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Effect of key factors on hydrogen production from cellulose in a co-culture of *Clostridium thermocellum* and *Clostridium thermopalmarium*

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ABSTRACT

A cellulolytic, hydrogen-producing bacterium (*Clostridium thermocellum* DSM 1237) and a non-cellulolytic, hydrogen-producing bacterium (*Clostridium thermopalmarium* DSM 5974) were co-cultured at 55 °C, using cellulose as the sole substrate. At a low load of cellulose (filter paper, 4.5 g/L), yeast extract had a significant effect on cellulose degradation and hydrogen production. The extent of cellulose utilization and hydrogen production displayed a linear relationship with the logarithm of the yeast extract concentration, and the optimal weight ratio of yeast extract to cellulose was 1:1. At a high load of filter paper (9 g/L), an alkali chemical was required to maintain efficient cellulose degradation. As the $KHCO_3$ concentration increased from 0 to 60 mM, the utilized cellulose increased from 1.23 g/L (13.5%) to 8.59 g/L (94.3%), and maximum hydrogen production (1387 ml/L of culture) occurred at 40 mM $KHCO_3$. Increasing the inoculation ratio of *C. thermopalmarium* to *C. thermocellum* from 0.05:1 to 0.17:1 had little influence on hydrogen production, probably because of the limited availability of soluble sugar in the medium during the early stages of the co-culture.

1. Introduction

Hydrogen is the cleanest energy carrier in existence, with a heating value as high as 3042 cal/m^3 (Das and Veziroglu, 2001). Several groups have attempted to develop a suitable biohydrogen production system (Hallenbeck and Benemann, 2002; Levin et al., 2004) using fermentation substrates ranging from monosaccharides to polysaccharides, hexose, and pentose (Adav et al., 2009; Levin et al., 2006; Lo et al., 2008; Maeda et al., 2007; Taguchi et al., 1996). As cellulose, the main component of biomass, is the most abundant renewable natural resource and substrate (Demain et al., 2005), the conversion of cellulose to hydrogen has attracted attention as a means of biological hydrogen production.

Several cellulolytic bacteria with the capacity for hydrogen production, including *Clostridium thermocellum*, which efficiently degrades cellulose, have been used in the biohydrogen fermentation of cellulose (Levin et al., 2006; Ren et al., 2007). However, the use of cellulolytic bacteria for hydrogen production is often limited by low hydrogen yields, owing to poor bacterial growth rates, and pH sensitivity (Desvaux, 2005, 2006). The co-culture of cellulolytic strains and hydrogen producers, by taking advantage of their specific metabolic capacities, offers a promising new way to improve the conversion efficiency of cellulose to hydrogen. Recently, the co-culture of a cellulolytic strain (*C. thermocellum* JN4) and a hydrogen-producing strain (*Thermoanaerobacterium thermosaccharolyticum* GD17) was found to improve the degradation of cellulose and increase the yield of hydrogen compared with the monoculture of *C. thermocellum* JN4 (Liu et al., 2008), with an increase from 0.8 to 1.8 mol of H_2/mol of glucose equivalent. As more cellulolytic and hydrogen-producing strains become available, optimal co-culture conditions for various combinations should be determined with regard to the biohydrogen fermentation of cellulose. Moreover, characterization of the key factors influencing hydrogen production in co-culture and identification of the ecological relationship between the organisms will contribute to improvements in the hydrogen-producing efficiency of this approach.

In this study, a typical cellulolytic, hydrogen-producing strain, *C. thermocellum* DSM 1237, and a non-cellulolytic hydrogen producer, *C. thermopalmarium* DSM 5974 (Soh et al., 1991), were co-cultured at 55 °C, for biohydrogen production from cellulose. The effects of the inoculation ratio, yeast extract concentration, and alkalinity on the co-culture system were investigated, and the dynamics of the abundance of the two strains during co-culture were monitored using real-time PCR.

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2. Methods

2.1. Microorganisms and media

Clostridium thermocellum DSM 1237 (corresponding to ATCC 27405) and *C. thermopalmarium* DSM 5974 were obtained from the German collection of microorganisms and cell cultures. A fresh *C. thermocellum* culture was maintained by the routine transfer (every 3 days) of a 10% (v/v) inoculum into fresh modified 1191 medium (composition provided by the ATCC) containing Whatman No. 1 filter paper (α-cellulose content, >98%) at 4.5 g/L. A fresh *C. thermopalmarium* culture was maintained by the routine transfer of a 1% (v/v) inoculum into fresh modified 1191 medium containing glucose (0.5 g/L) instead of filter paper.

