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Water Impact Statement

Recovering renewable H_2 from cellulosic wastewater and biomass plays a critical role in the renewable energy portfolio, but the dominant dark fermentation process showed limited H_2 yield due to product inhibition. By using anaerobic membrane gas extraction in thermophilic fermentation reactors, we found H_2 production by *Clostridium thermocellum* was significantly increased compared to conventional anaerobic fermentation.

Anaerobic membrane gas extraction facilitates thermophilic hydrogen production from *Clostridium thermocellum*

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Abstract

Clostridium thermocellum is among the most efficient bacteria to convert cellulosic biomass into H_2 during dark fermentation. However, despite great progress the H_2 yield and rate are still not satisfactory for large scale applications. The purpose of this study was to evaluate whether in-situ gas extraction using membrane bioreactors would increase H_2 production from *Clostridium thermocellum* when compared to a conventional anaerobic fermentation setup in thermophilic conditions. *C. thermocellum* DSM 1313, a cellulotyic, thermophilic bacterium was grown on cellobiose and Avicel in an anaerobic-fermenter (AF) and an anaerobic-membrane-bioreactor (AnMBR). Compared to the AF, the AnMBR increased cumulative H_2 production by 63%, from 25.8 to 42.1 mmols, increased the max H_2 production rate by 24%, from 3.4 to 4.2 mmol/hr, and increased yield by 58%, from 0.43 to 0.68 mmol H_2/mmol hexose, on cellobiose. Likewise, on Avicel, the AnMBR increased cumulative H_2 production by 59%, from 46.8 to 74.6 mmols, increased the max H_2 rate by 87%, from 3.1 to 5.8 mmol/hr, and increased the yield by 59%, from 0.76 to 1.21 mmol H_2/mmol hexose. These results show that anaerobic membrane gas extraction can be an effective approach to increasing both rate and yield of fermentative H_2 production.

Key words: *Clostridium thermocellum*, membrane bioreactor, fermentative hydrogen production, partial pressure, hydrogen yield, cellulose

1. Introduction

Hydrogen gas is a clean and efficient renewable energy carrier that provides great potential for addressing fossil fuel dependence and climate change concerns.[1-3] Hydrogen derived from biomass is appealing because it is considered sustainable and can be used directly in fuel cell vehicles for transportation to displace petroleum.[4-6]It is estimated that approximately 50 billion tons of cellulose could be produced annually from lignocellulosic residues, so the abundant availability of waste cellulose makes it an ideal renewable resource for renewable H_2 production.[7] Anaerobic fermentation has been a primary approach for bio-H_2 production, but the low H_2 molar yield from cellulosic substrates has been a challenge. This is partially due to its theoretical ceiling of 4 mol H_2 mol^{-1} hexose, but a more common issue is the low hydrolysis rate that limits fermentation kinetics.[8-9] Of all known cellulolytic microorganisms, *Clostridium thermocellum* displays one of the highest known rates of cellulose degradation.[10-13] One advantage of *C. thermocellum* is that it grows at 60 °C, which significantly increases the conversion rate, and it reduces the chances of contamination by precluding the growth of predominant mesophilic microorganisms. In addition, because the solubility of gases decreases with higher temperatures, the high temperature promotes more efficient removal of the product gases such as H_2 and CO_2.[9-10, 14]

It is known that high H_2 partial pressure has a negative effect on H_2 production because it inhibits the forward reaction and decreases hydrogenase activity, which makes the H_2 production reaction thermodynamically unfavorable.[15] To limit the impact of H_2 partial pressure, researchers have used techniques like sparging bioreactors with inert gases (carbon dioxide, nitrogen, and argon), vigorously shaking culture flasks, or increasing the stirring rate.[16-20]

The availability of CO_2 also affects H_2 yield, because cells synthesize succinate and formate via CO_2, pyruvate and NADH via the hexose monophosphate pathway. [21] Timely removal of CO_2 can prevent NADH consumption and in turn increase H_2 yield. [21] Compared with sparging and stirring, direct removal of produced gas using membrane bioreactors (MBRs) can be a promising yet under-investigated approach. MBRs have been used in aerobic and anaerobic wastewater treatment, and have demonstrated good effluent quality and low footprints by using hydrophilic membranes for water separation. [22-25] Recently researchers have started to combine H_2 fermenters with membrane technology in order to replicate the benefits of MBRs in wastewater treatment, because membrane bioreactors can increase H_2 yield and production rate by increasing the retention time of the solid substrate and the concentration of microorganisms. [26-29]

Table 1 summarizes the hydrogen fermentation MBR studies reported so far. While different studies focused on various aspects of the technology, including substrates, microbial strains, membrane materials, and reactor configurations, most studies demonstrated that employing membranes in fermenters increased H_2 production yield and rate (Table 1). For example, studies using a mixed culture in mesophilic conditions showed that varying an AnMBR's HRT influences H_2 production, with the highest H_2 yields occurring at longer HRTs while the highest volumetric H_2 production rates occurred at the shortest HRTs. [30-32] Other mixed culture studies showed that H_2 rate and typically H_2 yield increases linearly with an AnMBR's organic loading rate (OLR). [33-35] Some AnMBR studies also employed hydrophilic membranes to continuously remove fermentation carbon co-products, such as volatile fatty acids (VFAs), which in high enough concentrations can suppress H_2 production. [36, 37] Aside from using

membranes for increased cell retention and VFA extraction, numerous studies also evaluated which membrane materials and operating conditions are best suited for purifying H_2 from biogas mixtures. [38-41]

Table 1 indicates that to date, no study has investigated the benefits of using gas extracting hydrophobic membranes in pure-culture fermentation reactors, which are different from hydrophilic membranes used for water separation. Moreover, despite the findings that thermophiles demonstrated higher H_2 yield and rate, no study has reported AnMBR operation in thermophilic pure-culture conditions. With this knowledge gap in mind, this study evaluated whether continuous membrane gas extraction facilitates H_2 production from *C. thermocellum*. In order to maximize mass transfer, we submerged a hydrophobic polypropylene tube membrane inside a fermenter to extract gas produced in-situ during dark fermentation (AnMBR). We compared system performance with a no-membrane anaerobic fermentation (AF) control using two cellulosic substrates (Cellobiose, a cellulose-derived sugar, and Avicel). The batch experiments were carried out using a pure-culture of *C. thermocellum Δhpt DSM* 1313.[42] The H_2 yield and H_2 rate were compared between the AF and the AnMBR setups, with the latter showing increases in both rate and yield of H_2 production, highlighting the importance of H_2 removal to maximize its productivity.

