International Biodeterioration & Biodegradation 108 (2016) 122–126

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Short communication

Biodegradation of biodiesel and microbiologically induced corrosion of 1018 steel by *Moniliella wahieum* Y12

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ARTICLE INFO

Article history:
Received 24 August 2015
Received in revised form
22 October 2015
Accepted 30 November 2015
Available online 28 December 2015

Keywords:
Microbiologically influenced corrosion
Biodiesel
Biodegradation
Corrosion
Moniliella wahieum Y12
MIC

ABSTRACT

A putatively novel basidiomycetous fungus termed *Moniliella wahieum* Y12^T was isolated from a 20% biodiesel blend. The strain maximally degraded biodiesel at a rate of 3.56×10^{-2} mg/h during log phase growth. Induction of metal corrosion by the strain in a mass loss procedure using 1018 metal coupons showed total mass reduction exceeded that in controls by 70% through 30 days. Enhanced corrosion was observed at the pellicle and due to medium acidification. This is the first investigation of a *Moniliella* sp. and its impact on biodiesel stability and 1018 steel corrosion. (*M. wahieum* Y12^T = ATCC MYA-4962).

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1. Introduction

Analysis, control and mitigation of microbial contamination of biofuels are important operational considerations. Such contamination may reduce fuel stability, enhance biofouling, and induce corrosion of fuel-related components. Biodiesel is also more likely to be contaminated by microbes than is petroleum diesel (Zhang et al., 1998; Lee et al., 2009; Achten et al., 2010; Stamper et al., 2011). Diverse *Fungi* and *Bacteria* have been implicated in biofuel contamination (Chao et al., 2010; Bücker et al. 2011), a phenomenon directly attributed to biodiesel's hygroscopicity (Passman et al. 2009). Biodiesel's water adsorption and subsequent phase separation promotes microbial growth. Moreover, biodiesel's chemical and biological hydrolysis to fatty acids provides labile carbon for sustained growth, which in turn results in acidification and microbiologically influenced corrosion (MIC) (Passman, 2003; Leung et al., 2006; Corseuil et al., 2011; Passman, 2013). Enhanced oxidation rates through formation of anoxic environments and biofilms exacerbate MIC (Dzierzewicz et al., 1997; Rajasekar, 2010: Satoh et al., 2009: Stamper et al., 2011). We describe here the isolation of a putatively novel basidiomycete, *Moniliella wahieum* Y12^T from a 20% biodiesel blend (B20), and the strain's impact on biodiesel stability, and 1018 steel corrosion (Active Standard ASTM A1018/A1018M).

2. Materials and methods

2.1. Sample description

One liter of biodiesel (B20) blend with visible water contamination was collected from an in use fuel storage tank by the City and County of Honolulu. The sample provided for the work described here had visibly separated into an upper fuel layer, an observable film at the interface, and lower opaque, brown water layer.

2.2. Isolation and identification of *M. wahieum* Y12^T

The film at the interface of the fuel and aqueous layer was aseptically transferred to and serially diluted to 10^{-8} in YM broth

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http://dx.doi.org/10.1016/j.ibiod.2015.11.027
0964-8305/Published by Elsevier Ltd.

(containing per liter of distilled water: 10 g dextrose, 5 g peptone, 3 g malt extract and 3 g yeast extract, pH 7.0). Sub-samples (100 μl) from each dilution were spread-plated onto YM agar (as YM broth, but with 15 g per liter Difco agar) in 100 mm diameter Petri plates, and incubated at 30 °C for one week. Single putative bacteria and fungi colonies that arose were picked and streaked onto fresh YM agar plates and incubated for one week at 30 °C. A strain designated Y12 was then aseptically transferred to 2 ml of YM broth in a 15 ml polypropylene tube, and incubated with shaking at 30 °C for 1 week.

Genomic DNA was extracted from 1 ml of the Y12 culture described above. Biomass was collected by centrifugation at 5000 *g* for 5 min; genomic DNA was extracted from the pelleted biomass in the MoBio UltraClean® Microbial DNA extraction kit according to the manufacturer's instructions. Extracted DNA was used as the template in polymerase chain reactions (PCR) with the primer pairs D1/D2 (NL1/D1, and D2/NL4) (Kurtzman and Robnett, 1997), with Roche *Taq* DNA polymerase in 25 μl reactions: Initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 min, 50 °C for 1 min, 72 °C for 45 min, and a final extension at 72 °C for 7 min. PCR products were checked for size on a 1% TBE agarose gel in TBE buffer and stained with ethidium bromide. Fragments of the expected size (~680 bp) were visualized under UV light, excised and purified in the Qiagen QIAquick® gel extraction kit and sequenced in the 'Advanced Studies in Genomics, Proteomics and Bioinformatics' core facility at the University of Hawai'i at Mānoa. Sequence chromatograms were manually edited and consensus sequences obtained in Vector NTI. The nearest 100 phylogenetic neighbors were determined through a BLAST comparison of the 26S consensus sequences (Altschul et al., 1997).

