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Biodiesel from Microalgae, Yeast, and Bacteria: Engine Performance and Exhaust Emissions

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Supporting Information

ABSTRACT: Biodiesels (fatty acid methyl esters) derived from oleaginous microbes (microalgae, yeast, and bacteria) are being actively pursued as potential renewable substitutes for petroleum diesel. Here, we report the engine performance characteristics of biodiesel produced from a microalgae (*Chaetoceros gracilis*), a yeast (*Cryptococcus curvatus*), and a bacteria (*Rhodococcus opacus*) in a two-cylinder diesel engine outfitted with an eddy current brake dynamometer, comparing the fuel performance to petroleum diesel (#2) and commercial biodiesel from soybeans. Key physical and chemical properties, including heating value, viscosity, density, and cetane index, for each of the microbial-derived biofuels were found to compare favorably to those of soybean biodiesel. Likewise, the horsepower, torque, and brake specific fuel consumption across a range of engine speeds also compared favorably to values determined for soybean biodiesel. Analysis of exhaust emissions (hydrocarbon, CO, CO_2, O_2, and NO_x) revealed that all biofuels produced significantly less CO and hydrocarbon than petroleum diesel. Surprisingly, microalgae biodiesel was found to have the lowest NO_x output, even lower than petroleum diesel. The results are discussed in the context of the fatty acid composition of the fuels and the technical viability of microbial biofuels as replacements for petroleum diesel.

INTRODUCTION

Biodiesel is a renewable liquid transportation fuel that can be used to displace petroleum-derived diesel fuel without significant modifications to existing engines or fuel distribution networks. Biodiesel consists of alkyl esters of fatty acids and are typically produced from triglycerides (e.g., soybean oil) and an alcohol (e.g., methanol) in the presence of either a base or acid catalyst.[1−3] This process, called transesterification, forms fatty acid methyl esters (FAME) where the properties of the biodiesel are dependent upon the fatty acid composition of the feedstock oil.[4−6] Biodiesel derived from bio-oils has several properties that make it a good renewable liquid fuel. It is fully miscible with petroleum diesel and can be blended at any ratio. Biodiesel consistently shows reduced exhaust emissions compared to petroleum diesel; many studies have concluded that biodiesel use results in the reduction of unburned hydrocarbon, particulate, and CO emissions.[7] In contrast to these improvements in emissions for biodiesel, most emissions studies have found that biodiesel produces more NO_x emissions than petroleum diesel.[8]

The majority of current biodiesel used in the United States (U.S.) is derived from oilseed crops (e.g., soybeans), thereby competing with food products and requiring quality farmland. At current production levels, traditional crops (corn or soybean) are only capable of meeting a small fraction of the U.S. transportation fuel demand.[9] This highlights the need for alternative feedstocks that could be more productive than traditional oilseed crops and utilize marginal land unsuitable for cultivation. Oleaginous microorganisms, such as microalgae, yeast, and bacteria, are being actively investigated as promising potential feedstocks for biodiesel production.[10−12]

There is considerable interest in the use of microalgae for biofuel production.[13,14] Some strains of microalgae are capable of accumulating a significant quantity of triacylglycerol (TAG), amounting to 20−50% of their cellular dry weight (CDW).[13,15−17] Microalgae, like plants, utilize the energy of the sun and carbon from CO_2 to grow and make lipids. Although potential productivity estimates for microalgal biodiesel vary widely, many reports agree that microalgae has the potential to yield more oil than the most productive oilseed crops.[16,18] An important advantage that microalgae have over plants is their ability to be cultivated on marginal land with water that is unsuitable for irrigation (e.g., saltwater, saline aquifer, and wastewater).[19] This feature alone could allow for widespread microalgae cultivation that does not compete with traditional agriculture for limited land and water resources. Although promising, several technical challenges remain to be resolved before widespread use of microalgae derived fuels can be achieved, including more economical methods for harvesting and dewatering, and a better understanding of how microalgae fuels will perform in engines.

In addition to microalgae, a few select species of oleaginous yeast and bacteria are also capable of accumulating oil in very high concentrations, approaching 80% CDW.[12,20,21] Yeast and bacteria require a reduced source of carbon to meet their energy needs. Many of these strains are capable of metabolizing a diverse range of carbohydrates, such as residual sugars from food production (e.g., molasses or lactose)[22,23] or biomass hydrosylate.[24] The diverse metabolism of oleaginous microbes

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permits the production of a significant amount of oil for biofuel use without impacting the food supply. A substantial amount of low-cost carbon is available to produce biodiesel through fermentation with oleaginous microorganisms without competing with food production.[25]

The properties of biodiesel produced from vegetable oil, animal fats, and individual pure methyl esters have been described extensively,[26] while FAME produced from microbial sources has been characterized to a much lesser extent. A recent report has concluded that biodiesel produced from the green algal strains *Ankistrodesmus braunii* and *Nannochloropsis* sp. performs comparably to petroleum diesel fuel in a diesel engine.[27] However, the authors did not compare the performance of microalgal biodiesel to commercially available and widely used biodiesel. It is also unknown how the emissions of a diesel engine might change when using microbial derived fuels. Further, not all microalgae are likely to perform identically given their large diversity in fatty acid profiles. Few reports exist that discuss the properties of biodiesel fuel produced from either yeast or bacteria and none to our knowledge that describe their performance in a diesel engine.[28−30] The lack of available data for biodiesel produced from microalgae has lead researchers to speculate what properties these fuels might have based on the known fatty acid profiles of several species of microalgae and the individual properties possessed by the corresponding FAME.[31−33]

The performance and emissions of biodiesel fuels from many plant or animal sources have been evaluated.[8] Oleaginous microorganisms represent an underutilized source of oils to produce biodiesel and could be significant in reducing our dependence on petroleum fuels. Therefore, it is important to establish the quality of biodiesel produced from microbes. We report here the properties, engine performance, and emissions for biodiesel produced from the microalgae *Chaetoceros gracilis*, the yeast *Cryptococcus curvatus*, and the bacterium *Rhodococcus opacus*. Key physical properties of each biodiesel fuel were determined and compared with commercial soybean biodiesel. Each fuel was then used to operate a two-cylinder indirect injection diesel engine attached to an eddy current dynamometer. The horsepower and torque output from the engine under load is reported for each microbial biofuel and compared to outputs obtained with both diesel fuel and soybean biodiesel. Key emissions data were collected for each biofuel without load at a steady 3500 rpm, allowing comparison to emissions from petroleum diesel and soybean biodiesel.