107 **Table 1:** Summary of all anaerobic H_2 MBR studies. [30-41]

Organism	Substrate	Temperature ˚C	Reactor Configuration	Study	Max Yield mol H2/mol hexose	Max Rate L/(L-d)	Improvement	Reference
E. coli (XL1-BLUE)	Formate	37	CSTR coupled with a membrane module	How CSTR HRT impacts H2 production and how to separate H2/CO2 using a HF membrane	0.26 mmol H2/ mmol formate	0.13	Rate = N/A Yield = N/A	Bakonyi, 2012
R. capsalatus T. kirishi	Lactate Glucose		Side-stream MBR	Influence of membrane material in purifying H2/CO2 streams	N/A	N/A	Rate = N/A Yield = N/A	Teplyakov,2002
Mixed Culture	Agrowaste	35	Externally-submerged MBR	How removing VFAs, mixing, TMP and fouling affected H2 production	1.10	5	Rate = N/A Yield = N/A	Trad, 2015
Mixed Culture	Glucose	37	Double side-stream MBR	Evaluating H2 production at different HRTs and ability of PDMS to purify H2	1.13 (HRT=92 hrs)	6.11 (HRT=12 hrs)	Rate = 140% Yield = 232%	Bakonyi, 2015
Mixed Culture	Glucose	35	Gas Separation MBR and CSTR	Comparing H2 production between a GSMBR and a CSTR	1.91	9.20	Rate = 25% Yield = 21%	Bakonyi, 2017

Mixed microflora	Tofu processing waste	60	CSTR and MBR	Comparing H2 production between a CSTR and MBR on tofu processing waste	1.87 (HRT = 8 hrs)	12.81 (HRT=8 hrs)	Rate = 57% Yield = 56%	Kim, 2011
Mixed Culture	Glucose, Sucrose Fructose	35	Side-stream hollow fiber MBR	Impact of HRT, substrate, and reactor configuration on H2 production	At HRT (Hrs) = 4,2, 2 1.72, 1.51, 1.55	At HRT (Hrs) = 4,2, 2 1.02, 1.67, 1.87	Rate = 580%, 351%, 345% Yield(s)=64%, 13%, 4%	Lee, 2006
Mixed mesophilic microflora	Glucose	35	submerged MBR	Influence of SRT on continuous H2 production in a submerged MBR	1.19 (SRT=12.5 d)	5.8 (SRT=12.5 d)	Rate = N/A Yield = N/A	Lee, 2010
Mixed Culture	Food waste	55	HF-MBR	Influence of organic loading rates on H2 production from a HF-MBR	111.1 mL-H2/g-VS added	2.2	Rate = N/A Yield = N/A	Lee, 2014
Mixed Culture	Glucose	35	Side-stream hollow fiber MBR	Impact on H2 production when H2 and CO2 are extracted.	0.93	3.0 mmol H2/ g VSS hr	Rate = 10% Yield = 15%	Liang, 2002
Mixed consortia	Glucose	35	submerged anaerobic MBR	Impact on H2 production when VFAs are removed from the medium	1.58	2.47	Rate = 51% Yield = 11%	Noblecourt,2017

Mixed Culture	Glucose	N/A	Side-stream ceramic cross-flow MBR	Compare H2 production between a chemostat and MBR at different SRTs	1	7.68 ± 0.48	Rate = 25% Yield = 3%	Oh, 2003
Mixed Culture	Glucose	23	CSTRs and MBRs	Influence of organic loading rates on H2 production	1.78	4.74	Rate = 53% Yield = 84%	Shen, 2009
Mixed Culture	Synthetic wastewater	23	HPMBR	Influence of organic loading rates on biomass, EPS, and H2 production	0.004-0.008 mol/g COD	4.77 ± 0.36	Rate = N/A Yield = N/A	Shen, 2010
N/A	N/A	23	Sidestream MBR	Using PDMS and SAPO 34 membrane modules to separate H2/CO2 streams	N/A	N/A	Rate = N/A Yield = N/A	Ramirez-Morales, 2013
N/A	N/A	37-55	Membrane Module	How different process variables impact a polyimide membranes ability to purify H2	N/A	N/A	Rate = N/A Yield = N/A	Bakonyi,2013

2. Materials and Methods

2.1 Preparation of Inoculum and Media

All reagents and chemicals for media and substrates were obtained from Sigma Chemical Co. and Fisher Scientific. *C. thermocellum* DSM 1313 *Δhpt* derived strains were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. [42] Inoculum was cultured in 26 ml balch tubes (Bellco Glass Co.) containing 10 mL of CTFUD rich media and 5 g/L filter sterilized cellobiose. These tubes were incubated at 55°C and shaken at 125 rpm inside a MaxQ 4000 incubator. Each tube was air sealed with butyl rubber stoppers. 10% by volume of the actively growing culture was successively transferred and grown in 100 ml then 200 ml glass bottles (Bellco Glass Co.) containing 5 g/L filter sterilized cellobiose and CTFUD rich media prior to being grown in the 4.25 L polycarbonate batch reactors. The CTFUD rich media contained (per liter of distilled deionized water): $Na_3C_6H_5O_7 * 2H_2O$, 3.0 g; $(NH_4)_2SO_4$, 1.3 g; KH_2PO_4, 1.5 g; $CaCl_2 * 2H_2O$, 0.13 g; L-Cysteine-HCl, 0.50; MOPS sodium salt (adjust pH to 7.0 after addition of MOPS), $MgCl_2 * 6H_2O$, 2.6 g; $FeSO_4 * 7H_2O$, 0.001 g (use 1000 fold dissolved liquid concentration); Yeast Extract, 4.5 g; Resazurin 0.2% (w/v), 0.5 ml/l. [14] Culture was grown at 60 °C until it reached late exponential phase.

2.2 AF and AnMBR Reactors Setup

As shown in Figure S1 and Figure S2 (supplemental information), batch culture fermentations were carried out using two different bioreactor setups that were custom designed for this study: an anaerobic fermenter (AF) as a control and an anaerobic-membrane-bioreactor (AnMBR) as the active reactor. In total the bioreactors had a total volume of 4.25 L

and a working volume of 2 L. The AF was operated atop a magnetic stir plate (Fisher-Scientific) at a stir rate of 60 rpm. The AF was maintained at 60 °C using an electronic heating jacket connected to a temperature controller (ElectroLab 240). The reactor pH was maintained at 7.0 by using a pH controller (ElectroLab 260) delivering 2M NaOH solution. The AF was kept anaerobic by constantly delivering argon gas from a gas canister which subsequently passed through a mass flow controller (AALBORG GFC17), through a 0.2 µm PTFE membrane filter (Gelman Nylon Acrodisc) and into a gas dispersion tube (Pyrex, pore size 40-60 µm). The entire gas delivery setup was connected by platinum cured silicon tubing (Cole-Parmer). The AF setup also had a headspace gas line that dropped into a beaker of water that constantly vented gas into the beaker to eliminate pressure buildups in the reactor vessel. The head-plate of the reactor also contained a condenser unit used to cool down headspace gas before it traveled into the gas sampling line that led to the Gas Chromatography unit. This condenser unit was connected to a water faucet using platinum-cured silicon tubing and cold tap water was circulated through the unit.

