Hydrothermal Liquefaction of Bacteria and Yeast Monocultures

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Supporting Information

ABSTRACT: We hydrothermally treated monocultures of *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* at isothermal (350 °C for 60 min) and fast (rapid heating for 1 min) liquefaction conditions. Fast hydrothermal liquefaction (HTL) of *P. putida* and *S. cerevisiae* produced the highest biocrude yields of 47 ± 13 and 48 ± 9 wt %, respectively. Biocrudes generated via fast HTL were always richer in O and N and had a higher yield of hexane-insoluble products. Isothermal HTL of all microorganisms always produced an aqueous phase richer in NH₃ than the aqueous phase from fast HTL. Up to 62 ± 9% of the chemical energy in the biomass could be recovered in the biocrude product fraction. These results demonstrate the feasibility of applying HTL to produce high yields of biocrude from bacteria and yeast that are high in protein (>80 wt %, dry and ash-free basis (daf)) and low in lipids (<3 wt %, daf). Such microorganisms could serve as a renewable feedstock for biofuels.

INTRODUCTION

Bacteria and yeasts are widely used in industrial biochemical processes that convert feedstock substrates into finished products, such as specialty chemicals, food products, and biofuels. Although there is significant value in the aforementioned products, the microbial biomass itself is usually discarded as a waste. Biochemical processes are also used in wastewater treatment, and they create a secondary sludge that is rich in microorganisms, which are often collected and discarded. This microbial biomass can have heating values comparable to that of microalgae, which has received tremendous attention as a feedstock for biofuels. Hydrothermal liquefaction (HTL), a high-temperature and high-pressure process, can convert whole wet biomass into energy-dense bio-oils, also referred to as biocrude. HTL of bacteria and yeasts may create an opportunity to produce a renewable biofuel from low-value materials.

The HTL of microbial communities in sludge is not new. HTL of sludge from biologically treated cornstarch and pulp/paper waste resulted in biocrude yields of 15–30 and 42–65 wt %, respectively. HTL of anaerobically digested sludge from municipal sources has been less successful, producing biocrude yields of ≤10 wt %. Recent studies of HTL of microorganisms have focused on microalgae as a feedstock and produced biocrude yields as high as 66 wt %. Although a majority of the algal biomass is converted into biocrude, the aqueous co-product also contains some organic carbon along with nutrients, such as nitrogen-and phosphorus-containing substrates. This aqueous co-product can be used for cultivating additional biomass. Using it to grow more algae can be difficult, as several studies have shown that it can be toxic or nutrient-limited. Moreover, recycling this water, which contains organic carbon, to an open pond for algae growth may substantially increase the risk of invasion of contaminating heterotrophs into the algae culture. However, Nelson et al. have recently demonstrated the feasibility of cultivating *Escherichia coli* and *Pseudomonas putida* monocultures using this aqueous phase with minimal dilution and nutrient supplementation. Adding a microbial cultivation step in an algae HTL biorefinery to use the organic carbon in the aqueous phase can enhance ease of its recycling to an open pond and provide additional biomass for HTL. To the best of our knowledge, HTL of microbial monocultures, such as bacteria and yeast, has not been examined.

We cultivated *E. coli*, *P. putida*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* and subjected the biomass to hydrothermal treatment at fast (rapid heating for 1 min) and isothermal (350 °C for 60 min) liquefaction conditions. *E. coli* and *S. cerevisiae* are bacterial and yeast species, respectively, that are frequently used in industrial bioprocesses. We selected *P. putida* because it is known to metabolize a diverse array of substrates, which makes it a good candidate for growth on complex waste streams. *B. subtilis*, a widely studied Gram-positive bacterium, was included to investigate the impact of its differing cellular composition (particularly in the peptido-glycan-abundant cell wall) on liquefaction products when compared to the other two Gram-negative bacteria (*E. coli* and *P. putida*). We report herein the results of how microorganism selection, growth media, cellular structure (Gram-positive versus Gram-negative), and hydrothermal treatment conditions affect the yield and composition of the different product fractions from HTL.

