

ORIGINAL ARTICLE

Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* speciesZ. Ren¹, T.E. Ward¹, B.E. Logan^{1,2} and J.M. Regan^{1,2}

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biomass, cellulose, clostridia, fermentation, hydrogen.

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Abstract**Aims:** To characterize cellulolytic, hydrogen-producing clostridia on a comparable basis.**Methods and Results:** H₂ production from cellulose by six mesophilic clostridia was characterized in standardized batch experiments using MN301 cellulose, Avicel and cellobiose. Daily H₂ production, substrate degradation, biomass production and the end-point distribution of soluble fermentation products varied with species and substrates. All species produced a significant amount of H₂ from cellobiose, with *Clostridium acetobutylicum* achieving the highest H₂ yield of 2.3 mol H₂ mol⁻¹ hexose, but it did not degrade cellulose. *Clostridium cellulolyticum* and *Clostridium populeti* catalysed the highest H₂ production from cellulose, with yields of 1.7 and 1.6 mol H₂ mol⁻¹ hexose from MN301 and 1.6 and 1.4 mol H₂ mol⁻¹ hexose from Avicel, respectively. These species also achieved 25–100% higher H₂ production rates from cellulose than the other species.**Conclusions:** These cellulolytic, hydrogen-producing clostridia varied in H₂ production, with *Cl. cellulolyticum* and *Cl. populeti* achieving the highest H₂ yields and cellulose degradation.**Significance and Impact of the Study:** The fermentation of cellulosic materials presents a means of H₂ production from renewable resources. This standardized comparison provides a quantitative baseline for improving H₂ production from cellulose through medium and process optimization and metabolic engineering.**Introduction**

To address the global concerns of energy-related environmental pollution, hydrogen has been proposed as a clean and efficient energy carrier of the future. Containing the highest energy content per unit weight (143 GJ tonne⁻¹), it offers a potential substitute for current transportation fuels, and it is also the only fuel whose oxidation products do not contain carbon dioxide and do not contribute to ozone depletion or acid rain (Nath and Das 2004). Moreover, H₂ can be produced biologically from renewable resources, including biomass, which is environmentally sustainable and can reduce dependence on fossil fuels and decrease net carbon dioxide emissions (Winsche *et al.* 1973; Logan 2004).

Cellulose, which accounts for 30–50% by weight of biomass, is the most abundant biopolymer on earth. Approximately 30 billion tons of terrestrial cellulose are produced each year (Cox *et al.* 2000). The quantity and availability of cellulose make it a major renewable resource (Schwarz 2001), and the fermentation of its glucose subunits to H₂ offers one means of combining biomass degradation with energy production. The current bottleneck of this utilization is the hydrolysis of cellulose, and considerable research has been focused on accomplishing this efficiently through physicochemical techniques such as steam explosion and dilute-acid pretreatment, as well as enzymatic hydrolysis (Lynd *et al.* 2002; Elam *et al.* 2003).

There are many microbial consortia capable of both cellulose hydrolysis and H₂ production, such as ruminal

and soil communities, and certain members of the genus *Clostridium* possess both capabilities and are often found dominant in cellulolytic, H₂-producing reactor systems (Leschine 1995; Hawkes *et al.* 2002). Their use could significantly reduce physicochemical or enzymatic pretreatment costs associated with fermentative H₂ production from cellulose. The maximum theoretical yield of H₂ from such a fermentation is 4 mol H₂ mol⁻¹ hexose, with the corresponding production of acetate and carbon dioxide. However, this theoretical yield cannot be achieved experimentally because microbes use some electrons to produce more biomass, certain strains produce additional soluble fermentation products that are more reduced than acetate, or the H₂ produced may be reconsumed by uptake hydrogenases of the H₂ producer or other community members in a mixed culture (Hallenbeck and Benemann 2002). Strategies to address this latter H₂ loss that apply various selective pressures to repress the activities of H₂ consumers, such as low pH, short retention time and heat or alkaline treatment have been demonstrated (Oh *et al.* 2003; Cai *et al.* 2004).

