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An improved test for the evaluation of hydrocarbon degradation capacities of diesel-contaminating microorganisms

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ABSTRACT

The development of a test to evaluate the degradation of semi-volatile fuels as diesel by microorganisms is presented. This method is based on the principles described in the CEC-L-103 standard procedure that is exclusively meant for testing the biodegradability of non-volatile lubricants. Therefore, significant modifications involve aseptic conditions for testing specific microorganisms and conducting the test in closed vessels avoiding evaporation losses, while fuel quantification using gas chromatography-flame ionization detection (GC-FID) is retained. It is suggested that the modified procedure should enable routine application for semi-volatile hydrocarbon-based fuels. GC-FID provides additionally valuable information on the alteration of fuel component patterns during biodegradation. The procedure was successfully tested using two bacteria (*Pseudomonas aeruginosa* and *Sphingomonas* sp.) and two yeasts (*Moesziomyces* sp. and *Candida* sp.) isolated from real diesel contamination cases. All tested microorganisms caused a significant degradation of diesel fuel achieving hydrocarbon degradation percentages ranging from 23% to 35%. Specific aspects on the test modification and prospects for further modification regarding targeted investigations in the field of fuel contamination by microorganisms are briefly discussed.

1. Introduction

Microbial contamination of fuels leads to decreased product quality and can compromise the performance of fuel systems and engines by biofouling and microbiologically influenced corrosion processes (Passman, 2013). Since admixtures of biodiesel are more conductive to microbial contamination (Sorensen et al., 2011; Soriano et al., 2015), fuel biodeterioration becomes ever more relevant as biodiesel-containing fuel formulations are increasingly used (Schleicher et al., 2009; Passman, 2013).

Numerous studies in the last two decades have demonstrated that a wide variety of bacteria and fungi, including molds and yeasts, may colonize fuels such as diesel, biodiesel and kerosene (Gaylarde et al., 1999; Rauch et al., 2006; Yemashova et al. 2007; Rodriguez-Rodriguez et al., 2009, 2010; Sorensen et al., 2011; Suflita et al., 2012; Martin-Sanchez et al., 2018). In addition, many of these microbes may actually degrade contaminated fuels (Itah et al., 2009; Junior et al., 2009; Buddie et al., 2011; Soriano et al., 2015). Generally, microbiological reports have been focused on microbiomes and their effects on fuel properties. However, testing methods for fuel biodegradation are needed to assess the deteriorative potential of microbial contaminants as well as their potential use in bioremediation of contaminated environments.

Multiple approaches have been used to investigate the ability of microorganisms to degrade fuels. Culture-based approaches varied regarding the type of salt solution (Bushnell and Haas, 1941; Richard and Vogel, 1999), the ratio of salt solution and fuel typically added as sole carbon source, inoculum preparation, and incubation conditions (Zhang et al., 1998; Itah et al., 2009; Junior et al., 2009; Schleicher et al., 2009; Buddie et al., 2011; Sorensen et al., 2011; Bücker et al., 2014; Gassen et al., 2015; Soriano et al., 2015). Hydrocarbon degradation was investigated via CO_2 evolution (Zhang et al., 1998; Richard and Vogel, 1999; Soriano et al., 2015) and/or oxygen consumption (Soriano et al., 2015), using gas chromatography (Richard and Vogel, 1999; Buddie et al., 2011) or gravimetry (Itah et al., 2009). Additionally, fuel properties (Schleicher et al., 2009; Rodriguez-Rodriguez et al., 2010; Bücker et al., 2014; Soriano et al., 2015) and biomass growth (Rodriguez-Rodriguez et al., 2010; Sorensen et al., 2011; Bücker et al., 2014; Martin-Sanchez et al., 2018) were frequently monitored in this kind of studies by using different techniques. However, these procedures do not allow assessing the impact of isolated microorganisms on the chemical composition of volatile fuels such as diesel by means of a straight

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forward procedure. In addition, there is no harmonized protocol appropriate for diesel degradation testing of isolated microbes despite it is considered as a particularly vulnerable fuel to microbial deterioration (Yemashova et al. 2007; Passman 2013, Schleicher et al., 2009).