MATERIALS AND METHODS

This section first describes the cultivation and analysis of the biomass feedstocks and then describes the procedures for the hydrothermal treatment and the collection, separation, and analysis of the product fractions.
Biomass Cultivation and Harvesting. We grew all four organisms in various “rich” media containing high concentrations of complex biologically derived materials, such as yeast extract and peptone, to maximize the biomass yield per volume of culture. For the rich media, we used Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) for P. putida, terrific broth medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 2.31 g/L L glucose, 1 g/L NH₄Cl, 6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 mM Mg₂SO₄, and 0.1 mM CaCl₂) for E. coli and B. subtilis, and yeast peptone dextrose medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose) for S. cerevisiae. We also grew E. coli in a “minimal” medium, which contained only chemically defined substrates and nutrients. For the E. coli minimal medium, we used M9 medium (20 g/L glucose, 1 g/L NH₄Cl, 6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 mM Mg₂SO₄, and 0.1 mM CaCl₂). Both seed cultures and final cultures were grown in the same media. E. coli grown in terrific broth and M9 minimal medium will be referred to as E. coli TB and E. coli MM, respectively.

To grow the biomass feedstock, we first obtained cryogenically preserved (−80 °C) stocks of E. coli K12 MG1655, P. putida KT2440, B. subtilis SB491, and S. cerevisiae S288C and grew a 20 mL seed culture in a 50 mL test tube. We incubated the seed culture for 24 h at 30 °C and agitated it at 250 rpm in a New Brunswick Excella E24 incubator. We then added the seed cultures to fresh growth media at a ratio of 20 mL seed culture/2 L medium in 1.5−2.0 L cultures and grew them for 24−48 h in a New Brunswick I26 or Innova 4400 incubator agitated at 120 rpm. Incubation temperatures ranged from 30 to 37 °C, and growth vessels were capped with a sterile filter to allow for oxygen transport.

We harvested cell cultures by centrifuging them in 1 L vessels at 5000 relative centrifugal force (rcf) for 15 min in a Beckman Coulter Avanti J-20XP or Thermo Scientific Sorvall Lynx 6000 centrifuge. To “wash” the biomass to remove potential substrates remaining from the media, we discarded the supernatant media, resuspended the cell pellets in an equal volume of deionized water, and then reformed the pellet again with a 20 min centrifugation. We then discarded the supernatant water, resuspended each cell pellet in 10 mL of water, transferred this slurry to 50 mL centrifuge tubes, and centrifuged it once more at 5000−12 000 rcf in an Allegra 21R or Eppendorf 5810R centrifuge. We discarded the supernatant water from these tubes and considered the resulting cell pellets raw wet biomass. The collected biomass was always at least a 12 wt % slurry.

Biomass Feedstock Analysis. We dried biomass samples in a 70 °C oven for 48 h to completely remove the water. To determine the ash content, we measured 50 mg of the dried biomass into a preweighed aluminum weigh boat. A Ney Vulcan 3-130 muffle furnace heated the sample to 250 °C from room temperature at a rate of 10 °C/min. After a 30 min holding period, the temperature was increased at a rate of 20 °C/min to 550 °C and then held for 30 h. After removing the samples from the furnace, we cooled them in a desiccator to room temperature and then recorded the mass of ash. We sent dried samples of the biomass feedstock to Atlantic Microbials, Inc. for analysis of C, H, N, and S contents. We calculated the O content as the difference between 100 wt % and the combined contents of C, H, N, S, and ash.

We developed a lipid analysis method by combining practices from Levine et al. and Lewis et al. and purchased chemicals of >99% purity, from Fisher Scientific. We measured approximately 20 mg of dried biomass into a glass test tube and then added 2 mL of a 5% (v/v) solution of acetyl chloride in methanol and a magnetic stir bar. We vigorously stirred the reaction mixture (>100 rpm) for 90 min at 100 °C using a magnetic stir plate and temperature-controlled heating block. After the holding period, we quenched the reaction by adding 1 mL of room-temperature deionized water. After the solution cooled for 10 min, we added 4 mL of n-heptane and agitated each tube for 10 min on a vortexer set to 1000 rpm. We centrifuged the mixture for 3 min at 1500 rcf to separate and then collect the heptane layer for gas chromatographic analysis. We injected 1 μL of sample, with a 2:1 split ratio, into an Agilent 7890 gas chromatograph equipped with an Agilent DB-FFAP column (30 m × 320 μm × 0.25 μm). Helium at a column flow of 1 mL/min was the carrier gas. The injector temperature was 250 °C. The oven temperature was maintained at 60 °C until the injection and then increased to 200 °C at a rate of 20 °C/min and then to 240 °C at a rate of 5 °C/min. The final temperature was held for 3 min. We generated calibration curves using a RESTEK Marine Oil mixture of 20 fatty acid methyl esters as an external standard.

