Two-step Process for Production of Biodiesel Blends from Oleaginous Yeast and Microalgae¶

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ABSTRACT

Biodiesel produced from oleaginous microorganisms shows promise in displacing use of petroleum diesel fuel, however, low biodiesel yields and rigorous processing have thwarted large-scale commercialization. Here, we report a simple and efficient two-step process for generating biodiesel blends from microbial biomass, which eliminates the need for solvent extractions, distillations, or additional purifications. In the present work, diesel fuel was utilized to extract biodiesel produced from direct transesterification of the yeast, *Cryptococcus curvatus*, and microalgae, *Scenedesmus dimorphus*, thus generating a blend of microbial biodiesel and diesel fuel. Up to 93% and 83% of the produced biodiesel is extracted from both yeast and microalgae, respectively, whereas the majority of pigments are excluded. A B20 blend produced from yeast meets key ASTM fuel requirements including flash point, viscosity, sulfur, oxidation stability, and acid number. Integration of experimental data into system models reveals a 25% reduction in the net energy ratio (NER) with the process presented here compared to traditional solvent extraction.

Keywords: extraction, biodiesel, diesel, yeast, microalgae

1. INTRODUCTION

Several approaches to producing renewable liquid fuels biologically have already been developed and demonstrated at large scale to replace petroleum-derived fuels [1]. One such biofuel is biodiesel, which has been produced from various feedstocks including oils derived from land-based crops, microbes, and waste grease [2]. Biodiesel may displace the middle distillate of petroleum, diesel fuel, which is the most-utilized liquid fuel worldwide and its use is expected to rise due to industrialization of developing nations [3,4]. Chemically, biodiesel is composed of fatty acid alkyl esters, which are most commonly produced through either acid or base-catalyzed transesterification of triacylglycerides (TAG) and fatty acids using short chain alcohols.

Several issues have limited the potential of biodiesel as an alternative to petroleum diesel. First, the most commonly used feedstocks, land-based crops (e.g. soybeans, canola), generate low oil yields per acre [5]. Second, the downstream processing of these oils and subsequent conversion to biodiesel have high energy demand, generate oil losses, and require large amounts of water and chemicals [6]. Third, due to high gel point, low oxidative stability, and inferior fuel economy of biodiesel, blending with diesel fuel is usually required for use in most engines [7]. In order to mitigate the shortcomings of current biodiesel production, studies have investigated the minimization of the energy inputs of downstream processing for biodiesel production. One report investigated the direct transesterification of macroalgae using hexane as a co-solvent to eliminate the need for an additional step of extraction [8]. Also, studies have demonstrated the potential of diesel fuel as a solvent for enzyme-catalyzed transesterification of TAG thus generating a blend in order to eliminate the need for a solvent separation process [9]. The use of diesel fuel has also been shown to have good extraction efficiency at extracting triglyceride oils from peanuts using diesel-based reverse-micellar microemulsions [10]. These are novel techniques which contribute to efficient means of biofuel production, however the feasibility of these processes are limited by the materials and reagents required as well as the feedstocks utilized in these processes.

Oleaginous microbes, which produce high levels of lipids, are gaining momentum as potential feedstocks for biodiesel production. Lipids accumulated in the cell, mostly in the form of TAG, are ideal biodiesel precursors owing to their high energy density. A means of producing biodiesel directly from dried microalgae via direct transesterification has recently been developed. In this methanolysis process, methanol and sulfuric acid are reacted with dry microbial biomass to simultaneously extract and convert lipids into fatty acid methyl esters (FAME), the constituents of biodiesel. This process not only transesterifies neutral lipids but an additional yield of 5-10% is obtained due to transesterification of phospholipids and the esterification of free fatty acids that cannot be reasonably extracted with conventional solvent extraction techniques [11]. However, the presence of contaminants introduced by the biomass (i.e., pigments) makes solvent extraction of the produced biodiesel and its purification necessary [12]. In spite of these limitations, it would be advantageous to demonstrate a biodiesel production process that is compatible with highly productive feedstocks, such as microbes, while not requiring extensive processing and purification of the biofuel. In order to reduce the energy inputs of purification concurrent with producing a biodiesel blend that is compatible with current fuel infrastructure, here we demonstrate and optimize the use of petroleum diesel fuel as an extractant of biodiesel produced from yeast and microalgae.

