Title

Coupling gas purging with inorganic carbon supply to enhance biohydrogen production with Clostridium thermocellum

Authors

Kim, Changman

Wolf, Isaac

Dou, Chang

et al.

Publication Date

2023

DOI

10.1016/j.cej.2022.141028

Date: Nov 5th, 2022

For submission to *Chemical Engineering Journal*

Coupling gas purging with inorganic carbon supply to enhance biohydrogen production with *Clostridium thermocellum*

Changman Kim[1,2], Isaac Wolf[1], Chang Dou[1], Lauren Magnusson[3], Pin-Ching Maness[3], Katherine J. Chou[3], Steven Singer[4,5], Eric Sundstrom[1,5,*]

[1] Advanced Biofuel and Bioproducts Process Development Unit, Lawrence Berkeley National Laboratory, Emeryville, CA, 94608, USA

[2] Department of Biotechnology and Bioengineering, Chonnam National University, Gwangju, 61186, Republic of Korea

[3] Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA

[4] Joint BioEnergy Institute, 5885 Hollis street, 4th floor, Emeryville, CA, 94608, USA

[5] Biological System Engineering Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA, 94702, USA

[*]**Corresponding author**

Eric Sundstrom, PhD

Address: Advanced Biofuel and Bioproducts Process Development Unit, Lawrence Berkeley National Laboratory, Emeryville, CA, 94608, USA

E-mail address: esundstrom@lbl.gov

Abstract

Clostridium thermocellum is a desirable biocatalyst for biohydrogen production, with a native ability to simultaneously saccharify cellulose and to metabolize released cellodextrins for hydrogen production. During fermentation with *C. thermocellum*, partial pressures of two gases - CO_2 and H_2 - are critical drivers of overall reaction kinetics. Biohydrogen production is enhanced by maintaining a low hydrogen partial pressure, while high concentrations of dissolved CO_2 promote microbial biomass synthesis. Our study evaluates the inherent trade-offs between hydrogen stripping and inorganic carbon supply for optimized biohydrogen synthesis. We find that nitrogen sparging at low flow rates increases hydrogen production when compared with an equivalent nitrogen overlay, but that high rates of nitrogen sparging inhibit cell growth and hydrogen production. Decreasing dissolved hydrogen partial pressure via nitrogen sparging also lowers production of reduced metabolites, including lactate and ethanol. To address potential stripping of inorganic carbon from the production medium, we supplemented CO_2 to the sparging gas and co-optimized for gas flow rate and for the CO_2 fraction of the sparging gas. Total hydrogen production increased from 50 $mmol \cdot L^{-1}$ in the base condition, when the bioreactor was sparged with 0.1 LPM of pure nitrogen, to 181.3 $mmol \cdot L^{-1}$ when sparged with 1.3 LPM of 33% CO_2, demonstrating that biohydrogen production is highly sensitive to both parameters. Fine sensitivity of biohydrogen production to sparging conditions highlights the critical importance of bioreactor design and operation to achieve maximum H_2 removal without compromising inorganic carbon supply to bacterial central metabolism.

Keywords: *Clostridium thermocellum*; Biohydrogen; Gas sparging; Avicel

1. Introduction

Biological hydrogen production is an intriguing technology for conversion of complex organic waste streams into a renewable, dispatchable, and fungible energy carrier [1, 2]. Dark fermentation using lignocellulosic biomass is particularly appealing, as it allows for cost-effective and profitable use of agricultural and forestry residue while avoiding the adverse air pollution impacts associated with waste biomass combustion [3, 4]. Along with hydrogen, dark fermentation produces a high-quality CO_2 stream that can be sold, sequestered, or upgraded for further use, effectively decoupling energy release from biomass degradation and the resulting CO_2 emissions [5, 6]. However, to make biohydrogen production economically viable, conversion rates and yields must be improved, and these improvements must be consistent with conditions found in larger-scale bioreactors. During bioreactor scale-up, partial pressures of three gases (H_2, O_2, and CO_2) are critical factors impacting hydrogen productivity, titer, and yield from cellulosic biomass [1].

Oxygen inhibits both native hydrogenase activity and the central metabolism of hydrogen-producing anaerobic bacteria. To maintain anoxic conditions, most reactor configurations apply nitrogen or argon to remove dissolved oxygen, with associated impacts on dissolved hydrogen and carbon dioxide concentrations [7, 8]. High hydrogen partial pressure can thermodynamically shift the reaction away from hydrogen generation and toward hydrogen uptake [9, 10]. In such cases, hydrogenases can oxidize hydrogen by transferring electrons to reduce cellular redox co-factors, including NAD^+ and ferredoxin ([11]). At present, two opposing ideas describe the impact of CO_2 partial pressure on biohydrogen fermentations. *Park et al.* demonstrated that headspace CO_2 removal enhances secondary autotrophic metabolism by acetogens in a mixed community of microorganisms [12], while *Kim et al.* proposed CO_2 sparging as thermodynamically beneficial for H_2 production [13].