We estimated the protein content (wt %) of the biomass by multiplying its N content (wt %) by 6.25. We calculated the carbohydrate content as the difference between 100 wt % and the sum of the lipid, protein, and ash contents.

HTL. We constructed Swagelok reactors using 3/4 in. port connectors fitted with a cap on one end and a 3/8 in. union on the other end. The union allowed for the attachment of a 15 000 psi-rated High Pressure Equipment Co. valve, with grafoil packing, for sampling the gas products. The valve was attached via 8.5 in. of 1/4 in. outer diameter tubing. The nominal volume added by the valve was...
Table 1. Elemental and Biochemical Compositions (wt %) and Higher Heating Value (MJ/kg) of the Biomass

<table>
<thead>
<tr>
<th>Feedstock</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>S</th>
<th>O</th>
<th>ash</th>
<th>lipid</th>
<th>protein</th>
<th>carbohydrate</th>
<th>HHV</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli TB</td>
<td>46.54</td>
<td>6.69</td>
<td>13.70</td>
<td>0.67</td>
<td>25.58</td>
<td>6.82 ± 0.02</td>
<td>0.57 ± 0.36</td>
<td>86</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>E. coli MM</td>
<td>47.32</td>
<td>6.88</td>
<td>13.17</td>
<td>0.58</td>
<td>27.15</td>
<td>4.9 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>82</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>P. putida</td>
<td>46.58</td>
<td>7.08</td>
<td>13.23</td>
<td>0.55</td>
<td>21.48</td>
<td>11</td>
<td>2.7 ± 0.7</td>
<td>83</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>42.65</td>
<td>6.56</td>
<td>11.45</td>
<td>0.43</td>
<td>25.91</td>
<td>13.0 ± 0.4</td>
<td>0.55 ± 0.03</td>
<td>72</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>46.47</td>
<td>7.31</td>
<td>12.04</td>
<td>0.47</td>
<td>29.03</td>
<td>4.68 ± 0.01</td>
<td>2.7 ± 0.6</td>
<td>75</td>
<td>17</td>
<td>22</td>
</tr>
</tbody>
</table>

0.5 mL. The total volume of the reactor and valve is approximately 2.2 mL (depicted in Figure 1).

For conventional, isothermal HTL, we loaded 1.35 g of 12 wt % biomass slurry to each Swagelok reactor. At this loading, water would fill 95% of the reactor volume at the reaction conditions. We sealed the reactors and placed them into a Techne fluidized sand bath set at 350 °C. The reactors were submerged in the sand bath and agitated using a Burrell Wrist Action shaker for 60 min. For fast HTL reactions, performed with rapid heating, we loaded the reactor with 0.30 g of 12 wt % biomass slurry. This water loading matched previous experiments in our laboratory. After sealing the reactors, they were placed in a 600 °C sand bath for 1 min. As previously described by Faeth et al., we used dummy reactors (depicted in Figure 2) fitted with a thermocouple to record the temperature and calculate the heating rate. In both cases, after the desired holding time had elapsed, we removed the reactors from the sand bath and quenched them in a room-temperature water bath.

Recovery and Analysis of HTL Product Fractions. We followed the procedure published previously to collect and separate the solids, aqueous-phase products, light biocrude (hexane solubles), heavy biocrude (hexane insoluble and dichloromethane soluble), and gas product fractions from each reactor.\(^\text{5,10}\) We report the gravimetric yield of each product fraction as its mass divided by the mass of biomass loaded into the reactor on a dry basis (wt %). We also report the biocrude yields on a dry and ash-free basis (daf) because the ash content cannot contribute to the yield of biocrude.

Solvent-free samples of the light and heavy biocrude were sent to Atlantic Microlab, Inc. for measurement of the weight percent of C, H, N, S. The O content in the light and heavy biocrudes was calculated by difference. We report elemental distribution as the mass of an element in each of the product fractions per the total mass of that element in the biomass. We diluted the aqueous phase 1:600 with deionized water and measured NH3 in the aqueous phase using methods described previously.\(^\text{9}\)

The hexane-soluble product, also referred to as the light biocrude, was analyzed with an Agilent 5973 mass spectrometer. We injected 1 μL into a 300 °C split injection port using a split ratio of 2:1 onto an Agilent HP-5 capillary column (50 m × 200 μm × 0.33 μm). The oven was set to 100 °C, and the temperature increased to 300 °C at a rate of 5 °C/min immediately after injection. The samples exited the column into an electron ionization mass spectrometer. We used matching software to tentatively identify molecular constituents in the biocrude sample based on mass spectra.