Researchers have tested various inocula for H₂ production from cellulose, but the H₂ yields were generally very low. For example, using an uncharacterized, heat-shocked soil inoculum, Logan *et al.* (2002) found the H₂ yield from cellulose was very low (0.005 mol H₂ mol⁻¹ hexose added) compared with glucose (0.92 mol H₂ mol⁻¹ hexose) due to poor cellulose hydrolysis. Lay (2001) observed a maximum H₂ yield of 0.35 mol mol⁻¹ hexose, which occurred at a cellulose concentration of 12.5 g l⁻¹ when inoculated with heat-shocked sludge. Using microcrystalline cellulose, Miller and Wolin (1995) reported a H₂ yield of 0.007 mol H₂ mol⁻¹ hexose added with a pure culture of the ruminal bacterium *Ruminococcus albus*, and a very small amount of H₂ when coupling with a H₂-consuming acetogen. With *Clostridium cellulolyticum* and MN301 cellulose, Desvaux *et al.* (2000) observed that the H₂ yield and the extent of cellulose degradation were highly dependent on the initial cellulose concentration, with the H₂ yield first increasing to 1.66 mol H₂ mol⁻¹ hexose at an initial hexose concentration of 44.1 mmol l⁻¹, and then decreasing to 0.33 mol H₂ mol⁻¹ hexose at an initial hexose concentration of 179.6 mmol l⁻¹. The degree of cellulose degradation continuously decreased with increasing substrate concentration, from more than 85% at the low substrate concentration down to 45% at high substrate concentration. There are numerous other reports of isolated clostridia that hydrolyse cellulose and produce H₂ (Ueno *et al.* 1995, 2001), but quantitative data collected from comparable experimental conditions are lacking.

The objective of this research was to quantitate H₂ production from cellulose for several selected *Clostridium*

species. There has been considerable research on the characterization and genetic manipulation of cellulolytic clostridia for enhanced solventogenesis, but this has not been explored for H₂ production. A matrix of batch experiments was performed using six species and three types of cellulosic substrates, with daily monitoring of H₂, substrate, biomass and end-point monitoring of soluble fermentation products. These data provide a comparable characterization of the cellulolytic and hydrogen-producing capabilities of these strains, which can serve as a baseline for improving cellulose utilization and H₂ production efficiency through strain selection, medium and process optimization, and potentially metabolic engineering.

Materials and methods

Cultures and media

Six mesophilic species of *Clostridium* were selected to evaluate cellulose degradation and H₂ production. These species included *Clostridium acetobutylicum* (ATCC 824), *Cl. cellulolyticum* (ATCC 35319), *Clostridium phytofermentans* (ATCC 700394), *Clostridium cellobioparum* (DSM 1351), *Clostridium celerecrescens* (DSM 5628), and *Clostridium populeti* (DSM 5832). Strain selection was based on obtaining physiologic variety and including species on which extensive background research has been performed, with strains having partially or fully sequenced genomes preferred to facilitate subsequent genetic analyses and modification. The optimum reported growth temperatures for these species ranged from 30°C to 37°C. *Cl. acetobutylicum* was included as a negative control because it is a well-characterized species that does not grow on cellulose (Nolling *et al.* 2001). Strains were purchased from ATCC (Manassas, VA, USA) or DSMZ (Braunschweig, Germany), cultured in CM3 medium (Weimer and Zeikus 1977) with modifications as detailed below, and preserved as frozen stocks at -80°C in 15% glycerol. The components of modified CM3 medium (per litre of deionized water) were 5 g cellobiose, 1.3 g (NH₄)₂SO₄, 1.5 g KH₂PO₄, 2.9 g K₂HPO₄·3H₂O, 0.2 g MgCl₂·6H₂O, 2.5 mg FeSO₄·7H₂O, 0.075 g CaCl₂·2H₂O, 0.5 g L-cysteine, 1 mg resazurin, 1 ml mineral solution and 2 g yeast extract. Mineral solution contained (per litre of deionized water) 1.5 g FeCl₂·4H₂O, 70 mg ZnCl₂, 100 mg MnCl₂·4H₂O, 36 mg H₃BO₃, 190 mg CoCl₂·6H₂O, 2.0 mg CuCl₂·2H₂O, 24 mg NiCl₂·6H₂O and 36 mg Na₂MoO₄·2H₂O. The medium was buffered with 0.03 mol l⁻¹ 2-(N-morpholino) ethanesulfonic acid monohydrate and adjusted to pH 6.5 using 1 mol l⁻¹ NaOH, and then was made anaerobic by sparging with nitrogen gas following the technique of Oh *et al.* (2003).

Before each batch experiment, aliquots of the frozen stocks were cultured in medium containing cellobiose and used as inocula when the cultures reached exponential growth conditions. Modified CM3 medium was used for the batch experiments, with cellobiose (the primary soluble product of cellulose hydrolysis) used as a substrate control for H₂ production without the requirement for cellulolytic activity, and equal-mass replacements of cellobiose with MN301 (a combination of amorphous and microcrystalline cellulose; Macherey-Nagel, Duren, Germany) or Avicel (microcrystalline cellulose; PH 105, gift from FMC Corp., Philadelphia, PA, USA) to examine H₂ production from cellulosic substrates.