Bioconversion of lignocellulosic biomass can be typically viewed as a two-step process: 1) the polysaccharides are broken down into monomeric sugars or soluble sugar oligomers; 2) the soluble sugars are fermented and converted to the target products. In many engineered and natural systems, mixed microbial cultures accomplish this dual role of deconstruction and fermentation via division of labor. Although mixed cultures can metabolize complex and recalcitrant lignocellulosic feedstocks, mixed culture approaches often result in lower productivity of specific target products due to increased metabolic complexity [3, 4]. In contrast to mixed microbial systems, the Gram-positive, thermophilic, and obligate-anaerobic biocatalyst *Clostridium thermocellum* is capable of achieving biomass conversion to products in pure culture, owing to its natural ability to produce cellulosomes: multi-enzyme complexes bearing cellulase and xylanase enzymes highly effective in depolymerizing cellulose and hemicellulose into their soluble hexose and pentose sugar constituents. *C. thermocellum* can therefore hydrolyze and ferment complex cellulosic biomass in one integrated process known as consolidated bioprocessing (CBP) [14]. This process results in production of hydrogen, ethanol, and organic acids with acetate typically produced in greater quantities than lactate and formate [15, 16]. *C. thermocellum* has three [Fe-Fe] hydrogenases and one ferredoxin-dependent [NiFe] hydrogenase which are presumably correlated to anaerobic redox metabolism [17, 18]. Previous reports have successfully demonstrated biohydrogen production from cellulosic biomass (avicel) and xylose using *C. thermocellum* [19]. However, further development is required to improve both hydrogen yield and productivity under industrially relevant conditions [15, 16].

Active CO_2 fixation metabolism during cellulose assimilation has been reported in *C. thermocellum*, a unique feature of this organism [20]. Although the relationship between

CO_2 metabolism and hydrogen production was not clearly elucidated, Xiong et al. demonstrated that bicarbonate addition can improve not only carbon recovery, but also essential cell physiological activities including amino acid synthesis and cell growth. Therefore, while enhanced hydrogen removal via gas stripping may boost the hydrogen production metabolism, it may simultaneously strip CO_2 from the solution, negatively affecting amino acid synthesis and cell growth.

In this study, we aim to develop gas sparging strategies for improved biohydrogen fermentation from cellulose-based biomass by *C. thermocellum* by effectively balancing hydrogen removal and CO_2 supply. We focus on examining the potential trade-off between N_2 sparging rates and CO_2 partial pressures, and their relative impacts on cellular metabolism, cell biomass production, and hydrogen production. In addition, we evaluate these parameters in fermentations with high concentrations of cellulose (45 g/L), creating metabolite profiles, mixing conditions, and culture viscosities representative of high-intensity biohydrogen production conditions. Taken together, we show that the effects of H_2 removal and inorganic carbon supply on the bacteria are coupled, and we demonstrate that engineering interventions -including CO_2 supplementation during gas stripping - can substantially improve hydrogen production in high-solids fermentations without compromising *C. thermocellum* central metabolism.

2. Materials and methods

2.1. Strain and materials

Clostridium thermocellum KJC315 (a DSM1313 derived strain, Δ*hpt*) was provided

by NREL [20]. In this study, two different media were used for microbial cell growth and biohydrogen production. CTFUD rich media includes (per liter in D.W.): 3 g $Na_3C_6H_5O_7 \cdot H_2O$, 1.3 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.13 g $CaCl_2H_2O$, 2.6 g $MgCl_2 \cdot 6H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 11.56 g MOPS sodium salt, 0.5 g L-cysteine HCl, 5g cellobiose, 4.5 g yeast extract, 0.5 mL of 1000x resazurin solution. MTC contains the following components (per liter in D.W.): 5 g MOPS sodium salt, 2 g $Na_3C_6H_5O_7 \cdot H_2O$, 1 g K_2HPO_4, 1.25 g $C_6H_8O_7 \cdot H_2O$, 1 g Na_2SO_4, 2.5 g $NaHCO_3$, 1.5 g NH_4Cl, 2 g urea, 1 g, $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 1 g L-cysteine HCl, 20 mg pyridoxamine dihydrochloride, 4 mg PABA, 2 mg D-biotin, 2 mg vitamin B_{12}, 0.5 mL of pre-made resazurin solution (44 mM). Prior to inoculation, both medium was adjusted to pH 7.0 using 2 N NaOH and 10 % H_2SO_4. MTC media was supplemented with 5 g/L D-(+)-Cellobiose. MTC media was also used in all bioreactor fermentations, and was supplemented with 30 g/L of Avicel PH-101 (~ 50 μm particle size, Sigma Aldrich, USA) ,or 20 g/L of D-(+)-Cellobiose as specified in the results section. CTFUD rich media was used for bacteria storage in -80 ℃, growth, and reactivation of seed 1 cultures. For pre-culture 2 and main fermentation, MTC media was applied.

2.2. Bioreactor operation

Seed culture cultivation was performed using two consecutive seed trains at 10 mL and 50 mL culture volume (in 20- and 125-ml serum bottles, respectively) incubated in a rotary shaker at 60 °C and 150 rpm for 24 h. Initial seed cultures were reactivated from frozen stock in CTFUD rich media, before transferring to a second seed cultivated in MTC media containing 5 g/L D-(+)-Cellobiose. Main cultivation was performed with 0.95 L MTC media

and 0.05 L inoculum in 2 L jacketed BIOSTAT® B (Sartorius AG., Goettingen, Germany) benchtop bioreactors with dual Rushton impellers and constant gas flow regulator. Process parameters were 60 °C and 100 rpm agitation with pH controlled at 7.0 using 2 M NaOH. A default flow rate of 0.1 liters per minute (LPM) was utilized for nitrogen, carbon dioxide and mixed gases unless otherwise specified. All bioreactors were sparged with a ring sparger sitting below the lowest impeller and containing 14 holes, with each hole 1mm in diameter. Gases were supplied either directly to the gas headspace of the bioreactor (overlay), or via sparging into liquid culture (sparged). Manual 5 mL samples were taken throughout the cultivation and stored at 4 °C for further analysis.