We calculated the higher heating value of the light and heavy biocrude using the Boie formula\(^\text{29}\) and the elemental composition data (wt %) on a dry basis.

\[
\text{Higher heating value (MJ/kg)} = 0.3516C + 1.16225H + 0.00628N + 0.10465S - 0.11090
\]

We used the heating values and gravimetric yields of the biocrude product fractions to estimate the energy recovered in the biocrudes from the original biomass. We report the average of at least three replicate experiments, unless otherwise noted. When available, we report experimental error as 1 standard deviation.

Control Experiments. We added approximately 160 mg of dried biomass to a glass test tube, which is similar to the biomass loading in the reactor for isothermal liquefaction. We then added 9 mL of dichloromethane and agitated the samples on a vortexer set at 1000 rpm for 1 h. After mixing, we added 1.2 mL of deionized water, which mimics the water loading in a reactor, and agitated the samples for another 1 h. We then followed the workup procedure described previously to collect and measure the yield of each of the product fractions, except for gas.\(^\text{9}\) This control experiment provides the yields of each product fraction available from the biomass simply by wet extraction without HTL.

**RESULTS AND DISCUSSION**

This section first reports the characteristics of the microorganisms that we cultivated and then reports the results of the hydrothermal treatment of the biomass. The latter section describes the yield, elemental composition, and selected molecular composition of the product fractions. We also report the heating value and energy recovery of the biocrudes and compare results among the various microorganisms.

Feedstock Analysis. Table 1 shows the elemental and biochemical contents of each of the biomass feedstocks. There are but modest variations in the elemental compositions of C, H, N, S. The O content in the light and heavy biocrudes was calculated by difference. We report elemental distribution as the mass of an element in each of the product fractions per the total mass of that element in the biomass. We diluted the aqueous phase 1:600 with deionized water and measured NH3 in the aqueous phase using methods described previously.\(^\text{9}\)

Although the two E. coli cultures were cultivated using different growth media, their C, H, N, and O contents were within 6% relative difference of those reported previously.\(^\text{30,31}\) However, the yeast and bacteria had lipid values <0.6 wt %.

E. coli grown with MM, S. cerevisiae, and P. putida had the highest lipid contents, but they were all ≤2.7 wt %. E. coli grown with TB and B. subtilis had lipid values ≤0.6 wt %. E. coli TB was grown in a nutrient-rich condition; therefore, it is reasonable that the cells would accumulate less lipids than the E. coli MM. Likewise, Gram-positive organisms, such as B. subtilis, have fewer lipids possibly because of the lack of an outer membrane in the cell envelope compared to Gram-negative organisms. The carbohydrate content of each biomass sample varied between 4 and 17 wt %. All biomass samples were rich in protein (≥72 wt %). In comparison to microalgae feedstocks typically subjected to HTL, the yeast and bacteria...
have a much lower lipid content and much higher protein and N content.\textsuperscript{32}

**Yields of Product Fractions.** As a control experiment, we exposed dried, unreacted biomass to fresh solvents at room temperature. After exposure to dichloromethane and water, ≤4 wt % of the biomass partitioned to the organic phase as biocrude. The remaining biomass partitioned to the solid product fraction. Therefore, solvent extraction alone does not generate the high biocrude yields that are typical of hydrothermal treatment.

Figure 3 shows the daf yields of light and heavy biocrudes for each organism at both hydrothermal treatment conditions. Yields of biocrudes are presented on a dry basis in Table S1 of the Supporting Information. The isothermal treatment at 350 °C for 60 min is common practice;\textsuperscript{8,11,33−35} therefore, the present results from the yeast and bacterial biomass can be compared to those from HTL of other feedstocks. We also used rapid heating or fast HTL, which can increase the biocrude yield.\textsuperscript{16} The average maximum temperature observed in the dummy reactor during fast HTL was 276 ± 41 °C, and the average heating rate of the reactors at fast liquefaction...
Table 4. Tentative Identities and Relative Abundance of Different Compounds in the Light Biocrude