EXPERIMENTAL SECTION

Materials and Reagents. Petroleum diesel No. 2 (abbreviated diesel #2) was obtained from a local fuel distributor and was verified to be free of fatty acid methyl esters by gas chromatography with detection by mass spectrometry as described below. Soybean biodiesel (B100) was used as the biodiesel reference fuel for this study and was kindly provided by Dal Soglio, Inc. (Midvale, UT). Reagent-grade chemicals were used for the conversion of microbial lipids to biodiesel. Methanol was obtained from Pharmco-AAPER (Brookfield, CT) and concentrated sulfuric acid was obtained from EMD Chemicals (Gibbstown, NJ). Chloroform used in the purification of microbial FAME was obtained from Fisher Chemicals (Fairlawn, NJ).

Strains and Culture Conditions. The microalgae *Chaetoceros gracilis* (UTEX LB 2658), a diatom, was obtained from The Culture Collection of Algae at the University of Texas at Austin (UTEX). *C gracilis* was grown as described previously[15] with a modification of the method for inoculating the larger raceway culture. Larger raceway cultures (220 L), initially containing 100 L of media, were inoculated with 10 L of dense (∼500 mg dry weight (DW) L^{-1}) *C. gracilis* culture. Raceways (Separation Engineering, Escondido, CA) were constructed of fiberglass and were mixed with an attached paddle wheel. The raceways were equipped to maintain a constant pH by the introduction of CO_2. After the initial raceway culture had reached an approximate density of 0.5 g DW L^{-1}, additional media was added to bring the total volume of the culture up to 220 L. *C. gracilis* cultures were grown in either batch or sequential batch mode. In both modes the culture was harvested once the density reached an approximate value of 0.8−1 g DW L^{-1}. In batch mode the total culture volume was collected by centrifugation using a continuous centrifuge (LE model, CEPA, Lahr, Germany). For sequential batch mode operated cultures, ∼70% of the culture was harvested, leaving the rest as an inoculum. Fresh media was then added to remaining culture, starting the process over. Sequential batch cultures were operated for 2−3 cycles. Collected algal biomass was immediately frozen then dried by lyophilization (Freezone 4.5, Labconco, Kansas City, MO).

The yeast *Cryptococcus curvatus* (ATCC 20509) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). *C. curvatus* was preserved at −80 °C in YPD[34] media with 20% (% v/v) glycerol and generally cultured on YPD media. Growths for lipid accumulation were performed in a 50-L fermenter with an aeration rate of 3 standard cubic feet per minute (SCFM) at a temperature of 30 °C using media described previously.[35] The fermenter agitator consisted of 3 marine blades rotating at 225 rpm. Culture was inoculated with an overnight culture equal to 5% of the fermenter volume and allowed to grow for 5 days. Cells were collected by centrifugation, frozen, and then lyophilized.

The bacterium *Rhodococcus opacus* PD630 (DSM 44193) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained as instructed by DSMZ. Cultures were grown on defined phosphate media adapted from Kurosawa et. al and Chartrain et al.[30,36] Growths for lipid production were initiated with a 10% (% v/v) starting inoculum at an OD_{660} of 0.4 and were supplemented with either 6.43 or 9.65 g L^{-1} sodium nitrate and 80 or 120 g L^{-1} sucrose, respectively. It was found that successful growths depended highly on the dissolved oxygen content, which became limited in growths with larger amounts of sucrose due to the greater oxygen demand of higher cell densities. Although growths of *R. opacus* generated excessive amounts of foam, addition of antifoam was found to be deleterious to growth and was omitted. Growths reached maximum lipid and biomass production after 120 h. Large-scale growths were performed in a 50-L fermenter as described above for *C. curvatus* with increased agitation (700 rpm) and the addition of a secondary containment vessel to capture the excess foam produced from the growths.

Biodiesel Production from Microbial Biomass. Microbial lipids are extracted and converted to biodiesel using a direct transesterification approach developed at Utah State University.[15] Biodiesel production from yeast, bacteria, or microalgae was initiated by the addition of 0.5−1 kg of dried microbial biomass to methanol containing 2% (% v/v) H_2SO_4 at a ratio of 20 L of methanol for every kg of biomass. Reactions were carried out in a 20-L pilot reactor (Chemglass Scientific, Vineland, NJ) maintained at 62 °C for 6 h with continuous overhead stirring at 400 rpm. Once the transesterification reaction was complete, the residual biomass was removed by vacuum filtration and methanol filtrate was returned to the reactor. The residual biomass was then washed with chloroform equal to 10% (% v/v) of the reaction volume to recover residual FAME. The chloroform filtrate was then mixed with the filtered methanol in the 20-L reactor and then water equivalent to the initial methanol volume was added to force a phase separation. The methanol/water/chloroform mixture was allowed to separate into a lower organic (chloroform and biodiesel) and an upper aqueous (methanol, H_2SO_4, and water) phase. The bottom organic layer, containing the crude biodiesel, was then removed for further processing. The chloroform was removed from the organic phase through distillation by heating the solution to a temperature of 65 °C. Residual vapors were subsequently removed by blowing air across the crude biodiesel. The FAME was recovered from the crude mixture by utilizing vacuum distillation. The crude FAME

Table 1. Fatty Acid Composition of Biodiesel Fuels

	fatty acid chain length (% of total fatty acids)[a]						degree of unsaturation (% of total fatty acids)[b]		
biodiesel fuel	C14	C15	C16	C17	C18	C20	mono	poly	mono + poly
soybean *G. max*	0	0	11	0	88	<1	24	61	85
yeast *C. curvatus*	0	0	16	0	83	<1	60	6	66
bacteria *R. opacus*	2	5	43	22	27	0	51	0	51
microalgae *C. gracilis*	10	<1	72	0	11	6	34	28	62