2. Materials and Methods

2.1. Chemicals and Reagents

Reagents used in this study include ACS grade sulfuric acid, chloroform, and glycerol, which were purchased from Fisher Scientific (Hampton, New Hampshire), as well as ACS grade methanol from Pharmco-AAPER (Brookfield, CT). Diesel fuel was acquired from Big West Oil (Logan, Utah) and was confirmed to contain no biodiesel by GC-MS analysis. FAME standards, monopalmitin, dipalmitin, and tripalmitin were obtained from Nu-chek Prep Inc. (Elysian,

Minnesota). N-Methyl-N(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich. All nutrients and chemicals for media were purchased from Alfa Aesar (Ward Hill, Massachusetts) unless stated otherwise.

2.2. Strains and culture conditions

The yeast, *Cryptococcus curvatus* (ATCC 20509), was obtained from the American Type Culture Collection (ATCC, Manassas, VA). *C. curvatus* was maintained and grown as previously described except that a 100 L fermenter (Bioflow 610, New Brunswick, Enfield, Connecticut) was utilized with the medium modified to include sucrose in place of glucose [13]. The culture was incubated for 3 days at 30 °C, with dissolved oxygen set at 20%. The pH was maintained at 5.0 with 5M sodium hydroxide. Foam was controlled by addition of propylene glycol. Culture was concentrated using a continuous centrifuge (Z41 model, CEPA, Lahr, Germany). Collected microbial paste was snap-frozen in liquid nitrogen and broken into small pieces and dried by lyophilization (Freezone 4.5, Labconco, Kansas City, MO). After drying, the biomass was crushed with mortar and pestle and further homogenized by blending (Waring 701 IHg, Conair, Stamford, CT). The yeast biomass from a single 100L growth was utilized throughout all experiments conducted.

The microalgae *Scenedesmus dimorphus* (UTEX 417), was obtained from The Culture Collection of Algae at the University of Texas at Austin (UTEX). All cultures were grown in BG-11 media modified to contain 0.3 g L⁻¹ sodium nitrate in order to stress for lipid production [14]. Algal culture was grown as previously described except that four 5L flasks were used to inoculate 220L raceways [11]. Raceway cultures were harvested when optical density at 750nm did not increase for two consecutive days. The total culture volume was concentrated using a tangential-flow concentrator (AG Technologies, Needham, MA) and pelleted to a wet paste by centrifugation. Collected algal paste was frozen, dried, and homogenized in the same manner as

the yeast. The same homogenized biomass from two raceways under identical conditions was used throughout this study.

2.3. Direct Transesterification

Samples of yeast and algal biomass were converted via direct transesterification according to Wahlen *et al.* except that conversions were performed in screw cap test tubes (16mm x 100mm, Pyrex, Tewksbury, MA) on a heat block (digital dry block heater 210W, VWR, Radnor, PA) maintained at 80 °C for six hours, and mixed by vortexing every 15 minutes [11].

2.3.1. Reaction Solvent Volume to Biomass Conversion Efficiency

For reaction solvent volume to biomass conversion efficiency experiments, 100 mg of either yeast or microalgal biomass was added to the appropriate volume of reaction solvent. The reaction solvent contained 2% sulfuric acid (% v:v) in methanol. Samples containing FAME and unconverted lipids were exhaustively extracted and prepared as previously described [11].

2.3.2. Diesel Extraction

For diesel extraction experiments, 1 g of either yeast or microalgae biomass was converted in 10 mL of reaction solvent (10:1 reaction solvent volume:biomass) identical to that for the reaction solvent volume experiments. The produced reaction mixture was then subjected to the addition of diesel fuel at various volumes to test certain aspects of the diesel extraction process.

2.4. Diesel Extraction Procedure

The direct transesterification reaction was added to graduated test tubes (17mm x 120mm, Cole-Parmer, Vernon Hills, IL) and were tared. The appropriate volume of diesel for the particular diesel to reaction solvent volume ratio was added to the test tubes and the mass of the diesel was recorded. The test tubes were then wrapped in parafilm to prevent evaporation of

methanol or volatile compounds present in the diesel and were vortexed at high speed for 5 minutes. Additional mixing time or mixing speed was found not to improve extraction efficiency. The test tubes were then centrifuged at 2515 rpm (Clay Adams Dynac, Parsippany, NY) to force separation of the diesel and FAME in the top layer and the methanol, sulfuric acid, and biomass in the bottom layer. The volume of the diesel extract was recorded and the diesel extract was analytically removed with a gas-tight syringe and placed into tared test tubes such that the percent recovery of the diesel could be measured gravimetrically. Samples of the diesel extracts were stored at -20 °C until analysis could be carried out.