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>% Total Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-methylphenol</td>
<td><img src="image" alt="Ethylphenol Structure" /></td>
<td>2.43 - 1.82 1.4 5.7 - 1.14 -</td>
</tr>
<tr>
<td>4-ethylphenol</td>
<td><img src="image" alt="Ethylphenol Structure" /></td>
<td>1.98 - 1.41 1.41 4.09 - 0.58 -</td>
</tr>
<tr>
<td>Piperidin-2-one</td>
<td><img src="image" alt="Piperidin-2-one Structure" /></td>
<td>0.8 - 0.79 0.44 2.72 - 0.29 -</td>
</tr>
<tr>
<td>2H-azepin-2-one</td>
<td><img src="image" alt="2H-azepin-2-one Structure" /></td>
<td>0.81 - 0.56 0.4 2.66 - 0.31 -</td>
</tr>
<tr>
<td>Indole</td>
<td><img src="image" alt="Indole Structure" /></td>
<td>0.75 - 0.49 1.32 0.57 1.04 1.65 1.78 0.12 -</td>
</tr>
<tr>
<td>(3,5, or 7)-methylindole</td>
<td><img src="image" alt="Methylindole Structure" /></td>
<td>1.46 - 1.07 1.08 0.55 3.32 1.24 0.56 0.49</td>
</tr>
<tr>
<td>N-alkylbenzamine</td>
<td><img src="image" alt="N-alkylbenzamine Structure" /></td>
<td>1.67 - 1.59 1.24 1.68 0.85 0.67</td>
</tr>
<tr>
<td>2,4-dimethylindole</td>
<td><img src="image" alt="2,4-dimethylindole Structure" /></td>
<td>0.57 - 0.44 0.57 1.21 - 0.51 -</td>
</tr>
<tr>
<td>N-(2-phenylethyl)ethanamide</td>
<td><img src="image" alt="N-(2-phenylethyl)ethanamide Structure" /></td>
<td>0.42 - 1.3 1.01 2.24 1.61 0.22 0.91</td>
</tr>
<tr>
<td>Dodecanoic acid</td>
<td><img src="image" alt="Dodecanoic acid Structure" /></td>
<td>0.58 2.77 0.52 1.07 0.58 3.03 - 0.37 0.77</td>
</tr>
<tr>
<td>2-(indol-3-yl)acetaldehyde</td>
<td><img src="image" alt="2-(indol-3-yl)acetaldehyde Structure" /></td>
<td>0.64 - 1.6 0.61 0.67 0.43 -</td>
</tr>
<tr>
<td>1-[(3-methylindol-1-yl)ethane]</td>
<td>![1-<a href="image">(3-methylindol-1-yl)ethane Structure</a></td>
<td>0.57 - 0.53 0.69 - 0.41 -</td>
</tr>
<tr>
<td>1-phenylmethyl-2-pyrrolidnone</td>
<td><img src="image" alt="1-phenylmethyl-2-pyrrolidnone Structure" /></td>
<td>0.3 - 0.28 0.36 0.28 - 0.96 -</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td><img src="image" alt="Tetradecanoic acid Structure" /></td>
<td>1.39 3.24 2.17 4.86 1.15 12.41 14.24 1.19 2.2</td>
</tr>
<tr>
<td>Tetradecanamide</td>
<td><img src="image" alt="Tetradecanamide Structure" /></td>
<td>0.52 0.53 0.28 0.64 0.64 - 0.28 -</td>
</tr>
<tr>
<td>9H-carbazole</td>
<td><img src="image" alt="9H-carbazole Structure" /></td>
<td>0.56 0.96 0.44 0.96 0.44 0.44 0.44 0.44 0.44 0.44</td>
</tr>
<tr>
<td>Hexadecanenitrile</td>
<td><img src="image" alt="Hexadecanenitrile Structure" /></td>
<td>0.81 0.84 1.41 1.24 0.63 - 0.84 0.62</td>
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<td>Cycloheptanindole</td>
<td><img src="image" alt="Cycloheptanindole Structure" /></td>
<td>0.58 0.38 0.17 0.58 0.38 0.17 0.58 0.38 0.17 0.58</td>
</tr>
<tr>
<td>9H-carbazole</td>
<td><img src="image" alt="9H-carbazole Structure" /></td>
<td>0.56 0.96 0.44 0.96 0.44 0.44 0.44 0.44 0.44 0.44</td>
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<tr>
<td>Hexadecanenitrile</td>
<td><img src="image" alt="Hexadecanenitrile Structure" /></td>
<td>0.81 0.84 1.41 1.24 0.63 - 0.84 0.62</td>
</tr>
<tr>
<td>Cycloheptanindole</td>
<td><img src="image" alt="Cycloheptanindole Structure" /></td>
<td>0.58 0.38 0.17 0.58 0.38 0.17 0.58 0.38 0.17 0.58</td>
</tr>
</tbody>
</table>
conditions was $216 \pm 37 \, ^\circ\text{C/min}$, with the uncertainty representing 1 standard deviation of the population. 