[a]The fatty acid composition of each biodiesel is categorized as the % of the total fatty acids with a given number of carbon atoms, where C*xx* notation represents the carbon (C) chain length (*xx*). The complete fatty acid composition of each biodiesel fuel can be found in Supporting Information Table 1. [b]Percent of the total fatty acids that are monounsaturated and polyunsaturated.

was heated to a temperature of 180 °C in the process. The vacuum was generated by a rotary vane pump (Adixen Pascal 2005SD, Pfeiffer Vacuum, Milpitas, CA). A glass cold trap immersed in a dry ice−ethanol bath was placed between the distillation apparatus and the vacuum pump to prevent solvent from reaching the vacuum pump. The reaction vessel was allowed to cool before removing the vacuum to prevent combustion of FAME as the reaction vessel was heated above the autoignition temperature of biodiesel (177 °C). Purity of each fuel was verified by gas chromatography as described previously.[15]

Fuel Properties. The energy density of each fuel type was measured in triplicate using a combustion calorimeter (C 2000 basic version 1, IKA, Wilmington, NC). The calorimeter was operated in isoperibolic mode with the initial temperature set at 25 °C. Benzoic acid was used for decomposition vessel calibration. For each test, approximately 500 mg of liquid fuel was combusted with oxygen at 435 pounds per square inch (psi) in a quartz crucible placed inside the decomposition vessel. Fuel density was determined using a hand-held digital (Densito 30 Px, Mettler Toledo, Columbus, OH) density meter according to standard methods (ASTM D4052). Densities recorded at room temperature were converted to density at 15 °C using the method of Lapuerta et al.[37] Kinematic viscosity measurements were made according to standard methods (ASTM D445) using a Cannon-Fenske viscometer (Cannon Instrument Company, State College, PA). Biodiesel cetane index values were calculated according to Lapuerta et al.[37]

Compositional Analysis of Fuels. The fatty acid composition of each fuel was determined by GC/MS analysis, using a gas chromatograph (model 2010, Shimadzu Scientific, Columbia, MD) equipped with a programmable temperature vaporizer (PTV), split/splitless injector, flame ionization detector (FID), mass spectrometer (MS)(GCMS-QP2010S, Shimadzu Scientific, Columbia, MD), and autosampler. Samples were prepared by diluting ∼20 mg of the final biodiesel product from each source with chloroform to a final biodiesel content of ∼1 mg mL^{-1}. One μL of each sample was injected into the split/splitless injector set to a split ratio of 1:2. Analytes were separated using a stabilwax column (30 m, 0.25 mm ID, and 0.10 μm film thickness, Restek, Belafonte, PA) and detected using a quadrupole mass spectrometer (GCMS-QP2010S, Shimadzu Scientific, Columbia, MD) set to maintain an interface and ion source temperature of 240 and 200 °C, respectively. Helium was used as the carrier gas set to a constant velocity of 50 cm s^{-1}. The injector temperature was set at 235 °C and the column was initially set at a temperature of 100 °C for 1 min then increased to a temperature of 235 °C at a rate of 10 °C min^{-1} then held at this temperature for 10 min. The mass spectrometer scanned a mass range of 35 to 900 m/z at a rate of 2000 scans s^{-1}. Each individual fatty acid methyl ester (FAME) was identified by comparing the retention times of resolved compounds with that of FAME standards and by comparing the mass fragment pattern of each resolved peak to the National Institute of Standards and Technology (NIST) 2005 mass spectral library (NIST, Gaithersburg, MD) using the software GC/MS postrun analysis v2.3 (Shimadzu Scientific, Columbia, MD). FAME standards used included methyl myristate, methyl palmitate, methyl palmitoleate, and methyl oleate (Nu-Chek Prep, Inc., Elysian, MN).

Engine Setup. Tests were performed with a naturally aspirated indirect injection combustion ignition engine (Kubota Z482-ES04, Lincolnshire, IL) controlled with an autostart module (DSE 4110, Deep Sea Electronics, North Yorkshire, England). The fuel and starting system were controlled using an externally switched dual coil solenoid (SE-3204, Woodward, Niles, IL). Power from the engine was transferred using a power takeoff (PTO) with 6.5-in. high efficiency (HE) clutches (North American Clutch & Driveline, Rockford, IL). Load application was accomplished using an electronically controlled (eddy control 96-DC at 120-AC, Land & Sea, Concord, NH) eddy current type absorber (20-96 V, Land & Sea, Concord, NH). A load cell was used to measure the load application (LC703-300, Omegadyne, Sunbury, OH). Engine speed measurements were made using a remote optical sensor (ROS-P, Monarch Instruments, Amherst, NH). Fuel flow rate was measured using an ultrasonic flow meter (Atrato Ultrasonic 710, JLC International, New Britain, PA). Environmental conditions were monitored throughout the engine tests to allow for correction to SAE J1349 standards (dyno weather station, Land & Sea, Concord, NH). Ambient temperature readings were made using a thermistor (ST-100, Apogee Instruments, Logan, UT). K-type thermocouples were used to monitor exhaust gas temperatures (430-440, Land & Sea, Concord, NH) and engine oil temperature (HSTC-TT-K-24S-36, Omega, Stamford, CT). Engine coolant temperature was measured using an engine temperature thermistor (430-457, Land & Sea, Concord, NH). Surface temperatures of the engine block and eddy current brake were measured using infrared radiometers (S1-121, Apogee Instruments, Logan, UT) paired with a data logger (CR1000, Campbell Scientific, Logan, UT). The data logger was controlled using software developed for use with the CR1000 (Loggernet 4.1, Campbell Scientific, Logan, UT). All measurements were captured and recorded using the "Pro" full function 28 channel harness (Land & Sea, Concord, NH). Dyno runs were controlled, recorded, and reported using the DYNO-MAX 2010 version 10.15 software (Land & Sea, Concord, NH).