2.5. Gas Chromatography

For all quantitative and qualitative analyses, 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 was utilized.

2.5.1. FAME Quantitation

Fatty acid methyl ester (FAME) content of each sample from direct transesterification and diesel extraction experiments was determined according to Wahlen *et al.* [11].

2.5.1.1. Diesel to Reaction Solvent Volume FAME Extraction Efficiency

Diesel extract samples produced at various diesel to reaction solvent ratios were diluted such that each FAME peak fell within the detector calibration. The amount of FAME extracted at different diesel to reaction solvent volumes was quantified by integrating peaks with retention times identical to those determined to be FAME by the reaction solvent volume to biomass experiments as well as by GC-MS analysis 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). Diesel fuel confirmed not to contain biodiesel was diluted identically to the diesel extracts and run alongside the diesel extracts on the GC to confirm that the quantified FAME peaks were not components of diesel fuel. The total mass of FAME in the diesel was calculated using the volume determined during separation as well as dilution factors. The percent extracted was then calculated as the total mass of FAME in the diesel out of the total mass of FAME as calculated with the original known mass of yeast or microalgae biomass used in the direct transesterification reaction.

2.5.2. Total Glycerin

Diesel extract samples for free glycerin were prepared similar to Munari *et al.* by derivatizing 100 mg of diesel extract with 100 μ L of MSTFA for 30 minutes at room temperature [15]. The volume was then diluted to 5 mL with chloroform in a volumetric flask and mixed before a 1 mL sample was taken for GC-MS analysis. Derivatized glycerol was resolved by GC-MS using an XTI column (30 m, 0.32 mm ID, and 0.25 μ m film thickness) (Restek, Bellefonte, PA). Samples were run directly on the column and the oven temperature program started at 40 $^{\circ}$ C for five minutes followed by a temperature ramp of 10 $^{\circ}$ C per minute to 180 $^{\circ}$ C for five minutes. The amount of derivatized glycerol was determined by linear regression analysis using standards ranging from 0.002 mg mL⁻¹ to 0.1 mg mL⁻¹ of glycerol similarly derivatized with MSTFA. Samples for quantitation of monoglycerides, diglycerides, and triglycerides were prepared and run similar to Munari *et al.* as previously described. The detector response was calibrated using standards of monopalmitin, dipalmitin, and tripalmitin with concentrations ranging from 0.001 mg mL⁻¹ to 0.05 mg mL⁻¹ similarly derivatized with MSTFA.

2.5.3. Methanol Quantitation

The methanol content of undiluted diesel extracts was determined by GC-MS as previously explained with the split/splitless injector connected to a XTI-5 column with a split ratio of 150:1.

Standards of diesel spiked with various amounts of methanol ranging from 0.1 to 4% (% g/g) were used to calibrate the mass spectrometer response.

2.5.4. *Qualitative Analysis of Diesel Extracts*

For qualitative analysis of diesel extracts, analytes were separated using an HT5 column (30 m, 0.25 mm ID, and 0.10 μm film thickness) (Forte HT5 column; SGE Analytical Science, Austin, TX) and detected with a mass spectrometer set to maintain an interface and ion source temperature of 340 and 200 $^{\circ}\text{C}$, respectively. Helium was used as the carrier gas set to a constant velocity of 50 cm s^{-1} . The injector temperature was set at 370 $^{\circ}\text{C}$ and the column was initially set at a temperature of 60 $^{\circ}\text{C}$ for 1 minute. The oven temperature was then increased to a temperature of 200 $^{\circ}\text{C}$ at a rate of 5 $^{\circ}\text{C}$ per minute and then further increased to 340 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C}$ per minute and held for 5 minutes. The mass spectrometer scanned a mass range of 35 to 500 m/z at a rate of 1000 scans s^{-1} .