Batch culture experiments

Batch H₂-production experiments were conducted with each strain using a large headspace, intermittent gas-release method (S.E. Oh, unpubl. data). This is a modification of the Owen method (Owen *et al.* 1979), using a small liquid volume relative to the headspace volume to reduce inhibition caused by H₂ accumulation (Hallenbeck and Benemann 2002) without the need for continuous gas release and monitoring by respirometry (Logan *et al.* 2002). In an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA), 120 ml serum bottles (Wheaton Scientific, Millville, NJ, USA) were each filled with 18 ml anaerobic modified CM3 medium (without the growth substrate) and sealed with a butyl rubber stopper (Bellco Glass, Vineland, NJ, USA) and aluminum seal. Concentrated carbohydrate stocks (10×) were prepared similarly in separate bottles. After autoclaving all the bottles for 15 min, 2 ml of the carbohydrates were transferred aseptically into respective reaction vessels to give a final concentration of 5 g l⁻¹. Bottles were inoculated with the respective pure culture, adjusting the inoculum volume based on OD₆₀₀ values to introduce approximately 2 × 10⁸ cells into each bottle. The batch experiments were performed at 35°C in a shaker with agitation at 100 rev min⁻¹. Controls without inocula for each substrate (abiotic) and without carbohydrate for each clostridial species (biotic) were also included. All tests (except controls) were run in triplicate with results averaged.

Analyses

Gas production volume was measured and released daily using glass syringes (5–50 ml). H₂ concentration was measured by injecting 0.1 ml of headspace gas using a gastight syringe (Hamilton, Reno, NV, USA) into a gas chromatograph (Model 310; SRI Instruments, Torrance, CA, USA) equipped with a thermal conductivity detector

and a molecular sieve column (Alltech Molesieve 5A 80/100.6 × 1/8 × 0.085; Alltech Associates Inc., Deerfield, IL, USA) with argon as the carrier gas. Carbon dioxide was analysed similarly except that a different molecular sieve column (Alltech Porapak Q 80/100.6 × 1/8 × 0.085) was used with helium as the carrier gas (Oh *et al.* 2003).

Biomass was determined by cellular protein measurement. Samples (0.2 ml) were centrifuged (5900 g, 5 min), washed using phosphate-buffered saline (130 mmol l⁻¹ NaCl and 10 mmol l⁻¹ Na₂HPO₄, pH 7.2), and resuspended in 0.1 ml of 0.2 mol l⁻¹ NaOH solution. After another equivalent centrifugation, protein in the supernatant was quantified spectrophotometrically at 750 nm using the DC protein assay (BioRad Laboratories, Hercules, CA, USA), with bovine gamma globulin as a standard. The remaining pellets not dissolved by NaOH in the MN301 and Avicel experiments were stored at -20°C for subsequent cellulose analysis. Residual cellulose after the protein extraction was then solubilized in 67% sulphuric acid for 1 h at 30°C as described by Updegraff (1969) and quantified using the phenol-sulphuric acid method for sugars with glucose as the standard (Huang and Forsberg 1990). Standard curves were also determined for cellobiose, MN301 cellulose and Avicel using these assay conditions and they were found identical. Cellobiose concentration was determined similarly after filtration of the NaOH supernatant through a sterile 0.45 µm syringe filter.

For metabolite analysis, samples were centrifuged and the supernatant was filtered through 0.45 µm membranes and stored at -20°C. The concentrations of organic acids (formate, acetate, propionate and butyrate) and solvents (acetone, methanol, ethanol, *n*-propanol and butanol) were determined by gas chromatography (Agilent 6890N; Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and a fused silica capillary column (DB-FFAP) with helium as the carrier gas at a 38.8 ml min⁻¹ flow rate.

Culture purity determination

At the end of the experiment, culture purity in each bottle was tested by ribosomal intergenic spacer analysis (Iyer *et al.* 2004). A final sample was withdrawn from each bottle and centrifuged to obtain cell pellets. DNA was extracted and purified from pellets using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Purified genomic DNA was PCR amplified using the eubacterial primer set 926f (16S rDNA, 5'-AAACTYAAAAGGAATTGACGG-3') and 115r (23S rDNA, 5'-GGGTTBCCCCATTCRG-3') (Lane 1991). The thermal profile involved an initial 2 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 56°C and 1.5 min at

72°C; and a 5-min final extension at 72°C. The PCR products were analysed by agarose gel electrophoresis.

Statistical analysis

Mean H₂ yield values for each substrate were analysed by one-way analysis of variance (ANOVA) and Tukey's test at the 5% level of significance (Kuehl 1999).