Unless otherwise stated, the modified complex medium contained 1.43 g of KH_2PO_4, 7.2 g of $K_2HPO_4{\cdot}3H_2O$, 0.5 g of NH_4Cl, 4.5 g of yeast extract (Oxiod LP0021), 1.0 ml of resazurin (0.25 mg/ml), 0.5 ml of vitamin solution, and 1 ml of mineral solution per liter of distilled, deionized water. The pH was adjusted to 7.0. After the medium was flushed with N_2 and autoclaved, sterile cysteine-HCl (30 g/L) was added to a final concentration of 0.5 g/L.

The vitamin solution contained 50 mg each of P-aminobenzoic acid, nicotinic acid, thiamine, riboflavin, and lipoic acid (thioctic acid); 20 mg of biotin; 20 mg of folic acid; and 10 mg of cyanocobalamin per liter of distilled water. The mineral solution contained 20.2 mg of trisodium nitriloacetate; 2.1 mg of $FeCl_3{\cdot}6H_2O$; 2.0 mg of $CoCl_2{\cdot}6H_2O$; 1.0 mg each of $MnCl_2{\cdot}4H_2O$, $ZnCl_2$, and $NiCl_2{\cdot}6H_2O$; and 0.5 mg each of $CaCl_2{\cdot}2H_2O$, $CuSO_4{\cdot}2H_2O$, and $Na_2MoO_4{\cdot}2H_2O$ per liter of distilled water.

2.2. Experimental design

All batch experiments were performed in 40-ml anaerobic tubes and at 55 °C. Filter paper was cut into 3-mm strips to serve as the cellulose source. For low cellulose loading (filter paper, 4.5 g/L), 30 ml of culture medium were used. The inoculation ratio of *C. thermocellum* in mono- or co-culture was fixed at 1/10 of the medium volume; thus, 3 ml from 3-day-old cultures of *C. thermocellum* were inoculated into 30 ml of medium. For co-culture, 1.5, 150, or 500 μl of overnight cultures of *C. thermopalmarium* were inoculated into co-culture medium along with *C. thermocellum*, to give *C. thermopalmarium*/*C. thermocellum* inoculation ratios of 0.0005:1, 0.05:1, and 0.17:1, respectively. For high cellulose loading (filter paper, 9 g/L), the volume was reduced to 20 ml, because we feared that the strips might have been flushed out of the medium by the produced biogas and then jam the syringe needle. The inoculation ratio of *C. thermocellum* in this co-culture was also fixed at 1/10 the volume, and the *C. thermopalmarium*/*C. thermocellum* ratio was 0.05:1. Inoculations were performed in an anaerobic chamber (Thermoelectric Corp., Houston, TX, USA). The anaerobic tubes were sealed with rubber stoppers and a layer of high-vacuum silicone grease and 60-ml syringes were inserted to collect and measure the evolved biogas production.

2.3. Chemical analysis

The biogas composition was analyzed using a GC7900 gas chromatograph (Techcomp, Shanghai, China) equipped with a thermal conductivity detector, as reported previously (Tao et al., 2007). Volatile fatty acids and ethanol were quantified by gas chromatography (Shimadzu 17A, Kyoto, Japan) using a flame ionization detector and a 30-m FFAP capillary column (Lanzhou Atech, Lanzhou, China). The temperatures of the injector and detector were 250 and 260 °C, respectively. The column was held at 70 °C for 2 min, heated to 200 °C at a rate of 8 °C/min, and maintained at 200 °C for 1 min. The carrier gas was nitrogen with a flow rate of 70 ml/min. Lactate and formate were separated by liquid chromatography (Agilent 1100 series, Böblingen, Germany) using a Zorbax SB-C18 column at a flow rate at 0.8 ml/min, detected by measuring the absorbance at 215 nm, and quantified using a standard curve.

Total cellulose, residual cellulose, and soluble sugar were measured using the phenol–sulfuric acid method described by Ren et al. (2007). The amount of cellulose per gram of Whatman No. 1 filter paper, as the carbon source, was 1.012 g of glucose equivalent.