Engine Test Procedure. The engine was started and idled (1300 rpm) for 3 min and the throttle was then increased to 3500 rpm and run for 1 min. The eddy current brake (ECB) was conditioned by applying a 25% load for 10 s, and then disengaged and the engine returned to idle. At 5 min total run time, the engine was turned off and allowed to heat soak. The pretest conditioning was performed to minimize variability due to engine conditions for each fuel being tested. Prior to each test the engine oil and oil filter were changed and replaced with fresh 10W-30 oil preheated to 85 °F. Testing of the fuels took place once the ECB reached 5 °F above room temperature. The surface temperature of both the engine block and the ECB were monitored to within 0.2 °C with an infrared radiometer (Apogee Instruments, Logan, UT). To begin each test, the engine was first started and idled for 30 s. The throttle was then increased to full (3800 rpm) and run for an additional 30 s. An electronically controlled down-sweep load was then applied to the engine via the ECB to load the engine from 3800 rpm down to 2450 rpm at a rate of 150 rpm s^{-1}. For each fuel, the test procedure was performed in triplicate. After each run, the engine was brought immediately to an idle (1300 rpm) and turned off. The ECB and engine were allowed to cool until they reached the initial temperature of the previous run. Data presented represent the average of the three runs of each fuel. Data were

Table 2. Properties of Fuels

fuel	density at 15 °C (g cm^{-3})[d]	kinematic viscosity at 40 °C (mm^2 s^{-1})	heating value (kJ g^{-1})	volumetric energy density (kJ cm^{-3})	cetane number (minimum value)	biodiesel cetane index[f]
petroleum diesel						
ASTM standard (D975)		1.9−4.1			40	
#2 (this study)[a]	0.818[e]	2.1 (±0.06)	46.10 (±0.036)	37.7		
biodiesel						
ASTM standard (D6751)	0.86−0.90	1.9−6.0	NA		47	
soybean[b] *Gycine max*	0.884	3.9 (±0.1)	39.97 (±0.093)	35.3		54
microalgae *C. gracilis*[c]	0.885	3.4 (±0.06)	39.51 (±0.006)	35.0		51
yeast *C. curvatus*[c]	0.876	4.5 (±0.1)	39.33 (±0.289)	34.5		67
bacteria *R. opacus*[c]	0.895	4.1 (±0.05)	37.31 (±0.252)	33.4		41

[a]Petroleum diesel #2 obtained from a local fuel distributor, verified to not contain fatty acid methyl esters by GC/MS analysis. [b]Commercially available soybean biodiesel obtained from a local distributor, used as unblended B100. [c]Biodiesel obtained by the direct transesterification of the respective organisms. [d]Density values are the result of two independent measurements measured at according to ASTM D4052 as described in materials and methods. Density values obtained by direct measurement were converted to density at 15 °C according to Lapuerta et al. 2010. Although each independent measurement was identical for each fuel the method has an accuracy of ±0.001 g cm^{-3}. [e]Density of petroleum diesel was measured at 19.2 °C. [f]Biodiesel cetane index calculated according to Lapuerta et al. 2010.

corrected to SAE J1349 standards (85% mechanical efficiency, 77 °F inlet air temperature, 29.38 in Hg at 32 °F, and dry air) by the dynamometer software (DYNO-MAX 2010, Land & Sea, Concord, NH). Upon completion of triplicate engine performance tests, emissions data for each fuel were collected by operating the engine without load at 3500 rpm for 3 min, using a 5-gas analyzer (CO_2, CO, NO_x, unburned hydrocarbon, and O_2) (emissions analyzer part 5002-5, Land & Sea, Concord, NH). Reported data were obtained by averaging an identical 60-s interval for each exhaust gas and fuel where the gas composition had reached steady state. Prior to conducting engine performance and emissions tests for the next fuel, the engine oil and oil filter were changed as described above and the fuel delivery system was completely evacuated and cleaned with isopropanol. The fuel system was then refilled with the new fuel and bled to remove all air. This was done to ensure tests were performed using only the fuel to be tested. The engine test method was then repeated for each subsequent fuel.

RESULTS

Molecular Properties of Biodiesel Fuels. The fatty acid composition of each biodiesel fuel was determined by GC/MS analysis (Supporting Information (SI) Table 1). The results of this compositional analysis are summarized in Table 1. Each fuel is described according to the proportion of fatty acids of a given carbon chain length and the degree to which the fuel consists of unsaturated fatty acids. The notation C*xx* (e.g., C14) is used to represent individual fatty acid groups where "C" refers to carbon and "*xx*" specifies the number of carbon atoms.

Consistent with previous reports of soybean biodiesel,[26,38] 88% of FAMEs are derived from C18 fatty acids. The majority (85%) of the soybean FAME exists as unsaturated FAME, where 24% contains a single double bond (monounsaturated) and 61% contains more than one (polyunsaturated). The most prominent FAME found in soybean biodiesel is methyl linoleate (C18:2, 54%, SI Table 1). In terms of the fatty acid chain length, biodiesel obtained from the yeast *C. curvatus* was found to be the most similar to soybean biodiesel with 83% of FAME being derived from C18 fatty acids. Yeast biodiesel differs significantly, however, from the soybean fuel as to its degree of unsaturation (66% of FAME). Yeast biodiesel is composed primarily of methyl oleate (C18:1, 60%) and is almost exclusively monounsaturated (60%), containing only 6% polyunsaturated FAME. Biodiesel produced from the microalga *C. gracilis* differed substantially from the other fuels. Microalgae biodiesel contained a significant amount of shorter chain FAME derived from myristic acid (C14:0, 10%). FAME derived from C16 fatty acids (72%) were the most common component of microalgae biodiesel, differing significantly from soybean and the other microbial fuels. The amount of unsaturated FAME (62%) in microalgae biodiesel was less than that in both the yeast and soybean biodiesels but greater than that in the bacterial biodiesel. Unlike each of the other biodiesel fuels, microalgae biodiesel contained nearly equivalent amounts of monounsaturated and polyunsaturated fatty acids (34% and 28%, respectively). The other biodiesel fuels contained a majority of either polyunsaturated or monounsaturated FAME. The biodiesel produced from the bacterium *R. opacus* was the only other fuel to contain shorter chain fatty acids (14 or fewer carbon atoms) although in much lower quantities. Bacterial biodiesel was also the most diverse in terms of carbon chain length, containing a significant amount of FAME derived from C16 (43%), C17 (22%), and C18 (27%) fatty acids. Bacterial biodiesel also contained the least amount of unsaturated FAME (51%), all of which was monounsaturated. The bacterial biodiesel was also found to differ substantially in its molecular structure from the other fuels. GC/MS analysis of the bacterial biodiesel revealed several compounds that correlated well with fatty acids modified by either methylation or hydroxylation (SI Table 1). Additional analysis will be required to definitively identity each of these unusual FAME molecules.

