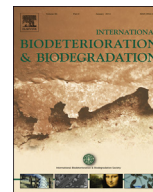




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Short communication

Biodegradation of biodiesel and microbiologically induced corrosion of 1018 steel by *Moniliella wahieum* Y12Travers H. Ching^{a,b}, Brandon A. Yoza^{a,*}, Ruijin Wang^{a,b}, Stephen Masutani^a, Stuart Donachie^c, Lloyd Hihara^d, Qing X. Li^b^a Hawaii Natural Energy Institute, 1680 East West Rd. POST #109, University of Hawai'i at Mānoa, HI 96822, USA^b Department of Molecular Biosciences and Bioengineering, 1955 East West Rd. Ag. Science #218, University of Hawai'i at Mānoa, HI 96822, USA^c Department of Microbiology, 2538 McCarthy Mall, Snyder Hall 207, University of Hawai'i at Mānoa, HI 96822, USA^d Department of Mechanical Engineering, 2540 Dole St. #302, University of Hawai'i at Mānoa, HI 96822, USA

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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; Bückner 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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(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 (MgSO₄ (1 M, 2 ml) and CaCl₂ (1 M, 100 µl) in 780 ml distilled water, 200 ml (5X) M9 salts (comprising Na₂HPO₄ · 7H₂O, 64 g; KH₂PO₄, 15 g; NaCl 2.5 g; NH₄Cl 5.0 g) pH 7.2) and re-suspended 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

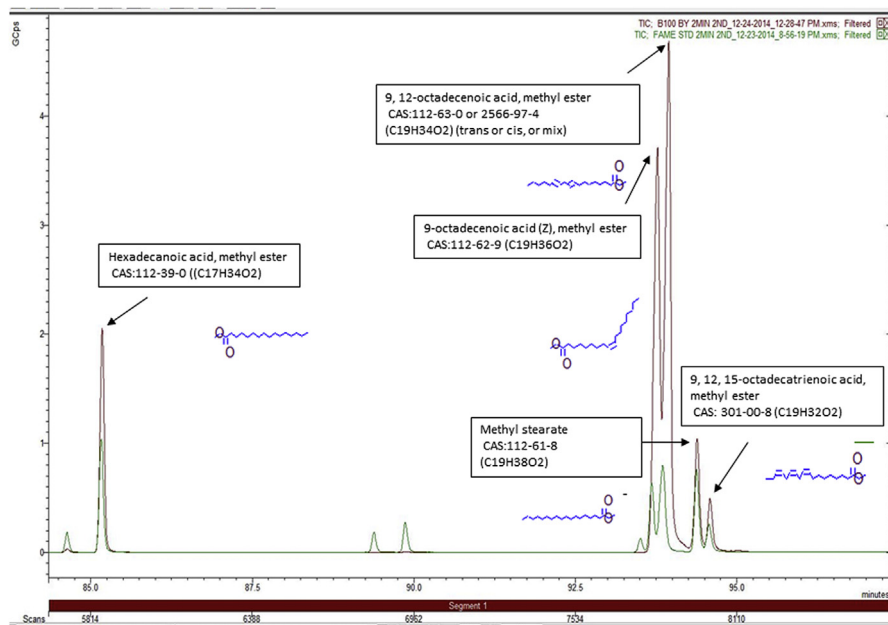


Fig. 1. Comparison of B100 from Pacific Biodiesel against a FAME reference standard (FAMQ-005, AccuStandard Inc., New Haven, CT, USA) in a Scion Bruker 400-GC Series GC/MS with a 15 m x 0.25 BR-5 guard column; 60 m x 0.25 BR-1701 analytic column, source temperature 250 °C, and transfer temperature 260 °C. FAMES are principally composed of C17 and C19 hydrocarbons.



Fig. 2. Maximum likelihood tree showing the relationship of *Moniliella wahieum* Y12^T with type strains of species in the same and related genera on the basis of approximately 90% of the nucleotides in the 26S D1/D2 region. Sequences from two *Tilletia* spp. were used as outgroups. Bootstrap values for 500 replicates are shown at each branch. Scale bar represents 5 substitutions per 100 nt.

stearate and 9, 12, 15- octadecatrienoic acid methyl ester (Fig. 3). This biodiesel was degraded at a maximum rate of 3.56×10^{-2} mg/h during log phase growth. By 70 h, biodiesel in solution was completely utilized and logarithmic cell growth ceased. Degradation rates for all fatty acid methyl esters in the biodiesel sample were not determined. However, dominant esters in this fuel sample correlated with those comprising 17 and 19 carbon atoms, with varying degrees of saturation.