2.3. Biodiesel degradation

Biodiesel (B100) was obtained directly from a local manufacturer, Pacific Biodiesel (1003 Makepono St., Honolulu, Hawai'i). Pacific Biodiesel was then the only large-scale commercial supplier in Hawai'i, and also provided biodiesel to the City and County of Honolulu. The company's biodiesel is produced from waste cooking oils. Blend B100 was initially profiled in a Scion Bruker 400-GC Series GC/MS with a 15 m × 0.25 BR-5 guard column; 60 m × 0.25 BR-1701 analytic column, source temperature set at 250 °C, and transfer temperature at 260 °C. Hydrocarbons were compared against a FAME reference standard (FAMQ-005) from AccuStandard Inc. (New Haven, CT, USA) (Fig. 1).

A loop of Y12 was transferred from YM agar to 3 ml of YM broth and incubated with shaking for 24 h at 30 °C. Cells were then harvested by centrifugation at 5000 × *g* for 1 min. Labile carbon was removed by washing the cells three times in a modified M9 minimal medium ($MgSO_4$ (1 M, 2 ml) and $CaCl_2$ (1 M, 100 μl) in 780 ml distilled water, 200 ml (5X) M9 salts (comprising $Na_2HPO_4 \cdot 7H_2O$, 64 g; KH_2PO_4, 15 g; NaCl 2.5 g; NH_4Cl 5.0 g) pH 7.2) and resuspended in 3 ml of M9 minimal medium. 20 μl of the washed cell suspension was aseptically transferred to each of three 25 × 150 mm glass test tubes containing 1 ml of modified M9 minimal medium and 2% v/v of biodiesel that was pre-sterilized by filtration through a 0.22 μm PTFE membrane, and incubated at 30 °C with shaking.

Every 24–48 h through 1 week a sub-sample of the medium below the fuel layer was collected and spread on YM agar for CFU determination. The fuel remaining at the end of the week was extracted by addition of 1 ml of hexane. Qualitative reduction of biodiesel was determined in a Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionization detector (GC/FID) and Agilent DB-5 analytic column, using the following program; 2 min at 80 °C, ramped at 15 °C/min to 300 °C, and held for 5 min. Controls comprised non-inoculated modified M9 medium containing 2% v/v biodiesel. Percentage loss of the biodiesel remaining after the incubation was calculated through peak area reduction.

Relative content loss was determined by calculating mean reduction of the three largest peaks in the GC/FID analysis. Peaks used were observed at retention times 14.194, 16.060 and 16.126 min. The method did not have the resolution observed in GC/MS, and resulted in co-elution, but analyte reduction was clearly evident. As determined by standard peak comparisons, elution times utilizing GC/FID correlated with the GC/MS chromatogram as follows; 14.194 min. – hexadecanoic acid methyl ester; 16.060 min. – co-elution of 9,12- octadecenoic acid methyl ester and 9-octadecenoic methyl ester; 16.126 min. – co-elution of methyl stearate and 9, 12, 15- octadecatrienoic acid methyl ester.

2.4. Oxidation of 1018 metal coupons

Metal corrosion was determined through gravimetric loss by oxidation of 1018 steel coupons. Triplicate metal coupons were weighed on a Mettler analytical balance prior to placement in individual 100 ml autoclaved jars containing 40 ml of modified M9 minimal medium with 2% v/v biodiesel. Y12 was grown overnight in 3 ml of YM broth and harvested by centrifugation at 5000 × *g* for 1 min. Labile carbon was removed by triple washing in a modified M9 minimal medium, as described above. Cells were resuspended in modified M9 medium and used to inoculate replicates, including non-inoculated controls. Of the suspension of washed cells, 1 ml was used to inoculate each experimental jar. Controls were inoculated with 1 ml of sterile M9 medium. Aerobic conditions were maintained in the jars through a 0.22 μm filter inserted into a hole in the cap. Jars were shaken at 50 rpm at 30 °C.

Mass loss was determined after incubation as described above for 30 and 60 days. Metal coupons were removed and submerged in a solution of diammonium citrate (200 g in 1 L distilled water) at 80 °C for 20 min (International Organization for Standardization, method ISO 8407:1991 designation C3.4). Coupons were air dried before weighing and mass loss calculation.

3. Results

3.1. Isolation and identification

A filamentous fungus was cultivated from a film observed at the interface of the fuel and aqueous layers in contaminated biodiesel (B20) obtained from the City and County of Honolulu. A BLAST comparison of a 565 nt fragment of the D1/D2 region of the 26S rRNA gene showed the strain shared 98% sequence identity with its nearest described relative, *Moniliella suaveolens* var. *nigra* CBS 542.78^T (AF335524) (Altschul et al., 1997). Strain Y12 has been tentatively assigned to the genus *Moniliella* as the type strain of *M. wahieum* Y12^T (ATCC MYA-4962) (Fig. 2).

3.2. Biodiesel degradation

Biodiesel degradation during cultivation of Y12^T was determined through loss of chromatographic peaks from B100 biodiesel obtained from a local manufacturer (Pacific Biodiesel, 1003 Makepono St., Honolulu, Hawaii). Three dominant chromatograph peaks determined by GC/FID were used in the loss determination and plotted against cell density (Fig. 3A, B).

According to standard peak comparisons, elution times in GC/FID correlated with the GC/MS chromatogram as follows: 14.194 min. – hexadecanoic acid methyl ester; 16.060 min. – co-elution of 9,12- octadecenoic acid methyl ester and 9-octadecenoic methyl ester; 16.126 min. – co-elution of methyl