2.4. Real-time quantitative PCR

Real-time PCR was performed using the Rotor-Gene 3000 system (Corbett Robotics, Brisbane, Australia). The abundance of each strain in the co-culture system was measured by quantifying the copy number of a gene from each strain: *C. thermocellum cipA* (cellulosomal scaffolding protein gene) (Stevenson and Weimer, 2005) and *C. thermopalmarium galK* (annotated as a galactokinase-coding gene). For *cipA*, amplification using the forward primer 5′-GTAAC GGCAGCTACAACGGAAT-3′ and reverse primer 5′-CTTTACCCCAT-ACAAGAACACC-3′ resulted in a 272-bp fragment. For *galK*, amplification with the forward primer 5′-TTCTTTGGAAATATCCCAAATG GTG-3′ and reverse primer 5′-CATCTAACTTTATAGGAACGTATTC-3′ gave a 264-bp fragment. A SYBR Premix Ex Taq kit (Takara, Shiga, Japan) was used under the following reaction conditions: 95 °C for 10 s followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 15 s. All assays were performed at least in triplicate. Those products with copy numbers between 10^2 and 10^8 were used as gradient templates to generate standard curves. Genomic DNA was extracted from co-cultured fermentation liquor at various times and used as the template for quantitative PCR.

2.5. Data analysis

The volume of biogas produced by batch fermentation was corrected to the standard conditions (273 K, 101 kPa). The cumulative hydrogen production per liter of culture was calculated according to the biogas produced from 30 or 20 ml of fermentation medium in each batch test and the corresponding hydrogen composition of the collected biogas. The hydrogen yield from cellulose was estimated as described in Eq. (1). The extent of cellulose utilization was calculated as in Eq. (2).

$$\mathrm{H_2\ yield} = \frac{\text{Net } H_2 \text{ production}}{\text{Glucose equivalent utilized}} \tag{1}$$

$$\text{Extent of cellulose utilization} = \frac{\text{Total cellulose} - \text{Residual cellulose} - \text{Soluble sugar}}{\text{Total cellulose}} \times 100\% \tag{2}$$

Linear regressions of the extent of cellulose utilization and hydrogen production on the logarithm of the yeast extract concentration were performed using the least-squares method. The abundance of *C. thermopalmarium* was determined from the copy number of *galK* (%) in the co-culture as described in Eq. (3). Student's *t*-test was performed with Excel 2003 software, employing a two-tailed test. All data are presented as the average of triplicate experiments.

$$\text{Abundance of } galK = \frac{galK \text{ copy number}}{galK \text{ copy number} + cipA \text{ copy number}} \times 100\% \tag{3}$$

3. Results and discussion

3.1. Effect of inoculation ratio on cellulosic hydrogen production

In previous studies, the inoculation ratio of *C. thermocellum* was 1/10 the medium volume (Levin et al., 2006; Liu et al., 2008). During co-culture, cellulolytic bacteria hydrolyzed cellulose which provided glucose or cellodextrin as a carbon source for their hydrogen-producing partner; therefore, the abundance of cellulolytic bacterium in the inoculum was typically higher than that of its hydrogen-producing partner (Liu et al., 2008). In this study, hydrogen production was compared among cultures with *C. thermopalmarium*/*C. thermocellum* inoculation ratios of 0:1 (monoculture of *C. thermocellum*), 0.0005:1, 0.05:1, and 0.17:1 (v/v) (Fig. 1). In all cases, the cumulative hydrogen production reached its highest value at about 60 h after inoculation. In the monoculture of *C. thermocellum*, hydrogen production was 176 ml/L of culture, which is nearly the same as that reported by Levin et al. (2006) under similar fermentation conditions (168 ml/L). Hydrogen production increased greatly with the addition of *C. thermopalmarium* to the monoculture of *C. thermocellum*. Hydrogen production was not significantly different (Student's *t*-test, $p = 0.73$) between cultures with *C. thermopalmarium*/*C. thermocellum* inoculation ratios of 0.05:1 and 0.17:1; however, compared with these two ratios, the lowest ratio (0.0005:1) resulted in slightly lower hydrogen production. At *C. thermopalmarium*/*C. thermocellum* inoculation ratios greater than 0.05:1, the enhancement of hydrogen production was limited. Thus, an inoculation ratio of 0.05:1 was used in our subsequent studies.