2.3. Analysis

The concentrations of sugars and other byproducts (e.g., formic acid, lactic acid, acetic acid, ethanol, and glycerol) in the fermentation samples were quantified using a high-performance liquid chromatography (HPLC) system (Ultimate 3000, Thermo Fisher Scientific, Waltham, MA) equipped with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm, Hercules, CA, USA). The mobile phase (4 mM of H_2SO_4 solution) flowed at a rate of 0.4 mL/min and the column oven temperature was set at 40 °C for HPLC operation. The off-gas of the fermentation was monitored using a magnetic sector MS analyzer (Prima BT Bench Top Process Mass Spectrometer, Thermo Fisher Scientific, Waltham, MA) by which the concentrations of N_2, CO_2, H_2, O_2 were monitored in real time. H_2 production rates were quantified by comparison with known flow rates of inert N_2 supplied to the bioreactor with the following equation.

$$H_2\ production\ rate\ (LPM) = \frac{N_2\ flow\ rate\ (LPM) \times Measured\ H_2\ concentration\ (\%)}{Measured\ N_2\ concentration\ (\%)}$$

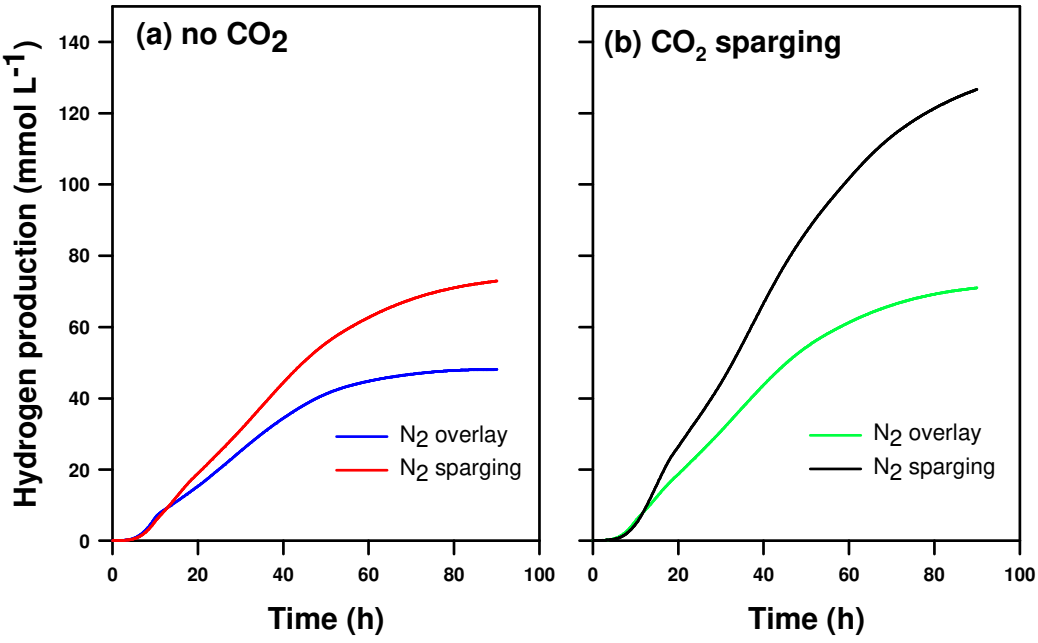
All analyses were conducted in duplicate or triplicate and presented as the average value.

3. Results and discussions

3.1. Gas purging strategies in biohydrogen production

Strict anaerobic conditions are required for optimal *C. thermocellum* growth, and these conditions are typically maintained by continuous purging with biologically non-reactive gases, including nitrogen and argon. In agitated bioreactor fermentations there are two potential modes of gas purging: gas overlay with inert gas supplied to the reactor headspace and gas sparging with inert gas sparged directly into the liquid culture. To evaluate the differences in *C. thermocellum* metabolic response to these strategies, bioreactors were operated with either overlay nitrogen or sparged nitrogen supplied at 0.1 LPM into 1 L of liquid culture for comparison. Data from Fig. 1 (a) shows hydrogen production profiles between overlay and sparged bioreactors purged with 0.1 LPM of nitrogen and supplied with 30 g/L of avicel. Under these conditions, sparged bioreactors produced more hydrogen (72.9 mmol · L^{-1}) compared with overlay bioreactors (48.1 mmol · L^{-1}). Generally, nitrogen sparging improves the thermodynamic preference for biohydrogen production by decreasing both the hydrogen partial pressure in the reactor headspace and by reducing the dissolved hydrogen concentration in media [8, 21]. In this study, both overlay and sparging reactors should have similar headspace hydrogen partial pressure because the continuous purging gas diluted the produced hydrogen gas (maintained below 2 % in both reactors), even if gas

bubbling increased the gas-liquid interface area. However, because nitrogen sparging can extrude dissolved hydrogen in the media, sparged cultures are likely subjected to lower hydrogen partial pressures in the aqueous phase.



185

186 **Figure 1.** Hydrogen production profiles (a) without CO_2 and (b) with CO_2 sparging between
187 N_2 sparging and overlay reactors, at a flow rate of 0.1 LPM (n = 2), using 30 g/L avicel as a
188 substrate.

189 To determine the effects of nitrogen sparging on dissolved hydrogen in the media,
190 an abiotic experiment was conducted, confirming elevated hydrogen stripping rates in the
191 nitrogen sparging condition (Fig S1). According to the results, direct sparging accelerated
192 hydrogen stripping when compared to overlay purging. Therefore, in isolation, relatively low
193 dissolved hydrogen concentrations tend to boost *C. thermocellum* biohydrogen generation.