2.6. *Pigment Content*

The amount of pigment, chlorophyll from the microalgae and carotenoids from the yeast, were quantified spectrophotometrically using a Varian 50 Bio UV–Visible spectrophotometer (Varian Inc., US). The samples were diluted as necessary to give a value under one absorbance unit. The concentration of either chlorophyll or carotenoids in the diesel extract compared to the methanol layer was calculated according to Lichtenthaler and Wellburn, using the following set equations [16,17]:

$$\text{Chlorophyll } a \text{ (}\mu\text{g mL}^{-1}\text{)} = 15.65(A_{666}) - 7.34(A_{653})$$

$$\text{Chlorophyll } b \text{ (}\mu\text{g mL}^{-1}\text{)} = 27.05(A_{653}) - 11.21(A_{666}) \quad (1)$$

$$\text{Total Carotenoids (}\mu\text{g mL}^{-1}\text{)} = (1000A_{470} - 2.86C_a - 129.2C_b)/245$$

The methanol layers were blanked against the reaction solvent, which contained 2% sulfuric acid in methanol (% v/v). The diesel extracts were blanked against diesel fuel blended with purified biodiesel produced from yeast or microalgae to the appropriate blend based upon the diesel to reaction solvent volume ratio being tested. The purified biodiesel was produced and purified according to Wahlen *et al.* for both yeast and microalgae [13]. The values are represented as the percent of the pigments extracted into the diesel extract over the various diesel to reaction solvent volume ratios tested. The percent pigment extracted is calculated as the amount of pigment in the diesel extract out of the total pigment in the diesel extract and the methanol layer.

2.7. Production of a B20 Blend from Direct Transesterification of Yeast Biomass

In order to produce enough of an approximate B20 diesel extract for fuel testing, 100 g of yeast biomass ($67.1 \pm 0.8\%$ FAME content) was subjected to the direct transesterification in 1 L of 2% sulfuric acid in methanol (% v:v). The reaction was maintained at 62 °C and mixed continuously with a magnetic stir plate in a two-neck 2 L round bottom flask for six hours. The round bottom flask was attached to a reflux condenser in order to prevent evaporation of the methanol. After the reaction was complete, 300 mL of diesel (0.3:1 diesel to reaction solvent volume ratio) was added to the reaction and was mixed for 15 minutes. The total volume was then poured into teflon centrifuge bottles (Thermo Scientific, Waltham, MA), sealed with sealing cap assemblies (Thermo Scientific, Waltham, MA) designed for the bottles, and centrifuged at 5,000 rpm. The diesel extract was then pulled off and subjected to heating in a clean 2 L round-bottom flask at 100 °C for 15 minutes to remove residual methanol. The biodiesel content of the blend was confirmed by the concentration of FAME in the diesel extracts as previously described.

2.8. American Society of Testing and Materials (ASTM) Tests

The produced B20 blend was subject to the following ASTM sanctioned tests and were performed by Bently Tribology (Sparks, NV) according to ASTM D7467: Diesel Blends B6 to

B20. Flash Point was measured according to ASTM D93 by Pensky Martens closed cup and given in degrees Celsius ($^{\circ}\text{C}$). Viscosity was measured according to ASTM D445 at 40°C and given in centistokes (cSt). Sulfur content was measured according to ASTM D5453 by UV fluorescence and given in parts per million (ppm). Oxidation stability was measured according to EN15751 rancimat and given in hours (hrs). Acid number was measured according to ASTM D664 and given in milligrams of potassium hydroxide per gram of sample (mg KOH/g).

2.9 Net Energy Ratio (NER) Calculations

An evaluation of the life cycle energetics of the studied recovery system compared to a traditional solvent recovery system was evaluated. The two pathways were compared on the metrics of net energy ratio (NER). The NER is defined here as the energy in over the energy produced with a NER of less than 1 desirable. The foundational modeling work involves the construction of an engineering system model that accurately tracks energy and mass flow between sub-process models. The sub-process models were validated with experimental and literature data and are intended to be representative of a large-scale industrial processing facility. The modeling effort is based on a yeast feedstock with a 70% lipid content and dried to 98% moisture. The primary assumptions for the sub-process models of the presented experimental system are: *in situ* transesterification: 98% conversion efficiency requiring $5.27 \text{ MJ kg-oil}^{-1}$ and $0.09 \text{ kWhr kg-oil}^{-1}$ heating and electrical energy; diesel extraction: 1.5:1 diesel:reaction volume ratio, 98% extraction efficiency, and 98% product recovery [18,19]. The solvent recovery pathway includes the solvent recovery of lipids, base-catalyzed transesterification, and a solvent biofuel recovery [18,20–23]. Primary assumptions for the three sub processes are: solvent extraction: 2:1 solvent to biomass ratio (vol:vol), $2.64 \text{ MJ kg-oil}^{-1}$ and $0.04 \text{ kWhr kg-oil}^{-1}$ heating and electrical energy, and a 95% extraction efficiency [18,20–22]; transesterification: 98% conversion efficiency, and $3.75 \text{ MJ kg-oil}^{-1}$ and $0.1 \text{ kWhr kg-oil}^{-1}$ heating and electrical energy [20,24]; biodiesel recovery: 99% recovery efficiency, 1:1 solvent to mixture ratio (vol:vol), 2.3