The AnMBR reactor was setup similar as the AF reactor except that a 2.44 m long coiled hydrophobic polypropylene microfiltration tube membrane (3M Membrana, Accurel PP V8/2 HF) was submerged in the 2L liquid. The V8/2 membrane had a pore size of 0.2 um, a wall thickness of 1550 um $\pm$ 150 um, an inner diameter of 5500 um $\pm$ 300 um, and a melting point of 160 C. The submerged membrane had an active surface area of 600 cm^2 and was connected to a pressure gauge module using Tygon SE-200 tubing (Cole-Parmer). Black Norprene chemical tubing (Cole-Parmer) attached to the other side of the pressure gauge module was run through a peristaltic pump (Watson Marlow 505 Du) and connected to a gas bag (Kynar Bag 12X12” Dual

Valve). The AnMBR was maintained at the same pH, temperature, and stir rate as the AF. A peristaltic pump was used to apply a vacuum to the membrane and was operated at a continuous pumping rate of 0.2 rpm. The gas bag collected the product gas that permeated through the membrane and the condenser unit cooled down the headspace gas on its way for GC measurements. The pressure gauge was used to record the trans-membrane-pressure throughout the fermentation.

2.3 AF and AnMBR Reactor Operations

Prior to the startup of batch cellobiose experiments, the bioreactors were filled with 1700 ml of CTFUD rich media, the media was adjusted to a pH=7.0, and the vessels were sterilized by autoclave. Prior to the startup of batch Avicel experiments the bioreactors were filled with 1800 ml of CTFUD rich media and 5 g/L Avicel, the media was adjusted to a pH=7.0, and then the reactors were sterilized by autoclave.

For the AF reactor, the air in the reactor liquid and headspace was replaced with argon after 30 minutes of sparging. Once the AF was shown to be anaerobic on the u-GC the sparge rod was pulled from the reactor liquid and up into the reactor headspace for the duration of the fermentation. For the AnMBR reactor, the membrane was cleaned by soaking the module in 122 ˚C autoclaved water for 10 minutes prior to submerging it in the freshly autoclaved bioreactor inside of a laminar hood. After assembling the AnMBR inside of the laminar hood, the reactor was allowed to cool to 60 ˚C and the Argon flow, temperature control, pressure gauge and gas bag was hooked up to the reactor. The air in the reactor liquid and headspace was replaced with argon by sparging for 30 minutes. Once the AnMBR was shown to be

anaerobic on the u-GC the sparge rod was pulled from the reactor liquid and up into the reactor headspace for the duration of the fermentation.

For cellobiose experiments, when the reactors reached 60 ˚C and the pH was adjusted to 7.0 with NaOH, the reactor was inoculated with 200 ml of actively growing *C. thermocellum* culture along with 100 ml of 100 g/L filter-sterilized cellobiose. A peristaltic pump was turned on for AnMBR gas extraction at a constant rate of 0.2 rpm. The 2L reactor culture was grown in batch mode on 5 g/L cellobiose for 24 hours. For Avicel experiments, when the reactors reached 60 ˚C and the pH was adjusted to 7.0 with NaOH, the reactor was inoculated with 200 ml of actively growing *C. thermocellum* culture and grown in batch mode on 5 g/L Avicel for 27 hours. Throughout testing, the reactors were kept anaerobic by sparging the headspace with 20 standard cubic centimeters of argon and maintained at a pH of 7.0, a temperature of 60 ˚C, and a stir rate of 60 rpm.

2.8 Analytical procedures

Cell growth during cellobiose experiments was measured as a function of optical density (OD) by spectrophotometry (DU800; Beckman Coulter) at OD_{600}. An OD_{600} of 1 correlated to 1.04 g/L cell dry weight (R^2=0.9918). The composition of *C. thermocellum* biomass was determined to be $\mathrm{C_5H_8NO_2}$ by elemental analysis. Briefly, *C. thermocellum* was grown to stationary phase on 5 g/L cellobiose; the biomass was pelleted and washed three times before drying overnight at 105 ˚C and subsequently sent to Huffman Labs for analysis of carbon, hydrogen, nitrogen, oxygen, and sulfur. Cell growth on the insoluble Avicel substrate was determined indirectly by measuring the total protein content of samples using a modification of

196 the Bradford method.[43] Samples (10 ml) taken during the Avicel experiments were centrifuged
197 (8000 x g for 15 min) and the supernatant was removed. Pellets were washed with 0.9%
198 (wt./vol.) NaCl and resuspended in 2 mL of 0.2N NaOH. Samples were then incubated in a
199 boiling water bath to hydrolyze the cells from the solid substrate. After the bath, the samples
200 were cooled, centrifuged (8000 x g for 15 min), then supernatants were collected for protein
201 content analysis as described.[44,45] The leftover pellets were stored in a -80 °C freezer for 24
202 hours before getting lyophilized for 48 hours. Post lyophilization the samples were weighed and
203 the Avicel degradation values were recorded.
204 Headspace gas concentrations of H_2 and CO_2 in the AF and AnMBR were measured by
205 automatic sampling using a 2-channel uGC (490 Micro GC, Agilent Technologies). Channel 1
206 contained a Poraplot U column with argon as carrier gas and operated at an oven temperature
207 of 100 ˚C. Channel 2 contained a Molsieve column with helium as carrier gas, operated at an
208 oven temperature of 65 ˚C. Peak areas were compared with a standard curve, considering both
209 temperature and pressure. The membrane effluent gas extracted from the reactor liquid of the
210 AnMBR was fed to a gas collection bag (Kynar Bag 12X12" Dual Valve) after passing through a
211 desiccator.
212 Cellobiose, lactate, formate, acetate, and ethanol were measured by HPLC (1200 series;
213 Agilent Technologies) with a mobile phase of 4 mM H_2SO_4 using an Aminex HPX-87H column
214 with a Micro Guard Cation H Cartridge. The column temperature was set to 55 ˚ C and the flow
215 rate was 0.6 mL/min. All data points shown represent the average of two independently
216 replicated experiments, each with multiple batches. [46,47]
217