The goal of this study was to develop a straight-forward procedure to test the diesel degrading ability of specific microorganisms, which should be robust enough to be the basis of a future standard test method. The protocol needs to address crucial features such as enabling the testing of semi-volatile substrates, quantification of degradation and providing information of changes in chemical composition due to microbial activity. Gas chromatography with flame ionization detection (GC-FID) is used for substrate quantification by the standard test method CEC-L-103 (Völz, 2012; CEC, 2014), which is explicitly applicable to non-volatile lubricants only. While the potential of GC-FID to provide detailed information on compositional changes is limited in case of the lubricants, changes of distinct features in diesel fuel can be monitored with this method. Thus, the principle of CEC-L-103 was modified to allow testing semi-volatile fuels with the added value of characterizing structure-related degradation of different substrate components. The resulting protocol was evaluated by testing two bacteria and two fungi which were isolated from real diesel contamination cases.

2. Materials and methods

2.1. Chemicals, nutrients and materials

Culture media: Tryptic soy broth, glucose monohydrate and peptone from soymeal were from Merck (Darmstadt, Germany), malt extract and Agar-Agar Kobe I were from Carl Roth GmbH (Karlsruhe, Germany).

Salts for mineral substrate (MS): KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $ZnSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, $CuSO_4{\cdot}5H_2O$, and $FeSO_4{\cdot}7H_2O$ were from AppliChem GmbH (Darmstadt, Germany), $K_2HPO_4{\cdot}3H_2O$ was from Merck KGaA (Darmstadt, Germany), $Na_2HPO_4{\cdot}7H_2O$ was from Honeywell Riedel-de Haën AG (Seelze, Germany), NH_4Cl (Merck KGaA), H_3BO_3 was from Scharlau Chemie S.A. (Barcelona, Spain), $CoSO_4$ and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Chemicals for testing and analysis: $MgSO_4{\cdot}7H_2O$ and tetrahydrofuran (THF, Chemsolute p. A., 99.9%) were from Th. Geyer (Remmingen, Germany), anhydrous Na_2SO_4 and concentrated sulfuric acid were from Merck (Darmstadt, Germany), *n*-heptane (picograde) was from LGC Promochem (Wesel, Germany), *n*-tetracontane was from Fluka (Buchs, Switzerland).

Ultra-low sulfur diesel (ULSD) was purchased at a nearby service station, which, according to the European Standard EN590, may contain up to 7% fatty acid methyl esters. Mineral oil light (CAS-No. 8042-47-5) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) was used as reference oil. ProClin 150 biocide (1.5% active microbiocidal compounds, 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one) was from Sigma-Aldrich Co. (St. Louis, MO, USA).

Materials: 100 mL Borosilicate 3.3 Erlenmeyer flasks from Isolalab Laborgeräte GmbH (Wertheim, Germany) with screw caps with PTFE seals from Bola, Bohlender GmbH (Grünsfeld, Germany).

2.2. Microorganisms

Two bacteria and two fungi, isolated from contaminated diesel tanks in Malaysia, were used in this study (Table 1). Some preliminary culture experiments qualitatively showed their ability to grow better in presence of fuels such as kerosene and ULSD (data not shown).

Molecular identification of microorganisms was performed by PCR and sequencing of ribosomal markers, 16S rDNA gene (16S) for bacteria, and rDNA internal transcribed spacers (ITS) for fungi. Genomic DNA extraction, primers and PCR conditions, as well as Sanger sequencing, were performed as described elsewhere (Martin-Sanchez et al., 2018). Obtained sequences were submitted to the European Nucleotide Archive (ENA, EMBL-EBI) under accession numbers LT897777-LT897780 (https://www.ebi.ac.uk/ena/data/view/LT897777-LT897780). Identification of strains was based on comparison of their rDNA sequences with GenBank using the BLASTn algorithm from EzBioCloud's Identify Service for bacteria or the National Center for Biotechnology Information (NCBI) for fungi.