Results

H₂ production

With MN301 cellulose, *Cl. cellulolyticum* produced the most H₂ (15.7 ± 1.2 ml after 9 days) (Fig. 1a). The corresponding H₂ yield was 1.7 ± 0.1 mol H₂ mol⁻¹ hexose consumed (Fig. 2). By comparison, *Cl. populeti* produced 15.2 ± 1.1 ml H₂ in 8 days (Fig. 1a), with a corresponding yield of 1.6 ± 0.1 mol H₂ mol⁻¹ hexose consumed (Fig. 2). The H₂ yields of *Cl. cellulolyticum* and *Cl. populeti* on MN301 were higher than the other four species ($P < 0.05$), and there was no significant difference between these two species within a 95% confidence interval. *Cl. celerecrescens* produced 20% less H₂ volume on MN301 than *Cl. cellulolyticum* (13% less on a yield basis). *Cl. cellobioparum* and *Cl. phytofermentans* grew slowly and produced considerably less H₂ (41 and 38% less H₂ volume, respectively, than *Cl. cellulolyticum*) (Fig. 1a). As expected, *Cl. acetobutylicum* did not degrade cellulose; the negligible H₂ volume produced by *Cl. acetobutylicum* can be attributed to cellobiose carry-over with the inoculum.

The trends with Avicel were similar to MN301, but the amounts of gas produced were smaller and the lag times were longer (Fig. 1b). *Cl. cellulolyticum* again produced the highest H₂ volume (13.9 ± 0.9 ml) and yield (1.6 ± 0.1 mol H₂ mol⁻¹ hexose consumed) (Fig. 2). *Cl. populeti* produced a comparable amount of H₂ (13.2 ± 0.4 ml), but the yield was lower (1.4 ± 0.1 mol mol⁻¹) due to greater Avicel degradation. As with MN301, the H₂ yields of *Cl. cellulolyticum* and *Cl. populeti* on Avicel were both higher than the other four species ($P < 0.05$), and there was no significant difference between these two species. The yields of H₂ from Avicel with *Cl. cellobioparum*, *Cl. celerecrescens*, and *Cl. phytofermentans* were 29, 21, and 38% less than *Cl. cellulolyticum*, respectively (Fig. 2). Again, *Cl. acetobutylicum* did not produce gas from Avicel.

The pattern of H₂ production from cellobiose was quite different (Fig. 1c). Cellobiose is a soluble hydrolysis product of cellulose, and all the bacteria except *Cl. phytofermentans* completed gas production in 3 days, accompanied by more than 93% cellobiose consumption. The highest H₂ volume (26.5 ± 2.1 ml) and yield

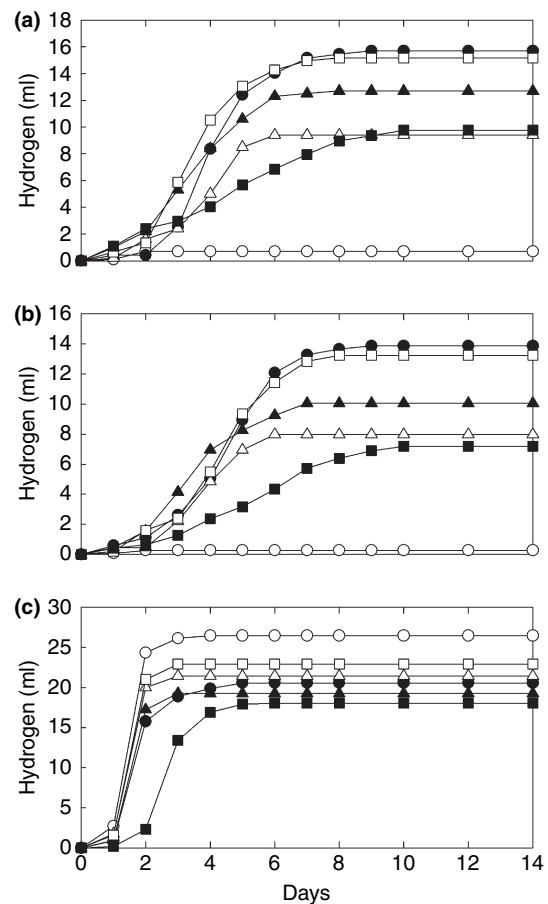


Figure 1 Cumulative hydrogen production by six *Clostridium* strains on MN301 cellulose (a), Avicel (b) and cellobiose (c) (values shown are averages of triplicate vials). Strains: *Clostridium acetobutylicum* (○), *Clostridium cellulolyticum* (●), *Clostridium cellobioparum* (△), *Clostridium celerecrescens* (▲), *Clostridium populeti* (□) and *Clostridium phytofermentans* (■).

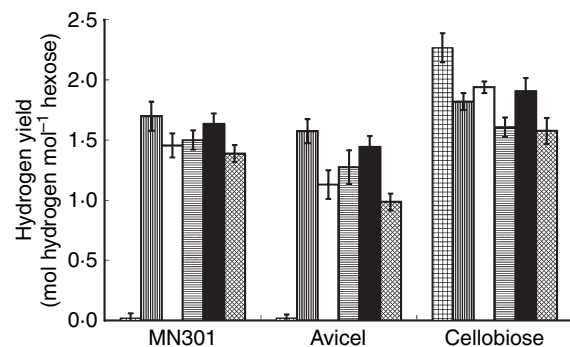


Figure 2 H₂ yield comparison of the six *Clostridium* species for each substrate. Strains: *Clostridium acetobutylicum* (□), *Clostridium cellulolyticum* (●), *Clostridium cellobioparum* (△), *Clostridium celerecrescens* (▲), *Clostridium populeti* (■) and *Clostridium phytofermentans* (■).