3. Results and Discussion

3.1 Cumulative Gas Totals and Rates

Data from Figure 1 shows the cumulative H_2 production from each reactor configuration and substrate type with respect to time. The AnMBR gas extraction environment facilitated substantial increases in cumulative H_2 production from both the cellobiose and cellulose substrates when compared to the AF configuration. No loss of reactor liquid was observed during the gas extraction process. On cellobiose, the AnMBR headspace produced 31.9 ± 12.3 mmols of H_2, which is an improvement of near 24%, when compared to the AF cumulative headspace amount of 25.8 ± 3.35 mmols of H_2. Figure 1 (A) reveals that 10 mmols of H_2 were extracted from the reactor solution and collected in the gas bag, which brings the cumulative total amount of H_2 produced from cellobiose using the AnMBR to 42.1 ± 12.6 mmols of H_2, a 58% increase in total H_2 compared to the AF. On Avicel, the AnMBR headspace produced 52.5 ± 3.87 mmols of H_2, which is an improvement of 12% more H_2 when compared to the AF cumulative headspace amount of 46.8 ± 1.41 mmols of H_2. Figure 1 (B) reveals that 22.1 mmols of H_2 were extracted from the reactor solution and collected in the gas bag, which brings the cumulative H_2 produced from Avicel using the AnMBR to 74.6 ± 6.7 mmols, which is a 59% increase compared to the AF.

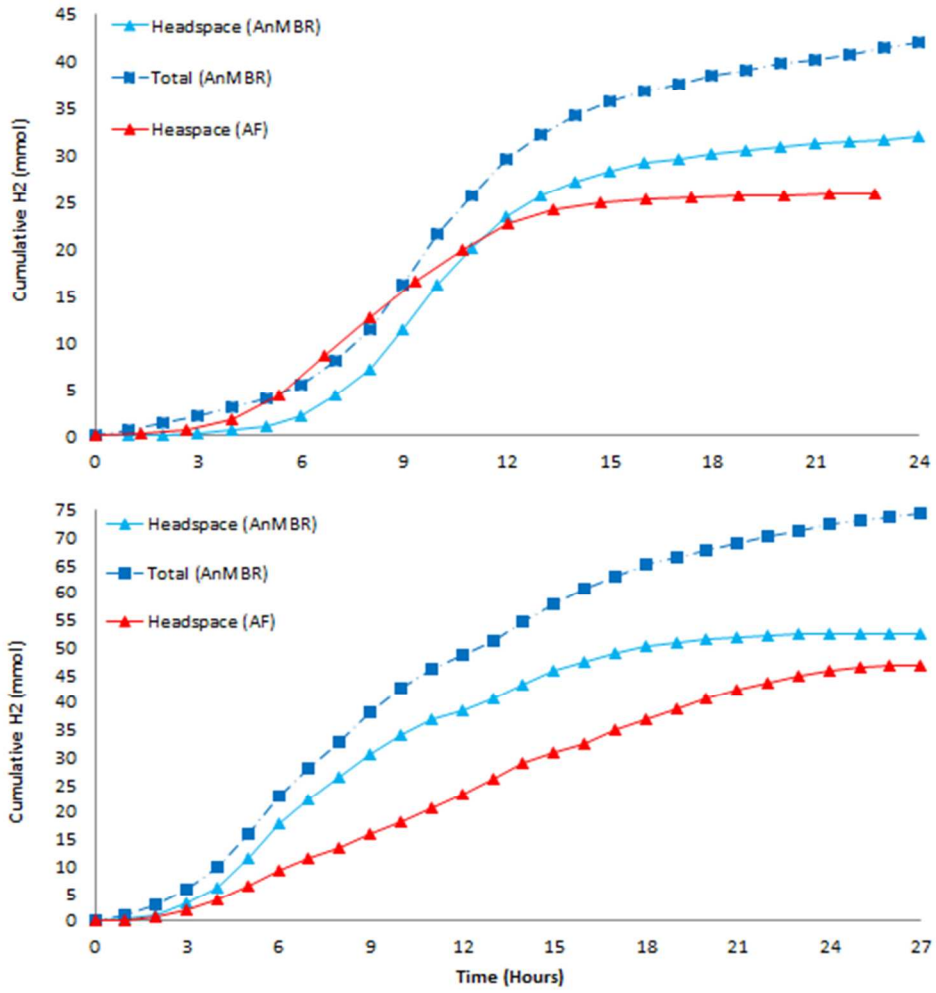


Figure 1: Cumulative H_2 production from Cellobiose (A) and Avicel (B) from the AF and AnMBR over time.

Data from Figures S3 and S4 (supplemental information) reveal the membrane permeate, headspace, and total (permeate + headspace) biogas compositions as H_2/CO_2 from the cellobiose and Avicel AnMBR experiments were 3.8 (334 mL/87 mL), 1.0 (1063 mL/1112 mL), 1.2 (1459 mL/1198 mL), and 4.0 (735 mL/184 mL), 0.71 (1752 mL/2471 mL), 0.94 (2488 mL/2655 mL), respectively. The significant difference between the membrane and headspace H_2/CO_2 ratios indicates that the hydrophobic membrane was more selective to H_2 than CO_2. Since the total biogas compositions as H_2/CO_2 for cellobiose and Avicel were fairly close in value

to each other, 1.2 and 0.94 respectively, it suggests the gas extraction process was relatively uniform between the two substrates. Figure S3 also shows how membrane resistance from cellular growth influenced the pressure gradient over the course of the different AnMBR growth experiments. On Cellobiose, the vacuum pressure during the fermentation was well correlated to the optical density graph provided in Figure 2. At t=0, t=4, t=8, and t=24 the corresponding vacuum pressures are -0.70, -3.2, 0.90, and -5.8 psi for the AnMBR cellobiose experiments. The vacuum pressure increased from t=0 to t=4 correlated to the lag phase in cellular growth and an increase in biomass being present in the reactor. The vacuum pressure decrease from t=4 to t=8 was the result of peak gas production through the membrane which correlated with the exponential phase of cellular growth. The large increase in vacuum pressure from t=8 to t=24 was caused by substantial increases in biomass concentration and increased biofilm formation incurred by the stationary and death phases of *C. thermocellum's* growth. At t=0, t=4, t=9.5, and t=27 the corresponding vacuum pressures were -0.70, 0.90, 0.45, and -4.5 psi for the AnMBR Avicel experiments. The decrease in vacuum pressure and positive pressure readings from t=0 to t=9.5, revealed that enough gas was produced through the membrane during the lag and exponential phases of growth to overcome vacuum resistance produced from biofouling. The increase in vacuum pressure from t=9.5 to t=27 was the result of increased resistance resulting from membrane adhesion of the solid substrate and biofilm formation incurred from high biomass concentrations during the stationary and death phases of *C. thermocellum's* growth. Further studies are needed to better examine membrane biofilm formation, but the changes in vacuum pressure over time suggested biofilm growth on membrane surface. Several studies in the literature reveal that the increases in membrane