*B. subtilis* showed the lowest total yield of biocrude for both conventional isothermal ($17 \pm 3 \, \text{wt} \, \%$, daf) and fast ($4 \pm 1 \, \text{wt} \, \%$, daf) liquefaction conditions. These low yields may be linked to its cellular structure. One contributing factor may be the different molecular composition of the cell envelope. Gram-positive bacteria, including *B. subtilis*, have a thick layer of peptidoglycans, which are polysaccharides cross-linked by polypeptides. These biomacromolecules likely hydrolyze to simple sugars and amino acids that would reside within the aqueous-phase product fraction. Past research has shown that continued hydrothermal processing of water-soluble amino acids and carbohydrates can form organic-solvent-soluble products.\textsuperscript{36,37} The 1 min reaction time for fast HTL may have been long enough to form these water-soluble products yet too brief to allow for their repolymerization into biocrude components. Such processes could account for the higher biocrude yield at isothermal HTL conditions observed in Figure 3 for *B. subtilis*.

*Fast hydrothermal treatment of *P. putida* and *S. cerevisiae* produced the most biocrude with yields of $47 \pm 13$ and $48 \pm 9 \, \text{wt} \, \%$, respectively. The higher yield of biocrude from *S. cerevisiae* may also be linked to the cell structure of the yeast, in this case improving the yield, unlike the result from *B. subtilis*. The larger standard deviation in the yields from fast HTL probably arises from the lower biomass loadings. There is less material to recover in these experiments, and transfer losses become more significant, on a relative basis, compared to conventional liquefaction.\textsuperscript{16} The reaction conditions did not affect the total yield of biocrude for both types of *E. coli*. Figure 3 shows that fast liquefaction always produced a higher fraction of heavy biocrude than light biocrude, regardless of organism. This trend is also true for the fast liquefaction of the microalga *Nannochloropsis* sp.\textsuperscript{16} Figure 3 shows that, with the exception of *B. subtilis*, the yield of total biocrude produced at conventional HTL conditions did not vary much, ranging between 26 and 35 wt %, daf for the different microorganisms.

Table 2 shows the yield of solids (water and dichloromethane insoluble), aqueous-phase products, and gas for both isothermal and fast HTL of each organism. The aqueous phase was translucent amber in color for all of the different microorganisms. More than 42 wt % of the biomass is converted to aqueous-phase products. The solids were gray powders that settled at the bottom of the test tube. Solid yields were always <9 wt %, with the exception of *E. coli* MM treated with fast HTL. *E. coli* differentially expresses genes that affect the cell structure when grown in minimal versus rich medium.\textsuperscript{38} The milder reaction conditions in fast HTL may not have been sufficient to break down certain components in the *E. coli* cell, especially when grown in MM. The data in Table 2 suggest that...
the residual solids remaining after fast liquefaction likely form mostly aqueous-phase products when reacted under the harsher isothermal HTL conditions.

Table 2 shows that <7 wt % of the biomass is converted to gas. The differences in the composition of the gas products from HTL of each biomass were not statistically significant. Therefore, Figure 4 shows the average composition of the gas phase from isothermal and fast HTL. The gas phase from isothermal HTL was on average 93 ± 6 mol % CO₂ with the balance being H₂, CO, CH₄, C₂H₄, and C₂H₆. The gas from fast HTL had significantly less CO₂, 64 ± 15 mol %, and significantly more CO, CH₄, C₂H₄, and C₂H₆. At harsher reaction conditions, CO₂ is a common product from the hydrothermal decomposition of amino acids.³⁹