($2.3 \pm 0.5 \text{ mol mol}^{-1}$) were achieved by *Cl. acetobutylicum* ($P < 0.05$). Other strains had H_2 yields between 1.6 and $1.9 \text{ mol H}_2 \text{ mol}^{-1}$ hexose consumed using cellobiose (Fig. 2). *Cl. phytofermentans* showed a longer lag phase and gradual H_2 production, and the final volume of H_2 was only 18.0 ml coupled with 88% consumption of cellobiose.

There was no measurable H_2 production in the uninoculated bottles containing carbohydrates or in the bottles lacking added carbohydrates, even though they contained yeast extract and a small amount of medium carryover from the inoculum.

Substrate degradation and biomass accumulation

In addition to gas production, substrate degradation and biomass accumulation were measured daily (Fig. 3 for the species with the highest H_2 yield obtained for each substrate). The consumption of substrate and production of

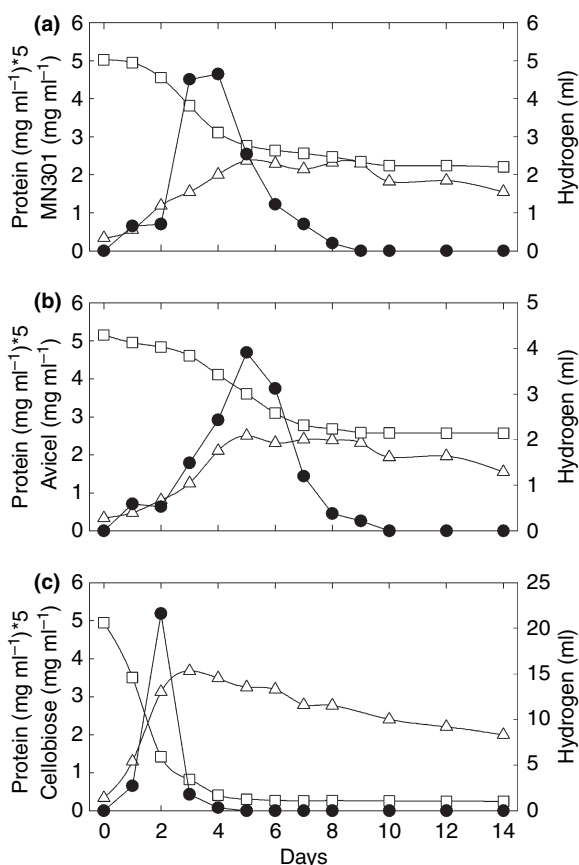


Figure 3 Daily hydrogen production (●), substrate accumulation (□) and biomass accumulation (△) for the species with the highest H_2 production on each substrate. (a): *Clostridium cellulolyticum* on MN301 cellulose; (b): *Cl. cellulolyticum* on Avicel; (c): *Clostridium acetobutylicum* on cellobiose.

biomass coincided with the production of H_2 . Compared with over 90% (4.5 g l^{-1}) cellobiose consumption in 2 days (Fig. 3c), MN301 and Avicel degradation was slower and less complete, with *Cl. cellulolyticum* converting 56% (2.8 g l^{-1} , Fig. 3a) and 46% (2.3 g l^{-1} , Fig. 3b), respectively, after approximately 10 days. *Cl. populeti* hydrolysed the greatest percentage of cellulose, with the conversion of 62% (3.1 g l^{-1}) MN301 and 52% (2.6 g l^{-1}) Avicel. Microscopic observations showed that most cells were adhering to cellulose fibres during the exponential growth phase, while most cells were present in the supernatant at the end of the experiment (data not shown), suggesting they were no longer participating in efficient cellulolysis.

The maximum-specific daily H_2 production rates, normalized by protein measurements, were calculated for each strain and each substrate (Fig. 4). Strains with higher H_2 yields also showed faster specific H_2 production. Hydrogen production rates from cellulose varied between 0.74 and $2.06 \text{ ml H}_2 \text{ mg}^{-1} \text{ protein day}^{-1}$, which was much lower than from cellobiose ($2.41\text{--}3.14 \text{ ml H}_2 \text{ mg}^{-1} \text{ protein day}^{-1}$). However, the molar growth yields normalized by substrate consumed were similar, around $32 \pm 5.4 \text{ g cells mol}^{-1}$ hexose, with *Cl. populeti* usually on the high side ($35.6\text{--}37.1 \text{ g cells mol}^{-1}$ hexose), and *Cl. cellobioparum* commonly low ($25.4\text{--}28.9 \text{ g cells mol}^{-1}$ hexose).