When the *C. thermopalmarium* ratio was increased from 0.0005:1 to 0.17:1 in co-cultures, the butyrate concentration also increased, with butyrate replacing ethanol and acetate as the main end product. In contrast, in mono-cultures of *C. thermocellum*, ethanol and acetate were present at higher concentrations and were the main end products (Levin et al., 2006). The increased butyrate concentration in co-cultures suggests that the contribution of *C. thermopalmarium* to biohydrogen production in the co-culture gradually improved. The decreased production of ethanol in co-culture may be the main cause of the increase in hydrogen production, because the ethanol-producing pathway competes for NADH with the hydrogen-producing pathway and should be avoided in hydrogen production (Levin et al., 2004).

3.2. Dynamics of the abundance of each strain in co-culture during hydrogen production

To explain why an inoculation ratio of 0.05:1 (*C. thermopalmarium* to *C. thermocellum*) was sufficient for active hydrogen production, the abundance dynamics of the two strains during the co-culturing process were quantified using real-time PCR. *galK* (annotated as a galactokinase-coding gene) and *cipA* (cellulosomal scaffolding protein gene) are specific single-copy genes in *C. thermopalmarium* and *C. thermocellum*, respectively. The copy number of each gene represented the abundance of the respective strain in co-culture (Fig. 2).

In the curve showing the abundance dynamics of *galK*, a positive slope indicates faster growth of *C. thermopalmarium*, whereas a negative slope indicates faster growth of *C. thermocellum*. The OD_{660} curves based on the co-culture and monoculture of *C. thermocellum* also helped to distinguish the growth phases. During the first 16 h after inoculation, *C. thermopalmarium* grew faster than *C. thermocellum*, but from 16 to 32 h, *C. thermocellum* grew faster. After 32 h, *C. thermopalmarium* again grew faster and continued to grow, gradually increasing in abundance until the co-culture reached the plateau phase. However, during the entire co-culturing process, *C. thermocellum* dominated *C. thermopalmarium*, with the abundance of *C. thermopalmarium* being less than 20%.

In co-culture, *C. thermocellum* was able to grow on cellulose, whereas *C. thermopalmarium* depended on soluble sugar for growth. In the early phase of co-culture (0–16 h), the small amount of soluble sugar present mainly in the inoculum or in the yeast extract in the medium enabled *C. thermopalmarium* to grow faster than *C. thermocellum*. However, the soluble sugar in the medium was consumed quickly, limiting the growth of *C. thermopalmarium*

