidoia et al., 2010). Biological parameters such as activities of enzymes like peroxidase, dehydrogenases and esterases, density of microbial biomass, the rate of CO_2 release or oxygen consumption, are considered to be sensitive indicators of decomposition of organic inputs and the detoxification of xenobiotics as well as the remediation progress (Pieper et al., 2004; Aparna et al., 2010; Hawrot-Paw et al., 2010; Lisiecki et al., 2014).

In this work, we attempted to estimate the influence of tert-butylhydroquinone (TBHQ) on biodiesel biodegradation in soil. For the purpose of the investigation, changes in: dehydrogenase activity (assayed by a modified method of Lester Earl Casida with TTC (2,3,5 triphenyltetrazolium chloride) (Casida et al., 1964), esterase activity (assayed by a modified method of Margesin using p-nitro phenol (pNP) acetate as substrate (Margesin and Schinner, 2005), FFA (free fatty acid) concentration (quantified by a method developed by Kwon and Rhee) (Kwon and Rhee, 1984), and germination and early plant growth (using Phytotoxkit®) were determined.

2. Materials and methods

2.1. Fuels

Two types of commercial biodiesel were used in this study. One of them was supplemented with TBHQ (amount of antioxidants added to biodiesel by the manufacturer - 210 mg L^{-1}) while the second did not contain this antioxidant. Both the fuels were produced from rapeseed oil according to EN 14214, and purchased from a local supplier in Poland. The fatty acid methyl ester (FAME) profile of the biodiesel used in this study: C16:0 Hexadecanoic ME 5%, C18:0 Octadecanoic ME 2%, C18:1 Octadec-9-enoic ME 64%, C18:2 Octadeca-9,12-dienoic ME 22%, C18:3 Octadeca −9,12,15-trienoic ME 1%, C20:0 Eicosanoic ME 1,1%, C20:1 Eicos-11-enoic ME 0,9% C22:0 Docosanoic ME 1%.

2.2. Microorganisms and inoculum preparation

2.2.1. Selection of bacteria

Six strains of bacteria (Table 1) isolated in the Institute of Technical Biochemistry (ITB) from environmental samples of soil polluted with petroleum or FAME's derivatives in Poland, were evaluated for biodegradation of biodiesel fuel. All bacterial strains belonged to a pure culture collection of the IBT, Lodz University of Technology (LUT). The biodegradability of biodiesel by selected microorganism were verified using the technique based on the redox indicator 2,6-dichlorophenol indophenol (DCPIP) (Hanson et al., 1993). 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 (Roy et al., 2002; Pirollo et al., 2008; Soares et al., 2009). The ability of microorganisms for fuel degradation was determined based on the change in the color of the culture medium containing DCPIP after 12, 18 and 24 h of incubation (Miranda et al., 2007; Soares et al., 2009). For each tested bacteria three replicates were made. Only two strains, *Sarcina* spp. and *A. xylosoxidans* G21, were selected for further the experiments. After 12 and 18 h of incubation at 30 °C the strains almost completely discolored the culture medium. Other strains discolored the culture medium after 24 h or more (Table 1).

Table 1
Time (h) of DCPIP indicator decolorization.

Microorganism	Fuel	
	B100 with TBHQ	B100 without TBHQ
A. xyloxosidans G21	**20**	**18**
Sarcina spp.	**19**	**24**
Pseudomonas spp. G-4B	29	33
Bacillus subtilis P31	24	38
Acientobacter spp.	49	72
Ochrobactum anthropi R51	25	29

2.2.2. Inoculum preparation

Bacterial strains: *Sarcina* spp. and *A. xylosoxidans* G21 from the pure culture collection at the Institute of Technical Biochemistry (ITB) of the Lodz University of Technology (LUT), were maintained on “A” medium: glucose 2 g, yeast extract 2 g, disodium hydrogen phosphate 1,5 g, ammonium chloride 2,5 g, agar 25 g per liter of tap water, stocks at 4 °C. Parameters of tap water: pH 7.0, conductivity: 433 μs mL^{-1}, Ca: 70 mg mL^{-1}, Mg: 30 mg mL^{-1}, Na: 5.61 mg mL^{-1}. To prepare the inoculum, the stock suspension (1 mL) was transferred to a 500-mL flask containing 40 mL of sterile mineral medium "A" (without agar) and cultivated for 24 h at 30 °C on an orbital shaker (120 rpm). Before sterilization by autoclaving (121 °C, 20 min) pH of the medium was adjusted to 6.8.

2.3. Bioremediation of soil

2.3.1. Soil samples

Sandy loam soil with low organic matter content collected from an uncontaminated residential area in Lodz province, Poland was used in the experiment. The sample was taken from a depth of 10–50 cm as a bulk and sieved at 2 mm to remove large debris and ensure homogeneous mixing. The sieved soil was stored at 4° C until used (Olk et al., 2006). Details of some physicochemical and biological characteristics of the experimental soil: Clay (%) (≤2 μm) 16, Silt (%) (2–50 μm) 19, Sand (%) (≥50 μm) 65, Bulk density (g cm^{-3}) 1.52, pH (1:3) H_2O 5.5, EC (mS cm^{-1}) 0.62, TOC (%) 2.1, Total N (%) 0.14, C:N 15:1, Avail. P (mg kg^{-1}) 37.7 ± 0.26, Ca (mol kg^{-1}) 2.00 ± 0.15, Mg (mol kg^{-1}) 1.00 ± 0.10, K (mol kg^{-1}) 0.45 ± 0.01, Na (mol kg^{-1}) 0.11 ± 0.01, Al^{3+} 0.95 ± 0.03 (mol kg^{-1}) (Bouyoucos, 1962; Nelson and Sommers, 1982; Franzluebbers et al., 1994; Sparks et al., 1996; Pietrzyński, 2000; Reeuwijk van, 2002).

2.3.2. Determination of the effect of TBHQ on the dynamics of biodiesel biodegradation

Bioremediation processes were conducted for 60 days in laboratory conditions using 2 L glass vials containing 1.8 kg of soil contaminated with biodiesel with TBHQ or biodiesel without TBHQ in the 5% w/w initial concentration. Higher concentration of biodiesel in soil may cause a reduction in FAME degradation, regardless of TBHQ presence. In the previous work ratios of the inoculum to pollutant have been verified to ensure that they were sufficient for effective biodegradation (Wieczorek et al., 2015). The soil was inoculated (40 mL, OD_{600nm} of 0.3 ± 0.01) with a one-day liquid culture of *Sarcina* spp. or *A. xylosoxidans* G21, prepared as described