MJ kg-oil⁻¹ and 0.03 kWhr kg-oil⁻¹ heating and electrical energy [18,20–22,24]

A detailed description of the inputs and assumptions for the modeling effort are presented in the supplemental materials. The life cycle modeling system boundary includes the energy requirements associated with the processing of yeast biomass to fuel through either the presented diesel extraction pathway or a traditional solvent pathway and is defined to facilitate a direct comparison between the two pathways.

3. Results and Discussion

3.1. Optimization of Reaction Solvent Volume to Biomass

In order to minimize the amount of solvent necessary to fully convert yeast and microalgae biomass, various reaction solvent volume to biomass ratios were tested including 2:1, 5:1, 10:1, and 20:1 (volume:weight). It was found that a reaction solvent volume to biomass ratio of 10:1 was sufficient to fully convert all saponifiable lipid and free fatty acids into FAME in both yeast and microalgal biomass as the higher ratio of 20:1 did not increase quantified FAME (**Figure 1**). These results demonstrate that less solvent can be utilized in comparison to other studies for direct transesterification, and appears to be dependent on the amount of biomass utilized in the reaction rather than the total FAME content [11,25]. This is apparent as a 10:1 ratio was sufficient for both the yeast biomass which contained $67.1 \pm 0.8\%$ FAME content and the microalgae which contained $26.2 \pm 0.9\%$ FAME content. This information is relevant since the amount of methanol required for conversion would have significant effect on the energy input in maintaining reaction temperature and the distillation of the methanol for its recapture. Likewise, the reaction volume of the direct transesterification could have an impact on the efficacy of diesel as an extractant of the produced FAME as well as the partitioning of pigments introduced by the biomass.

3.2. Effect of Diesel Volume added to the Direct Transesterification

The effect of the volume of diesel on the characteristics of the produced diesel extracts was evaluated by testing various diesel volumes added to the direct transesterification of yeast and microalgae including 0.08:1, 0.3:1, 0.7:1, 1.5:1, and 4:1 (volume:volume). Chromatograms showing diesel fuel before and after extraction of FAME produced from the direct transesterification of yeast and microalgae are shown in **Figure 2**.

3.2.1. FAME extraction

The amount of FAME extracted from the direct transesterification was evaluated against various volumes of diesel fuel. For the reaction of yeast biomass, the diesel to reaction solvent volume ratio of 1.5:1 maximized FAME extraction with $93.0 \pm 3.3\%$, however, even the lowest ratio of 0.08:1 did very well at extracting $77.2 \pm 5.6\%$ of available FAME. For microalgae, the diesel to reaction solvent volume ratio of 1.5:1 also maximized FAME extraction with $82.6 \pm 1.5\%$, however, lower ratios suffered low FAME extraction with as low as $30.4 \pm 1.6\%$ for the 0.08:1 ratio (**Figure 3**). It is likely that the microalgal biomass introduces emulsifying compounds, which interfere with proper separation of the diesel and methanol layers. This phenomenon is supported by the fact that the amount of diesel recovered is reduced at lower diesel to reaction solvent volume ratios for microalgae (**Table 1**). Overall, diesel fuel demonstrates good extraction efficiency of FAME from direct transesterification of yeast and microalgae biomass without the need for copious amounts of solvent or the addition of water as demonstrated in other FAME/lipid solvent extraction techniques [26,25].

3.2.2. Diesel Recovery

The amount of diesel recovered during extraction would likely be a key determinant in optimization of the two-step diesel extraction process. The loss of diesel fuel during extraction due to emulsification would likely lead to large energy inputs in its recapture. It was found that for both the direct transesterification of yeast and microalgae, lower diesel to reaction solvent

volume ratios led to a more pronounced emulsification of the diesel fuel in the methanol layer. However, at larger diesel to reaction solvent volume ratios, between 98 and 99% of the introduced diesel is recovered (**Table 1**). This demonstrates that the use of diesel fuel acts much like other solvents in solvent-based extraction processes like that used for hexane soybean oil extraction [27].