Fermentation products and carbon and electron recovery

In addition to gaseous metabolites and biomass, the fermentation of carbohydrates generates various soluble products, including volatile fatty acids and solvents. The distribution of fermentation products varied for the different strains, but the major products from each strain were generally similar for all three substrates (Table 1).

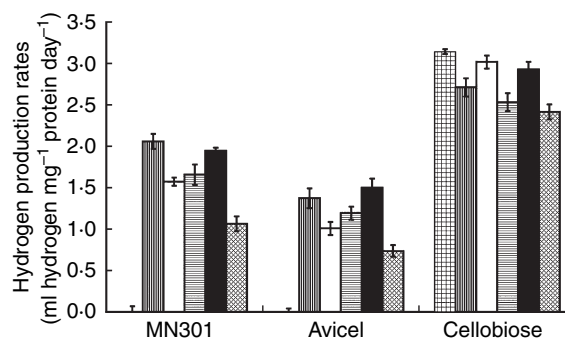


Figure 4 Maximum daily specific hydrogen production rates. Strains: *Clostridium acetobutylicum* (▨), *Clostridium cellulolyticum* (▧), *Clostridium cellobioparum* (▩), *Clostridium celerecrescens* (▤), *Clostridium populeti* (■) and *Clostridium phytofermentans* (▦).

Table 1 Molar-based metabolites variation and carbon and electron recovery*

<i>Clostridium</i> species	Substrate	Electron recovery (fraction)	Carbon recovery (fraction)	Degraded substrate	H ₂	Biomass	CO ₂ ‡	Acetone	Ethanol	<i>n</i> -Propanol	Acetate	Formate	Butyrate
<i>Cl. acetobutylicum</i>	MN301	ND†	ND	ND	<4	<1.2	ND	ND	ND	ND	ND	ND	ND
	Avicel	ND	ND	ND	<4	<1.2	ND	ND	ND	ND	ND	ND	ND
	Cellulose	0.89	0.89	23.1	52.3	9.5	26.8	<0.2	1.7	<0.2	14.2	<0.2	6.4
<i>Cl. cellulolyticum</i>	MN301	0.82	0.82	18.3	31.0	6.1	19.8	<0.2	4.1	0.8	17.2	<0.2	<0.1
	Avicel	0.82	0.80	17.4	27.4	6.2	17.9	<0.2	5.2	0.6	14.5	<0.2	<0.1
	Cellulose	0.80	0.77	22.3	40.6	6.2	24.8	<0.2	8.2	<0.2	18.6	0.2	<0.1
<i>Cl. cellobioparum</i>	MN301	0.72	0.76	12.8	18.6	4.2	15.0	<0.2	2.6	<0.2	8.9	<0.2	0.8
	Avicel	0.70	0.73	13.9	15.8	4.1	15.4	<0.2	4.8	<0.2	7.8	<0.2	1.0
	Cellulose	0.81	0.78	21.8	42.3	6.7	26.0	<0.2	7.8	<0.2	15.9	<0.2	0.6
<i>Cl. celerecrescens</i>	MN301	0.74	0.74	16.7	25.1	5.7	17.2	0.3	5.0	<0.2	8.5	<0.2	1.5
	Avicel	0.77	0.79	15.6	19.9	4.9	17.4	0.2	5.7	<0.2	7.2	<0.2	2.6
	Cellulose	0.77	0.76	23.7	38.0	5.5	26.9	0.7	7.9	<0.2	16.6	<0.2	2.0
<i>Cl. populeti</i>	MN301	0.76	0.82	18.4	30.0	6.1	21.4	<0.2	<0.2	<0.2	6.5	<0.2	7.8
	Avicel	0.74	0.82	18.1	26.2	6.1	22.1	<0.2	<0.2	<0.2	3.3	<0.2	9.1
	Cellulose	0.79	0.83	23.8	45.3	7.5	27.8	<0.2	<0.2	<0.2	16.5	<0.2	6.7
<i>Cl. phytofermentans</i>	MN301	0.83	0.83	13.9	19.3	4.9	14.7	<0.2	6.1	<0.2	10.6	1.2	<0.1
	Avicel	0.73	0.72	14.1	14.2	4.0	12.9	<0.2	7.3	<0.2	8.0	1.1	<0.1
	Cellulose	0.80	0.77	22.6	35.6	5.9	27.1	<0.2	13.6	<0.2	13.2	<0.2	<0.1

*Unless noted, the units for all data are mmol l⁻¹.

†ND, Not determined.

‡CO₂ is the sum of headspace CO₂ and dissolved CO₂ (all species).