3.3. Oxidation of 1018 metal coupons

Composition and carbon content of 1018 steel coupons used here approximately matches that of material used to build many fuel containment vessels. Our data show this carbon steel is

oxidized at an elevated rate during biodiesel degradation in the presence of *M. wahieum* Y12^T (Fig. 4). Metal coupons in the presence of only biodiesel and medium showed an average mass reduction of 0.49 ± 0.22 mg after 30 d, and 0.85 ± 0.07 mg after 60 days. Mass loss from coupons in the same medium in the presence of Y12^T exceeded that in controls by 70% after 30 days, and 50% after 60 days (mass reduction of 0.85 ± 0.06 mg, and 1.30 ± 0.14 mg after 60 days). The pH of the negative control remained the same (pH 7), whereas that of the B100 had fallen to 5.

4. Discussion

Biological contamination of fuels has been known for some time (cf. Gaylarde et al. 1999). The fundamental interaction in such

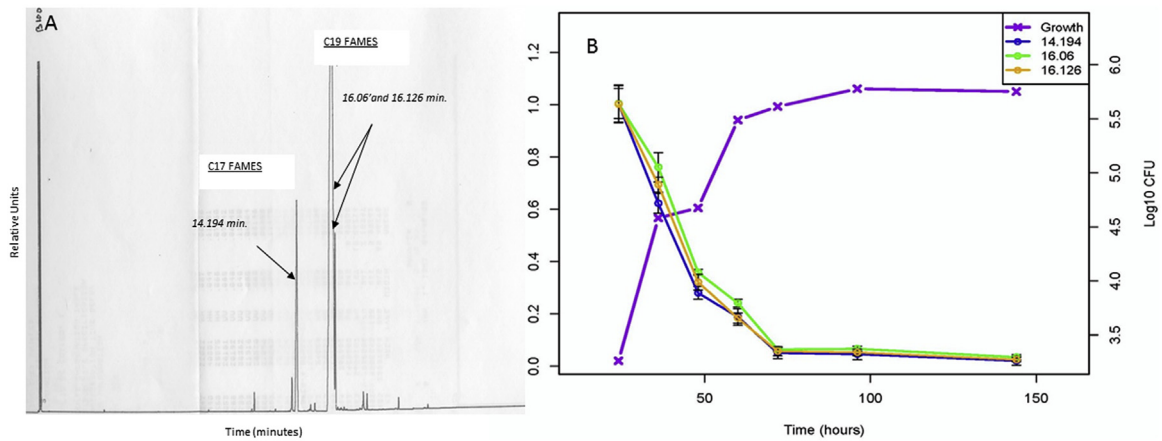


Fig. 3. A) GC chromatogram of B100. The program used was 2 min at 80 °C, ramp at 15 °C/min to 300 °C, and hold for 5 min. The three peaks used for evaluation are observed at 14.194, 16.06 and 16.126 min. B) Biodiesel degradation by *Moniliella wahieum* Y12^T. Relative concentration of biodiesel, determined by reduction of the three most prominent chromatogram peaks, is displayed on the y1 axis. Maximum CFU/ml occurred at 96 h (y2 axis).