267 pressure displayed over time like the ones shown in Figure S3 is a good proxy for resistance
268 caused from biofilms. [22, 48-50]

269 Data from Table 2 summarizes *C. thermocellum's* gas production metrics from each
270 reactor configuration and substrate type with results averaged from two independent runs. The
271 data reveal that the highest rate of H_2 production from the AF was 3.4 mmol hr^{-1} on cellobiose
272 and 3.1 mmol hr^{-1}on Avicel, respectively. In comparison, the highest rate of H_2 production
273 from the AnMBR was 4.2 mmol hr^{-1} on cellobiose and 5.8 mmol hr^{-1}on avicel. Both Table 2
274 and Figure 1 clearly reveal that reducing the partial pressure of dissolved gases via membrane
275 gas extraction increased the rate of H_2 production by 24% on cellobiose and by 87% on Avicel,
276 respectively. Table 2 shows the AnMBR also increased the CO_2 production rate by 218%
277 compared to AF, from 0.95 ± 0.13 mmol hr^{-1} to 2.8 ± 0.06 mmol hr^{-1} on cellobiose, and by
278 64%, from 2.8 ± 0.50 mmol hr^{-1} to 4.6 ± 0.64 mmol hr^{-1} on Avicel. The H_2 and CO_2 total gas
279 volume was also increased using the AnMBR, with the H_2 volume increasing by 63% and 46%,
280 and the CO_2 volume increasing by 218% and 78%, on cellobiose and Avicel, respectively.

281 **Table 2.** Gas metrics with respect to substrate type and reactor configuration.

Substrate	Reactor Mode	Total H_2 (mmol)	Max H_2 Rate (mmol/hr)	H_2 Yield (mmol H_2 H_2/mmol Hexose)	Total CO_2 (mmol)	Max CO_2 Rate (mmol/hr)
Cellobiose (5 g/L)	AF	25.8 ± 13%	3.4 ± 0%	0.43 ± 11%	11.3 ± 4%	0.95 ± 14%
	AnMBR	42.1 ± 30%	4.2 ± 16%	0.68 ± 30%	35.9 ± 21%	2.8 ± 2%
	% Increase	63%	24%	58%	218%	195%
Avicel (5 g/L)	AF	46.8 ± 3%	3.1 ± 7%	0.76 ± 3%	44.8 ± 12%	2.8 ± 18%
	AnMBR	74.6 ± 9%	5.8 ± 9%	1.21 ± 15%	79.6 ± 9%	4.6 ± 14%
	% Increase	59%	87%	59%	78%	64%

282

The increases in all H_2 and CO_2 related metrics associated with the AnMBR is to be expected according to Le Chatelier's principle which states that the equilibrium of *C. thermocellum's* fermentation reaction will shift to the right if one or both of the gaseous products of the reaction are removed from solution.[51] Decreasing the partial pressure of dissolved gases in solution reduces the total pressure of gas in solution, allowing *C. thermocellum* to increase both H_2 and CO_2 production. A study performed by Tanisho et al. using *Enterobacter aerogenes* fermenting molasses as the substrate demonstrated that the amount of NADH, a likely electron donor supporting H_2 evolution, was increased by 107% when Ar(g) was blown into solution to remove accumulating CO_2.[21] A study involving the H_2 producer *C. cellobioparum* found that removing H_2 from solution by gassing out the growth flask with CO_2 increased total H_2 production by 80%, 107%, and 165% when the cells were grown on glucose at concentrations of 0.2 %, 0.4%, and 0.6%, respectively.[16] Liang et al. grew a mixed mesophilic culture in a membrane bioreactor that had a side-stream hollow fiber membrane module operated under a vacuum of -10.8 kPA.[28] The operation facilitated hydrogen evolution rate by 10% and hydrogen yield by 15% when compared with a CSTR operation. The literature confirms that decreasing the partial pressure of dissolved gases in solution can promote cumulative H_2 increases anywhere between 15-165% and H_2 production rate increases anywhere between 12.5%-130%. All the cumulative gas totals and production rates carried out using *C. thermocellum* in this study are consistent with findings reported in the literature, indicating that the application of an AnMBR is an effective strategy to increase H_2 production.

3.2 Cell growth and substrate degradation

Figure 2 shows the influence of reactor type and substrate on the optical density (OD) of *C. thermocellum*. In both reactor configurations, *C. thermocellum* achieved a higher overall cell biomass concentration and grew at a faster rate when grown on the soluble substrate (cellobiose) compared to the solid substrate (Avicel). The cells grew to a higher overall biomass concentration and at a quicker rate when they were grown on cellobiose in the AnMBR when compared to the AF. The cells also grew at a faster rate but achieved the same final OD when grown on Avicel in the AnMBR when compared to the AF.

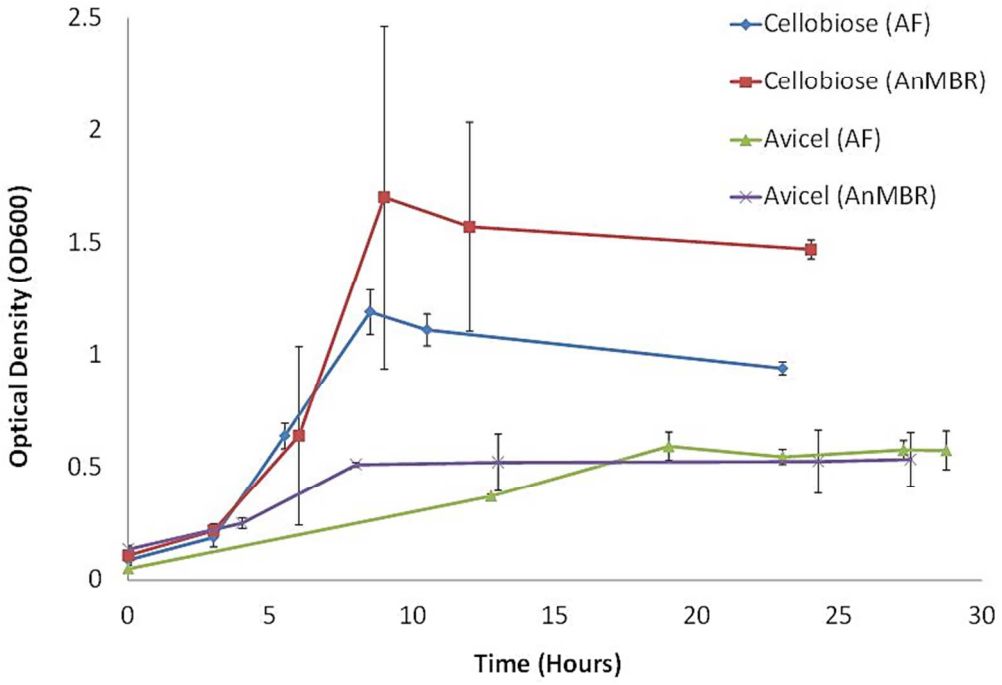


Figure 2: Optical Density (OD600) of *Clostridium thermocellum* on Cellobiose and Avicel in the AF and AnMBR over time.