Physical Properties of Biodiesel Fuels. The biodiesel industry has well-defined standards used to certify biodiesel fuels for commercial sale. The standards, set by ASTM International, look at all aspects of the fuel that might be important for commercial resale and may not necessarily relate to the performance of the fuel in an engine. To describe the physical properties of the fuels most related to combustion, we chose a reasonable subset of the ASTM standards that describe the energy content of the fuel, its density, viscosity, and biodiesel cetane index (Table 2). Density of the biodiesel fuels ranged from 0.876 g cm^{-3} for yeast at the low end to 0.895 g cm^{-3} for bacterial biodiesel at the upper end, within the range

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specified by ASTM for biodiesel (0.86−0.90 g cm^{-3}). Microalgae and soybean biodiesel fuels were found to have densities (0.885 and 0.884 g cm^{-3}, respectively) in the middle of this range. The biodiesel standard (ASTM D6751) specifies that the kinematic viscosity for biodiesel fuels must fall within the range of 1.9−6.0 mm^2 s^{-1}. All fuels tested here were within the acceptable range for kinematic viscosity. Microalgae biodiesel had the lowest measured kinematic viscosity of any of the biofuels in this study at 3.4 mm^2 s^{-1}, and yeast biodiesel measured the highest at 4.5 mm^2 s^{-1}. An important parameter for the quality of a fuel is its energy density, expressed as the heating value (kJ g^{-1}) of the fuel. Fuels with a higher energy density can accomplish more work than an equal amount of lower energy density fuels. Soybean biodiesel had the highest heating value (39.97 kJ g^{-1}) of the biodiesel fuels tested, although it was substantially lower (13%) than diesel #2 (46.10 kJ g^{-1}). Biodiesel from both microalgae and yeast were found to have heating values slightly less than soybean biodiesel (39.51 and 39.33 kJ g^{-1}, respectively). The bacterial biodiesel heating value (37.31 kJ g^{-1}) is significantly lower than that of soybean biodiesel and the other two microbial biodiesel fuels.

A biodiesel cetane index (BCI) developed by Lapuerta et al.[37] relating the density of fatty acid ester fuels with their cetane number was used to predict the biodiesel cetane number of each biofuel used in this study. The density of these fuels is influenced by both the chain length and degree of unsaturation of its constituent fatty acids.[37] Biodiesel produced from the yeast *C. curvatus* had the highest BCI, followed by soybean and microalgae biodiesel with BCI of 54 and 51, respectively. The bacterial biodiesel had the lowest BCI (41). Although this is lower than the cetane number limit minimum of 47 specified by ASTM D6751, it should be noted that the bacterial biodiesel contains a substantial amount of branched-chain fatty acids as well as a small amount of hydroxylated fatty acids. The biodiesel cetane index is based on straight-chain fatty acid esters and may not accurately predict the cetane number of a fuel that contains a large proportion of branched-chain fatty acids. Therefore the predicted cetane number for bacterial biodiesel might not explain differences found in its performance or emissions from the other fuels.

Performance of Biodiesel Fuels in a Diesel Engine. The comparison of the performance of an engine operated with an alternative fuel is an important tool in evaluating the potential of the fuel to replace the traditional fossil fuel. Using engines equipped with measurement instrumentation such as dynamometers, soybean biodiesel has been established as a suitable replacement for diesel #2.[39−41] Here, microbial biodiesels, prepared from oleaginous yeast, bacteria, and microalgae, were tested in a diesel engine coupled to a dynamometer and the performance output for each microbial fuel was compared to that achieved with both soybean biodiesel and diesel #2. The specifications for the engine setup are summarized in Table 3. For this engine, peak horsepower for each fuel was achieved at 3500 rpm (Figure 1). The highest power output (8.5 hp) of all fuels tested was achieved with diesel #2. Soybean biodiesel registered a power output of 8.2 hp, 96.5% of the value obtained for diesel #2. Of the microbial fuels tested, bacterial biodiesel had the lowest power output at 7.8 hp, still producing 92% and 95% of the output achieved with diesel #2 and soybean biodiesel, respectively. Power output for the engine operated with both yeast and microalgae biodiesel was similar for each fuel achieving 93% and 96% of outputs for diesel #2 and soybean biodiesel, respectively. The power output observed was relatively high for the biodiesel fuels given their lower heating value.