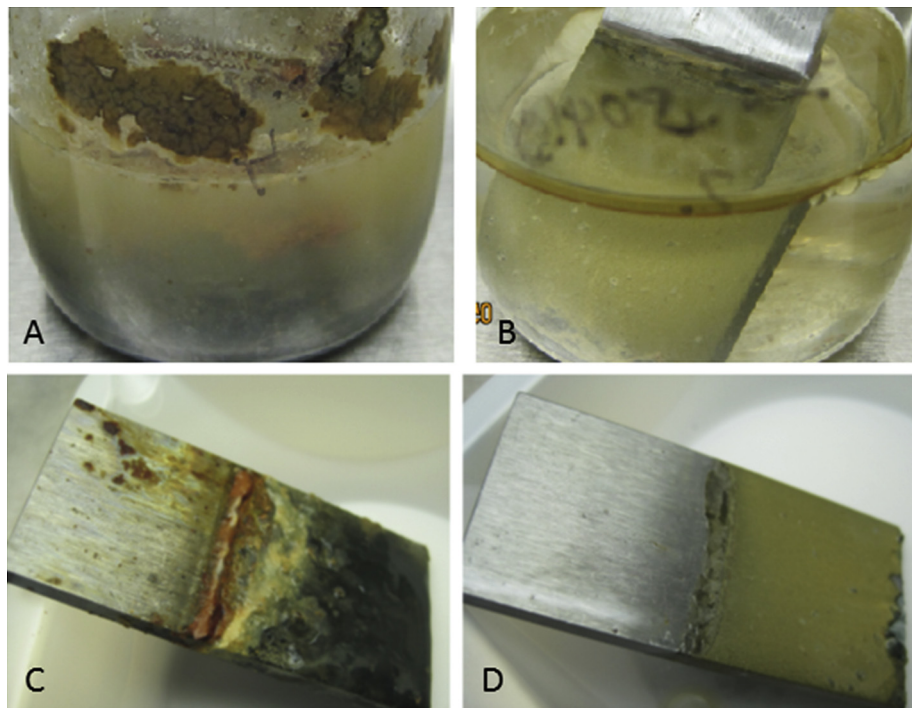


Fig. 4. Microbiologically Induced Corrosion of a 1018 metal coupon by *Moniliella wahieum* Y12^T, with biodiesel as the carbon source. A) Inoculated sample containing 2 ml biodiesel and 40 ml modified M9 minimal medium. Significant growth was observed on the side of the containers and occurs also on the 1018 coupon. B) Control sample and fuel layer after 60 days incubation. C) Visible MIC on the metal coupon after 60 days incubation. D) Chemical oxidation of the control coupon after 60 days incubation.

contamination is the ability of a microorganism to utilize a chemical component as a growth substrate. Transfer of carbon through the production of metabolites can subsequently support other metabolisms, and also chemically impact the local environment (Passman, 2013). Here, we investigated the ability of *M. wahieum* Y12^T to utilize FAMES as sole carbon sources, and the subsequent impact on corrosion. Many microorganisms thrive on biodiesel as their sole carbon source (Zhang et al., 1998). Of the three isolates purified from our sample described here, *M. wahieum* Y12^T was the only one considered potentially novel on the basis of the rRNA gene nucleotide sequence. Since to our knowledge no *Moniliella* sp. has been characterized as a fuel contaminant, we chose to focus our investigation of microbiologically induced corrosion on this

organism. Moreover, due to the complexity of interactions that occur in a natural community we chose also to study this process in a pure culture of *M. wahieum* Y12^T. The degree and rate of biodiesel degradation are dependent on the component hydrocarbons' saturation and fatty acid chain length (Knothe, 2005). *M. wahieum* Y12^T did not appear to prefer a specific hydrocarbon, and complete utilization of both saturated and unsaturated FAMES was observed at nearly identical rates (Fig. 3B). Differences between biodiesel biodegradability could also be explained by differences in their physicochemical properties, mainly viscosity. Higher viscosity results in decreased bioavailability and slower biodegradation (Corseuil et al. 2011). Consistent with this observation, degradation rates we determined were also limited by bioavailability. Available

carbon occurs only at the phase interface through increased surface area due to emulsion formation, or smaller droplet sizes due to mechanical disruption in aqueous media. While all of our experiments were performed in a shaker incubator, the degradation rates depended on how well the biodiesel dispersed. Our isolates were obtained only in aerobic conditions. Oxygenic conditions also have an impact on biodiesel degradation rates and has been shown to also occur at high rates during anaerobic respiration (Aktas et al. 2010). Oxidation and acidification are typically observed during diesel fuel degradation (Prankl and Schindlbauer, 1998); biofilm formation is also recognized as a contributory factor in metal corrosion (Achten et al., 2010; Damon, 1941). A reduction in pH was observed during Y12^T growth here, with medium pH falling from 7.0 to 5.3 after 30 days; this may arise through products of beta-oxidation including acetic acid, smaller fatty acids and ultimately CO₂. We also observed film formation at the fuel/water interface on the metal coupon, and on inner surfaces of the jar (Fig. 4). Further studies will determine the nature of this film.

5. Conclusion

The capacity to degrade biodiesel and corrode 1018 steel coupons was investigated in *M. wahieum* Y12^T cultivated from a contaminated biodiesel (B20) blend. Biodiesel can be utilized as the sole carbon in a minimal medium in aerobic conditions, resulting in acidification of the medium. Although many isolates are capable of thriving on biodiesel as their sole carbon source, *Moniliella* spp. have not thus far been described as being involved in fuel contamination. Indeed, few environmental isolates have been used to simultaneously investigate biological corrosion and degradation rates.

The presence of *M. wahieum* Y12^T in retail fuel system components or underground storage tanks would be detrimental through direct degradation of fuel and the development of biofilms that result in biofouling and metal corrosion. It is important to consider that metabolite production by *M. wahieum* may also provide bioavailable carbon to species unable to directly metabolize biodiesel. Such transfer of carbon could support additional biological processes with the potential to facilitate sustained corrosion, enhanced degradation and biofouling. While analysis of natural communities is important, primary metabolism in the sequence of carbon transfer is supported by the ability to first utilize biodiesel as bioavailable carbon. Investigating this metabolism will determine how we can reduce the negative impacts of this community. Studying the whole genome of *Moniliella* and differential expression of its proteome should provide insights that will facilitate rapid identification and prevention of contamination. Few isolates have been specifically described in terms of their ability to degrade biodiesel, so *M. wahieum* Y12^T has been deposited in the ATCC as MYA-4962 with such investigations in mind.

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