194 Xiong et al. demonstrate that the addition of sodium bicarbonate correlates with a
195 higher *C. thermocellum* cell biomass when using cellobiose as the carbon source [20]. To
196 evaluate the impact of sparging conditions on inorganic carbon availability, abiotic bioreactor
197 operations with 0.1 LPM nitrogen sparging were examined in combination with the addition
198 of a 47.6 mmol sodium bicarbonate bolus. Fig S2 depicts CO_2 release profiles in abiotic

reactors, with approximately 85 % of inorganic carbon (40.7 mmol) stripped out within 8 hours during the reactor deoxygenating and pre-conditioning stage. The water solubility of CO_2 is only 0.58 g/L under the *C. thermocellum* fermentation condition (1 atm at 60 ℃). MTC media contains 2.2 g/L of HCO_3^- (2.5 g/L of $NaHCO_3$); however theoretically around 1.5 g/L of bicarbonate was discharged from the media via gas sparging prior to inoculation, leaving only 0.5–0.7 g/L of dissolved CO_2 species at the time of inoculation.

To compensate for this gas stripping effect and provide a more stable supply of inorganic carbon, two additional conditions were evaluated in which pure nitrogen was substituted with a mixed purging gas containing approximately 33% CO_2 (0.033 LPM of 100 % CO_2) and 67% N_2 (0.067 LPM of 100 % N_2), ensuring constant partial pressure of CO_2 throughout the fermentation. In these reactors, CO_2 supplementation resulted in 1.7 and 1.5-fold higher hydrogen production (126.7 and 71.0 mmol in N_2 sparging and overlay reactor, respectively) (Fig 1(b)), compared with reactors receiving pure N_2 (Fig 1 (a)).

Cell growth experiments were also conducted to determine the effects of gas purging using D-(+)-cellobiose as a substrate instead of Avicel, as 30 g/L Avicel media solution is too milky for effective OD measurement (Fig 2). Pure nitrogen sparging significantly inhibited bacterial growth at a flow rate of 0.1 LPM. Mixed N_2 and CO_2 sparging enabled *C. thermocellum* to fully utilize cellobiose in both the 0.1 and 0.2 LPM reactors. Nitrogen overlay resulted higher cell growth but lower hydrogen production, because dissolved CO_2 and H_2 could be converted to biomass. Additional supply of CO_2 in the 0.1 LPM overlay reactor presented additional inorganic carbon uptake due to the abundant CO_2 species in the media. These experiments confirm the need for a consistent supply of inorganic carbon in *C. thermocellum* biohydrogen production, particularly when CO_2 produced during

cellulose metabolism is rapidly stripped from the solution as part of a hydrogen removal strategy. Notably, while 72.9 mmol · L^{-1} hydrogen production was observed with 0.1 LPM pure nitrogen sparging in the avicel condition presented in figure 1, no production at all was observed under equivalent conditions with cellobiose feedstock. This variability is likely attributed to the high viscosity of the avicel-containing cultivation medium, which dampens mixing and liquid-gas mass transfer, diminishing the rate of CO_2 stripping from the system.

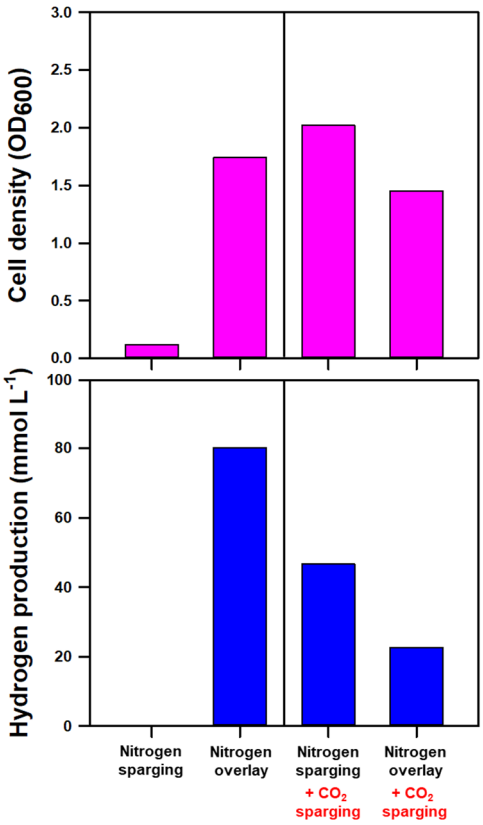


Figure 2. Cell density and hydrogen productions for *C. thermocellum* after 48 hours of growth using cellobiose (5 g/L) as a substrate. Overlay and sparged gases were supplied at a total flow rate of 0.1 LPM.

3.2. Metabolic characterization of *C. thermocellum* hydrogen production

To further understand the effect of gas purging strategies on bacterial metabolism during Avicel fermentation, metabolites were analyzed from four different bioreactor configurations: 1) N_2 sparging, 2) N_2 overlay, 3) N_2 and CO_2 sparging and 4) N_2 overlay and CO_2 sparging. N_2 sparging reactors (conditions 1 and 3) should exhibit enhanced hydrogen removal rates when compared with N_2 overlay reactors (conditions 2 and 4). CO_2 sparging reactors (3 and 4) contained higher concentrations of dissolved CO_2 when compared with non-CO_2 sparging reactors (1 and 2). Fig 3b and Table 1 present the production of key fermentation byproducts and the metabolic pathways leading to their production in *C. thermocellum.*