The faster growth rate and higher optical density (OD) observed in the cellobiose AnMBR is consistent with results published in the literature. Chung et al. found that the H_2-

producing *C. cellobioparum* grew to a higher optical density and at a faster rate, when H_2 was continuously removed from the growth culture compared to the control of no H_2 removal.[16] Since the AnMBR continuously removed H_2 as it was produced, we assume it reduced cellular feedback inhibition, making the hydrogenase reaction thermodynamically favorable in *C. thermocellum*, which allowed the cells to achieve higher OD's with this setup when compared to the AF. The error bars on the cellobiose AnMBR curve are much greater than the error bars on the cellobiose AF curve because the membrane surface could give rise to biofilm formation and increased cell density variability between AnMBR experiments.

Intense biofilm formation may provide one explanation as to why the measured Avicel AnMBR final optical density was not greater than the Avicel AF final biomass density or cellobiose AF and AnMBR biomass densities. During the Avicel AnMBR experiments, the solid substrate was observed to accumulate on the surface of the membrane, which in turn facilitated microbial growth on the membrane surface. Since liquid samples were taken from reactor liquid in the middle of the reactor instead of near or from the coiled-membrane located at the edges of the reactor they may under-report the actual cell density of *C. thermocellum* grown on Avicel in the AnMBR.

Substrate degradation rates for cellobiose and Avicel are illustrated in Figure 3. As can be seen in Figure 3 (A), the degradation rate of the liquid substrate was similar between the two reactor setups. It appears that the partial vacuum environment had negligible benefits when it came to the cells degrading the cellobiose but since the cells readily metabolize soluble substrates this behavior is not too surprising. As can be seen in Figure 3(B) the AF achieves faster degradation of the Avicel substrate during the first 8 hours and the AnMBR achieves

faster degradation of the substrate after the first 8 hours. The large error bar on the second data point of the AnMBR plot in Figure 3 (B) indicates there was considerable variability at this time point, however, with this variability this graph suggests that the AnMBR did increase the overall degradation rate of the solid substrate when compared to the AF.

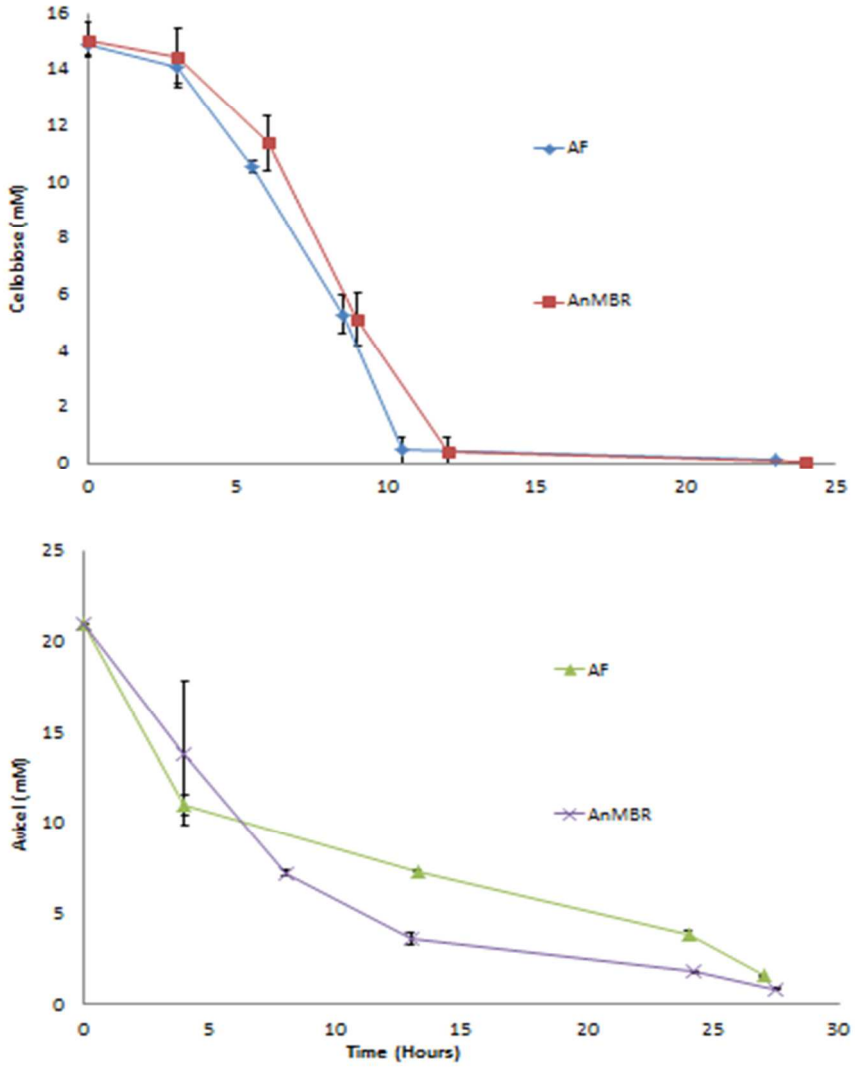
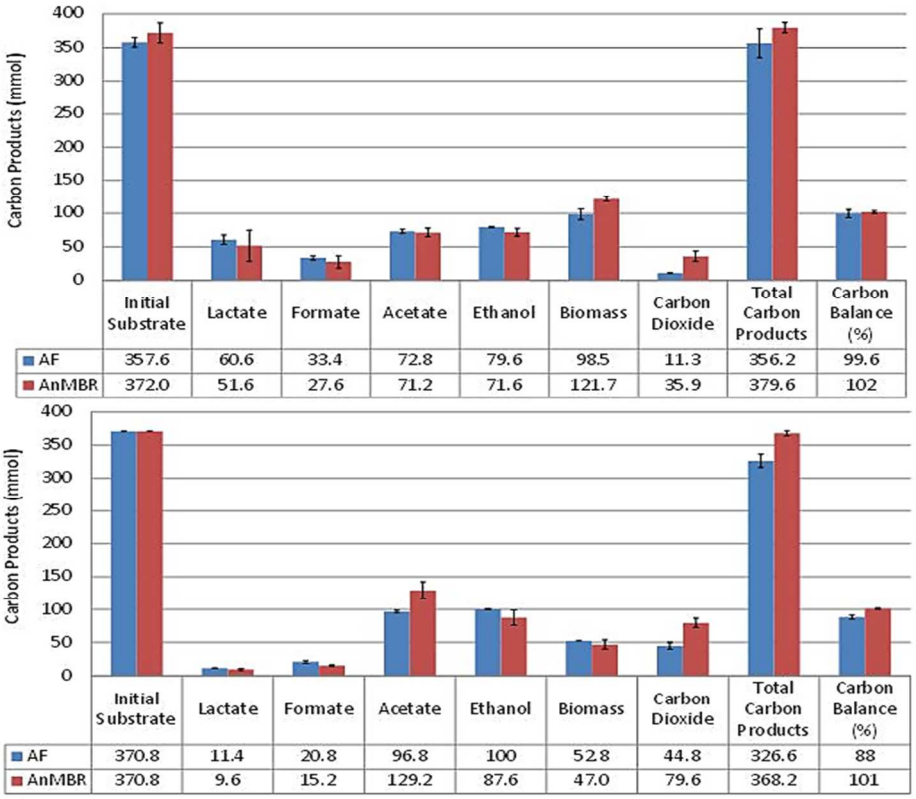