Table 3. Engine Specifications

engine model	Kubota Z482-ES04
number of cylinders	2
engine type	indirect injection naturally aspirated 4-stroke diesel, liquid-cooled
displacement	29.23 $in.^3$
bore	2.64 in.
stroke	2.68 in.
compression ratio	23.5:1
fuel injection type	nozzle
injection pressure	1991 psi
injection timing	20° BTDC
continuous rated output	10.8 hp SAE (7.9 kW)
rated speed	3600 rpm
dynamometer type and model	eddy current absorber (#20 96 V, Land & Sea, Inc.)
controller	eddy control 96-DC
control program	Dyno-max 2010 version 10.15

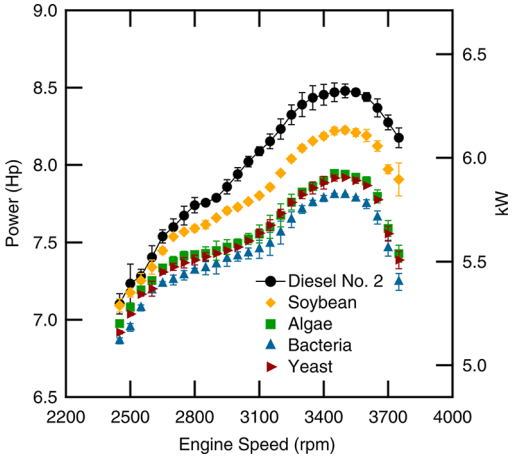

Figure 1. Diesel engine power output (horsepower or kW) as a function of engine speed (rpm) for diesel #2, soybean biodiesel, and the three microbial biofuels. Run conditions are described in the Experimental Section.

Less of a difference was observed in the torque output between the traditional fossil fuel and the alternative biodiesel fuels (Figure 2). A similar trend in the torque output is visible with diesel #2 consistently producing the highest torque throughout the rpm range, followed by soybean, yeast, microalgae, and bacterial biodiesel in order of decreasing torque output. Exhaust gas temperature data were collected for each fuel throughout the operation of the engine with each fuel (Figure 3). Exhaust gas temperatures were highest for diesel #2, followed by soybean, microalgae, yeast, and bacteria (in order of decreasing temperatures). BSFC for each fuel was calculated continuously throughout the test procedure (Figure 4). The biofuels showed higher BSFC across the rpm range of the test compared to diesel #2, which is consistent with their lower energy content. Very small differences in BSFC between the biodiesel fuels were observed.

Comparison of Emissions of Microbial Biodiesel to Soybean Biodiesel and Petroleum Diesel. After the collection of engine performance data, the engine was allowed operate at 3500 rpm with no load applied for the collection of emissions data. Emissions data were collected without applying

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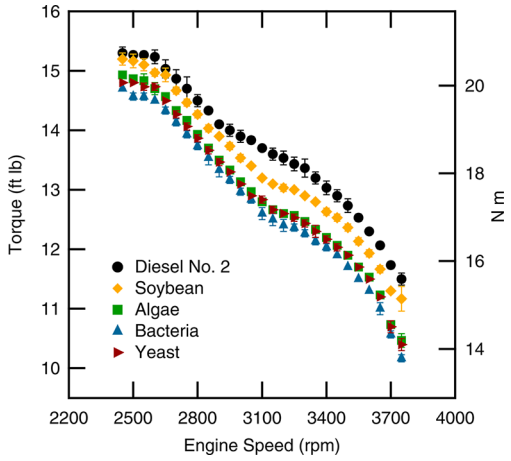

Figure 2. Diesel engine torque (ft-pounds or N-m) output as a function of the engine speed (rpm) for the tested fuels.

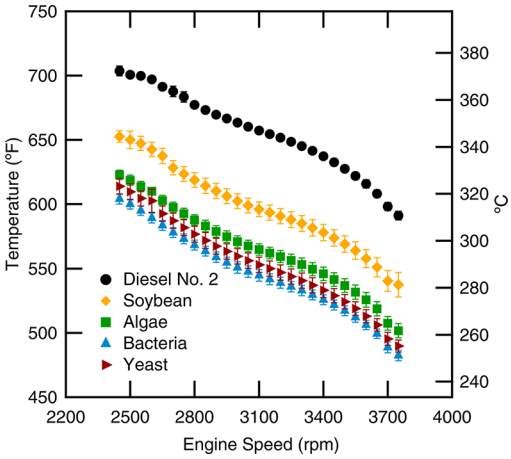

Figure 3. Engine exhaust gas temperature (°F or °C) as a function of engine speed (rpm) for the tested fuels.

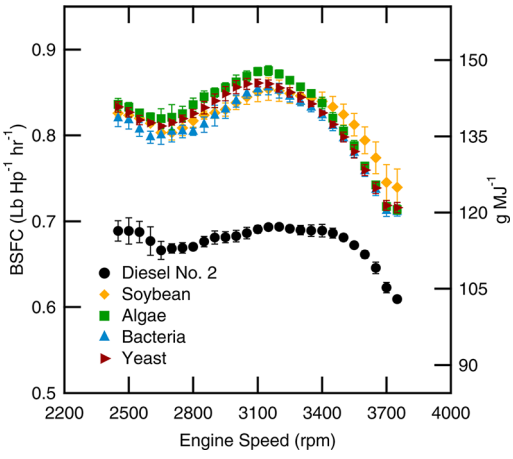

Figure 4. Brake specific fuel consumption (BSFC; Lb/Hp/h or g/MJ) as a function of engine speed (rpm) for the tested fuels.

a load due to equipment limitation. The air-cooled ECB rapidly overheated during the steady state load applications required for collecting emissions data. Data were collected for CO_2, CO, unburned hydrocarbons (HC), NO_x, and O_2 for 3 min and the data from an identical 60 s interval where concentrations of exhaust gas had reached steady state were averaged and are reported in Table 4. Raw data from exhaust emissions experiments are displayed in SI Figure S1. Diesel #2 had slightly lower CO_2 emissions than the four biodiesel fuels. All biodiesel fuels were found to produce significantly lower CO emissions compared to diesel #2. The yeast and bacterial biodiesel fuels produced less than half the CO (0.05% of total exhaust gas) observed for the diesel #2 and 30% less than soybean biodiesel. Microalgae biodiesel had the highest CO emissions of all the biodiesels tested, but it was still 17% lower than diesel #2.