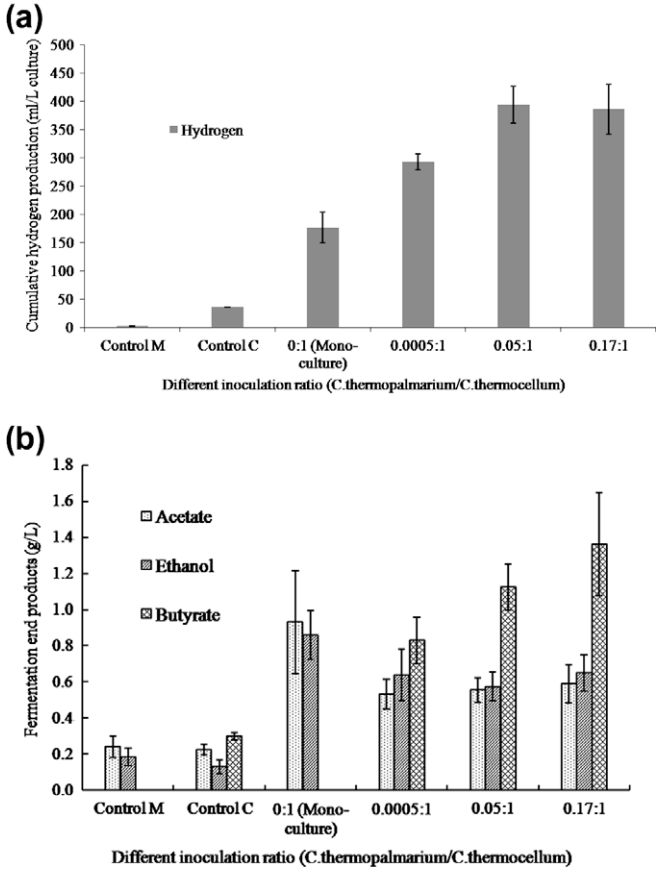

Fig. 1. Production of metabolites during the fermentation of cellulose in a monoculture of *C. thermocellum* and a co-culture of *C. thermopalmarium* and *C. thermocellum* at various inoculation ratios. (a) Cumulative hydrogen production per liter of culture; (b) the concentrations of acetate, ethanol, and butyrate; control M: no carbon source in monoculture of *C. thermocellum*; control C: no carbon source in co-culture at a *C. thermopalmarium*/*C. thermocellum* inoculation ratio of 0.05:1; 4.5 g/L filter paper were used as the carbon source in the other treatments.

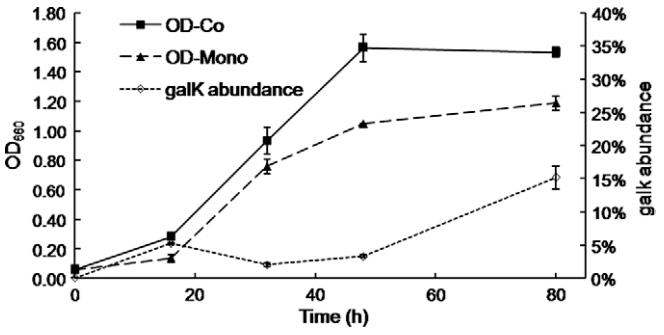

Fig. 2. Relative abundance of the two strains in co-culture. OD-Co and OD-Mono refer to the OD660 of the co- and mono-cultures, respectively; *galK* abundance refers to the copy number of *galK*, representing the abundance of *C. thermopalmarium* in the co-culture; 4.5 g/L filter paper were used as the carbon source.

to about 5%, even if additional *C. thermopalmarium* cells were added. This explains why the ratio of *C. thermopalmarium* to *C. thermocellum* in the inoculum did not significantly affect hydrogen production in the co-culture (Fig. 1a).

Between hours 16 and 32 of culturing, *C. thermocellum* grew faster because it was able to form a "cellulose-enzyme-microbe" complex (Lu et al., 2006), which allowed the highly efficient utilization of cellodextrin from cellulose (Zhang and Lynd, 2005), and perhaps provided a few amount of soluble sugar for the growth of *C. thermopalmarium*. After 32 h, owing to the growth of *C. thermocellum* and the increased cellulose hydrolytic activity, more soluble sugar was produced in the medium, supporting the growth of *C. thermopalmarium*. At this stage, *C. thermopalmarium* grew faster than *C. thermocellum*, and the corresponding fermentation of soluble sugar caused a drop in the pH of the co-culture medium. The lower pH (<6.0) might have caused a further decrease in the growth of *C. thermocellum*, but only slightly influenced the growth of *C. thermopalmarium* (Desvaux, 2006; Ozpinar and Ozkan, 2007; Soh et al., 1991). Nevertheless, *C. thermocellum* dominated *C. thermopalmarium* during the entire fermentation process when the inoculation ratio was 0.05:1.

3.3. Effect of yeast extract on co-cultured cellulosic hydrogen production

Yeast extract significantly influenced cellulose utilization and hydrogen production in the co-culture using 4.5 g/L filter paper as the carbon source. Hydrogen production increased from 83 to 396 ml/L of culture as the concentration of yeast extract increased from 0.56 to 4.50 g/L (Fig. 3). At the same time, the utilized cellulose increased from 1.21 (26.5%) to 3.81 g/L (83.7%), and the final pH decreased gradually from 6.65 to 6.02. The cellulose utilization extent and hydrogen production both exhibited a positive, linear relationship with the logarithm of the yeast extract concentration in the observed range (R^2 values were 0.958 and 0.996, respectively) (Fig. 4). The logarithmic-linear relationship reflected that in order to achieve higher cellulose utilization extent and hydrogen production, the demand of yeast extract would increase exponentially.