Figure 3. Cellobiose degradation (A) and Avicel degradation (B) in the AF and AnMBR over time.

3.3 Organic End-Product Synthesis and Carbon Balance

Figure 4 (A) shows that when *C. thermocellum* was grown in the AnMBR on cellobiose, the cells produced 15% less lactate, 17% less formate, 2% less acetate, 10% less ethanol, and 24% more biomass when compared to the organic end-products produced in the AF. Similarly, Figure 4 (B) shows that when *C. thermocellum* was grown in the AnMBR on Avicel, the cells produced 16% less lactate, 27% less formate, 33% more acetate, 12% less ethanol, and 11% less biomass when compared to the organic end-products produced in the AF.

The shift in metabolites facilitated by continuously removing gas via extraction, sparging, shaking, and stirring is well established in the literature.[16, 18, 19, 31] Compared to the control, increases in H_2 production are accompanied by increases in CO_2 and acetate and decreases in the more reduced metabolites, such as ethanol and lactate.[9] The best H_2 production runs from this study were also paired with the highest production rates of CO_2 and acetate as indicated by Table 2 and Figure 4. The combination of Avicel with the AnMBR achieved the highest total amount of H_2 produced, 68.6 $\pm$ 8.9 mmols, and was accompanied by the highest amounts of CO_2 and acetate production, which were 79.6 $\pm$ 7.2 mmols and 129.2 $\pm$ 12.9 mmols, respectively.



365

366 **Figure 4:** Distribution of carbon products and carbon balance recovery on Cellobiose (A) and

367 Avicel (B) using the AF and AnMBR.

368

369 **3.4 Yields, Carbon Balance, and Shifted Metabolism**

370 Due to membrane gas extraction, the H_2 yield increased from 0.43 $\pm$ 0.05 to 0.68 $\pm$ 0.20

371 mol H_2 mol hexose^{-1}, the CO_2 yield increased from 0.19$\pm$ 0.01 to 0.58 $\pm$ 0.12 mol CO_2

372 mol hexose^{-1}, and the acetate yield decreased slightly from 0.61$\pm$ 0.02 to 0.57 $\pm$ 0.05 mol

373 acetate mol hexose^{-1}, on Cellobiose. Similarly, as the result of membrane gas extraction, the

374 H_2 yield increased from 0.76 $\pm$ 0.02 to 1.21 $\pm$ 0.14 mol H_2 mol hexose^{-1}, the CO_2 yield

375 increased from 0.72 $\pm$ 0.09 to 1.29 $\pm$ 0.12 mol CO_2 mol hexose^{-1}, and the acetate yield

376 increased from 0.72 $\pm$ 0.01 to 1.29 $\pm$ 0.13 mol acetate mol hexose^{-1}, on Avicel.

In order to determine whether the measured H_2 yields in this study were reasonable, the theoretical H_2 yield that could be generated from each experiment was calculated using carbon balance equations 1 and 2 along with the organic acids data from Figure 3.[8]

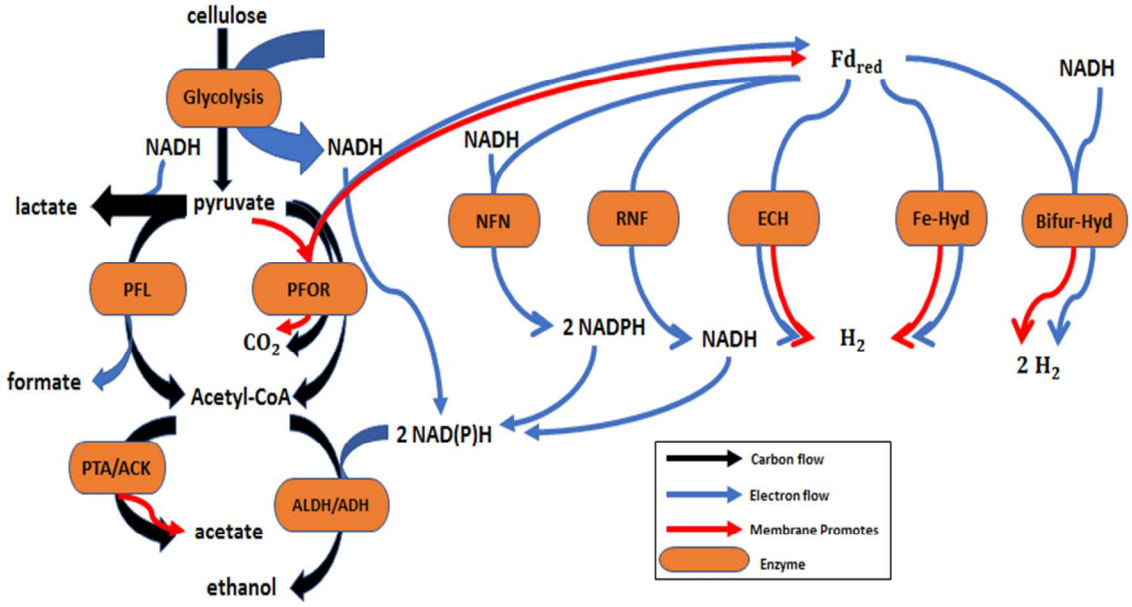
$$[CO_2] = [\text{Acetate} + \text{Ethanol} - [\text{Formate}] \quad (1)$$

$$[H_2] = [2*CO_2] + [\text{Formate}] - [2*\text{Ethanol}] \quad (2)$$

Table S1 reveals that the H_2 yield increases reported in Table S1 by the AnMBR are reasonable since the measured yields fall below the theoretically estimated yields for every experimental setting. Furthermore, Table S1 indicates that continuous gas removal using the AnMBR induces a metabolic response that pushes *C. thermocellum* further towards its theoretical maximum H_2 producing potential. This is demonstrated by the increase in H_2 and CO_2 yields on cellobiose, the increase in H_2, CO_2, and acetate yields on Avicel, and the decrease in undesirable branched pathway end-products that are illustrated in Figure 4.