Consistent with other reports,[42] diesel #2 had the highest HC emissions of all the fuels tested and there were significant differences found among the biodiesel fuels tested. Biodiesel produced from yeast and bacteria had the lowest HC emissions, registering 59% and 65% less than diesel #2, respectively. Microalgae biodiesel produced the highest HC emissions of the biofuels tested, but was still 30% lower than diesel #2 levels. Biodiesel has long been associated with higher NO_x emissions than diesel #2.[42] However, we observed significant variation in the NO_x output among the biodiesel fuels tested. Bacterial biodiesel recorded NO_x emissions that were 81% higher than that observed for diesel #2. Soybean and yeast biodiesel also recorded NO_x emissions higher than petroleum diesel (9% and 37% higher, respectively). Interestingly, microalgae biodiesel produced 24% lower NO_x emissions than diesel #2.

DISCUSSION

Biodiesel (FAME) can be a renewable, low CO_2-footprint replacement for petroleum-derived diesel. The majority of biodiesel used today is derived from oil seed crops, primarily soybeans in the U.S. A number of studies have established the functional properties of biodiesel derived from various sources to determine the potential to displace petroleum diesel.[39,40,43,44] Based on the volumetric energy density of biodiesel and diesel fuels, power output for biodiesel is expected to be ~8% lower than for diesel #2, largely due to the oxygen content of biodiesel. In practice the difference in power output can be higher or lower, because of other factors. Brake specific fuel consumption, which is a measurement of the rate of fuel consumption per power output, is increased for biodiesel fuels compared to diesel #2 and is correlated with the oxygen content of the fuel. Most biodiesel fuels are composed of only a few different compounds and thus their properties are largely influenced by the fatty acid composition of the fuel, which is identical to that of the parent oil (i.e., tryglyceride from feedstock oil). Affected physical properties may include density, cetane number, viscosity, heating value, and melting temperature.[4] In turn the physical properties of the fuel can influence the exhaust emissions and performance of the engine.[45]

While biodiesel derived from plant seed oils has advantages as a replacement for petroleum diesel, there is strong interest in the potential for biodiesel produced from microbial derived oils because of potential use of contaminated water, the diversity of oils that can be produced, use of marginal lands, and potential for higher oil yields per acre. Three different groups of microbes are known to produce high neutral oils including select microalgae, bacteria, and yeast. Here, we have selected a representative from each of these three groups, produced biodiesel, have characterized the properties of the fuels in comparison to biodiesel produced from plant oils.

Plant-based oils, commonly used to produce biodiesel (e.g., soybean, canola, and sunflower) are similar to one another in terms of fatty acid composition, containing primarily C16 and

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Table 4. Emissions for Biodiesel Fuels and Petroleum Diesel[a]

	emission parameters[b]				
fuel	CO_2 (%)	CO (%)	HC (ppm)	NO_x (ppm)	O_2 (%)
petroleum diesel #2[c]	3.700 (±0.000)	0.109 (±0.006)	28.96 (±1.08)	25.71 (±1.302)	15.3 (±0.000)
biodiesel					
soybean *G. max*[d]	3.881 (±0.000)	0.077 (±0.005)	17.38 (±0.59)	31.67 (±1.433)	15.4 (±0.000)
yeast *C. curvatus*	3.800 (±0.000)	0.048 (±0.004)	11.86 (±0.48)	39.67 (±1.394)	15.6 (±0.040)
bacteria *R. opacus*	3.891 (±0.032)	0.050 (±0.000)	9.87 (±0.49)	46.76 (±1.283)	15.5 (±0.000)
microalgae *C. gracilis*	3.797 (±0.016)	0.090 (±0.006)	19.75 (±0.94)	21.87 (±1.817)	15.6 (±0.000)

[a]Emissions data were collected from a diesel engine operating at 3500 rpm for the duration of 3 min. The average of data obtained from a 60 s interval where exhaust emissions were at steady state is shown along with the standard deviation (±). [b]Emissions data are represented as either parts per million (ppm) or as a percentage of the total gas. [c]Petroleum diesel #2 was obtained from a local fuel distributor. The absence of fatty acid methyl ester in this fuel was verified by GC/MS. [d]Soybean biodiesel was obtained and used as unblended B100.

C18 fatty acids with varying degrees of unsaturation.[17] Microbial oils, however, can differ substantially and may contain uncommon fatty acids that differ in both chain length and structure.[17,46] Each of the microbial sources of oil chosen for this study differ in one way or another from soybean oil, a common feedstock for biodiesel production. Biodiesel obtained from the microalga *C. gracilis*, contains a substantial amount of C16:1 (palmitoleic) methyl ester and C14:0 (myristic) methyl ester, both rarely found in plant oils. The presence of both fatty acids has been shown to be beneficial to biodiesel fuels by improving oxidative stability without sacrificing cold flow performance in the case of palmitoleic acid[45] and improved NO_x emissions in the case of shorter chain fatty acids such as myristic acid.[47] The biodiesel obtained from the bacteria *R. opacus* oil differs from plant oils in both the distribution and structure of fatty acids. The bacterial oil contains smaller chain fatty acids as well as fatty acids that are modified by hydroxylation or methylation. Studies examining the effect of branching in the alcohol portion of the ester indicate that this aids cold flow properties[4] without negatively affecting emissions. The study showed no increase in NO_x emissions from isopropyl esters compared to methyl esters.[48] Hydroxylated fatty acids can cause biodiesel fuels to have higher viscosity, but the proportion of this unusual fatty acid in the *R. opacus* oil is not high enough to significantly affect viscosity.[45] Biodiesel from the yeast *C. curvatus*, like plant oil, contains predominantly C16 and C18 fatty acids but lacks the polyunsaturated fatty acids commonly found in plant oils. The high amount of oleic acid in this oil should be advantageous for oxidative stability without greatly affecting cold flow properties.[45] Given the influence that fatty acid composition has on the performance of a biodiesel fuel, it is important to determine the physical properties of the biodiesel from the selected organisms and evaluate their performance using a dynamometer coupled to a diesel engine to determine the viability of using oleaginous microorganisms as feedstock for biofuel production.