It has been reported that *C. thermocellum* ATCC 27405 requires at least 0.6% yeast extract to efficiently convert 1% cellulose to ethanol (Sato et al., 1992). Yeast extract is a good complex nutritional source of amino-nitrogen, vitamins, and other unknown growth factors for many microbial organisms (Smith et al., 1975). The main functional components in yeast extract (Johnson et al., 1981) that are used by *C. thermocellum* are P-aminobenzoic acid, vitamin B12, pyridoxamine, and biotin. Yeast extract is also necessary for the growth of the hydrogen producer *C. thermopalmarium* (Soh et al., 1991). This may explain the significant effect of yeast extract on both cellulose degradation and hydrogen production in the co-culture.

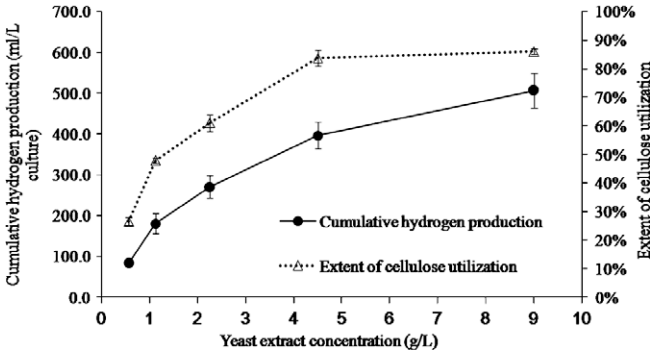

Fig. 3. Cumulative hydrogen production per liter culture and the extent of cellulose utilization at various yeast extract concentrations; 4.5 g/L filter paper were used as the carbon source.

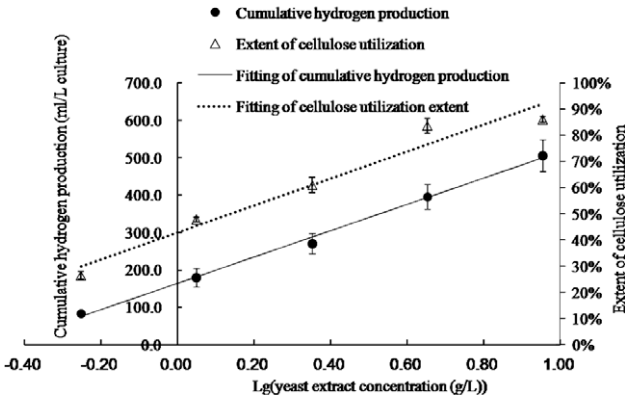

Fig. 4. Fitting of the hydrogen production and the extent of cellulose utilization to the logarithm of the yeast extract concentration, which varied from 0.45 to 9.00 g/L; 4.5 g/L filter paper were used as the carbon source.

As the yeast extract concentration increased from 4.50 to 9.00 g/L, hydrogen production continuously increased from 396 to 506 ml/L culture; however, the utilized cellulose only increased from 3.81 to 3.92 g/L. Therefore, equal amounts of cellulose and yeast extract, i.e., about 1 g of yeast extract per g of cellulose, were considered suitable for hydrogen production and cellulose degradation.

3.4. Effect of alkalinity on cellulose utilization and hydrogen production

Under the optimal fermentation conditions determined using 4.5 g/L filter paper as the carbon source, hydrogen production increased only slightly, to 535 ml/L of culture, when the filter paper concentration was increased to 9.0 g/L. Meanwhile, the utilized cellulose decreased to be only 1.23 g/L (13.5%), and the final pH dropped to 5.53. It has been reported that the cellulose-degradative ability and growth of *C. thermocellum* are seriously inhibited at pH levels below 6.00 (Desvaux, 2006). Therefore, the alkalinity was increased to delay a drop in pH, by adding autoclaved $KHCO_3$ to the co-culture medium at a final concentration of 0, 20, 40, or 60 mM. The fermentation end products, cellulose utilization extent, and final pH were determined at each $KHCO_3$ concentration (Table 1). As the concentration of $KHCO_3$ was increased from 0 to 60 mM, the utilized cellulose increased from 1.23 (13.5%) to 8.59 g/L (94.3%), while the final pH increased from 5.53 to 6.26. Hydrogen production peaked at 1387 ml/L of culture in the presence of 40 mM $KHCO_3$. The concentrations of by-products such as ethanol, acetate, and butyrate were also maximal at 40 mM $KHCO_3$, and the final pH was 5.86. These data indicate that the inhibitory effects of low pH on the growth and activity of *C. thermocellum* under high substrate loading were effectively relieved by the addition of 40 mM $KHCO_3$. In addition, no inhibition of bacterial growth or hydrogen production was detected in the co-culture when the pH remained above 5.8.