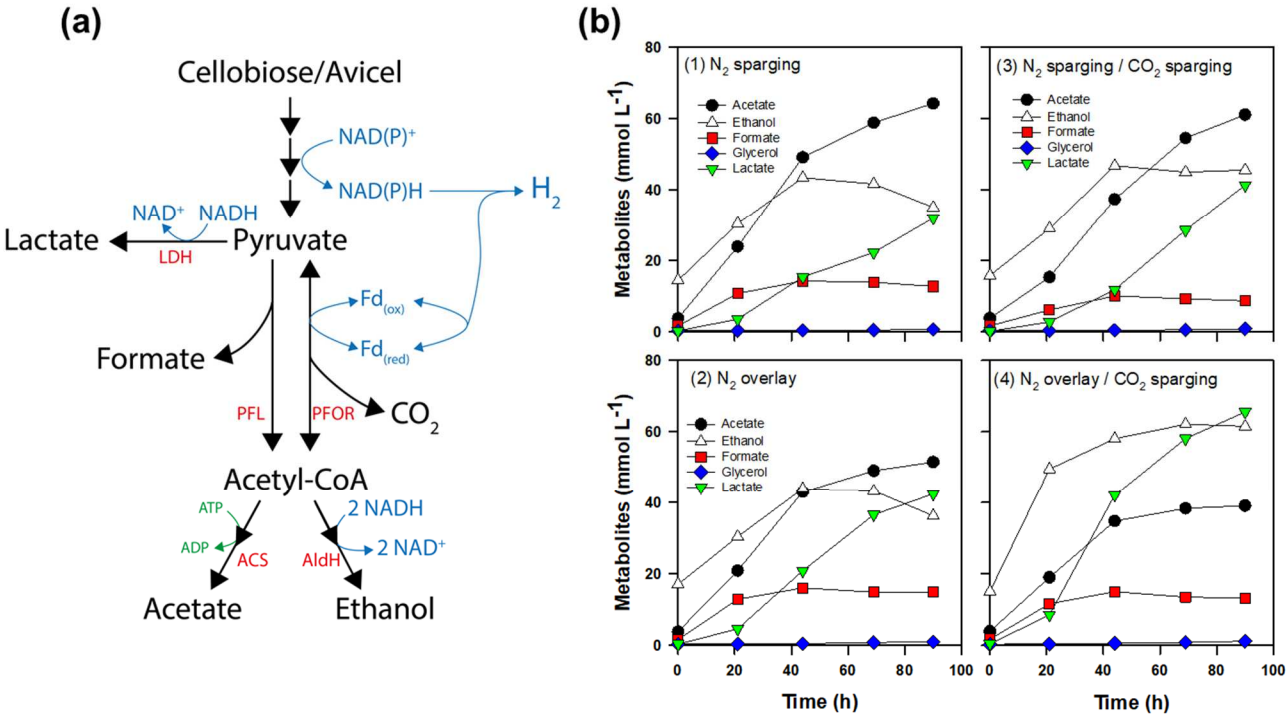


Figure 3. (a) Metabolic pathway from cellobiose/Avicel and (b) metabolites profiles of the

245 different gas purging reactors

246 **Table 1**. Comparison of metabolites produced when utilizing variable gas purging strategies

Purging gas		Metabolites (mmol L^{-1})					H_2/Acetate (mol/mol)
N_2	CO_2	H_2	Acetate	Ethanol	Formate	Lactate	
Sparging	-	72.9	64.3	35.1	13.1	32.4	1.1
Overlay	-	48.1	61.1	45.6	8.9	41.8	0.8
Sparging	Sparging	126.7	61.5	36.3	15.3	42.9	2.1
Overlay	Sparging	71	39.1	61.6	13.5	66.3	1.8

247

Hydrogen production is an "electron sink" for *C. thermocellum* metabolism when balancing the cellular redox state [22, 23]. Hydrogen stripped from the solution by nitrogen sparging effectively decreases the concentration of dissolved hydrogen in the media, shifting the thermodynamic equilibrium to favor additional hydrogen production. This process may therefore lead to a shift of metabolic carbon flux towards non-reducing pathways. As a result, N_2 sparged reactors produced fewer reduced metabolites, including lactate and ethanol, when compared to the N_2-overlay reactors in which additional CO_2 was supplied via sparging.