3.2.3. Pigment co-extraction

The introduction of non-polar pigments presented by the biomass in the direct transesterification reaction makes downstream purification processes necessary. Carotenoids in yeast and chlorophyll in microalgae are the most common contaminants in microbial biodiesel production since they are likewise non-polar and are co-extracted with solvent-based extraction [12]. Since products of both triglyceride and biodiesel extraction require decolorizing and other purification processes, developing a procedure that can avoid these energy inputs would be advantageous [6,28]. For the direct transesterification of yeast biomass, a diesel to reaction solvent volume ratio of 0.08:1 excludes greater than 96% of carotenoids in the produced diesel extract. Similarly, greater than 99% of chlorophyll is excluded in diesel extracts from microalgae (**Figure 4, Figure 5**). Carotenoids were not detected in the diesel extracts of microalgae biomass. It is likely that the acid present during reaction not only promotes cell lysis and acts as a catalyst for the transesterification, but may also contribute to the breakdown of chlorophyll and carotenoid pigments. It has been reported that acid may contribute to the hydrolysis of chlorophyll removing magnesium ion forming pheophytin, and it may also be further hydrolyzed such that the phytol constituent is released leaving only the protoporphyrin ring [29,30]. Similarly, it is known that carotenoids are prone to oxidative degradation under elevated temperatures in the presence of acid, which may be accelerated by the presence of compounds present after cell lysis [31]. In either case it is likely that any pigments present may

be structurally changed in such a way that they are partitioned into the more polar methanol layer and thereby excluded from the diesel extracts.

3.2.4. Methanol content

Methanol and diesel fuel are nearly immiscible in one another, which allows for proper separation of the two layers after centrifugation. However, the presence of a microemulsion of methanol in the produced extract would likely lower the flash point below acceptable ASTM standards as well as the energy density [32]. In order to quantify the amount of methanol in the produced extracts, samples from different diesel to reaction volume ratios were evaluated. Over the various diesel to reaction volumes tested, most of the methanol content in the produced extracts were around 1% by mass, however at lower diesel to reaction volume ratios these numbers increased to over 2% for yeast and over 3% for microalgae in the case of the 0.08:1 diesel to reaction solvent volume (**Table 1**). The presence of this microemulsion of methanol produced in the diesel extracts would likely require quick distillation of residual methanol to ensure the diesel blend quality.

3.2.5. Total Glycerin

The presence of glycerin derivatives including free glycerol and bound glycerides (i.e. monoglycerides, diglycerides, triglycerides) in the produced extracts would result from incomplete conversion and subsequent extraction by the diesel fuel. The presence of free and bound glycerides have been determined to lead to storage stability problems as well as causing detrimental effects on engines including injector fouling, deposits on internal engine parts, and higher aldehyde emissions [33,34]. Free glycerol was not detectable in any of the diesel extracts from either microalgae or yeast. The amount of bound glycerides (monoglycerides, diglycerides, and triglycerides) is minimal in both yeast and microalgae diesel extracts (**Table 1**).

3.3. ASTM Evaluation of B20 Biodiesel Blend from Yeast

In order to evaluate the blends produced from the two-step process, an approximate B20 blend produced from yeast was subject to a series of ASTM sanctioned tests. These tests include flash point, viscosity, sulfur, oxidation stability, and acid number. These tests were chosen as these parameters were thought to potentially implicate the quality of the produced blends using the two-step diesel extraction process. First, the detection of a microemulsion of methanol in the diesel extracts was considered to be a possible concern, as it would likely affect the flash point. Samples evaluated for flash point directly after extraction were found to have flash point values under the allowed minimum limit since some methanol is co-extracted into the diesel layer (**Table 1**). In order to produce a blend with an acceptable value, the B20 blend was subject to heating at 100 °C to distill off any residual methanol while not losing any of the diesel fuel as the distillation range of diesel fuel is between 200 and 300 °C [35]. The fact that most of these extracts contained around 1% methanol by mass demonstrates that the extra distillation step would not be energy intensive. Since 2007, diesel fuel has been mandated to contain less than 15 ppm sulfur, which requires hydrosulfurization [30,31]. Therefore, we saw it as necessary that the biomass introduced into the diesel fuel during extraction did not contaminate the fuel beyond the limits allowed. The ASTM test demonstrates that the sulfur values are well under the limit at 6.18 ppm. Oxidation stability is often a quality that pure biodiesel fuel fails due to the lack of antioxidants, which must be added after purification to prevent rancidification. Here, the B20 blend demonstrates that the oxidation stability well outlasts the minimum of 6 hours at nearly 36 hours. It is possible that the diesel fuel also co-extracts antioxidants such as tocopherols from the biomass, thereby making their addition unnecessary [33]. The direct transesterification contains sulfuric acid and/or the presence of unconverted fatty acids that could be co-extracted by the diesel. To determine the presence of any fatty acids or residual acids, the acid number was evaluated. The results show that the maximum value of 0.3 (mg KOH/g) is met by the blend at a