When the concentration of $KHCO_3$ was increased from 40 to 60 mM, the utilized cellulose increased from 8.19 (90.0%) to 8.59 g/L (94.3%). However, the production of hydrogen and other major metabolites such as butyrate, acetate, and ethanol decreased (Table 1). In addition, no lactate was detected, and the concentration of formate was around 0.30 g/L in the fermentation effluent at 60 mM $KHCO_3$. As *C. thermopalmarium* has been shown to form β-hydroxybutyrate-like inclusion bodies in the cytoplasm (Soh et al., 1991), some of the utilized cellulose might have been converted to

Table 1
Main end products, extent of cellulose utilization and pH under various alkalinity conditions.

Products	$KHCO_3$ concentration (mM)				
	0 (control)	0	20	40	60
Net H_2 production (ml/L culture)	90.8 ± 2.6	535 ± 102	1007 ± 69	1387 ± 233	1122 ± 12
Acetate (g/L)	0.260 ± 0.011	0.745 ± 0.037	0.934 ± 0.122	1.206 ± 0.202	0.981 ± 0.088
Butyrate (g/L)	0.456 ± 0.041	1.628 ± 0.165	1.859 ± 0.409	2.274 ± 0.479	1.571 ± 0.060
Ethanol (g/L)	0.038 ± 0.021	0.184 ± 0.037	0.284 ± 0.069	0.478 ± 0.137	0.422 ± 0.024
Formate (g/L)	0.082 ± 0.019	0.123 ± 0.038	0.382 ± 0.025	0.332 ± 0.043	0.052 ± 0.023
Lactate (g/L)	N.D.	N.D.	N.D.	N.D.	N.D.
Extent of cellulose utilization (%)	–	13.5 ± 0.1	53.1 ± 0.1	90.0 ± 0.1	94.3 ± 0.1
Initial pH value	6.80 ± 0.01	6.80 ± 0.01	6.85 ± 0.01	6.92 ± 0.01	6.98 ± 0.01
Final pH value	6.63 ± 0.01	5.53 ± 0.01	5.66 ± 0.01	5.86 ± 0.05	6.26 ± 0.06

Note: The working volume was 20 ml, with 9 g/L filter paper as the carbon source, and control was without filter paper added.
N.D. – not detectable.

β-hydroxybutyrate-like inclusion bodies at the higher pH with 60 mM $KHCO_3$.

The co-culture of *C. thermopalmarium* and *C. thermocellum* produced 1387 ml of H_2/L of culture under our optimized conditions, with an estimated hydrogen yield of 1.36 mol H_2/mol glucose equivalent. Although further increasing the concentration of cellulose may improve the cumulative hydrogen production per liter of culture, it may also result in a reduced hydrogen yield. For example, the co-culture of the cellulolytic strain X9 (a *C. acetobutylicum*-like strain) and the hydrogen producer *Ethanoigenens harbinense* B49 produced about 1810 ml of H_2/L of culture, but the hydrogen yield was less than 0.8 mol H_2/mol glucose equivalent, with 25 g/L microcrystalline cellulose as the sole carbon source (Wang et al., 2008). In contrast, the co-culture of *C. thermocellum* JN4 and *T. thermosaccharolyticum* GD17 converted 5 g of microcrystalline cellulose to 1232 ml of H_2, with a hydrogen yield of 1.8 mol H_2/mol glucose equivalent (Liu et al., 2008).

4. Conclusions

The co-culture of *C. thermocellum* and *C. thermopalmarium* produced nearly double the amount of hydrogen produced in *C. thermocellum* monocultures. Ethanol and acetate were the main metabolites in *C. thermocellum* monocultures, whereas the co-cultures produced butyrate as the main metabolite. Yeast extract greatly enhanced cellulose degradation and hydrogen production. As the cellulose concentration increased, the addition of an alkali chemical was required to keep the pH > 5.8. Increasing the *C. thermopalmarium* inoculation ratio from 0.0005:1 to 0.17:1 had only a limited effect on hydrogen production. The mechanisms of cooperation and competition between the two strains should be studied further by comparing gene expression in monocultures versus co-cultures.

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