Elemental Composition of the Light and Heavy Biocrudes. Figure 5 shows the C, N, O, and S contents (wt %) of the light and heavy biocrudes for each organism (exact values are included in the Supporting Information). Elemental ratios of H/C, N/C, O/C, and S/C in the light and heavy biocrudes are presented in the Supporting Information. Figure 5a shows that regardless of the conditions of the hydrothermal treatment, the light biocrude always had a higher weight percent of C than the heavy biocrude, similar to the results obtained previously for HTL of Nannochloropsis sp.⁹,¹⁶ Fast HTL biocrudes, both light and heavy, always had a lower weight percent of C than the biocrudes from conventional isothermal liquefaction. Panels b and c of Figure 5 show that the light and heavy biocrudes from fast HTL are always richer in N and O contents than their counterparts from isothermal HTL. Previous results for HTL of Nannochloropsis sp. showed that increasing the reaction severity, that is increasing the holding time and/or increasing the reaction temperature, reduces the O content in the biocrude.⁹ The results in Figure 5c show the same trend. Even so, the N and O contents in these biocrudes are roughly an order of magnitude greater than those in most petroleum crudes⁴⁰ and may present challenges to upgrade the biocrude to a hydrocarbon fuel. All of the biocrudes, however, have a reduced O content compared to the original biomass.

Figure 5d shows that the S content in the biocrude varies among the different organisms, but it is always <1 wt %, putting it within range of the S content of most petroleum crudes.⁴⁶ The heavy biocrude from fast liquefaction is always richer in S than the heavy biocrude from conventional liquefaction. The S content in the light biocrude from S. cerevisiae treated by fast liquefaction was the lowest at 0.15 wt %.

Ammonia in the Aqueous Phase. Figure 6 shows the percentage of the total N from the biomass that is present as dissolved ammonia in the aqueous phase. Isothermal HTL of the biomass favors the near complete conversion of water-soluble N products into ammonia. As reaction severity increases, more of the N in the biomass is converted into ammonia.⁹ Converting N-containing molecules into dissolved ammonia makes the nitrogen more bioavailable for use by most microorganisms. This conversion facilitates the recycling of N in a biorefinery, which is an important consideration for environmental sustainability.

Elemental Distribution. Knowing the gravimetric yields and the elemental composition of each product fraction allows one to calculate how the various elements are distributed among the product fractions. Carbon and nitrogen are of most interest because the C content strongly influences the heating value and N recycling is essential for a sustainable hydrothermal biorefinery. The present results (see Figures S5 and S6 of the Supporting Information) show that at most 64% of C in the biomass goes to the biocrude. B. subtilis had the lowest C distributed to the biocrude, while P. putida and S. cerevisiae had the highest distribution of C because of their higher yields of biocrude. Similar to the results from the HTL of microalgae, the majority of N (80% or more for conventional isothermal HTL) resides in the aqueous phase and solids.⁹ This outcome is desirable because it facilitates recycling the N-containing compounds as nutrients for cultivation of additional biomass. Less than 41% of the total N from the biomass is distributed to the biocrude.

Heating Value and Energy Recovery. Table 3 shows the higher heating values of the light and heavy biocrudes and the percentage of the chemical energy in the biomass that is recovered in the biocrude. The heating value of the light biocrude was always higher than that of the heavy biocrude, and biocrudes produced at isothermal HTL conditions had higher heating values than biocrudes produced at fast HTL conditions. These trends are simply a manifestation of the trends in the C and O contents of the various biocrude fractions. Regardless of the biomass feedstock processed, the variation in the heating value for a given biocrude (light or heavy) fraction from a given HTL approach (isothermal or fast) is always <2 MJ/kg. It appears that the processing conditions and product fractionation protocol play a larger role in determining the heating value than the biomass feedstock.⁵⁵

The last column of Table 3 shows the total energy recovered in the biocrude from the original biomass. S. cerevisiae and P. putida had the highest energy recovery values for both hydrothermal treatment conditions. B. subtilis had the lowest energy recovery (recall that it gave the lowest yields of biocrude). The energy recoveries in HTL biocrude from bacteria and yeast are not as high as those often observed from HTL of microalgae, where values exceeded 70% at the same processing conditions.⁹,¹⁶ Of course, microalgae typically have lipid contents that are an order of magnitude higher than those in the bacteria and yeast used in this study. Higher lipid contents tend to correlate with higher biocrude yields¹¹ and higher energy recoveries in the biocrude. Nevertheless, Table 3 shows that HTL can produce energy-dense biocrudes containing 40% or more of the chemical energy in the microbial biomass.

Molecular Composition of the Light Biocrude. We analyzed the light biocrude using gas chromatography–mass spectrometry (GC–MS). Table 4 shows the components in the light biocrude, for which the GC–MS software gave at least a > 50% match factor with a compound in its mass spectra library, and their relative abundance quantified by peak area. Not all of the compounds in the biocrude could be identified because of the large number of low-intensity peaks present in each chromatogram. All chemical identities in Table 4 remain tentative, because we used no authentic standards to obtain positive identities. The peak areas listed in Table 4 provide a qualitative representation of the relative abundance of that compound in the light biocrude.