Selected physical properties of the three microbial fuels in this study were found to be comparable to soybean biodiesel (Table 2) and are within ASTM (ASTM D6751) specification. A significant liability of biofuels is their higher oxygen content relative to fossil fuels, which contributes to lower energy density and higher BSFC. As expected, the heating value per unit mass of each biodiesel fuel was lower than that of petroleum diesel (on average 15%). However, the difference in volumetric energy density of the biodiesel fuels compared to petroleum diesel #2 reduces the difference in energy density on a per unit volume basis to 6% for soybean, 7% for microalgae, 8% for yeast, and 11% for bacteria. The difference in energy density was apparent in the power and torque outputs generated by diesel and biodiesel fuels. Soybean biodiesel had a power output 3% less than that of diesel #2, instead of the expected 6.3% reduction in power anticipated from its volumetric energy. Microalgae and yeast biodiesel fuels had power outputs (~ 6% lower than petroleum) that were much closer to the expected power production based on the difference in volumetric energy density (kJ cm^{-3}) for the microbial biodiesel fuels and diesel #2 (7.3% and 8.6%, respectively). The bacterial biodiesel fuel had the lowest energy density of the fuels tested (37.31 kJ g^{-1}; 33.4 kJ cm^{-3}), 6.7% lower than soybean biodiesel on a mass basis and 5.5% lower on a volume basis. The power output of this fuel was 5% lower than what was achieved with soybean biodiesel, which is consistent with the volumetric energy density. Ultimately the power generated by each fuel is directly correlated to the volumetric energy density of the fuel. Fuels with greater energy density, such as diesel #2, had a higher power output than less energy dense fuels (e.g., bacterial biodiesel). Microbial biodiesel fuels generated power close to what would be predicted based on their difference in energy density relative to diesel #2. The observed torque output for each fuel followed a similar trend with the most energy dense fuels achieving the highest torque output and the least energy dense fuels producing the least amount of torque. The power and torque output of biodiesel produced from oleaginous microbes indicate the ability of these fuels to operate as effectively as soybean biodiesel. Brake specific fuel consumption was calculated for each fuel. As expected, due to the oxygen content of the biofuels, the BSFC of each biodiesel fuel was significantly higher than that of diesel #2. The BSFC curve (Figure 3) for each biodiesel fuel was similarly shaped and no clear difference is apparent among the four biodiesel fuels tested. The performance indicators presented here indicate that oleaginous microbes would be effective alternative feedstocks to be used in place of more traditional oils.

An important advantage of using biodiesel in place of diesel #2 is improved emissions. Biodiesel has been shown to reduce many emissions, including CO and unburned hydrocarbons (HC).[49] It has been generally observed, however, that biodiesel increases emissions for NO_x relative to diesel #2.[49] Emissions data (Table 4) were collected for each of the fuels tested here including CO_2, CO, and O_2 as percentages of total gas and HC and NO_x as parts per million (ppm). Emission of CO_2 increased for each biodiesel fuel with respect to diesel #2 indicating improved combustion due to the biofuels' oxygen content. CO emissions were reduced by more than half for the microbial biodiesel fuels compared to petroleum diesel.

Hydrocarbon emissions were also substantially reduced for each biodiesel fuel relative to diesel #2 with yeast biodiesel registering the lowest value (9.87 ppm), nearly two-thirds lower than that for diesel #2. NO_x emissions for soybean, yeast, and bacterial biodiesel were higher than the measured levels for diesel #2, as expected. Surprisingly, however, NO_x emissions for algae biodiesel were lower than for diesel #2.

Emulsions of water and biodiesel have been shown to reduce NO_x emissions relative to biodiesel alone, where 5−10% water had a significant effect on emissions.[50] The water content of each fuel used in this study was measured by Karl Fischer titration. The European biodiesel standard (EN 14214) specifies that biodiesel should have a water content no greater than 500 ppm. Both commercial soybean biodiesel (580 ppm) and microalgae biodiesel (585 ppm) had water contents just slightly above the 500 ppm standard, whereas the water content of biodiesel fuels from yeast (362 ppm) and bacteria (448 ppm) were slightly below the standard. Given that the water content for all of the fuels was extremely low and similar, it is unlikely that the water could account for the differences observed in NO_x emissions between the fuels.

Fatty acid composition of a biodiesel fuel has a large effect on NO_x emissions. McCormick et al. found that NO_x emissions increased with increasing fuel density and with increasing amount of unsaturated fatty acids.[6] A study conducted with high oleic acid soybean biodiesel led to lower NO_x emissions relative to regular soybean biodiesel indicating that fewer polyunsaturated fatty acids contributed to the improvement.[38] Additional studies have found that oils containing less polyunsaturated fatty acids, such as palm or coconut oil, have lower NO_x emissions than soybean biodiesel.[39,40,51,52] Using neat methyl esters, Knothe et al. determined that unsaturated fatty acids as well as those with longer chain length (>C16) can increase NO_x emissions.[47] The low prevalence of polyunsaturated fatty acids and the predominance of shorter chain length fatty acids present in *C. gracilis* oil (Table 1) likely contribute to its low NO_x emissions.

In summary, the performance data presented here indicate that biodiesel derived from oleaginous microbes can be effective fuels to displace both petroleum diesel and biodiesel produced from plant oils. Microbial biodiesel fuels prepared from yeast, bacteria, and microalgae were found to generate similar amounts of power and torque when compared to soybean biodiesel and do not show an increase in BSFC relative to soybean biodiesel. Hydrocarbon and CO emissions are reduced from diesel #2 levels for all microbial and soybean biodiesel fuels. While NO_x emissions are elevated relative to diesel #2 in yeast, bacteria, and soybean biodiesel fuels, microalgae biodiesel fuel generated NO_x emissions that were significantly lower. This study demonstrates that microbial-derived biodiesel shows comparable properties in the parameters tested to soybean biodiesel. Future wide scale use of microbial oils as a source for biodiesel will require advances in large scale cultivation, dewatering, and oil extraction.

■ ASSOCIATED CONTENT

Supporting Information

A table showing the fatty acid composition of each fuel along with a figure showing hydrocarbon, NO_x, CO, and CO_2 output as a function of time. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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