The production of H_2 competes with the cellular electron pools of NAD(P)H and reduced ferredoxin that otherwise are used toward the production of reduced carbon byproducts including ethanol and lactate (Figure 5). Our observation of increased H_2 production coincided with a simultaneous decrease in both lactate and ethanol production. The AF and AnMBR carbon balances in Figure 4 (A) and Figure 4 (B) are 99.6%, 102%, 88%, and 101%, which indicates that almost all carbon from the substrates has been accounted for, demonstrating the high fidelity of this work. The increases in H_2 production are attributed to decreases in liquid lactate and ethanol production. The hypothetical mechanism by which the AnMBR facilitates increases in H_2, carbon dioxide, and acetate production is provided by the generalized

399 metabolic model for *C. thermocellum* in Figure 5. Figure 5 displays the various pathways,
400 enzymes, and metabolic reactions taking place as *C. thermocellum* converts sugars into
401 fermentation by-products. The black arrows show the carbon flux pathways, the blue arrows
402 show the electron flux pathways, and the red arrows hypothesize which pathways are
403 promoted in the AnMBR configuration compared to the AF configuration.
404



405

406 **Figure 5:** Metabolic pathway of *C. thermocellum* (PFL=pyruvate-formate lyase, PFOR=pyruvate
407 oxidoreductase, PTA=phosphotransacetylase, ACK=acetate kinase, ALDH= aldehyde
408 dehydrogenase, ADH=alcohol dehydrogenase, Fe-Hyd=iron hydrogenase, Bifur-Hyd=bifurcating-
409 hydrogenase, NFN=NADP (H) ferredoxin oxidoreductase, RNF=Ferredoxin: NAD (H)
410 Oxidoreductase, ECH=energy conservation hydrogenase). [52-54]
411

412 **3.5 AnMBR Performance Assessment**

413 According to Table 1, this is the first study in the literature to examine active anaerobic
414 membrane gas extraction under thermophilic pure-culture conditions. Comparing the
415 performance of the reactors in this study to reactors in the literature is difficult since the

operating conditions for each reactor varied substantially for each study. The two pure-culture studies listed in Table 1 by Bakonyi (2012) and Teplyakov (2002) investigated how well different membranes separated biogas mixtures into purified H_2 streams at mesophilic temperatures. With the highest H_2/CO_2 selectivity of Bakonyi's (2012) polyimide membrane study reaching 1.6 and the highest H_2/CO_2 selectivity of Teplyakov's (2002) PVTMS membrane study reaching 5.7, the H_2/CO_2 selectivity of 3.8-4.0 found in this study reveals that the V8/2 membrane has above average H_2 purification ability. The study that most closely resembles this study from Table 1 was conducted by Liang et al., who achieved a 10% increase in H_2 rate and 15% increase in H_2 yield, by using an anaerobic continuous gas extraction MBR. The 59-63% increase in H_2 rate and 58-59% increase in H_2 yield achieved by the AnMBR is this study not only surpass Liang et al.'s dark fermentation milestones but also reinforce the benefits of the membrane gas extraction process. Taking into account the MBR H_2 yield range of 0.93-1.87 mmol H_2/mmol hexose from Table 1, the AnMBR yields from this study of 0.68 ± 0.20 and 1.21 ± 0.18 mmol H_2/mmol hexose, appear to be on the lower side of values reported in the literature. All in all, the gas production metrics and membrane selectivity from this study appear to be reasonable, since the measured values are close to those from similar studies reported in the literature.

While the AnMBR setup design for this study was sufficient in demonstrating the proof of concept for using anaerobic membrane gas extraction to facilitate cellulosic hydrogen production in *C. thermocellum,* further studies are needed to optimize system performance and reduce cost. Before scaling up this process, operational factors such as membrane surface-area, selectivity, fouling, and cost need to be addressed in order to obtain higher H_2 yields. The V8/2 membrane used in this study was surface-area limited as the result of its tubular design, and

438 only portions of the coiled membrane not in direct contact with the reactor shell were active in
439 extracting gas. Increasing the membrane surface-area/reactor volume by replacing the tubular
440 coiled membrane in this study with a hollow-fiber membrane bundle, would allow the removal
441 of more produced gas, thereby increasing H_2 yields.[28] Although the V8/2 membrane in this
442 study already showed relatively high selectivity, 3.8-4.0 for H_2/CO_2, increasing the H_2 selectivity
443 of the membrane would not only help purify the product stream but also increase yields by
444 further reducing feed-back inhibition. Although membrane fouling was a minor issue in this
445 study, implementing membrane backwashing, re-cycling produced gas for sparging, and
446 utilizing granular activated-carbon (GAC), would all be beneficial techniques for mitigating
447 fouling and promoting higher H_2 yields. [23-24, 48-49]Implementing a jacketed reactor setup that
448 uses heated water coupled from an industrial process and recycling produced gas to use as a
449 sparging gas for maintaining an anaerobic environment are just a few ways operational costs
450 could be reduced for this setup.
451

452 **4. Conclusions**

453 This study demonstrates that anaerobic membrane gas extraction can be used to
454 promote H_2 production on both sugar and cellulosic solid substrates from *C. thermocellum. C.*
455 *thermocellum* converts more cellulose substrate to acetate, CO_2 and H_2 and grows to lower
456 optical densities when grown on Avicel when compared to cellobiose. The AnMBR increased
457 the rate of solid substrate degradation but did not increase the rate of liquid substrate
458 degradation. The AnMBR increased the cumulative H_2 production by 63%, the hydrogen
459 production rate by 24% and the overall H_2 yield by 58% when grown on 5 g/L cellobiose. The

AnMBR increased the cumulative H_2 production by 59%, the hydrogen production rate by 87% and the overall H_2 yield by 59% when grown on 5 g/L Avicel. The most ideal growth environment for *C. thermocellum* in this study involved growing the cells on Avicel in the AnMBR. This growth environment prompted the production of 74.6 ± 6.7 mmol's of H_2, a H_2 production rate of 5.8 ± 0.52 mmol hr^{-1}, and a H_2 yield of 1.21 ± 0.14 mmol H_2 mmol hexose^{-1}, which were the highest benchmarks of each H_2 metric from this study. The data also show that the AnMBR effectively partitions more electrons to the formation of desirable gaseous products over the formation of undesirable liquid products. This study demonstrates that anaerobic membrane gas extraction using the AnMBR can be an effective process to facilitate cellulosic hydrogen production by dark fermentation.

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