Acetate was the only soluble product common to all six species. *Cl. acetobutylicum*, which grew only on cellulose, also produced butyrate and a small amount of ethanol [2.5% of the degraded substrate as carbon (notation throughout text)]. *Cl. cellulolyticum* produced acetate as well as an average of five times more ethanol than *Cl. acetobutylicum* and a small amount of *n*-propanol (1.7–2.1%). *Cl. cellobioparum* produced primarily acetate, with a similar amount of ethanol as *Cl. cellulolyticum* (on average 12%) and a slight amount of butyrate. *Cl. celerecrescens* was the only strain that produced acetone, although only a small amount (0.6–1.5%); the major products were ethanol, acetate and butyrate. The fermentation product pattern of *Cl. populeti* was unique in that this strain produced more butyrate than the other species, especially from cellulose, and it did not produce detectable ethanol. *Cl. phytofermentans* produced acetate, a significant amount of ethanol (up to 23%), and formate (1.3–1.4%).

The carbon recovery ranged from 72.2% to 89.3%. This was calculated based on the measured production of biomass, measured CO₂ gas in the headspace, dissolved CO₂ and soluble fermentation products compared with the carbon associated with substrate utilization. There were some potential carbon species that were not measured in this experiment, such as lactate and pyruvate. The dissolved fraction of the produced CO₂ was

calculated based on pH, Henry's law and the Henderson–Hasselbach equation. Results showed that the dissolved CO₂ was less than 10% of the observed headspace CO₂. The percentage of carbon assimilated into biomass, assuming a protein content of 55% and a cell formula of C₄H₇O₂N (Guedon *et al.* 1999b), was approximately 20% for each of the strains, meaning that growth yields were comparable, as discussed above. The electron (redox) recovery ranged from 70% to 89%, accounting for electrons ending up in H₂, measured soluble products and biomass.

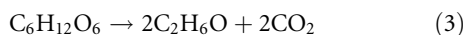
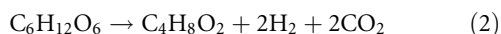
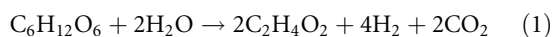
Confirmation of culture purity

After each experiment, ribosomal intergenic spacer analysis fingerprinting (Iyer *et al.* 2004) was used to assess the purity of the final cultures. Gels showed identical band patterns in the initial and final samples for each species (data not shown), indicating no contamination at detectable levels, which has been estimated to be 1% of the total DNA (von Canstein *et al.* 2002).

Discussion

The amount of H₂ produced from sugar fermentation depends on the catabolic pathways used by a bacterium (eqns 1–3). H₂ production is mainly coupled with the

acetate and butyrate production pathways in *Cl. acetobutylicum* (Girbal *et al.* 1995; Ren *et al.* 2007), with a greater H₂ molar yield associated with acetate than butyrate because the latter requires additional reducing equivalents. Stoichiometry alone reveals that H₂ production is not coupled directly with ethanol production.



Therefore, the theoretical maximum H₂ yield of 4 mol H₂ mol⁻¹ hexose would be achieved with the production of only acetate, and reduced H₂ yields would occur as the distribution of fermentation products shifts toward butyrate and ethanol. This is an important consideration in the selection of strains and the engineered redirection of metabolic flux toward increased H₂ yields. The acetate produced through this fermentation process can also be converted to H₂ using a newly developed microbial fuel cell-based process (Liu *et al.* 2005). By adding a small applied voltage (~250 mV) to a completely anaerobic system it is possible to convert acetate to hydrogen at yields of up to ~3 mol-H₂ per mol-acetate, at energy costs equivalent to 0.5 mol-H₂. This would achieve an overall increase of 5 mol of H₂ produced per hexose equivalent.

Of the strains tested in this study, *Cl. cellulolyticum* and *Cl. populeti* achieved higher H₂ production and yield on the cellulosic substrates than the other three cellulose-degrading strains, while *Cl. phytofermentans* showed the lowest H₂ production. These findings are consistent with the stoichiometry mentioned above, because *Cl. cellulolyticum* produced the highest percentage of acetate (30 ± 4.1%), *Cl. populeti* generated the most butyrate (29 ± 5.8%), and *Cl. phytofermentans* produced the most ethanol (21 ± 2.1%). These results are also consistent with other studies of fermentation products. Desvaux *et al.* (2001) found acetate was the main metabolite (39–58%) coupled with a large amount of H₂ when analyzing carbon flux distribution of *Cl. cellulolyticum*. Warnick *et al.* (2002) found 58% of the metabolites were ethanol when feeding *Cl. phytofermentans* with cellobiose, but no H₂ data were provided. This present study did not explore variability in cellulose hydrolysis and H₂ production among strains within clostridial species. It is possible that there are other strains with favourable properties beyond those tested here. Moreover, the performance of each strain might differ with other cellulose types. For example, it was demonstrated with *Clostridium lentocellum* that the acetate to ethanol ratio increased when crude biomass was used instead of pure cellulose, but no

H₂ data were reported (Ravinder *et al.* 2000; Lynd *et al.* 2002).