2.3. Diesel biodegradation test

Preparation of microbial inocula: Bacteria were cultivated on TSB overnight at 25 °C with orbital shaking (100 rpm). The resulting bacterial biomass was separated by centrifugation at 7800 rpm, washed and re-suspended using sterile MS (composition according to CEC-L-103, CEC, 2014). The optical density at 600 nm (OD_{600}) of bacterial solutions was fixed to 0.25–0.5 (equivalent to 2–4 × 10^8 cells·mL^{-1} in *Escherichia coli*). Fungi were cultivated on malt extract agar at 25 °C for five days. Similar to bacteria preparation, the fungal biomass was washed, homogenized and suspended in sterile MS. Fungal cell concentration was calculated using a Neubauer chamber. The colony forming units (cfu) for final inocula were calculated on tryptic soy agar and malt extract agar, for bacteria and fungi respectively. Final concentrations of inocula ranged from 2.2 to 5.2 × 10^8 cfu mL^{-1} for bacteria, and from 4.2 × 10^6 to 1.1 × 10^8 cfu mL^{-1} for fungi.

For each studied microorganism, twenty 100 mL-Erlenmeyer flasks were prepared as follows: Seven inoculated flasks with diesel (three for the initial time -day 0- and four for 21 days), seven inoculated flasks with reference oil (0 and 21 days), two control flasks with diesel and biocide, two control flasks with reference oil and biocide, and one neutral flask (nutrient solution only). An additional flask with diesel was included as microbiological control. All flasks were initially sterilized in the autoclave (121 °C for 15 min) and filled out with 50 mL of sterile MS. In the appropriate flasks were also added 100 µL of ULSD or reference oil solution in THF (25 mg mL^{-1} equivalent to 2.5 mg per flask), 100 µL of microbial inoculum, and 30 µL of ProClin 150 biocide (control flasks). Such small volumes (30–100 µL) were transferred using a Research Plus pipette and epTIPS Standard (Eppendorf, Wesseling-Berzdorf, Germany). The flasks for initial time (0 days) were immediately submitted to total hydrocarbon quantification. The other flasks were incubated in the dark at 25 °C for 21 days with continous orbital shaking at 100 rpm. Afterwards, the microbiological control flask was used for re-isolation of inoculated microorganism to exclude any contamination during the study. The remaining flasks were submitted to total hydrocarbon content quantification (see section 2.4).

To adapt this test for diesel, a semi-volatile fuel, an initial experiment evaluating the evaporation loss using different stoppers and set-ups was conducted including screw caps (i) closed, (ii) one opening after 10 days of incubation (1 h inside a class II biosafety cabinet), (iii) two such openings after 7 and 14 days of incubation, and (iv) permanent aeration using cotton plugs. A total of fifteen 100 mL-Erlenmeyer flasks (three replicates for 0 days and three replicates per set-up for 21 days), with 50 mL mineral substrate and 100 µL of ULSD solution, were included in this experiment. After this initial experiment, for each studied microorganism (Table 1), the degradation test was performed in two variants, using (i) closed screw caps or (ii) screw caps with two aeration times after 7 and 14 days.

Quality control/quality assurance during test development: All glassware was carefully cleaned to avoid contamination with hydrocarbons. Teflon cock in separation funnels were kept grease-free. $MgSO_4$ and $NaSO_4$ blank TPH content below detection limit. Dispensing of the THF solutions of ULSD and reference oil was done using a micropipette with a standard deviation of 0.8% (n = 10).

2.4. Quantification of diesel using GC-FID and identification of fatty acid methyl esters

Each flask was adjusted to pH 2 with H_2SO_4. Then, 8 g of