Table 4 shows that the fast HTL biocrude was less likely to contain heterocycles and aromatics. The nitrogen-containing heterocycles, such as substituted indoles and amines, possibly derived from the decomposition of porphyrins or the proteins that are abundant in the microorganisms.⁴¹ The nitrogen-containing heterocycles are not uncommon in petroleum crude, but the presence of such compounds in higher concentrations...
in the biocrude make it more difficult to upgrade to a hydrocarbon fuel. Free fatty acids appear in Table 4, and these are also common products in biocrudes from microalgae HTL. For the microbial biomass studied here, they are likely to be mostly derived from the cell membrane. The fast HTL biocrudes contain a higher percentage of fatty acids than the biocrudes from isothermal HTL, potentially showing that lipids in the cell hydrolyze faster than the other biomolecules. Fatty acid amides are more common in the isothermal HTL products, suggesting that the higher concentration of NH3 present in the reactor facilitates replacing the hydroxyl group in the fatty acid. Table 4 shows that more of the cyclic proline dimer,42 which is likely a decomposition product of proteins, is found in the fast HTL biocrude, suggesting the incomplete decomposition of proteins to amino acids at milder conditions.

CONCLUSION

We demonstrated the feasibility of using microbial monocultures as a feedstock for HTL to produce biocrude. The high N and O contents of the biocrude, for all organisms and all treatment conditions, necessitates additional treatment of the biocrude before its use as a liquid transportation fuel. The cultivation of bacteria with aqueous-phase byproducts from HTL of microalgae22 provides an opportunity to improve the overall use of nutrients and total biocrude output in an algal biorefinery.

E. coli cultivated in the TB (nutrient-rich) media was higher in ash and lower in lipid than the E. coli cultivated in MM. The growth media used to cultivate the bacterium did not significantly affect the elemental composition of the harvested biomass. The biocrude yields produced at both isothermal and fast HTL conditions were not significantly different for the two different cultivation media. This insensitivity of the HTL outcomes to the growth media suggests that the aqueous streams that are nutrient-depleted or contain substrates that are not easily metabolized22 may nevertheless be suitable for biocrude production via E. coli cultivation.

The Gram-positive organism, B. subtilis, provided the lowest yield of biocrude compared to all other microorganisms in this study. Its modestly lower lipid content is probably not fully responsible for the reduced yield because E. coli TB had a similar lipid content but significantly higher biocrude yields. The B. subtilis biomass did decompose during HTL, however, as the yield of residual solids was only about 4 wt %. The decomposition products were primarily water-soluble, which might be in part due to the cell envelope of this Gram-positive bacterium containing a thick peptidoglycan layer.

S. cerevisiae had a higher average yield of biocrude than the bacteria. The higher yield also resulted in a higher average recovery of the energy in the biomass. However, P. putida had similar yields and is capable of growing on media lacking nutrients and containing possible toxins that can inhibit growth, specifically the byproduct aqueous phase from the liquefaction of microalgae.22

As the literature and results presented herein indicate, fast HTL can, in many cases, lead to higher total biocrude yields than isothermal HTL.16 The shorter reaction time necessary for fast HTL would reduce reactor size and capital costs in an industrial process. However, in exchange for these benefits, fast HTL biocrudes appear to have a less desirable composition. For example, a higher percentage of the total biocrude exists as the heavy fraction. Fast HTL biocrudes also have higher O, N, and S contents, which are less desirable for use as a biofuel or biofuel precursor compared to isothermal HTL biocrudes. More N in the isothermal HTL aqueous phase is converted to NH3, making N preferable as a nutrient for algae cultivation.19 Further economic and environmental analyses of these trade-offs are required to determine which of these processes are preferable for the conversion of biomass to biocrude.

ASSOCIATED CONTENT

Supporting Information

Yields of light and heavy biocrude product fractions (wt %, dry basis) for each biomass and isothermal and fast HTL (Table S1), H/C (Figure S1), N/C (Figure S2), O/C (Figure S3), and S/C (Figure S4) atomic ratios in the light and heavy biocrudes, carbon (Figure S5) and nitrogen (Figure S6) distributions among the product fractions, and elemental composition (wt %) of the light and heavy biocrudes: (a) carbon, (b) nitrogen, (c) sulfur, (d) oxygen, and (e) hydrogen (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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