value of 0.26 (mg KOH/g). Lastly, the viscosity, which would reflect any residual triglycerides, was shown to fall well within the limits at 2.7 cSt [34]. All tests reported for the yeast B20 blend generated from the diesel extraction process had values which fell within ASTM requirements and gave similar values to a B20 blend produced from soybean biodiesel (**Table 2**).

3.4 Energetics of Microbial Biodiesel Production

In order to compare the two-step diesel extraction procedure presented here to traditional biodiesel production by solvent extraction and conversion methodologies, experimental data was integrated into engineering system models to evaluate the impact on the metric of NER. The initial input for each process flow starts with dry microbial biomass containing 70% lipid and ends with B100 utilizing traditional solvent extraction and conversion and a B5 blend generated from the two-step diesel extraction procedure (**Figure S1**). The results show a 25% energy savings with the two-step diesel extraction procedure compared to the traditional method. Diesel extraction gives a NER of 0.17 whereas traditional solvent extraction gives a NER of 0.24. Further detail and explanation of the NER calculations can be found in the Supplemental materials (Figures S1-S6 and Tables S1-S2).

4. Conclusions

Here we demonstrate that the use of petroleum diesel fuel selectively and efficiently extracts biodiesel produced from direct transesterification of yeast and algae biomass. The two-step diesel extraction procedure can bypass intermediate steps of traditional biodiesel production which reduces the NER of microbial biodiesel production and recovery by 25%. A B20 biodiesel blend produced from yeast passes select ASTM tests and may allow direct use in diesel engines. Future work evaluating the scalability of this process as well as co-extraction of other minor contaminants and their subsequent effect on the produced fuel blends will be necessary.

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FIGURE CAPTIONS

Figure 1. Reaction Solvent Volume to Biomass Transesterification Efficiency of Yeast and Algae. The FAME yield produced from direct transesterification of biomass from the microalgae, *Scenedesmus dimorphus* (○), and the FAME yield produced from the yeast, *Cryptococcus curvatus* (□), over several reaction solvent volume to biomass ratios including 2:1, 5:1, 10:1, and 20:1 (volume:weight). The reaction solvent contained 2% sulfuric acid in methanol (% v:v). The total FAME was normalized from the maximum amount of FAME produced from 100 mg of microbial biomass in each case. Error bars are shown with standard deviation of the mean.

Figure 2. GC Trace of Unblended Diesel Before and After Extraction of Fatty Acid Methyl Esters (FAME) Produced from Direct Transesterification of Yeast and Microalgae. **A.** Petroleum diesel blend verified to contain no biodiesel. **B.** Diesel blend after extraction of FAME produced from direct transesterification of the yeast, *Cryptococcus curvatus*. **C.** Diesel blend after extraction of FAME produced from direct transesterification of the microalgae, *Scenedesmus dimorphus*. Indicated compounds are methyl palmitate (1), methyl oleate (2), methyl stearate (3), and methyl tetracosanoate (4).

Figure 3. Effect of Different Diesel to Reaction Solvent Volume Ratios on FAME Extraction. The percent of FAME extracted from the direct transesterification reaction of the microalgae, *Scenedesmus dimorphus* (○) and the percent of FAME extracted from the direct transesterification reaction of the yeast, *Cryptococcus curvatus* (□), using different diesel to reaction solvent volumes including 0.08:1, 0.3:1, 0.7:1, 1.5:1, and 4:1 (v:v). The total available FAME for extraction is based upon the milligrams of FAME produced from the direct transesterification reaction using the optimal 10:1 reaction solvent volume to biomass ratio (volume:weight) for yeast and microalgae. Error bars are shown with standard deviation of the mean.