Cellulosome efficiency also affects the cellulose conversion process, and strains with high conversion kinetics allow smaller reactors with shorter retention times. Most strains produced 70–80% of the total H₂ in 4–5 days on cellulose, but *Cl. phytofermentans* produced H₂ at a constant rate for 12 days. On this basis, as well as H₂ yield, *Cl. cellulolyticum* and *Cl. populeti* are the two best wild type strains for hydrogen production (Figs 2 and 4). *Cl. populeti* initially produced H₂ faster than *Cl. cellulolyticum* from MN301, potentially because its cellulosome was synthesized more rapidly or was more efficient in adhesion.

Another reason for differing performances of the species may be due to the uniform experimental conditions. Although we attempted to address all of the nutritional requirements of each strain in the medium design, it is possible that the consensus medium was sub-optimal for some of the species. *Clostridium cellulovorans* (DSM 3052) was initially selected for inclusion in the experiments, but it failed to grow in the consensus medium and was removed from the study. As the original report for this species (Sleat *et al.* 1984) states that yeast extract can be substituted for the more commonly used trace mineral and vitamin components, we have no satisfactory explanation for its failure to grow.

As mentioned above, the extent of cellulose degradation was incomplete in these experiments, with approximately half of the cellulose hydrolysed and no further growth observed after 12 days. We attribute the lag phase for cellulose degradation observed in these experiments to the synthesis and assembly of the multi-enzyme cellulosome complex (Doi *et al.* 2003), given that culturing in cellobiose does not require cellulosome activity. After this lag, the pH in the bottles dropped to approximately 5.6 due to the accumulation of acidic metabolites, potentially contributing to limitation of cell growth. The inhibition of growth and H₂ production may also have been caused by the depletion of a particular nutrient from the culture medium (Kolter *et al.* 1993) or inefficiently regulated carbon flow that led to an accumulation of inhibitory intracellular compounds in the cells (Guedon *et al.* 1999a), as other studies showed that reinoculation with new cells led to further cellulose degradation (Desvaux *et al.* 2000). To address these limitations, running reactors in continuous mode and increasing the buffer capacity were reported as successful strategies to improve the degree of cellulose degradation (Guedon *et al.* 2000). The decline in cellulose hydrolysis rate and the significant amount of residual cellulose also may have been due to a more recalcitrant fraction of the initial cellulosic substrates and fewer new adherence sites on the cellulose fibres such that cells in

the supernatant could not participate in the degradation of the residual cellulose (Desvaux *et al.* 2000). This is consistent with the higher H₂ production obtained from MN301 cellulose than from Avicel, presumably because it was harder to hydrolyse the crystalline material more prevalent in Avicel.

Metabolic engineering provides an opportunity to improve H₂ production from cellulose. One strategy would be to start with an efficient cellulose-degrading strain and improve the hydrogen-producing enzymatic machinery. An analogous approach was used by Guedon *et al.* (2002) to enhance ethanol production from cellulose. They introduced *pdh* and *adhII* genes, organized as an artificial operon, into *Cl. cellulolyticum* to eliminate excess intracellular pyruvate accumulation and redirect the excess pyruvate toward ethanol production. An alternative approach would involve the use of *Cl. acetobutylicum*, a noncellulolytic clostridium, because it had a significantly higher H₂ yield from cellobiose than the other strains (Fig. 2). Genome sequencing has shown that *Cl. acetobutylicum* apparently possesses a complete cellulosomal gene cluster, but the genes do not allow the cells to grow on cellulose although several of them are expressed. It was speculated that this lack of growth on cellulose could be due to the absence or inactivity of the major catalytic components of the cellulosome, such as Cel48F and Cel9E homologues (Tyurin *et al.* 2000; Sabathe *et al.* 2002). Analysis of the genomic sequence of *Cl. acetobutylicum* to identify the defect(s), followed by introduction of functional copy(ies) of the defective gene(s), or introduction of a cellulosomal operon from an active cellulose-degrading strain, offer potential solutions to this problem with *Cl. acetobutylicum*. Thus, multiple opportunities exist for metabolic optimization through the integration of a high hydrogen-yielding central metabolism and an efficient cellulolytic activity.

To exploit the enormous potential of metabolic engineering for redirecting electron flux to H₂ production from cellulose, it is essential to identify the best target species. Higher H₂ yields and rates from cellulose and higher cellulose degradation are important factors. *Cl. cellulolyticum* and *Cl. populeti* showed significantly better performance than the other three cellulose-degrading strains in all of these criteria, indicating they would serve as good parent strains for research on biological hydrogen production from cellulose.

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