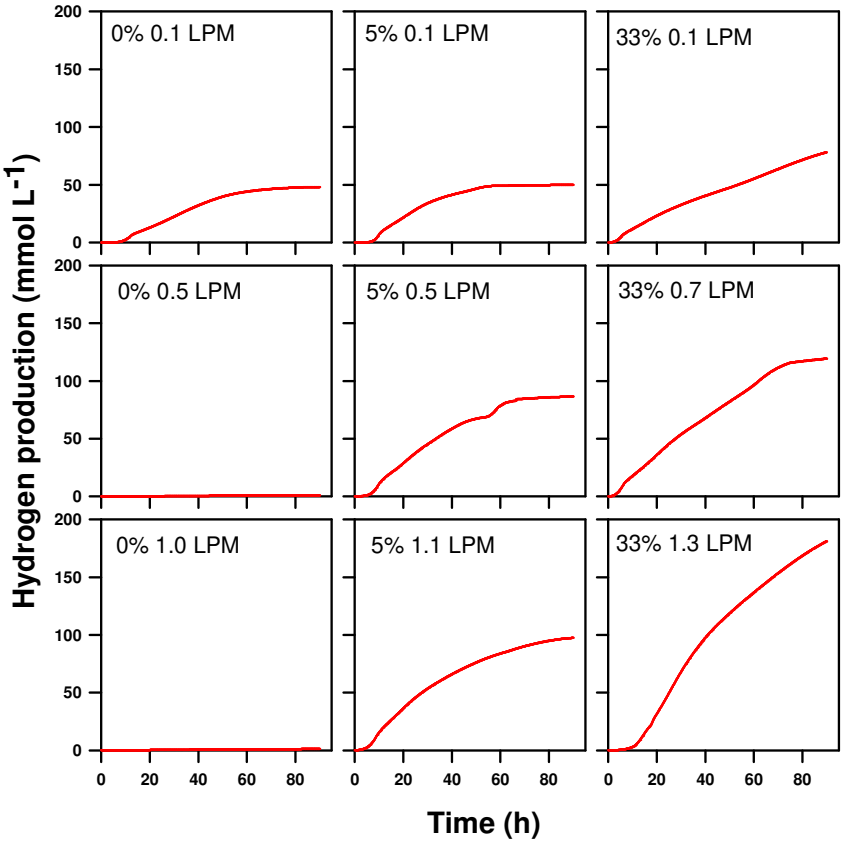
Pyruvate ferredoxin oxidoreductase (PFOR) can catalyze the reductive carboxylation of CO_2 and incorporate CO_2 to produce pyruvate from acetyl-CoA, even under cellulose-degrading conditions [20]. This reversed PFOR-driven CO_2 uptake pathway may be crucial for the biosynthesis of serine, methionine, and cell biomass via C1 metabolism. Continuous supply of CO_2 may increase the carbon flux toward pyruvate synthesis, further led to lactate production as overflow metabolism. This is consistent with higher lactate production in the CO_2 sparging reactors. Our data is also consistent in showing that production of lactate outcompetes production of ethanol during CO_2 sparging as acetyl-CoA, a precursor to form ethanol, was depleted to form pyruvate and subsequently routed to lactate instead.

Acetate is regarded as a primary byproduct in dark fermentation, often used to assess the H_2/Acetate ratio [24]. The theoretical equation ($C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2$) implies the ideal maximum ratio between hydrogen and acetate as 2. However, the true value varies with bacterial species and process conditions. Shen et al. proposed that relatively higher H_2/Acetate ratios could denote acetate oxidation or acetyl-CoA consumption, including CO_2 fixation by PFOR [24]. Formation of the reduced metabolites

lactate and ethanol, utilizing NADH as a co-factor, tends to decrease the H_2/Acetate ratio due to lowered hydrogen synthesis. When comparing replicates with CO_2 sparging, *C. thermocellum* with an N_2 overlay synthesized comparatively higher concentrations of lactate and lower concentrations of acetate, likely due to higher concentrations of dissolved hydrogen.

3.3. Optimization of gas sparging strategies

To further optimize biohydrogen production in bioreactor fermentations and deduce the interaction between hydrogen removal and CO_2 supply, we explored a variety of gas sparging rates and gas compositions in a full-factorial experimental design illustrated in Fig 4. These experiments were conducted with three sparging rates (0.1, 0.5, and 1.0 LPM for nitrogen gas) and three CO_2 concentrations (0, 5, and 33%), using modified MTC media containing lower concentration of vitamins (30% of the full recipe).



283

284 **Figure 4.** Hydrogen production profiles during Avicel (30 g/L) fermentation for variable gas
285 sparging conditions

286 Among 100% nitrogen sparging reactors, significant hydrogen production was only
287 identified at 0.1 LPM (48.1 mmol·L^{-1}) (Fig 4). Remarkably, higher sparging rates with 100%
288 nitrogen (0.5 and 1.0 LPM), resulted in no biohydrogen production nor Avicel metabolism
289 over 24 hours monitoring. As demonstrated in Fig S2, a higher nitrogen sparging rate
290 accelerates the CO_2 release rate. Thus, the media in these conditions may not maintain
291 sufficient inorganic carbon for essential microbial metabolism, highlighting the reliance of *C.*
292 *thermocellum* on CO_2 for growth on organic substrates. Hydrogen production, with a small
293 amount (~ 5% v/v) of CO_2 sparging, was measured at 50.0, 86.6 and 97.4 mmol·L^{-1} in 0.1, 0.5
294 and 1.1 LPM reactors, respectively. In contrast to the 100% nitrogen sparging condition, even

small amounts of CO_2 during gas sparging provided a sufficient inorganic carbon source, allowing *C. thermocellum* to generate the biomass required to metabolize Avicel and generate hydrogen. Higher concentration of CO_2 in the gas sparging stream resulted in further improvements to hydrogen production titers when compared with no/low concentration of CO_2 sparging reactors (78.2, 119.4 and 181.3 $mmol \cdot L^{-1}$ with 0.1, 0.5 and 1.0 LPM, respectively). As a result, we conclude that both high CO_2 partial pressures and high gas stripping rates are essential to maximize biohydrogen production as illustrated in Fig 3 (b). Notably, hydrogen production with the 0.1 LPM sparging rate was similar until early- to mid-stage production (45 hours) despite variable sparging gas compositions (36.3, 44.0 and 44.1 $mmol \cdot L^{-1}$ with 0, 5 and 33 % CO_2, respectively). This implies that addition of CO_2 gas at low sparging rates primarily serves to prolong productivity into the mid- to late- stages of fermentation. With lower sparging rates, biogenically released CO_2 appears sufficient to maintain central metabolic activity in the early stages of production. Supplemental CO_2 may prolong culture viability in later stages as metabolic activity slows, with an associated reduction in the availability of biogenic inorganic carbon.