Figure 4. Effect of Different Diesel to Reaction Solvent Volume Ratios on Percent of Pigments Extracted. The amount of chlorophyll and carotenoids extracted into the diesel extracts using different diesel to reaction solvent ratios including 0.08:1, 0.3:1, 0.7:1, 1.5:1, and 4:1 (v:v) to the direct transesterification reaction of the microalgae, *Scenedesmus dimorphus* (○) and the yeast, *Cryptococcus curvatus* (□), respectively. The amount of pigment in each layer was calculated spectrophotometrically according to Lichtenthaler *et al.* Error bars are shown with standard deviation of the mean.

Figure 5. Image Demonstrating the Separation of Diesel and Methanol Layers After Centrifugation. **Left.** Separation of the produced diesel blend after extraction FAME produced from direct transesterification of the yeast, *Cryptococcus curvatus*. **Right.** Separation of the produced diesel blend after extraction of FAME produced from direct transesterification of the microalgae, *Scenedesmus dimorphus*. Layers are indicated as the biodiesel/diesel layer, the reaction solvent containing methanol and sulfuric acid, and pelleted residual biomass.

Table 1. Blend properties based upon diesel to reaction solvent ratio (volume:volume)

Organism	Diesel:Reaction Volume (v:v) ^c	% Diesel Recovery ^d	% Methanol (% g/g) ^e	% Total Glycerin (% g/g) ^f
Microalgae ^a	0.08:1	54.1 ± 8.2	3.6 ± 0.3	0.046 ± 0.005
	0.3:1	82.7 ± 1.5	2.4 ± 0.1	0.017 ± 0.002
	0.7:1	93.5 ± 2.3	1.5 ± 0.4	0.006 ± 0.002
	1.5:1	95.6 ± 1.5	1.1 ± 0.2	0.003 ± 0.001
	4:1	97.8 ± 0.4	1.1 ± 0.3	ND ^g
Yeast ^b	0.08:1	75.7 ± 1.6	0.7 ± 0.4	0.009 ± 0.001
	0.3:1	80.7 ± 0.6	1.2 ± 0.1	0.003 ± 0.001
	0.7:1	93.3 ± 0.6	0.9 ± 0.3	0.002 ± 0.001
	1.5:1	97.0 ± 0.9	1.1 ± 0.5	ND ^g
	4:1	98.9 ± 0.4	0.7 ± 0.4	ND ^g

^aThe microalgae strain *Scenedesmus dimorphus*.

^bThe yeast strain *Cryptococcus curvatus*.

^cThe volumetric ratio of diesel added to the direct transesterification reaction.

^dThe mass of diesel recovered in the diesel extract.

^eThe percent mass of methanol in the diesel extracts as quantified by GC-MS analysis

^fThe percent mass of free and bound glycerides in the diesel extracts determined via GC-MS and GC-FID, respectively, and calculated according to Munari *et al.*

^gNot detectable with a detection limit of free glycerides at 0.01% (% g/g) and each bound glyceride at 0.005% (% g/g).

The average and standard deviation of the data are shown.

Table 2: Select fuel properties of a yeast biodiesel blend produced from two-step diesel extraction

Test Name (units)	Yeast B20 ^a	Soybean B20 ^b	ASTM Requirement ^c
Flash Point ^d (°C)	72.5	68	≥52
Viscosity ^e (cSt)	2.7	2.5	1.9 - 4.1
Sulfur ^f (ppm)	6.18	2.28	≤15
Oxidation Stability ^g (hrs)	35.9	3.04	≥6
Acid Number ^h (mg KOH/g)	0.26	0.12	≤0.3

^aApproximate B20 blend produced from two-step diesel extraction of the yeast *Cryptococcus curvatus*

^bFuel properties of a soybean B20 blend measured by Pala-En *et al.*

^cRequirements of ASTM D7467: Diesel Blends B6 to B20

^dMeasurement made according to test method ASTM D93 by Pensky Martens closed cup

^eMeasurement made according to test method ASTM D445 at 40°C.

^fMeasurement made according to test method ASTM D5453 by UV fluorescence

^gMeasurement made by rancimat according to test method EN15751

^hMeasurement made according to test method ASTM D664

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5