Metabolite production profiles under variable gas sparging regimes are shown in Fig S3. To understand the correlation between gas sparging rate, CO_2 concentration, metabolites, and hydrogen production, Pearson's correlation analysis was conducted (Fig S4). The results of this statistical analysis indicate that CO_2 concentration, hydrogen production, acetate production, and formate production have a positive and significant correlation ($P > 0.7$). Pyruvate formate lyase is the core enzyme for the conversion of pyruvate to acetyl-CoA and formate in the anaerobic cellulosic metabolism of *C. thermocellum*. The conversion of acetyl-CoA to acetate is a key ATP-synthesis step, which does not affect the cellular redox

state. Relatively optimized conditions, including sufficient gas sparging and sufficient inorganic carbon source, allowed *C. thermocellum* to metabolize Avicel for synthesis of acetate and formate. Similarly, lactate production was significantly correlated with both H_2 production and CO_2 concentration ($0.3 < P < 0.7$). Sparging rates have a moderate level of correlation with hydrogen production ($P = 0.4$) because of out-range results from non-hydrogen productive reactors (0.5 and 1.0 LPM with 100 % nitrogen). This result further illustrates that sparging impacts cannot be considered in isolation, without considering inorganic carbon availability.

The results of *C. thermocellum* fermentation with 100% CO_2 sparging were summarized in Fig S5. After 24 h, the hydrogen production rates of the 100% CO_2 sparging reactors (56.0, 56.4, and 43.7 $mmol \cdot L^{-1} \cdot day^{-1}$ for 0.1, 0.5, and 1.0 LPM, respectively) were significantly higher than those of the mixed gas sparging reactors (27.3, 43.7, and 45.7 $mmol \cdot L^{-1} \cdot day^{-1}$ for 0.1, 0.6, and 1.3 LPM, respectively). However, hydrogen production dropped after 1 day for all 100% CO_2 sparging reactors. A small amount of reduced ferredoxin remained may have still been available to serve as an electron donor for hydrogen production owing to the re-oxidation of ferredoxin catalyzed by reversed PFOR. Metabolite profiles presented significantly higher concentrations of formate within 1 day, as shown in Fig S5. This implies that a higher concentration of CO_2 sparging triggers the reverse reaction of PFOR, resulting in overflow metabolism. Oversaturated CO_2-based metabolic interruption is generally acceptable in various bacteria species [25]. To prevent supersaturation of CO_2 in the media, mixed gas should therefore be used to achieve optimal biohydrogen production in *C. thermocellum*. While this approach is highly effective at a bench scale, further scale-up of biohydrogen production will likely require the development of alternative gas stripping

methods to avoid energy costs for sparging and to maximize hydrogen concentration in the off-gas. These approaches could potentially include vacuum or membrane-based methods [26], to avoid the high costs associated with gas sparging and downstream separation of gaseous products. Such strategies must be optimized to maintain adequate inorganic carbon while minimizing both H_2 partial pressures and dilution of H_2 in the output gas stream.

4. Conclusion

In this study, we developed gas sparging strategies for optimal cellulosic biohydrogen production using *C. thermocellum* in 1 L bioreactors. Nitrogen sparging enhanced hydrogen removal and overall hydrogen productivity when compared to a nitrogen overlay. Continuous supply of inorganic carbon by CO_2 sparging combined with high overall gas sparging rates increased hydrogen productivity from 50 $mmol \cdot L^{-1}$ in the nitrogen sparging base condition to 181.3 mmol·L-1 when supplied with a high flow rate of 33% CO_2. This result demonstrates that a co-optimized sparging rate and CO_2 concentration can achieve rapid hydrogen removal while preserving inorganic carbon supplies, thereby maximizing biohydrogen generation.

5. Acknowledgement

This work was in part authored by the Lawrence Berkeley National Laboratory and National Renewable Energy Laboratory (NREL), operated by Alliance for Sustainable Energy, LLC, for the U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. Funding was provided by U.S. Department of Energy Office of Energy Efficiency Renewable

Energy Hydrogen and Fuel Cell Technologies Office. The views expressed in the article do not necessarily represent the views of the DOE or the U.S. Government. The U.S. Government retains and the publisher, by accepting the article for publication, acknowledges that the U.S. Government retains a nonexclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this work, or allow others to do so, for U.S. Government purposes. Authors declare no competing interests.

6. References

[1] A. Ghimire, L. Frunzo, F. Pirozzi, E. Trably, R. Escudie, P.N. Lens, G. Esposito, A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products, Applied Energy 144 (2015) 73-95.

[2] J.-H. Jung, Y.-B. Sim, J.-H. Park, A. Pandey, S.-H. Kim, Novel dynamic membrane, metabolic flux balance and PICRUSt analysis for high-rate biohydrogen production at various substrate concentrations, Chemical Engineering Journal (2020) 127685.

[3] W. Han, Y.Y. Hu, S.Y. Li, F.F. Li, J.H. Tang, Biohydrogen production from waste bread in a continuous stirred tank reactor: A techno-economic analysis, Bioresource technology 221 (2016) 318-323.

[4] Y.-C. Li, Y.-F. Liu, C.-Y. Chu, P.-L. Chang, C.-W. Hsu, P.-J. Lin, S.-Y. Wu, Techno-economic evaluation of biohydrogen production from wastewater and agricultural waste, International journal of hydrogen energy 37(20) (2012) 15704-15710.

[5] J.-H. Lu, C. Chen, C. Huang, H. Zhuang, S.-Y. Leu, D.-J. Lee, Dark fermentation production of volatile fatty acids from glucose with biochar amended biological consortium, Bioresource technology 303 (2020) 122921.

386 [6] A. Chalima, A. Hatzidaki, A. Karnaouri, E. Topakas, Integration of a dark fermentation
387 effluent in a microalgal-based biorefinery for the production of high-added value omega-3
388 fatty acids, Applied Energy 241 (2019) 130-138.
389 [7] P.T. Sekoai, K.O. Yoro, M.O. Daramola, Effect of nitrogen gas sparging on dark
390 fermentative biohydrogen production using suspended and immobilized cells of anaerobic
391 mixed bacteria from potato waste, Biofuels 9(5) (2018) 595-604.
392 [8] O. Mizuno, R. Dinsdale, F.R. Hawkes, D.L. Hawkes, T. Noike, Enhancement of hydrogen
393 production from glucose by nitrogen gas sparging, Bioresource technology 73(1) (2000) 59-
394 65.
395 [9] L. Beckers, J. Masset, C. Hamilton, F. Delvigne, D. Toye, M. Crine, P. Thonart, S.
396 Hiligsmann, Investigation of the links between mass transfer conditions, dissolved hydrogen
397 concentration and biohydrogen production by the pure strain Clostridium butyricum
398 CWBI1009, Biochemical Engineering Journal 98 (2015) 18-28.
399 [10] B. Laurent, H. Serge, M. Julien, H. Christopher, T. Philippe, Effects of hydrogen partial
400 pressure on fermentative biohydrogen production by a chemotropic Clostridium bacterium in
401 a new horizontal rotating cylinder reactor, Energy procedia 29 (2012) 34-41.
402 [11] Y. Zheng, J. Kahnt, I.H. Kwon, R.I. Mackie, R.K. Thauer, Hydrogen formation and its
403 regulation in Ruminococcus albus: involvement of an electron-bifurcating [FeFe]-
404 hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative hydrogen-
405 sensing [FeFe]-hydrogenase, Journal of bacteriology 196(22) (2014) 3840-3852.
406 [12] W. Park, S.H. Hyun, S.-E. Oh, B.E. Logan, I.S. Kim, Removal of headspace CO2
407 increases biological hydrogen production, Environmental science & technology 39(12) (2005)
408 4416-4420.

[13] D.-H. Kim, H.-S. Shin, S.-H. Kim, Enhanced H2 fermentation of organic waste by CO2 sparging, International journal of hydrogen energy 37(20) (2012) 15563-15568.

[14] L.R. Lynd, C.E. Wyman, T.U. Gerngross, Biocommodity engineering, Biotechnology progress 15(5) (1999) 777-793.

[15] D.B. Levin, R. Islam, N. Cicek, R. Sparling, Hydrogen production by Clostridium thermocellum 27405 from cellulosic biomass substrates, International Journal of Hydrogen Energy 31(11) (2006) 1496-1503.

[16] Q.-Q. Tian, L. Liang, M.-J. Zhu, Enhanced biohydrogen production from sugarcane bagasse by Clostridium thermocellum supplemented with CaCO3, Bioresource Technology 197 (2015) 422-428.

[17] H.-F. Li, B.L. Knutson, S.E. Nokes, B.C. Lynn, M.D. Flythe, Metabolic control of Clostridium thermocellum via inhibition of hydrogenase activity and the glucose transport rate, Applied microbiology and biotechnology 93(4) (2012) 1777-1784.

[18] T. Rydzak, P.D. McQueen, O.V. Krokhin, V. Spicer, P. Ezzati, R.C. Dwivedi, D. Shamshurin, D.B. Levin, J.A. Wilkins, R. Sparling, Proteomic analysis of Clostridium thermocellum core metabolism: relative protein expression profiles and growth phase-dependent changes in protein expression, BMC microbiology 12(1) (2012) 1-18.

[19] W. Xiong, L.H. Reyes, W.E. Michener, P.C. Maness, K.J. Chou, Engineering cellulolytic bacterium Clostridium thermocellum to co-ferment cellulose-and hemicellulose-derived sugars simultaneously, Biotechnology and bioengineering 115(7) (2018) 1755-1763.

[20] W. Xiong, P.P. Lin, L. Magnusson, L. Warner, J.C. Liao, P.-C. Maness, K.J. Chou, CO2-fixing one-carbon metabolism in a cellulose-degrading bacterium Clostridium thermocellum, Proceedings of the National Academy of Sciences 113(46) (2016) 13180-13185.

[21] D.-H. Kim, S.-K. Han, S.-H. Kim, H.-S. Shin, Effect of gas sparging on continuous fermentative hydrogen production, International Journal of Hydrogen Energy 31(15) (2006) 2158-2169.

[22] H.-S. Lee, W.F. Vermaas, B.E. Rittmann, Biological hydrogen production: prospects and challenges, Trends in biotechnology 28(5) (2010) 262-271.

[23] K.J. Chou, W. Xiong, L. Magnusson, M. Seibert, P.-C. Maness, Renewable Hydrogen From Biomass Fermentation, (2020).

[24] N. Shen, F. Zhang, X.-N. Song, Y.-S. Wang, R.J. Zeng, Why is the ratio of H2/acetate over 2 in glucose fermentation by Caldicellulosiruptor saccharolyticus?, International journal of hydrogen energy 38(26) (2013) 11241-11247.

[25] K.C. Peet, A.J. Freedman, H.H. Hernandez, V. Britto, C. Boreham, J.B. Ajo-Franklin, J.R. Thompson, Microbial growth under supercritical CO2, Applied and environmental microbiology 81(8) (2015) 2881-2892.

[26] S. Singer, L. Magnusson, D. Hou, J. Lo, P.-C. Maness, Z.J. Ren, Anaerobic membrane gas extraction facilitates thermophilic hydrogen production from Clostridium thermocellum, Environmental Science: Water Research & Technology 4(11) (2018) 1771-1782.

Graphical abstract